DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

TRANSCRIPTIONAL REGULATION OF EGR-1 GENE IN MURINE CELLS. TOWARDS THE UNDERSTANDING OF THE ROLE OF CHROMATIN

GEMA TUR ARLANDIS

UNIVERSITAT DE VALENCIA
Servei de Publicacions
2007

Aquesta Tesi Doctoral va ser presentada a Valencia el dia 7 de Juny de 2007 davant un tribunal format per:

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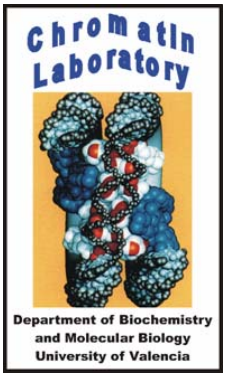
Depòsit legal:
I.S.B.N.:978-84-370-6880-0
Edita: Universitat de València
Servei de Publicacions
C/ Artes Gráficas, 13 bajo
46010 València
Spain
Telèfon: 963864115

Transcriptional Regulation of *egr-1* Gene in Murine Cells. Towards the Understanding of the Role of Chromatin.

Tesis Doctoral
Gema Tur Arlandis
València 2007



Transcriptional regulation of *egr-1* gene in murine cells. Towards the understanding of the role of chromatin.

GEMA TUR ARLANDIS

Valencia, 2007

Dissertation submitted to apply for PhD grade in Biochemistry from the University of Valencia.

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CERTIFY: that the present dissertation named "Transcriptional regulation of *egr-1* gene in murine cells. Towards the understanding of the role of chromatin", submitted to apply for PhD grade in Biochemistry, has been carried out under our direction by the M. Sc. in Biochemistry GEMA TUR ARLANDIS.

Signed. José Luis Rodríguez García

Signed. Gerardo López Rodas

"Si lo sabes de antemano, malo: sólo vas a decir lo que ya sabes, que es lo que sabemos todos. En cambio, si aún no sabes lo que quieres decir pero estás tan loco o tan desesperado, o tienes el coraje suficiente para seguir escribiendo, a lo mejor acabas diciendo algo que ni siquiera tú sabías que sabías y que sólo tú puedes llegar a saber, y eso a lo mejor tiene algún interés". *Javier Cercas, La velocidad de la luz.*

A Fernando, Gema, Vicent y Miguel

Com agrair a tots els que m'han ajudat a arribar fins ací? No serà fàcil no caure en el sentimentalisme, però espere que escrit sobre aquesta fulla o no, haja sabut donar-vos les gràcies abans. Si no ha sigut aixi, vos pregue em disculpeu, perquè sóc conscient de l'importància dels agraïments al principi de la tesis, però crec que es més important el dia a dia.

En primer lugar me gustaría agradecer al Prof. Luís Franco, porque gracias a él empecé no sólo en el mundo de la cromatina, sino en el apasionante mundo de la Bioquímica y a mis dos tutores de tesis: el Prof. José Luís Rodríguez y el Prof. Gerardo López. Gracias por hacerme crecer como científica y persona, aunque soy consciente que no siempre ha sido fácil.

I am particularly grateful to Prof. Myriam Gorospe, for gently giving me her advice and helpful antibodies in the beginning. I would also like to thank Prof. Andrea Grazziani for kindly let me be another member of his lab in Novara and introduce me in the fascinating world of RNA interference. Grazie mille a Paolo, per la sua pazienza con la spagnola strana; a Viola, Fede, Gian Luca, Sara, Samantha, Nicoletta et tutti l'altre che non ho menzionato ma qui me hanno fatto sentire Italia come la mia casa e qui serano sempre nel mio cuore.

Gràcies al Prof. Jordi Bernués i a tota la gent del seu laboratori, en especial al David, per acollir-me i ensenyar-me les meravelles del footprinting fins a altes hores de la matinada. Gracias al Prof. José Pertusa por "velozmente" ayudarme con el ImageJ y a Francisco José Rodríguez Fortuño por sus desarrollos matemáticos.

No se me olvidan todos los integrantes del Lab. de Cromatina, aquellos que están y aquellos que formarán siempre parte suya. La Prof. Maribel Rodrigo por sus consejos, Little John, "Gavin" y Andy por ser como son, y poner vida al lab. Nelo, Pepa, Halim, Elena Georgieva, Elena, Eliana, Maria, Elisa, Natalia, aquellos que se me olvidan y aquellos que seguirán llenando de vida el lab.

Gracias a todos los Miembros del Departamento de Bioquímica y Biología Molecular, sería muy difícil nombraros a todos, pero GRACIAS por haberme permitido compartir con vosotros las risas, los consejos y la cara de dormida por las mañanas. En especial a Maite y Paco por su paciencia y sus conocimientos de ordenador, sin los cuales dudo mucho de la existencia de esta tesis. Gracias a Loreto, Cris, Toni, Gon, Jorge, Mª Angeles y Vicent por ser la alegría en esta última parte del viaje. A Miquel, per fer realitat la història dels tallarins en la portada. A Ana del Servicio de Cultivos por ser el mejor "supermercado", a Maria y Toni de Secretaria por su eficiencia, a Juan Ramón y Pedro por estar siempre disponibles y a Paqui y Amparo por ser la primera sonrisa de muchas mañanas. Y cómo no, a Ángel y Sara por su inestimable ayuda con el confocal.

Por último, darles las gracias más especiales a: Aida, Amalia, Andrew, Ángela, Elisa, Helen, Inma, Paloma, Toya, Raúl, Sheila y Soni por apoyarme en los aciertos y equivocaciones y ser mi consciencia muchas veces durante este viaje, en especial a los que habéis compartido estos últimos días: Maria, Ibaixu y Maite. Nombraros a todos y decir lo importante de vuestro apoyo requeriría otra tesis, así que pedirme que os lo diga tomando una cervecita, si aún no os habéis enterado. Finalmente, agradecer a mi hermana Maria, mis "iaias" y "iaios" y mis padres; Gema y Miguel, per ser des de fan tants anys els meus millors fans.

INDEX

ABBREVIATIONS

ac	Acetylation
AP1	Activating Protein 1
AP2	Activating Protein 2
BRG-1	Brahma Related Gene 1
BRM	Brahma
BSA	Bovine Serum Albumin
CArG	$CC(A/T)_6GG$ DNA sequence
ChIP	Chromatin Immunoprecipitation
CIAP	Calf Intestine Alkaline Phosphatase
CIPed	Vector treated with CIAP
CoREST	REST Co-repressor
CRE	CREB Response Element
CREB	cAMP Response Element Binding Site
Chromodomain	Chromatin Organization Modifier
CTD	C-terminal domain
DAD	Deacetylase-Activating Domain
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMS	Dimethyl Sulphide
DMSO	Dimethylsulfoxide
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide Triphosphate
DSIF	DRB Sensitivity Inducing Factor
dsRNA	Double Stranded RNA
DTT	Dithiothreitol
EBS	Egr-1 Binding Site
EDTA	Ethylenediaminetetraacetic Acid
Egr-1	*Early growth response 1*

EGTA	Ethylenglycol-bis-(aminoethylether)-tetraacetic Acid
ERK	Extracellular-signal Regulated Kinase
ES	Embrionic Stem
Ets	E twenty six
FACT	Facilitate Chromatin Transcription
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GCN5	General Non-deprimible Coactivator
GCNF	Germ Cell Nuclear Factor
GFP	Green Fluorescent Protein
GPCRs	G Protein-coupled Receptors
GNAT	Gcn5-related N-acetyltransferase
GTF	General Transcription Factor
H	Histone
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIF	Hypoxia Inducible Factor
HPLC	High Performance Liquid Chromatography
HO-1	*heme oxigenase 1*
HMT	Histone Methyltransferase
HMG	High Mobility Group
IL	Interleukin
IP	Immunoprecipitated
ISWI	Imitation Switch

JAK-STAT	Janus Activated Kinase Signal Transducer and Activator of Transcription
JmjC	Jumonji-C domain
JNK	c-Jun N-terminal Kinase
K	Lysine
LB	Luria-Bertani Medium
LBA	Luria-Bertani Ampicillin Medium
LM-PCR	Ligated Mediated PCR
MAPK	Mitogen Activated Protein Kinase
Me	Methylation
MeCP2	Methyl CpG Binding Protein 2
MEM	Minimum Essential Medium
miRNA	micro RNA
MNase	Micrococcal Nuclease
MOPS	3-(N-morpholine) Propanesulfonic Acid
MSK-1	Mitogen and Stress-activated Kinase-1
MYST	Moz, Ybif-2/Sas3, Sas2 and Tip60
N-CoR	Nuclear Receptor Co-repressor
NES	Nuclear Export Signal
NF-κB	Nuclear Factor κB
NFP	Nucleosome Formation Potential
NGFI-A	*nerve growth factor-inducible gene A*
NLS	Nuclear Localization Signal
NP-40	Nonidet P-40
NuRD	Nucleosome Remodelling and Histone Deacetylation Complex
OD	Optical Density
PA	H3K10Phosphorilated H3K9Ac

PAD	Protein Arginine Deiminase
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pCAF	p300/CBP Associated Factor
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI3K	Phosphatidylinositol 3- phosphate kinase
PIC	Pre-initiation Complex
PKC	Protein Kinase C
PP1	Protein Phosphatase 1
PRMT	Protein Arginine Methyltransferase
P-TEFb	Positive Transcription Elongation Factor b
PTGC	Post-transcriptional Gene Silencing
RISC	RNA-induced Silencing Complex
RNase	Ribonuclease
RPD3	Reduced Potassium Dependency 3
RSK-2	Ribosomal Stress Kinase-2
SAGA	Spt-Ada-GCN5 Acetyltransferase
SAM	S-adenosil-L-methyonine
SAPK	Stress-activated Protein Kinase
SET	Su(var)3-9 enhancer of Zeste and Trithorax
scrbl	Scramble
SDS	Sodium Dodecyl Sulphate
ShRNA	Short hairpin RNA
SIR2	Silent Information Regulator 2
siRNA	Small interfering RNA
SMRT	Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor

SP1	Specificity Protein 1
SRE	Serum Response Element
SRF	Serum Response Factor
STAT	Signal Transducer and Activator of Transcription
SUMO	Small-related Ubiquitin Modifier
SWI/SNF	Switch/Sucrose Nonfermentor
Taq	Thermophyle DNA Polymerase
TBE	Tris-Borate-EDTA
t-BhP	Ter-butilhydroperoxide
TBP	TATA Binding Protein
TE	Tris EDTA
TEMED	N,N-tetramethylene Diamine
TF	Transcription Factor
TGS	Transcriptional Gene Silencing
TPA	13- acetate tetradecanoilphorbol
t-RNA	Transference RNA
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid
trx	*thioredoxin*
TSA	Trichostatin A
2XYT	2X Triptone Yeast extract rich medium
zif268	*zinc finger binding protein clone 268*

INTRODUCTION

1. TRANSCRIPTIONAL REGULATION

Transcriptional regulation of a gene is a highly sophisticated and precise mechanism, extremely accurate and efficient, to ensure not only normal cell life cycle but also quick responses to stimulus that threat cell survival. With the aim to achieve this goal eukaryotic organisms have developed many check-points to precisely regulate the transcriptional events.

The first level of transcriptional control will be achieved by selecting the right polymerase that is going to transcribe the gene. There are three different RNA polymerases, I, II and III, in the cell, which are in charge of transcribing the genetic information contained in the DNA into RNA. All three enzymes are highly conserved among species and they share common subunits as well as distinct subunits that confer them substrate specificity. RNApol I, formed by 14 subunits, transcribes large ribosomal RNA genes. RNApol II, composed by 12 subunits, is the complex that organizes the transcription of protein-coding DNA and some small structural RNA genes. Finally, RNApol III, with 17 subunits, is responsible for transcribing tRNA, 5S RNA and other small structural RNA genes.

The second level of transcriptional control is orchestrated by the binding of specific general transcription factors (GTFs), defined as the minimum factors necessary for *in vitro* transcription that regulate the pre-initiation complex (PIC) assembly. It is noteworthy PIC formation for the RNApol II, the main responsible of transcribing protein-coding genes.

A third level of transcriptional control comes by the fact that specific gene has to be turned on and off at the appropriate moment requiring the presence of sequence-specific DNA transcription factors that, by binding directly or indirectly through other proteins, can specifically activate mRNA synthesis. Three non-exclusive mechanisms may be found: 1) Stimulation of the basic transcriptional machinery recruitment, mainly RNApol II and GTFs. 2) Post-translational modifications such as phosphorylation and acetylation of the transcription factors and co-factors already present in the gene promoter, in order to modulate their

activity. 3) An important regulatory mechanism is achieved by modifying the DNA template through the interaction with chromatin modifying and remodelling complexes and their targeting to the promoters, either before or after PIC association.

Although the main regulatory points that initiate gene transcription are depicted above, to achieve a functional gene expression are also needed many other proteins to regulate the overall process. For instance, to initiate the polymerizing activity, RNApol II needs elongation factors as TFIIS, FCP1, P-TEFb, elongins, TREX, proteosome components or ELL family of proteins (Shilafard *et al.*, 2003 and Hartzog *et al.*, 2003) and some factors to ensure elongation through the nucleosomes such as FACT (Facilitate Chromatin Transcription), DSIF (DRB Sensitivity Inducing Factor), SPT6, CHD1 and ISW1 (Sims *et al.*, 2004). Nevertheless, since these additional regulations regarding the messenger RNA were not in focus of the present work, they will only be mentioned in this introduction.

1.1. TRANSCRIPTIONAL MACHINERY

When an intracellular or environmental signal affects the state of a regulatory protein, altering its nuclear localization, half-life and/or biological activity, it triggers multiple signals that finally converge in the promoters of the immediate-early genes activating or repressing the transcriptional machinery. As previously mentioned, in protein-coding genes, the main actors for the basal transcription are: RNApol II, GTFs (needed for the polymerase assembly into the PIC), the mediator complex (acting basically as a link between the activators and the RNApol II) and the DNA sequence-specific transcription factors. Although the general transcription machinery is similar for the three types of polymerase, there are subtle differences among the process that render each of them specific to a certain type of genes. We will focus now on the mechanism of action of the RNApol II since the group of genes that have been studied are transcribed by this polymerase.

1.1.1 RNA Polymerase II

RNA polymerase II is the enzyme responsible to transcribe DNA into messenger RNA. RNA polymerase II is indeed a relatively large complex formed by 12 polypeptide components and with a molecular weight higher than 500 kDa (Asturias, 2004). The X-ray analysis of the RNApol II complex captured in a RNApol II-DNA-RNA structure has revealed a feasible mechanism of initiation. The active side of the polymerase is located at the bottom of a cleft and is blocked by a mobile domain termed the "wall" that is not present in the free enzyme. This structure and the fact that, when the domain is present, the cleft is too narrow to accommodate double stranded DNA, suggest the idea that this domain will "close" the enzyme upon binding and it will be the key to the processivity of the enzyme (Cramer *et al.*, 2000).

RNApol II needs to be targeted to the promoter by specific transcription factors, and it is also necessary, the ordered recruitment of GTFs that will form the PIC is also necessary. Smale *et al.*, (2003), in an elegant experiment *in vitro* using core promoter sequences and purified GTFs, demonstrated that stable PIC formation was sufficient for basal transcription. The pathway for basal transcription requires TATA recognition by TBP (TATA Binding Protein) and the subsequent stabilization by TFIIA and TFIID. Later on, TFIIB binds TBP in a process that precedes TFIIF-RNApol II complex recruitment. At this point, TFIIE binds to the polymerase and serves as an anchor for TFIIH (Roeder, 2005; Baek *et al.*, 2006).

The answer to the question of why this complex is only enough for basal transcription is not yet clear as well as the whole requirements of the transcriptional machinery. Nevertheless, it seems that another counterpart in the transcription, the mediator complex, may emphasize the process either by facilitating RNApol II recruitment and PIC assembly (Yudkovsky *et al.*, 2000) or by facilitating the successive rounds of transcription (Mason *et al.*, 2005).

1.1.2 PIC and Mediator Formation

Regulation of transcription initiation requires a multisubunit "adaptor" that bridges specific DNA-binding transcription factors present in the promoter and/or enhancer with the general initiation factors bound to the RNApol II. This adaptor has been termed as *mediator complex* and it was first discovered in yeast and later in higher eukaryotes. Mediator complexes from both species is highly conserved and is also ubiquitously expressed (Conaway *et al.*, 2005).

The mediator complex of higher eukaryotes is formed by up to 25 different polypeptides. Some of them are highly homologue to the *Sacharomyces cerevisiae* mediator, (for instance SRB7, MED7, NUT2, SRB10, SRB11) but some others are so specific that it is even possible to distinguish between different complexes not present in yeast, such as TRAP/SMCC (Gu *et al.*, 1999), ARC, DRIP; CRSP (Taatjes *et al.*, 2002) or MED (Sato *et al.*, 2003). As an example of this specificity, MED1 is a subunit that mediates interaction with class I and II nuclear receptors and seems to be only necessary for activation of genes induced upon ligand binding like the thyroid hormone receptor (Zhang *et al.*, 2005).

The mechanisms by which mediator facilitates transcription are still under discussion. For instance, it is not yet clear if the mediator is targeted to the promoter before RNApol II, suggesting a role in the holoenzyme recruitment, or after the binding of the polymerase, indicating a further influence in PIC assembly and stability (Malik *et al.*, 2002; Reeves *et al.*, 2003). Wang *et al*., (2005) gave a clear example of mediator function after RNApol II binding by showing that MED23 (a subunit of the complex) directly binds to ELK1 and E1A transcriptional activators upon serum induction, and correlating it with the delay between polymerase recruitment and transcription process initiation. Struhl and *et al.*, (2005) even proposed that, since the mediator recruitment seems to be independent of SWI/SNF activity, holoenzyme phosphorylation or recruitment of elongating factors (as NELF or DSIF), the mediator could be involved in promoter melting or promoter clearance. On the contrary, Baek *et al.*, (2006) based on isolation of complexes

mediator-RNApol II, suggested that the function of the mediator may rather be in targeting the polymerase to the promoter.

Although the actual mechanism is not clear, Asturias (2004) has suggested a molecular mechanism in which the mediator is recruited to the promoter ahead of the RNApol II and exerts a function to anchor for the additional components of the PIC (Figure 1).

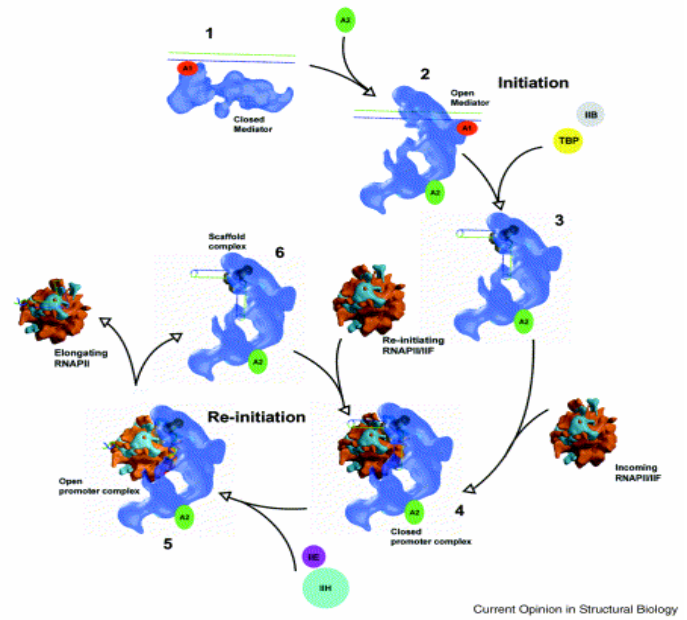


Figure 1. Structural model for Mediator initiation and re-initiation. Mediator is recruited to a promoter by activator A1 (1), and will suffer a conformational change (2) to allow the binding of transcription factors IIB and IID (TBP) (3) and RNAPII–IIF complex (4). DNA strand will reach the polymerase active site by the action of TFIIE and TFIIH (5). The holoenzyme will escape the promoter leaving the anchor for successive rounds of initiation (6, then back to 4). (Asturias, 2004).

Furthermore, to increase the complexity of the whole process, it have been characterized other factors that mediate the process of elongation, (as TFIIS, elongins, P-TEFb, proteasome components, etc.). Moreover, since the mechanism takes place in a chromatin environment, there are also components as FACT (Belotserkovskaya *et al.*, 2003), DSIF, SPT6, ISW1, and others (Sims III *et al.*, 2004) that are needed to facilitate the process.

To sum up, RNA polymerase activity in a promoter is a highly orchestrated mechanism, still to be uncovered, but for one sure thing which is the cooperation of multiple assistants.

1.1.3. Transcriptional Factor Binding

The mechanism by which trans-acting sequence-specific transcription factors regulate transcription by binding to cis-regulatory DNA sequences emerged in 1979 with the experiments of Tjian. This author purified the adenovirus-simian-virus S40 (SV40) T antigen protein and realized that it could bind to tandem palindromic sequences in gene promoters in a selective manner (Tjian, 1979). In the following years many other transcription factors (TFs) were discovered: Engelke et al., (1980) found out TFIIIA, which is specific for 5S genes, and Dynan et al., (1983) isolated the human SP1 transcription factor and located its binding sequence upstream of the transcriptional starting point of various genes. Nowadays, there are multiple transcription factor families known, classified depending on its DNA-binding motifs, arranged structural protein motifs that interact with exposed surfaces of the DNA bases and backbone.

Transcription factors are modular proteins, as first described by Brent et al., (1985) in experiments in which they constructed a chimera containing the prokaryotic transcriptional repressor *lexA* fused to *Gal4* activation domain. This construct is able to support the transcription of a yeast promoter containing the *lexA* operator. After those first experiments, it was showed that transcription factors also contain repression domains (Uno *et al.*, 2001), domains to interact with MAP

kinases, dimerization domains and domains that facilitate interaction with other proteins (Buchwalter *et al.*, 2004), giving a higher combinatorial complexity of the ways in which these factors can be regulated.

Transcriptional factors are usually classified according to their DNA binding domain which usually has characteristic amino acid sequences. As shown by X-ray crystallography, the most common recognition pattern between a trans-factor and a cis-sequence is the interaction of an α-helical domain with around 5 base pairs in the DNA major groove (Mueller, 2001). An example of transcription factor family sharing DNA binding domain is ETS (E twenty six) that contains the helix-turn-helix binding motive (Mo *et al.*, 2000), able to regulate gene transcription by binding to a purine rich sequence GGAA/T and by interacting with several specific factors (Oikawa *et al.*, 2003). Another highly abundant motif is the zinc finger, able to bind zinc ions through various cysteine and/or histidine residues (Klung *et al.*, 1995), which is present, for instance, in SP1 (Specificity protein 1, Uno *et al.*, 2001) and in EGR-1 (Early growth response- 1, Kachigian *et al.*, 1995). Other well known families are the basic helix-loop-helix proteins, as HIF (hypoxia inducible factors) (Kewley *et al.*, 2004), or the one that contains the basic leucine zipper motif, as CREB (cAMP response element binding, Acharia *et al.*, 2006) that usually facilitates dimmer formation.

Transcription factors are regulated by many non-exclusive mechanisms: 1) Certain factors are tissue-specific. For example the Germ Cell Nuclear Factor (GCNF) member of a nuclear receptor superfamily with restricted expression pattern in the adult. (Hummelke *et al.*, 2001). 2) The activity of the factors can be regulated by direct modification. For instance, Schiller *et al.*, (2006) demonstrated that phosphorylation of CREB at serine 133 is essential for *c-fos* induction. 3) A transcriptional factor can also be activated or inactivated when bound to ligands that influence the protein localization and/or its ability to bind DNA. This is the case of nuclear receptors as the retinoid X receptor (Moras *et al.*, 1998). 4) The availability of the factor can also vary, depending on the circumstances. For instance, some factors can dimerize with different partners that, depending on the companion, could facilitate transcriptional activation or inhibition. When CREB dimerizes with

CREM, though it can bind the DNA *cis*-sequence, it becomes inactive (Loriaux *et al.*, 1994). Moreover, it could also happen that the synthesis of one of them inhibits the synthesis of the other partner.

On the other hand, although the inhibition by transcription factors has been less studied than their role as activators, it has been demonstrated that in some cases the transcriptional inhibition takes place without interfering with the binding of other activators or GTFs.

1.2 CHROMATIN ROLE IN TRANSCRIPTION

Eukaryotic cells have solved the problem of fitting its large genome (around 2 m long) inside the nucleus (of around 10 μm of diameter) by creating a compact structure of DNA and histone proteins, called nucleosome, which is able to fold into more complex structures. The ensemble of DNA, histone and non-histone proteins is nowadays named chromatin, a formerly cytological term coined by Flemming (1882). Nevertheless, the chromatin structure creates an obstacle for the transcriptional machinery, as transcription factors have to gain access to the compact DNA template. In order to bypass the transcriptional constrictions, the cell has organized the binding of transcriptional activators that target chromatin enzymes to the promoters allowing changes in the chromatin accessibility. These enzymes can be classified into two main classes: chromatin modifier complexes and ATP-dependent chromatin remodelling complexes. Those of the first group covalently modify the histones, alter the overall charge of these highly basic proteins and disrupt the chromatin structure. Complexes of the second class use the energy of ATP hydrolysis to modify DNA-histone interactions, increasing the accessibility of nucleosomal DNA to transcriptional factors and to the transcriptional machinery. Furthermore, eukaryotic transcription can also be facilitated by alterations in the non-core histone protein content of chromatin (Becker *et al.*, 2002).

1.2.1 Chromatin Structure

Nucleosome, the building block of DNA packing, consists of around 200 bp (depending on the organism) a histone octamer formed by a tetramer of H3/H4, two dimmers of H2A/H2B and a copy of histone H1. The core nucleosome (Figure 2), obtained from extensive chromatin digestion with nuclease, has 147 bp that form 1.8 turns of superhelix around the histone octamer and lacks histone H1 (Thomas *et al.*, 1975, Richmond 1985 and Richmond *et al.*, 2003). The latter histone settles in the linker DNA, between two consecutive nucleosome cores.

Octamer histones are highly conserved basic proteins characterized by a structural domain composed of a long α-helix separated by β-sheet loops from two flanking α-helices called "the histone fold". This motif is responsible for the dimerization of histones through a hand-shake motif in which each monomer contacts the other in a head-to-tail orientation (Arents *et al.*, 1991 and Luger *et al.*, 1997). The N-terminal basic tails of the nucleosome core, rich in lysines and arginines, protrude from the octamer of histones and are susceptible of covalent modifications. These post-synthetic modifications in the histone tails (acetylation, phosphorylation and methylation) and outside the histone tails (ubiquitylation, glycosylation, SUMOylation and ADP-ribosylation) are important both for transcriptional regulation and for the higher-order chromatin structure condensation (Turner, 1993 and Spencer *et al.*, 1999). Zatlanova *et al.*, (1998) showed that these histone tails regulate the different folding degree states of chromatin fibres by interacting with neighbouring nucleosomes and other proteins as HMGs (High Mobility Group).

Nucleosomes arrange in a filament, which adopts a "beads on a string" shape (Figure 2). This filament is further folded to yield an irregular fibre of 30 nm of diameter. Although its structure is not fully understood, there is increasing evidence that it is shaped as a solenoidal arrangement of nucleosomes. The stability of the 30 nm fibre depends on the histone H1 and its variants, which control the higher-order structures and also stabilize nucleosomal positioning (Khochbin, 2001 and Bustin *et al.*, 2005).

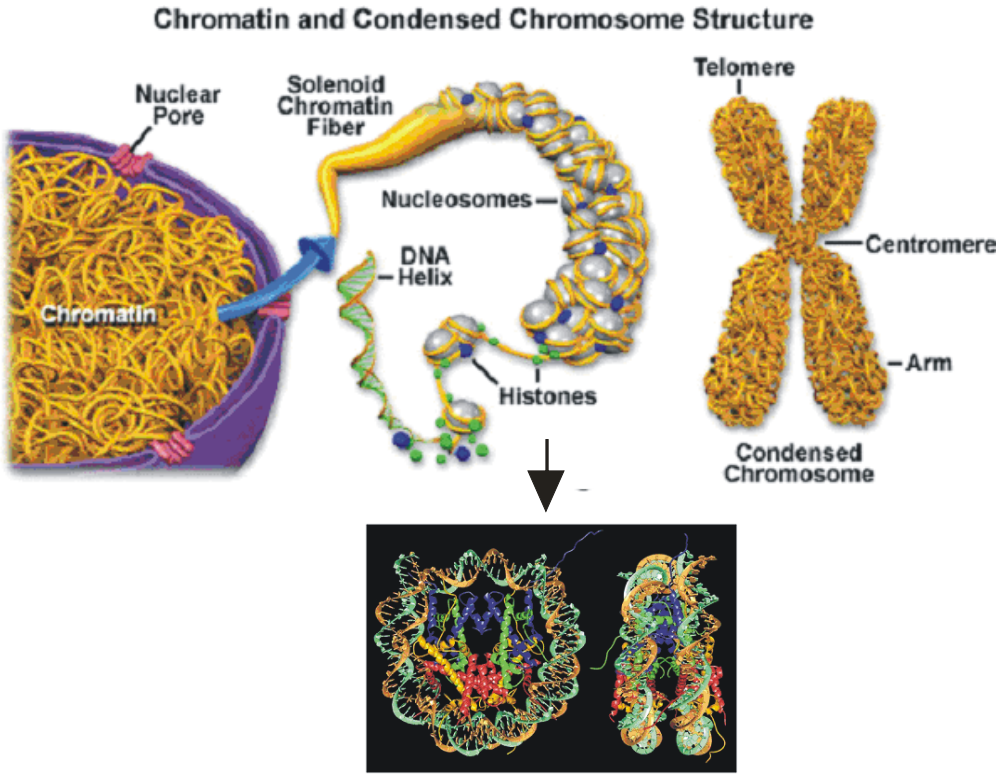


Figure 2. Chromatin compactation levels. DNA binds to the histone octamer to form the nucleosome (X-ray structure in black) and then is packed into higher-order structure. Adapted from Luger *et al.*, 1997 and Molecular Expressions™.

1.2.2. Chromatin Modifications: The Histone Code Hypothesis

As previously described, histones are subject to a huge number of post-translational modifications that can take place mainly at the N- terminus but also in the central domain and C-terminus of the proteins (Mersfelder *et al.*, 2006). This complex process has two consequences. On one side, since histone modifications alter the global protein charge modifying DNA-nucleosome interactions, it changes the nucleosomal dynamics, rendering the chromatin more fluid, and therefore

facilitating the protein accessibility to DNA. On the other side, these modifications are essential for transcriptional cofactors recruitment (Cosgrove *et al.*, 2004) and for transmission of epigenetic information (Lopez-Rodas *et al.*, 1993). Indeed, the fact that side-specific combinations of histone modifications correlate well with particular biological functions has lead to the hypothesis of an "histone modification code" (Strahl *et al.*, 2000, Jenuwein *et al.*, 2001 and Spotswood *et al.*, 2002) (see Table 1). For instance, the combination of H4K8 acetylation, H3K14 acetylation and H3S10 phosphorylation is usually associated with active transcription. Conversely, trimethylation of H3K9 correlates with transcriptional repression (Lachner *et al.*, 2001). Regardless of this, the term code may be misleading as, in contrast with the general conception of a code, the combination of histone marks does not always dictate the same biological function. As an example, H3K9 methylation can in some cases be associated with actively transcribed genes (Beisel *et al.*, 2002).

The hypothesis of a "histone code" is supported by the existence of histone modifying enzymes, such as histone acetyltransferases, histone deacetylases, histone methyltransferases and histone kinases that are specifically targeted to certain histone residues, with the combined action of cofactors containing binding domains able to specifically recognize these modifications. These chromatin modifying enzymes, whose substrate specificity is shown in Table 1), are targeted to the nucleosome by direct interactions with DNA sequence-specific transcriptional regulators. For instance, ETS 2 and SP1 act synergistically to recruit histone acetyltransferase p300 to the human *interleukin-12* promoter (Sun *et al.*, 2006). In the same manner, the modifications that affect the overall nucleosome structure and, therefore, the chromatin environment, have to be specifically "read" in order to delimit subdomains suitable to be either transcribed or repressed. This translating job is achieved by precise protein domains that are able to recognize and bind specifically to modified nucleosomes (Marmorstein, 2001). For example, bromodomains are able to recognize acetylated residues on the N-terminal tails of the histones, and are often associated with histone acetyltransferases and chromatin remodelling complexes (Zeng *et al.*, 2002). Similarly, chromodomains bind to methylated residues and are often associated to transcriptional repression by inducing chromatin remodelling (De la Cruz *et al.*, 2005). Finally, SANT domains (a

DNA binding domain present in SWI-SNF, ADA, NCoR and TFIIIB) show high affinity for unmodified histones (Boyer *et al.*, 2004) whereas SLIDE (SANT-like domain) domains interact with DNA (Saha *et al.*, 2006).

Table 1. Histone H3 and H4 modifications. Site-specific modifications of histones are depicted with the enzymes responsible of those modifications and their putative functions. Picture adapted from Peterson *et al.*, (2004).

Histone modification	Histone	Aminoacid	Enzyme	Function
Phosphorylation	**H3**	T3	?	Mitosis
		S10	TG2, AuroraB,MSK1, MSK2 SNF1	Immediate-early activation. Mitosis
		T11	DLK/ZIP/RSK2	Mitosis
		S28	MSK1,MSK2	Mitosis. Immediate-early activation
	H4	S1	?	Mitosis
Acetylation	**H3**	K4	ESA1, HPA2	Transcriptional activation
		K9	GCN5, SRC-1, PCAF	Transcriptional activation. Nuclear receptor coactivation
		K14	GCN5, SRC-1, TAF-1, PCAF, p300, TIP60, hTFIIC90	Transcriptional activation. Nuclear receptor coactivation
		K18	p300, CBP	Transcriptional activation. DNA replication
		K23	p300, CBP	Transcriptional activation
		K27	GCN5	Transcriptional activation
	H4	K5	HAT1, p300, TIP60, ATF2	Nucleosomal assembling, transcriptional activation
		K8	GCN5, p300, ATF2, PCAF, TIP60	Transcriptional activation
		K12	HAT1, TIP60	Histone assembling
		K16	MOF, GCN5, ATF2, TIP60	Transcriptional activation. DNA repair

Table 1. Histone H3 and H4 modifications. (Cont.)

Histone modification	Histone	Aminoacid	Enzyme	Function
Methylation	**H3**	R2	CARM1	In vitro methylation
		K4(diMe)	MLL4, SET1, MLL, SMYD3,SET7/9	Active euchromatin
		K4(triMe)		Transcriptional activation and elongation
		K9	SUV39h1 SUV39h2, ESET, G9A, EZH2, Eu-HMTase1	Pericentric heterochromatin DNA methylation, Rb mediated silencing. Transcriptional repression. X chromosome repression
		R17	CARM17	Transcriptional activation
		K27	EZH2, G9A	Transcriptional repression, Policomb repression. X chromosome inactivation
		K36	SET2	Transcriptional elongation
		K79	DOT1p	Transcriptional elongation
	H4	R3	PRMT1, PRMT5	Transcriptional activation
		K20 (monoMe)	PR-SET7	Transcriptional silencing
		K20(triMe)	SUV4-20	Heterochromatin
Ubiquitylation	**H3**	?	?	Spermatogenesis
SUMOylation	**H4**	?	UBC9	Transcriptional repression

A final and central aspect of the histone code is the interplay between modifications both within the same histone and between neighbouring ones (Figure 3). For instance, H3K4 methylation is able to block H3K9 methylation, as a *cis* effect by facilitating H3K9 acetylation and therefore generally induces gene expression (Zegerman *et al.*, 2002), whereas H2BK123 ubiquitylation precedes and facilitates H3K4 methylation (Sun *et al.*, 2002). As a result, the general idea would be that an inhibition signal would facilitate other inhibitory signals and the contrary would happen for the transcription activating signals.

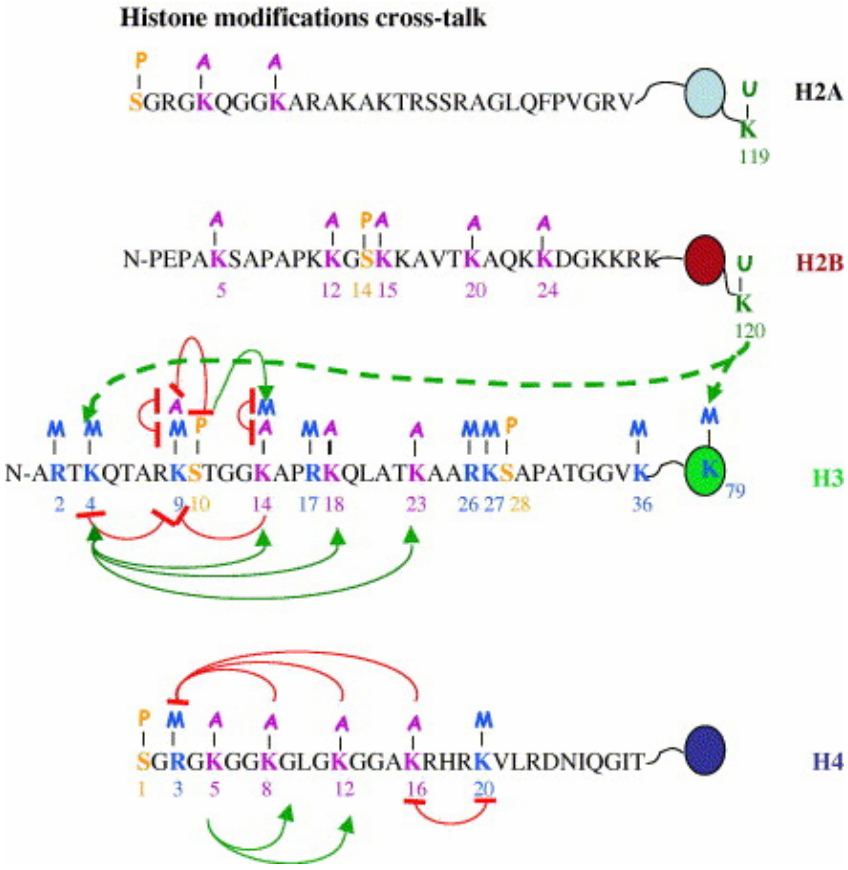


Figure 3. Cross-talk of histone modifications. Modifications that facilitate other modifications are represented with a green arrow, while those modifications that prevent others to occur are showed in red. Adapted from Santos-Rosa *et al.*, (2005).

1.2.2.1. Histone Acetylation: Histone Acetyltransferases and Histone Deacetylase Complexes.

Histone acetyltransferases (HATs) are enzymes that catalyze the transfer of an acetyl group from acetyl-coenzyme A to the ε-amino groups of conserved lysine residues that are present in histone tails (Figure 4). This process was generally linked to transcriptional activity (Kouzarides, 1999), as the neutralization of the

basic charge of the histone tails can alter the higher-order chromatin structure. Recently, it has been shown that there is also acetylation of the histones not in the tails but in the contact regions with DNA, and therefore it has been hypothesized that these modifications could alter the nucleosome stability and therefore facilitate the transcription process (Roth *et al.*, 2001). In general, the balance between acetylation and deacetylation is involved in processes such as replication and nucleosome assembly (Barman *et al.*, 2006), repair and apoptosis (Ikura *et al.*, 2003), higher-order chromatin packing (Horn *et al.*, 2002) and interactions of non-histone proteins with nucleosomes (Grant *et al.*, 1999). Even though the main role of HATs consist in acetylate histones at particular sites, they can also acetylate other proteins such as: transcriptional factors, like SP1, ELK or CtBP2 (Zhao *et al.*, 2006), structural proteins, as tubulin (Soucek *et al.*, 2006) or chromatin proteins, as HMG (Ueda *et al.*, 2006) and thus, regulate the stability, the ability to bind DNA and, the function of these proteins.

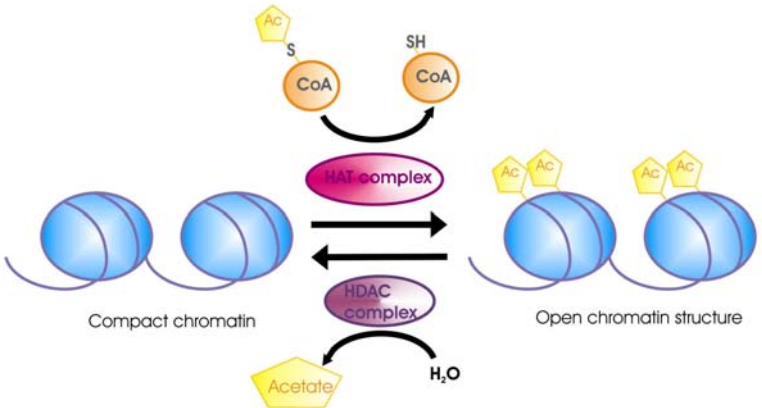


Figure 4. HAT and HDAC reactions. Schematic representation of nucleosomes in which HAT catalyzes the transfer of an acetyl group from acetyl-CoA to ε-amino groups of lysine side chain located in the histone tails. HDAC enzymes remove acetyl groups from acetylated lysines with the release of acetate.

HATs were initially classified according to the subcellular localization of the complexes that they form: type A was nuclear (Brownell *et al.*, 1996) and type B resided in the cytosol (Allis *et al.*, 1985), but recent studies from Poveda *et al.*, (2004) showed that also type B complexes can be found inside the nucleus. Nowadays HATs are classified into different families according to the homology of structural motifs that they posses (Table 2) (Kurdistani *et al.*, 2003).

Table 2. Classification and characteristics of HAT families. Table adapted from Sterner and Berger (2000); Roth *et al.*, (2001); Yang (2004).

	Catalytic subunit	**Function**	**Substrate**	**HAT complex**	**Interaction with other HATs**
GNAT superfamily	Gcn5	Coactivator Repair	H3/H2B H4	SAGA ADA A2 SLIK SALSA	p300/CBP
	PCAF	Coactivator	H3/H4	PCAF	p300/CBP
	Hat1	Histone deposition Chromatin assembly Gene silencing	H4/H2A	HATB	
	Elp3	Elongator/ Lysine demethylase?	H3/H4	Elongator	
	Hpa2	Unknown	H3/H4		
MYST family	Esa1	Cell cycle Repair Transcription	H2A/H4 H3	NuA4 Piccolo NuA4	
	Sas2	Gene silencing	H4	SAS	

Table 2. Classification and characteristics of HAT families. (Cont.)

	Catalytic subunit	Function	Substrate	HAT complex	Interaction with other HATs
MYST family	Sas3	Elongation	H3	NuA3	
	MOF	Coactivator	H4	MAF2	
	Chameau	PcG-dependent gene silencing			
	MOZ	Coactivator	H3/H4		
	MORF	Coactivator	H3/H4		
	Tip60	Co-regulator/ DNA repair/ Apoptosis	H3/H4 Androgen receptor	TIP60	
	Enok	Neuroblast proliferation			
	Hbo1	Replication	H3/H4	ORC	
P300/CBP	p300	Coactivator E4 ubiquitin ligase for p53	H2A/H2B /H3/H4 TFs, E1A		PCAF GCN5
	CBP	Coactivator	H2A/H2B /H3/H4 TFs		PCAF GCN5
Basal TFs	Nut1	Transcription initiation	H3>>H4	Mediator RNApol II	
	TAFII250	Transcription initiation/ Kinase/ Ubiquitin ligase		TFIID	
Specific TFs	TFIIIC	Transcription initiation	H3, H4>>H2A	TFIIIC	
CDY	ATF2	Specific DNA TF	H4/H2B		
Nuclear Receptor Cofactors	CDY	Histone to protamine transition during spermatogenesis	H4		
	SRC-1	Coactivator	H3/H4		p300/CBP PCAF
	ACTR	Coactivator	H3/H4		p300/CBP PCAF
	TIF2	Coactivator			

The GNAT superfamily (Gcn5-related N-acetyltransferase) is closely related to the *Sacharomyces cerevisiae* GCN5 enzyme. These HATs share four conserved domains: acetyl-CoA recognition and binding, nucleosomal recognition and binding, protein-interaction and an acetyl-lysine binding domain, the bromodomain (Zeng *et al.*, 2002; Ornaghi *et al.*, 1999). This family includes acetyltransferases linked to transcriptional initiation (GCN5 and PCAF), elongation (ELP3) and histone deposition and telomeric silencing (HAT1).

The MYST family (named for its founding members: MOZ, YBF2/SAS3, SAS2 and TIP60) is characterized by its methyl-lysine binding domain, the chromodomain, and by its domain bearing zinc-fingers as structural motifs (Brehm *et al.*, 2004). Members of the MYST family are involved in transcriptional elongation and replication (MOZ) and in transcriptional activation (ESA1) (Grant, 2001).

The p300/CBP family comprises the highly related p300 and CBP proteins that have a bromodomain and three cysteine/histidine rich domains (TAZ, PHD and ZZ) that serve as protein-protein interacting domains. Members of this family are coactivators for multiple transcription factors and they can also associate with other acetyltransferases, as an indication that multiple HAT enzymes can be recruited to the promoter to act synergistically during gene activation.

Other acetyltransferases are grouped as transcription factors, for example TAFII250 or ATF2, as it was described by Naar *et al.*, (2001).

As histone acetylation is a key feature in organizing gene expression, HATs should be tightly controlled by non-exclusive mechanisms. The easiest way of regulation is to control the availability of enzyme. For instance Tip60 expression increases during DNA-damage (Legube *et al.*, 2002). The regulation of the enzymatic activity is another mechanism that can be achieved by two means: post-translational modifications and interactions of the enzyme with other proteins. The histone acetyltransferase CBP is stimulated upon cyclin E/cyclin- dependent kinase 2 (Aitsi-Ali *et al.*, 1998) and its ability to bind CREB is diminished when methylated (Zanger *et al.*, 2001). HATs are usually part of large, multimolecular

complexes, which modulate the enzyme specificity by recognizing specific nucleosomal modifications (Table 2), but the modulation of this activity through the regulation of complex assembly has not yet been observed. On the contrary, there are many cofactors that can stimulate the HAT activity and that do not form part of the complex (f.i. CBP and p300 are stimulated by SP1, ZTA or ELK1 phosphorylated (Li *et al.*, 2003)).

On the other hand, histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from lysine residues in both histone and non-histone proteins (p53, E2F, MyoD) (Hubbert *et al.*, 2002). By this process they reverse the action of HATs and, through a subtle balance, they maintain the steady-state levels of histone acetylation. Broadly speaking, hypoacetylation has been associated with transcriptional repression and gene silencing (Forsberg *et al.*, 2001, Verdin *et al.*, 2003, Czermin *et al.*, 2003), whereas hyperacetylation is linked to gene activation.

Mammalian HDACs have been classified into three different subfamilies (I, II and III), based on their sequence homology to the yeast HDAC RPD3 (Reduced Potassium Dependency 3), HDA1 (Histone Deacetylase 1) and SIR2 (Histone Information Regulator 2) (Bjerling *et al.*, 2002 and Fishle *et al.*, 2002). Class I and II HDACs, from the different subfamilies, contain a zinc cation in the catalytic site and they are sensitive to the inhibitor trichostatin A (TSA), whereas class III HDAC are insensitive to TSA treatment and require the coenzyme NAD^+ as a cofactor (Sengupta *et al.*, 2004). A detailed list of the enzymes that form part of each group can be seen in Table 3.

HDACs have to be localized in the nucleus to be active, but some of them (class II HDACs, Table 3) can also be found in the cytosol. Targeting of HDACs takes place by a nuclear localisation signal (NLS) or by interacting with other proteins that translocate them to the nucleus. The return to the cytosol is achieved through a nuclear export signal (NES) (Ruijter *et al.*, 2003). For instance, HDAC1 and HDAC2 are exclusively nuclear, and HDAC3 even though has NLS and NES is always found in the nucleus. On the contrary, HDAC 4, 5 and 7 are able to shuttle in and out of the nucleus in response to cellular stimuli. HDAC5 can be found in the

nucleus during muscle cells proliferation, but during differentiation is localized in the cytoplasm (Bertos *et al.*, 2001).

Table 3. Histone deacetylase family members. HDAC families from yeast and humans are shown, and they are classified into subfamilies according to their homology of sequence. Adapted from Butler *et al.*, 2006 and Blander *et al.*, 2004.

HDAC FAMILY							
Origin	HDA1 subfamily			SIR2 subfamily			
	Class I	Class II	Unknown	Class I	Class II	Class III	Class IV
Yeast	Rpd3 Hos1 Hos2 Hos3	Hda1		Sir2 Hst1 Hst2 Hst3 Hst4			
Human	HDAC1 HDAC2 HDAC3 HDAC8	HDAC4 HDAC5 HDAC6 HDAC7 HDAC9 HDAC10	HDAC11	SIRT1 SIRT2 SIRT3	SIRT4	SIRT5	SIRT6 SIRT7

Regulation of HDACs is achieved by multiple mechanisms as it is expected for proteins that play essential physiological roles. First, HDACs are regulated by association with other proteins, which confer the ability to activate or inhibit their enzymatic activity (all purified HDACs, except HDAC8, are enzymatically inactive (Lee *et al.*, 2004)), and also to target them to the DNA or to the histones that they are going to modify (generally HDACs do not possess DNA-binding activity). For instance HDAC3 is activated upon binding to SMRT and N-CoR by the deacetylase-activating domain (DAD) (Guenter *et al.*, 2002). In general, the three proteins that can activate class I HDACs (MTA2, CoREST and SMRT/N-CoR) all possess the putative DNA-binding domain SANT. However, there are HDAC complexes that do not contain SANT domain proteins, as Sin3/HDAC complex, and therefore alternative components must substitute for the function of this domain. Some HDACs complexes can be seen in Table 4.

Table 4. Histone deacetylase complexes. Examples of HDACs complexes are depicted. Adapted from Rietveld *et al.*, (2002) and Hakimi *et al.*, (2005).

HISTONE DEACETYLASE COMPLEX						
Sin3/HDAC	**NCoR-1**	**NCoR-2**	**NCoR-3**	**Mi2/NuRD**	**MeCP2**	**Co-REST**
Sin3A	NCoR	NCoR	NCoR	Mi-2b	MeCP2	Co-REST
Sin3B	HDAC3	Sin3A/B	HDAC3	MAT2	Sin3A/B	HDAC1
HDAC1	BRG1	HDAC1	TBL-1	HDAC1	HDAC1	HDAC2
HDAC2	BAF170	HDAC2		HDAC2	HDAC2	Mi-2-like
RbAp48	BAF155	RbAp48		RbAp48	RbAp48	MTA-like
RbAp46	BAF47	RbAp46		RbAp46	RbAp46	BRAF35
SAP30	KAP	SAP30		MBD3	SAP30	BHC80
SAP18		SAP18			SAP18	

A second mechanism by which HDAC activity can be regulated is by post-translational modifications as phosphorylation or SUMOylation. Brosch *et al.*, (1992), showed that histone deacetylase 1 phosphorylation from germinating Zea mays embryos causes a change in the substrate specificity of the enzyme. Afterwards, Tsai *et al.*, (2002) showed that HDAC2 is phosphorylated at the C-terminus at serines 422 and 424, and these phosphorylations are needed for its association with the corepressors mSin3 and Mi2. As phosphorylation is a reversible reaction, HDACs can also interact with phosphatases to remove phosphate groups. Canettieri *et al.*, (2003) demonstrated that HDAC1 associates with PP1 and triggers dephosphorylation of CREB.

HDAC activity can be also regulated by conjugation with the small ubiquitin-related modifier (SUMO-1) that affects the subcellular localization, the protein-protein interaction and the enzymatic activity. HDAC1 (David *et al.*, 2002), and HDAC4 (Ling *et al.*, 2004), have been demonstrated to be specific targets of SUMO-1.

It has been also suggested that gene expression can affect HDAC activity. Consequently, Lagger et al., (2002) showed that HDAC2 and HDAC3 protein levels increased in HDAC1-deficient ES cells, suggesting a role for HDAC1 in gene expression regulation of other HDACs.

In the case of SIR2-like HDAC, enzymes that require NAD^+ for catalytic activity, their regulation can be easily achieved by the availability of this metabolic cofactor (Anderson *et al.*, 2003).

Finally, regulation of HDACs can be achieved by proteolytic processing too, as it is the case for hSIR3 that has to be processed by a matrix processing peptidase of the mitochondria to be active (Schewer *et al.*, 2002).

1.2.2.2 Histone Methylation: Histone Methyltransferases.

The lysine and arginine residues of the histones can be further modified by methylation, in a process carried by a family of enzymes called histone methyltransferases (HMTs). HMTs contain a SET domain that transfers methyl groups from S-adenosyl-L-methionine (SAM) to lysine residues (Baumbusch *et al.*, 2001 and Kouzarides, 2002) and to arginine residues (Bedford *et al.*, 2005) in a process that increments the hydrophobicity but does not alter the global charge of the N-terminus of the histones (Peters *et al.*, 2005).

There are two main families of HMTs depending on the residue that they modify (Table 5). Protein arginine methyltransferase (PRMT) alter arginine residues, and they are further classified into class I (rendering mono-methylation and asymmetric dimethylation) and class II (rendering mono-methylation and symmetric dimethylation). On the other hand, lysine residues can be mono-, di- and tri-methylated by HMTs, such as Suv39h1. Historically, methyl marks over the histones have been considered static modifications, because of the low levels of methyl-group turnover in chromatin. The recent identification of enzymes that antagonize or remove histone methylation has changed this view and now the dynamic nature of these modifications is being appreciated. The first experimental evidence for

"histone lysine demethylases" appeared when Shi *et al.* (2004) showed that LSD1 induces amine oxidation of mono- and di-methylated histone H3 lysine 4 to generate unmodified lysine and formaldehyde. Some of the demethylation reactions can be seen in Figure 5.

The finding of demethylase activity came from the identification of JmjC (Jumonji-C) domains, which are able to catalyze direct hydroxylation of the lysine methylamine group using iron and α-ketoglutarate as cofactors for a reaction that produces succinate and carbon dioxide. In this process, the hydroxymethyl group is spontaneously lost as formaldehyde to liberate one methyl group (Cloos *et al.*, 2006, reviewed by Klose *et al.*, 2007). Kose *et al.*, (2006) suggested that JHDM3A, an enzyme containing this domain, is a demethylase of H3 lysine 9 and lysine 36 that can also act over trimethyl-lysines. Moreover, the proteins of the JARDID1 family have been shown to remove the three methyl groups of H3K4 (Steward *et al.*, 2007, Lee *et al.*, 2007, Klose *et al.*, 2007 and Christensen *et al.*, 2007), and the same is true for the S*accharomyces cerevisiae* (Klose *et al.*, 2007) and *Droshophila melanogaster* homologues (Secombe *et al.*, 2007).

In addition to the recent demonstration of the existence of demethylases, several non-enzymatic mechanisms have been proposed to remove this mark. Jenuwein et al., (2001), for instance, proposed a cleavage of the histone N-termini; Ahmad (2002) suggested the possibility of histone variant exchange, and Banister (2002) hypothesized the destabilization by oxidation or radical attack.

Histone methylation has been associated to both transcriptional activation (Castellano *et al.*, 2006) and repression (Mal, 2006) and its function will depend on the methylated residue, the degree of methylation and on the interplay with other modifications (Lachner *et al.*, 2003). For instance, Dehe et al., (2006) showed that H3K4 tri-methylation is found in active genes, whereas mono- and di-methylation are marks mainly restricted to repression. Another association of methylation and repression was provided by Datta *et al.*, (2005), who demonstrated that DNA methyltransferase 3A can interact physically with BRG1 (a subunit of a chromatin remodelling complex) in mouse lymphosarcoma cells, and therefore promote gene

silencing. In general, transcriptional repression is correlated with high levels of tri-methyl H3K36, H4K20 (Kourmouli *et al.*, 2004 and Chadwick *et al.*, 2004), and H3K9 (Scripathy *et al.*, 2006) and low levels of H3K4 methylation and H3K9 acetylation. This modification pattern provides, for instance, the adequate environment for the HP1 interaction with the nucleosome to promote heterochromatin formation. Nevertheless, H3K4 tri-methylation has also been shown to facilitate NURF (an ATP-driven chromatin remodelling complex, see later) recruitment and the consequent gene expression (Wysocka *et al.*, 2006).

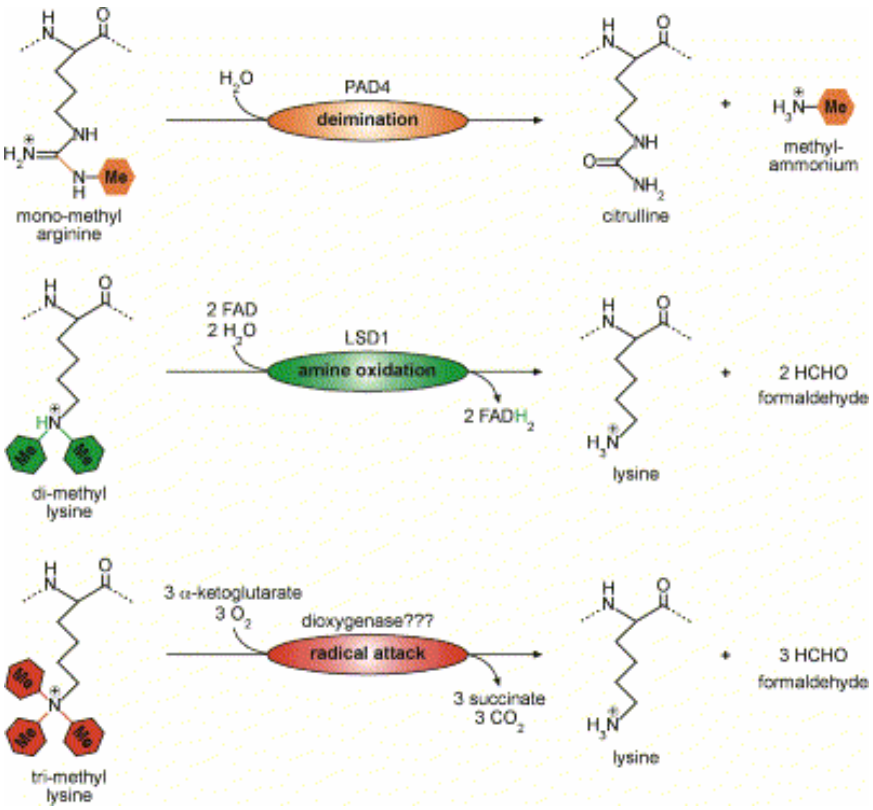


Figure 5. Enzymatic mechanism to remove histone methylation. Arginine methylation can be cleaved by protein arginine deiminases (PADs), orange reaction. Mono- and di-methyl lysines can be removed by amine oxidation (green reaction), whereas for tri-methyl lysines a non-experimentally proved radical attack has been proposed (red reaction). (Kubicet *et al.*, 2004)

Table 5. List of histone methyltransferases. The Table shows both families of histone methyltransferases and also the family of DNA methylases and the proteins that bind to methylated DNA. T stands for transcriptional. Adapted from Bedford *et al.*, 2005, Freitag *et al.*, 2005 and Turek-Plewa *et al.*, 2005.

Histone methyltransferase				
	Type	Enzyme	Substrate/ Function	Complex
Arginine methyl-transferase	Class I	PRMT1	H4/TFs/ T. activation	
		PRMT2	Unknown	
		PRMT3	TFs	
		CARM1	H3/ CBP/p300/ TFs T. Coactivator	AR, PCAF, NCOA2, P300, NUMAC
		PRMT6	PRMT6/ Fibrillarin	
	Class II	PRMT5	H4/H2A/H3 /Cell cycle	Methylosome
		PRMT7	Fibrillarin	
		PRMT8	Unknown	
Lysine methyl-transferase		Suv39h1	H3K9/H3K27/ T. repression	E2F1,E2F4
		Suv39h2	H3K9/H3K27/ T. repression	E2F1,E2F4
		G9a	H3K9/ T. repression	
		Eu-HMTase1	H3K9/ T. repression	E2F6
		ESET/SETDB1	H3K9/ T. repression	SET1/ASH2/ HCF1
		H3K4 MTases	H3K4/ T.activation	SET1, MENIN
DNA methyl-transferase		DNMT1	CpG maintenance	
		DNMT2	Weak activity	
		DNMT3A	CpG *de novo*	
		DNMT3B	CpG *de novo*	
		DNMT3L	DNMT3A-B cofactor	
		MBD1	T. repression	
		MBD2	T. repression	
		MBD3	Remodelling (Mi-2/NuRD)	
CpG-binding proteins		MDB4	DNA mismatch repair	
		MeCP1	T. repression	
		MeCP2	Co-repressor	
		Kaiso complex	Unknown	

1.2.2.3. Histone Phosphorylation

The core histones and histone H1 undergo phosphorylation of specific serine and threonine residues in the N-terminal domain and, in the case of histone H1, also in the C-terminal domain (Davie *et al.*, 1999). This post-translational modification has been linked to chromatin condensation (Sarg *et al.*, 2006), DNA repair (Hamada *et al.*, 2006) and transcriptional competence of immediate-early genes (Clayton *et al.*, 2003).

Histone kinases act mainly over serine 10 of H3, but this modification can have completely opposite effects on gene expression. On one hand, H3S10 phosphorylation is necessary to promote chromosome condensation in the pericentric heterochromatin (Zeitlin *et al.*, 2001). On the other hand, it leads to acetylation of H3K14 and subsequent transcription of immediate-early genes. In the literature two kinases of the MAPK pathway, which are directly responsible for this modification, have been described. They are called RSK-2 (Ribosomal Stress Kinase-2) and MSK-1 (Mitogen and Stress-Activated Kinase-1). The removal of the phosphate groups has been linked to PP1 (Protein Phosphatase 1) activity. In the same manner, a member of the PI3K (phosphatidylinositol 3- kinase) family has been related to phosphorylation of serine 139 of the histone variant H2AX (Takahashi *et al.*, 2005), which also leads to transcriptional activation.

1.2.2.4 Other Histone Modifications

As commented above, histones can be also modified in specific lysine residues by ubiquitin and ubiquitin-like proteins as SUMO. As these modifications have been less studied than those mentioned above, it is not clear whether their function consists in altering the nucleosomal structure (due to the large size of the modifiers) or if it consists in promoting or inhibiting interactions with non-histone regulatory proteins. Nevertheless, ubiquitination, a traditional proteins proteolytic mark, is generally linked to gene activation as assumed, for instance, by Briggs *et al*. (2002) and Osley (2004) showing that H2B monoubiquitination precedes H3K4

methylation and transcription in yeast. On the contrary SUMOylation is related to transcriptional repression by facilitating the association of some co-repressors as the Mi-2/NuRD remodelling complex (Gong *et al.*, 2006) or also competing with the ubiquitination.

1.2.3. Chromatin Remodelling

Eukaryotic transcriptional machinery faces multiple challenges during RNApol II assembly. One of them is to gain access to the DNA template, which is folded into compact chromatin fibres. To circumvent this problem, there are a family of chromatin remodelling enzymes that can alter histone-DNA contacts in the nucleosomal core using the energy of ATP hydrolysis. These complexes are indeed implicated in the overall dynamic chromatin structure since they have been linked to processes as replication (Zhou *et al.*, 2005), DNA repair and recombination (Shim *et al.*, 2005), sister chromatid cohesion (Hakimi *et al.*, 2002) and transposon integration (Bachman *et al.*, 2005).

All these complexes contain an ATPase subunit, that belongs to the SWI/SNF superfamily, but they are classified into three subclasses according to their sequence similarities and associated domains: SWI2/SNF2 (Switch/sucrose nonfermentors), ISWI (Imitation Switch) and CHD (Chromodomain, Helicase domain and DNA binding domain) (Allard *et al.*, 2004 and Varga-Weisz *et al.*, 2006) (for a detailed description of each subfamily, see Table 6). It is important to remark the specificity conferred by different subunits of the complex, as different experiments have indicated that they do not have redundant functions. Nevertheless, not all the complex subunits are indispensable, as in some cases null mice are viable.

ATP-dependent chromatin remodelling enzymes have also been found to associate with chromatin modifying enzymes such as HDACs, as it is the case of NuRD complex, or arginine methyltransferases, which can be stably coimmunoprecipitated with members of the SWI/SNF complex. Moreover, it has been reported that SWI/SNF can take part in the release of RNApol II from paused promoters (Serna *et al.*, 2006).

The mechanisms of regulation of these complexes still remains obscure, but it seems probably that phosphorylation of some of the subunits will change their properties, and although other modifications have not been reported so far, it is probable that they take place as well (Becker, 2002). On the other hand, it is also remarkable that SWI/SNF ATP-dependent remodelling machines can be tethered to gene promoters either by the action of co-factors or by direct interaction with the RNApol II or with the mediator machinery (Vignali *et al.*, 2000).

Table 6. Subfamilies of ATP-dependent chromatin remodelling complexes. The subunits of each of the complexes are indicated and also the origin of the complex. Adapted from Neely *et al.*, 2002.

ISWI subfamily of ATP-dependent chromatin remodelling complexes								
***Dros.* NURF**	***Dros.* CHRAC**	***Dros* ACF**	**Yeast ISW1**	**Yeast ISWI2**	**Human RSF**	**Human CHRAC**	**Human ACF**	**Human WCRF**
ISWI Nurf55 Nurf38/ iPPase p215	ISWI Acf1 CHRAC 14 CHRAC 16	ISWI Acf1	ISWI1 p74 p105 p110	ISWI2 Itc2/ p140	hSnf2h p3325	hSnf2h/ hSnf2L hACF1/ WCRF180 HuCHRAC 15 HuCHRAC 17	hSnf2h/ hSnf2L hACF1/ WCRF 180	hSnf2h hSnf2L WCRF 180 hACF1

SWI2/SNF2 subfamily of ATP-dependent chromatin remodelling complexes				
Yeast SWI/SNF	**Yeast RSC**	**Yeast INO80**	***Dros.* Brahma**	**Human SWI/SNF, RSC**
Swi2/Snif2 Swi1,3 Snf5,6,11 Swp82 Swp73/ Snf12 Swp61/Arp7 Swp59/Arp9 Swp29 $Taf_{II}30$ Tfg3	Sht1/Nps1 Sfh1 Rsc8/Swh3 Rsc11/Arp7 Rsc12/Arp9 Rsc6,1,2,4,14 Rsc3,30,5,13 Rsc7,9,10,15	Ino80 Arp4,5,8 Act1 Rvb1,2 p90, 100,32,26	Brm OSA/Eyelid Snr1 Bap155/Moira Bap55,60,111,74 β-actin/Bap47	hBRG1,hBRM p270/BAF250/hOSA hSNF5/INI1/BAF47 BAF170,155,60a,57 BAF53/ArpN β-actin BAF180 (hRSC complex only)

CHD subfamily of ATP-dependent chromatin remodelling complexes			
Human NURD	**Human NuRD**	**Human NRD**	***Xenopus* Mi-2**
CHD4/MI-2β CHD3/MI-2α HDAC1/NURD63 HDAC2/NURD59 RbAp48/NURD56 RbAp46/NURD55 MTA1/NURD70	CHD4/MI-2β HDAC1,2 RbAp48,46 MTA2, MBD3a, MBD3b	CHD3,4 HDAC1,2 RbAp48 p70,110	Mi-2 Rpd3, p66 MTA1-like MBD3, MBD3LF, RbAp48/p46

1.2.3.1 Mechanisms of Chromatin Remodelling

Movement of histone octamers can take place in *cis* (sliding of a nucleosome along the DNA), in *tras* (relocating the nucleosome into a stretch of DNA) (Lorch *et al.*, 1999 and Narlikar *et al.*, 2002) or even leading to the formation of di-nucleosome particles (Fry *et al.*, 2001). The mechanisms by which changes in nucleosomal position occur are still under discussion, and many hypotheses have been suggested. So far, it is well known how the remodelling complexes contact the DNA template but the mechanisms that they use to catalyze DNA exposure or hiding vary in the different complexes, probably due to their diverse subunit composition (Saha *et al.*, 2006). Among the mechanisms for remodelling, the most accepted ones are the *twist defect* diffusion and the *wave-ratched-wave*. The first one proposes small local alterations in average DNA twisting that propagate around the nucleosome. In contrast, the second model, suggests a DNA wave that is generated on the nucleosome surface by pulling DNA from the linker into the nucleosomes and which is propagated by diffusion to the distal linker (Saha *et al.*, 2006). Unfortunately, these mechanisms assume that the octamer remains unaltered, and therefore they do not explain the removal or exchange of dimmers from the octamer (Flaus *et al.*, 2004).

NURF, CHRAC and ISWI can move the nucleosomes *in cis* in 5' to 3' direction (Alexeev *et al.*, 2003), whereas SWI/SNF is also able to move them also *in trans* (Whitehouse *et al.*, 1999). SWI/SNF or RSC-disrupted nucleosome conformation leaves the histone octamer partially associated with the DNA, and therefore increases the accessibility of transcription factors and restriction enzymes. It can also render dinucleosome-like species, and the stability of the nucleosome particle is reduced. On the contrary, ISWI, NURF and CHRAC complexes seem to produce a gradual movement of nucleosomes along DNA, resulting in a more modest nucleosome alteration (Vignali *et al.*, 2000). Furthermore, ISWI complexes require the N-terminus of histone H4 for a productive ATPase cycle, whereas SWI/SNF remodelling complexes can deal with nucleosomes lacking these domains (Becker, 2002).

1.2.3.2 Integration of Remodelling Complexes and Other Regulatory Complexes

As already mentioned, chromatin remodelling complexes are required to modify the dynamics of certain chromatin regions to facilitate gene expression and/or repression. Nevertheless, histone modifying complexes are also required to fulfil that role. Therefore, the following question may be posed: for which reason remodelling and histone modification are coupled together? It seems that a single general response does not exist, but, rather, there is a specific answer for each gene. Chromatin modifying enzymes can interact with different proteins (for instance, K4H3 methyltransferase MLL1 interacts physically with the remodelling factor hSNF5/INI1 (Rozenblatt-Rosen *et al.*, 1998)), but also they can recognize previous chromatin modification carried out by other enzymes through different domains (see Table 7 for a detailed description of association with other complexes and recognition domains).

When the transcriptional state of a gene is changed, usually by gene-specific activator binding or repressor releasing, it triggers a cascade of reactions that will end in the appropriate chromatin state suitable for the transcriptional machinery access. The question arises as to whether the recruitment of chromatin modifying enzymes takes place in a specific order or not. This question is not an easy one to answer since there are examples in which first act the HAT complexes and then the remodelling ones and examples in the opposite way. Narlikar *et al.* (2002), trying to encompass both data, have suggested that each promoter will work using an order of action of these complexes that differs from promoter to promoter although ATP-dependent remodelling complexes and chromatin-modifying enzymes can influence each other.

Table 7. ATP-dependent nucleosome-remodelling complexes and associated modification enzymes. (Cosgrove *et al.*, 2004).

	Complex	Central ATPase	Associated Histone modification enzyme	Recognition domain
Activation	Swi/Snf	Snf2	SAGA-Gcn5	Bromo
	hSwi/Snf	Bgr1	P300-CBP	Bromo
	NURF	ISWI	P300-PCAF	Sant-Bromo
	INO80	ISWI-related	NuA4	
	RSC	Sth1	NuA4	Bromo
Repression	NuRD	Mi-2	HDAC1	Chromo
	ISW2p	ISWI	Sin3-RPD3	Sant
	hSwi/Snf	Brg1	Sin3-RPD3	Bromo
	hSwi/Snf	hBrm	Sin3-RPD3	Bromo
	NCoRC	Snf2H	HDAC1	Sant

1.2.4. Other Mechanisms that Regulate the Function of Chromatin

The eukaryotic gene expression, able to respond quickly to a great number of internal and external stimuli, can also be regulated by DNA methylation and by other less studied mechanisms, as replacement of histone variants or interference RNA. All these processes take place under certain circumstances to perform specific roles in gene regulation and their deregulation can cause many diseases. As an example, the disruption of normal DNA methylation has been suggested as a hallmark in tumorigenesis (Esteller, 2006).

1.2.4.1. DNA Methylation

DNA methylation, together with the histone modifications, is a component of a program or epigenetic process, that despite the fact that although does not alter the genetic material itself, can be inherited by mitotic cell divisions (Jaenish *et al.*, 2003). Methylation reaction occurs within CpG sequences at cyclic carbon atom 5 of cytosine, and it is carried out by a group of enzymes that are summarized on Table 5.

The vertebrate genomes contain between 60 to 90 % of CpGs base pairs in a methylated state and is noteworthy that methylation is also found at the promoter regions in the so called CpG islands (Heinrich, 1998). The pattern of methylation over the CpG islands has been related with transcriptional repression (reviewed in Fuks, 2005). Furthermore, DNA methylation is linked to some histone regulatory modification, as acetylation or methylation, since that DNA modification is easily recognized by methyl-CpG binding proteins, as MeCP2 (Methyl-CpG-Binding Protein-2), a protein that is a subunit of complexes containing SIN3A histone deacetylase, and establishing a cooperative system for transcriptional silencing (Urnov *et al.*, 2001).

1.2.4.2. Histone Variants

Histones H1, H2A, H2B, H3 and H4 are highly conserved basic proteins encoded, generally speaking, by multicopy, intronless genes, expressed during cell cycle S phase and transcribed into nonpolyadenylated mRNA. Except for H4, there exist non-allelic variants of the histones, which may exhibit some differences in the above mentioned properties of their genes. As there is some terminological confusion, we will designate the latter as histone variants, whereas the former will be further referred to as canonical histones. Histone variants occupy restricted and defined locations in the chromatin (Kamakaka *et al.*, 2006), and they are encoded by single-copy intron-containing genes, expressed throughout the cell cycle, and their transcripts are polyadenylated. Furthermore, in contrast to canonical histones, they are deposited in a replication-independent manner (Saha *et al.*, 2006).

All the histones except H4 have some specific variants. Histone H1 has sequence variants such as H1º or H5. Histone H2A has the largest number of variants, including H2A.Z, macroH2A or H2A-Bbd. Histone H2B, on the contrary, has few variants that appear to be restricted to the gametogenesis process. Finally, among histone H3 variants, the two major are H3.3 and CENP-A, the latter corresponding to the centromeric H3 (Pusarla *et al.*, 2005).

It seems to be obvious that the presence of histone variants allows new possibilities in the regulation of the genes by further modifying the chromatin context, but what is the precise function of these proteins? The answer seems to be complex, as each variant has a chromatin effect that is correlated with a different function. For instance, H3.3 is mainly found in highly transcriptional active genes, favouring a nucleosomal structure that facilitates RNA pol II processivity (Ahmad *et al.*, 2002), centromeric H3 variant is required for accurate chromosome segregation (Blower *et al.*, 2002) and H2A.X phosphorylation serves as a recruitment mark for the DNA repair machinery upon UV irradiation (Paul *et al.*, 2000).

The deposition of canonical histones takes place during the S phase with the help of the chromatin assembly factor CAF-1. On the contrary, histone variants are deposited into chromatin independently of DNA replication helped by proteins as NAP1, HIRA or HIF1 and by part of ATP-remodelling complexes. Histone H2A.Z can be deposited during and outside S phase helped by NAP1 and by SWR1, a SWI/SNF-like ATPase (Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004), showing a functional link between histone variants and chromatin modifying enzymes.

Finally, the presence of histone variants has profound consequences on the general nucleosome structure. They change the stability of the core nucleosome by changing the general interaction pattern within the octamer of proteins. For instance, a nucleosome containing a histone H2A-Bbd organizes only 118 bp of DNA (Bao *et al.*, 2004). They may also affect to the post-translational histone modifications as some of the histone variants have larger or shorter N-terminal tails. Moreover, nucleosomes with histone variant may expose different surface residues, as in the case of macroH2A, in which a long C-terminal tail extends away from the nucleosome facilitating an asymmetrical structure important for transcriptional repression (Abbot *et al.*, 2004). Undoubtedly, histone variants may also alter chromatin higher-order structures by changing the characteristic packaging of the chromatin.

1.2.4.3. Non-codifying RNAs: interference RNA and microRNAs

During the last decade, RNA has emerged as a central player in the regulation of both transcriptional processes and chromatin related events. On one side, RNA interference can drastically alter gene expression by post-transcriptional gene silencing and also by transcriptional gene silencing. On the other side, noncoding RNAs are intimately involved in processes as dosage compensation by heterochromatin formation and epigenetic changes by inducing DNA methylation (Lippman *et al.*, 2004, Tomari *et al.*, 2005 and Matzke *et al.*, 2005).

RNA interference is a process in which double-stranded RNA triggers gene silencing (Fire *et al.*, 1998) by mRNA specific degradation, translational inhibition, changes in the chromatin or a combination of these. Post-transcriptional gene silencing requires Dicer (RNAse III ribonuclease) processing of dsRNA, exogenously induced by experimental manipulation, viruses, endogenous parasitic elements or repetitive sequences. This enzyme, a member of the RNase III family of ribonucleases, converts dsRNA into small interfering RNAs (siRNAs) which will then be loaded into the RISC (RNA-induced silencing complex) machinery. The RISC is a multisubunit complex that has, among other proteins, Argonaute-2 that hold nuclease activity (Liu *et al.*, 2004 and Sen *et al.*, 2006) and releases fragments of ~21-23 nt (see Figure 6).

Furthermore, microRNAs (a family of ~21 nt regulatory RNAs encoded by endogenous precursor molecules (Filipowicz *et al.*, 2005; Pfeffer *et al.*, 2004)) regulate gene expression by imperfect base pairing to the 3'-untranslated region of target mRNAs and inhibit RNA synthesis by an unknown mechanism. Maturation of miRNA occurs via Drosha (a RNase III, see Figure 6) that processes in the nucleus the long structures into ~ 60-70 nt dsRNA-like hairpins that are further processed in the cytoplasm by Dicer into final duplex miRNAs of ~21 nt (Eckstein, 2005). miRNAs have been so far implicated in multiple biological processes such as stem cell differentiation, organ development, disease, cancer, etc. (reviewed in Zhang *et al.*, 2007).

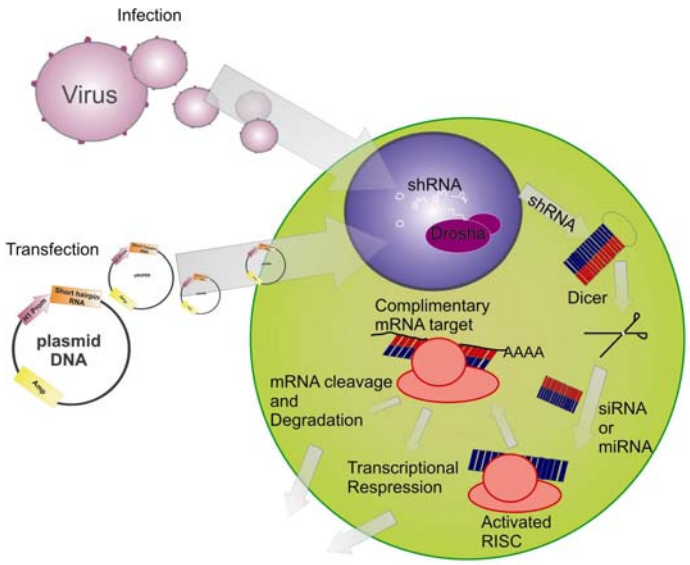


Figure 6. Scheme of RNA interference action. ShRNA is processed into the nucleus by Drosha and later in the cytosol by Dicer in a process that finally produces the mRNA translational repression.

There are many published papers showing that noncoding RNA affects other chromatin processes as dosage compensation (Bernstein *et al.*, 2005), centromere formation (Volpe *et al.*, 2002) or DNA elimination (Mochizuki, 2004). Although the precise mechanisms is still far to be completely known, in some cases it seems to involve histone methylases and other chromatin modifying enzymes. For instance, dosage compensation in mammals is a process achieved by random inactivation of an X chromosome that remains silent for the rest of the cell life. This inactivation is initiated at a single site (X inactivation centre) by the production of multiple noncoding RNAs. The process is followed by chromatin-remodelling events, generation of hypoacetylated forms for H3 and H4, methylation of K9 and K27 in H3 and K20 in H4 and by the exchange of histone H2A for macroH2A variant (Heard, 2004). How all these complex machineries are coupled together remains to be unveiled, but the fact that RNA regulation and chromatin function are linked can not be denied.

2. SIGNALLING PATHWAYS

The integrated function of the cells in an organism needs the existence of an interactive network of protein kinases and other messenger systems that respond quickly to extracellular signals as: cytotoxic drugs, light irradiation, heat-shock, osmotic stresses, reactive oxygen species, mitogens, growth factors, inflammatory cytokines and so on. Generally, these stimuli are detected by cell surface receptors that transmit the signal to cytoplasmatic kinases which by sequential stimulation of different signalling cascades specifically amplify the signal. The phosphorylation of several transcription factors in the cytoplasm and/or the translocation of protein kinases into the nucleus where they can modify both transcription factors and chromatin modifying complexes, will finally lead to a genetic regulation of cell growth, differentiation, apoptosis, development and so on.

There are many signal transducing pathways, but the principal ones involved in stress response are: the Mitogen-Activated Protein Kinases (MAPK) (Thiel *et al.*, 2005), Protein Kinase C (PKC), Protein Kinase A (PKA), Phosphatidylinositol 3 Kinase (PI3-K) and Janus Activated Kinase Signal Transducer and Activator of Transcription (JAK-STAT) (Jenkis *et al.*, 2004). As an example, Cuadrado *et al.*, reported the activation of the antioxidant gene *heme oxigenase* (*ho-1*) due to SP1 phosphorylation by PI3K and PKC.

2.1 MAP KINASES

Mitogen-activated protein kinases (MAPK) belong to a large highly conserved family of serine-threonine kinases that play a pivotal role in multiple biological processes and cellular responses to external stress signals by activating gene expression. They are ubiquitously expressed and proline-directed. In eukaryotes can be distinguished three distinctly regulated MAP kinase cascades: the extracellular-signal-regulated kinases (ERK), the c-Jun N-terminal or stress-activated protein kinases (JNK or SAPK) and p38 MAPK. Among the distinguishing features of MAP kinases are the absence of a regulatory subunit and the direct

activation by phosphorylation. There are usually two phosphorylation sites in the kinase activation loop, a tyrosine and a threonine, separated by a single variable residue (Pearson *et al.*, 2001).

Although there are different families of MAP kinases, there is an interaction among cascades to integrate responses and to moderate outputs, implying as a result that some MAPKs have overlapping substrate specificities (Lewis *et al.*, 1998). MAPKs form complexes that facilitate their activation, nuclear localization and specificity (Choi *et al.*, 1994).

2.1.1 ERK1 and ERK2

ERK1 and ERK2 are proteins of ~40 kDa ubiquitously expressed, although their relative abundance depends on the tissue. They are activated by serum, growth factors, cytokines, certain stresses and ligands for G protein-coupled receptors (GPCRs). The common activation pathway involves a tyrosine kinase receptor in the cell membrane that transduces the signal to a monomeric G protein, RAS, with the help of adaptor proteins such as SHC or GRB2. These, in turn, activate RAF, that leads to the ERK1/2 signal cascade. Diversity is achieved by ERK1 and ERK2 alternative spliced variants, and also by multiple classes of G protein that leads to cell type-specific mechanisms.

ERK1 and ERK2 are upstream activated upon dual phosphorylation by MEK1 and MEK2, generally speaking MKKs (Wu *et al.*, 1993), which can be upstream activated by kinases as RAF, generically termed MEKKs (Figure 7 for a more detailed cascade). These kinases can, in turn, act upon different transcription factors modifying its activity as ELK1, c-JUN, c-FOS and so on. MSK1, which is another kinase implicated in phosphorylation of histone H3, is also a substrate of ERK2, therefore implicating this pathway in chromatin regulation (Lee *et al.*, 2006).

To target ERK pathway to different biological events, specific kinase inhibitors like PD98959 and U0126 were used. The results demonstrated a role in processes such as neurite extension, fibroblast proliferation or circadian rhythm (Akashi *et al.*, 2000 and Klemke *et al.*, 1997). Strikingly, knockout mice for MEK1 are not viable, whereas those for ERK1 can survive with minimal phenotypic manifestations (Pearson *et al.*, 2001).

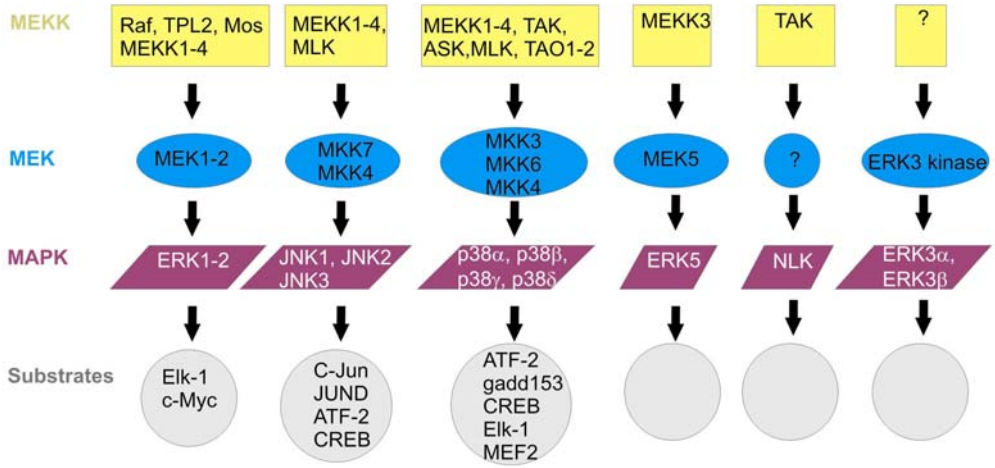


Figure 7. MAP kinase cascades. Adapted from Pearson *et al.*, 2001.

2.1.2 JNK/SAPK

JNK/SAPK kinases are a family encoded by three different genes (*mapk8*, *mapk9* and *mapk10*) with more than 10 alternative spliced variants. Like other MAP kinases they are activated by cytokines, certain ligands for GPCRs, serum, growth factors and agents that interfere with DNA and protein synthesis.

Activation of the kinases takes place after two phosphorylations on a tyrosine and a threonine separated by a proline in the activation loop. These phosphorylations are achieved by two different MEKs, MEK4 and MEK7 (Figure 7). MEK4 is thought to act on the tyrosine, whereas MEK7 would act on the threonine residue, suggesting an interaction between the two to achieve the proper signalling (Lawler *et al.*, 1998 and Lisnock *et al.*, 2000). Both MEKs can act over other substrates as p38, but they prefer JNK/SAP kinases. In the same manner, MEK7 has been found to coimmunoprecipitate with MEK1, but the nature and function of this interaction remains unclear (Holland *et al.*, 1997).

In parallel with ERK1/2, JNK seem to be essential for phosphorylation of activating protein 1 (AP1), due to cell stress. All the same, JNK/SAPK are responsible for the phosphorylation of transcription factors such as ELK1, c-JUN, MEF2, which will finally turn on a certain set of genes.

2.1.3 p38

p38 MAP kinase pathway shares many similarities with the other MAP kinase cascades above, as it can be activated by cytokines (IL-2, IL-7), hormones, growth factors (TGF-β) or different stresses (osmotic, shear or heat-shock). There are four p38 MAP kinases identified so far: p38α, p38β, p38γ and p38δ, being ubiquitously expressed only the first two. p38γ is expressed in skeletal muscle, and p38δ can be found predominantly in the lung, kidney, testis, pancreas and small intestine. All of them bear the aminoacid sequence Thr-Gly-Tyr in their activating loops and they are activated upon dual phosphorylation on threonine 180 and tyrosine 182, by upstream MAPK kinases: MKK3 (preferentially activating p38α and p38β), MKK6 and MKK4. The latter, shows higher preference for the JNK signalling pathway (Figure 7). A MKK independent mechanism of p38 activation involves TAK-1 (transforming growth factor-β-activated protein kinase 1).

Other p38 relatives that contain the TGY activation domain are: ERK6 reported by Lechner *et al.*, 1996, and stress activated protein kinase-3 (SAPK-3). Both of them appear to be homologous and are regulated as described by Cuenda et al., by MKK6 phosphorylation.

Despite the clear importance of p38 cascade in processes as inflammation, cell growth, cell differentiation or cell death, the physiological role of the different p38 isoforms that arise from differential splicing of three genes is still unclear. The use of inhibitors as pyridinylimidazole compounds (SB 203580/SB202190) that inhibit the catalytic activity of the kinase by competing for binding in the ATP pocket, has provided direct evidence of physiological substrates, and was also correlated with a blockade of cytokine translation, but with no appreciable effect on total DNA, RNA or protein synthesis, giving detail of the importance of p38 cascade in certain translational and transcriptional responses.

All this amplified response of the cell to a certain stimulus is aimed to modify a huge number of substrates, and in turn elicit the appropriate response for the cell survival. Among the targets of p38 cascade we can find: protein kinases (RSK1, RSK2, MSK1-2, MNK1-2, PRAK, MAKAPK-2/3), proteins (Tau, eEF2K, SAKS1, CDC25, SAP90, SAP97, α1-synthophin) and transcription factors (ELK1, CREB, ATF-2, CHOP, MEF2, MRF4, E47, NF-κB or p53). Simone *et al.*, showed that in absence of p38 MAKP signalling, the chromatin of the myogenin and MCK promoters was not remodelled in differentiation-promoting conditions. This was caused due to the inability of the ATPase subunits of the SWI/SNIF complex, BGR-1 and BRM to bind the promoter, although the acetylated state of the promoter did not change and neither did the recruitment of HATs as p300 or PCAF. This could be due to the need of phosphorylation of one or more of the subunits of the complex to be phosphorylated for its appropriate activity. It has also been proposed the importance of p38 MAPK in the regulation of the recruitment of RNA polymerase II at least to muscle-specific promoters. Alepuz *et al.*, (2003) showed that HOG1, the yeast homolog to p38 MAPK, interacts and helps in the recruitment of the RNA

polymerase II complex to yeast stress-responsive promoters such as STL1, by association with the Hot1 transcription factor.

Taken together, the substrate specificity of the different p38 isoforms is known to overlap, suggesting the existence of cell-type dependent and isoform-specific phosphorylation events.

2.2 OTHER SIGNALLING PATHWAYS

MAP kinases are the main responsible for stress response in the cell, but there are other families of kinases that can also trigger signalling cascades during cellular stress, like JAK/STAT or PI3K.

Janus Activated Kinase/Signal Transducer and Activator of Transcription family of transcription factors, was first involved with interferon and interleukin response, but recently it has been shown that they can also respond to biological stresses such as hypoxia, U.V. light and reperfusion, appearing to interact in this later function with p38 MAP kinase cascade (Dudley *et al.*, 2004). STATs comprise a family of seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5B and STAT6, whereas JAKs represent a family of four non-receptor tyrosine kinases: JAK1, JAK2, JAK3 and TYK2. Typically this cascade is initiated when a cytokine binds to its receptor, and consequently activates it and allows the sequential recruitment of STATs that become phosphorylated on tyrosine residues, dimerize and translocate to the nucleus where they activate specific target genes. JAK/STAT kinases have been linked to activation of transcription factors like SP1 or NF-κB (Kisseleva *et al.*, 2002).

Phosphoinositide 3-kinases (PI3K) are a family of second messengers that generate lipids able to control a wide variety of intracellular signalling pathways. They have been divided into three classes, but only those belonging to class I have been proved responsible for gene targeting. Class I enzymes are further divided into IA (containing p110 as catalytic subunit) and class IB (containing p100γ as catalytic

subunit). Type IA are coupled downstream to tyrosine kinases and Ras, whereas type IB are linked downstream to GTPase activating proteins and nucleotide exchange factors (Vanhaesebroeck *et al.*, 2005 and Foukas *et al.*, 2003).

2.3 PHORBOL ESTER SIGNALLING PATHWAYS

Diacylglycerols (DAGs) are glycerol derivatives in which two hydroxyl groups are substituted by fatty acids through ester bond formation, that differ ones from the others with respect to the type and degree of saturation of their fatty acid moieties. DAGs are essential second messengers in mammalian cells that act through different targets such as: PKCs, chimaerins, protein kinase D1 (PKD1), RasGRPs, Munc13s and DAG kinase γ (Brose *et al.*, 2002).

Phorbol esters are polycyclic esters used to mimic diacylglycerol-related biological processes, as oxidative stress, tumour progression (Geiger *et al.*, 2003), apoptosis (Chang *et al.*, 2006) and so on. Phorbols are polycyclic (four-ringed) compounds, and the most widely used are: 4β-12-O-tetradecanoylphorbol-13-acetate (TPA), 4β-phorbol-12.13-dibutyrate (PDBu) and phorbol 12-myristate-13-acetate (PMA), which differ only in their substitutions at positions 12 and 13 of ring B (Figure 8) (Silinsky *et al.*, 2003). The signalling cascade produced by these moieties was first identified with the help of inhibitory drugs, and it focused mainly in the activation of protein kinase C. Nevertheless, Rambaratsingh et al., (2003) found that phorbol ester signalling pathway involved also RasGRP1 in mouse epidermal keratinocytes, and Bauer *et al.*, (2005) also referred that the use of phorbol esters could act through MEK/ERK signalling pathway. Furthermore, Guo *et al.*, (2005) demonstrated that TPA activates p38 kinase through an autophosphorylation mechanism. All this controversy in the lack of a characteristic phorbol esters signalling pathway could be due to the fact that many cascades converge to achieve the final goal, that is to say the cell survival or death, or due to the fact that specific signalling will depend on the intrinsic cellular conditions.

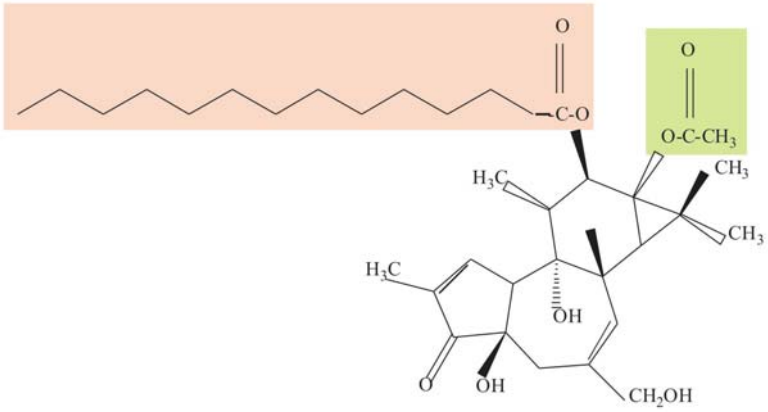


Figure 8. 4β-12-O-tetradecanoylphorbol-13-acetate (TPA).

3. *Egr-1* GENE

3.1 *Egr-1* AS AN EARLY-IMMEDIATE GENE

Egr-1 (early growth response gene 1), also known as *tis8* (tetradecanoyl phorbol acetate-induced sequence 8), *zif268* (zinc finger binding protein clone 268), *ngfi-A* (nerve growth factor-inducible gene A) or *krox-24* (gene containing sequences homologous to the Drosophila Kr finger probe) (Christy *et al.*, 1988, LeMaire *et al.*, 1988, Lim *et al.*, 1987, Milbrandt *et al.*, 1987 and Sukhatme *et al.*, 1988), is a member of the early growth response gene family of transcription factors, formed also by EGR-2, EGR-3 and EGR-4. All of them have high homology of their DNA-binding motif, a zinc finger domain. WT-1 (Wilm's Tumour 1) is a more distant related member of the family that is responsible, as it name indicates, with Wilm's tumour development in the kidney. *Egr-1* expression is quickly elicited in response to a wide variety of extracellular signals, including growth factors, cytokines, hormones, phorbol esters, irradiation and stresses of many kinds, and it shows a robust and transient pattern (usually at 3 h post-induction the mRNA is not longer detectable). *Egr-1* expression has been linked to cellular growth,

differentiation and apoptosis (Thiel *et al.*, 2002), and therefore serves as a link between extracellular signals and long-term cell responses by altering *egr-1* target genes transcription.

3.2 *CIS* ELEMENTS ON THE *egr-1* PROMOTER

Egr-1 promoter contains among other *cis*-elements, SREs (Serum response element), Ets (E-twenty six), CRE (cAMP response element), GCbox (which binds SP1-specificity protein 1-) and EBS (Egr-1 Binding site) in its 5' region (Figure 9). EGR-1 is able to bind to its own promoter and to interact with two co-repressors: NAB1 and NAB2. Furthermore, EGR-1 regulates *nab2* expression, and therefore is able to establish an autoregulatory loop that would control its own expression. Besides, Thiel *et al.*, (2002) demonstrated that NAB1 only needs to be recruited to the transcription unit either by protein-protein or by DNA-protein interaction to repress *egr-1* expression. Moreover, *egr-1* can be further induced by CREB and/or SRFs depending upon the stimulus that has elicited the response.

SRF is a transcription factor, often activated upon serum stimulation (Miano, 2003), that bounds as an heterodimer to the target sequence $CC(A/T)_6GG$ (CArG), and which needs to form a ternary complex with ETS to be fully active. ETS usually have their short DNA-specific binding sequence flanking SREs, so the ternary complex can be easily formed, and it also serves to confer specificity to the transcription factor. In the same manner, CREB is also capable of forming dimmers, and depending on the nature of these dimmers, it exerts its functions as an activating or as an inhibiting transcriptional factor. For instance, it can dimerize with CREM to act as transcriptional repressor, whereas CREB interaction with ATF2 or another CREB molecule enables the dimmer to act as a transcriptional activator (Carlezon *et al.*, 2005). In addition, all these transcription factors can be modified by acetylation and phosphorylation, as another regulatory way to modify and control their binding and activity properties.

TGGGCGCTTTGGCAGTGGCGGTTCCCTCGGGACTGCGGGGAAGGCCCAGGCCGCCGCGCC
TGCTCAGTTCTCCCTCACTGCGTCTAAGGCTCTCCCGGCCTGGCTCCGCGCCCAGCCCAG
ACTACGGGAGGGGGAACGTGGAGGCGACGGAAGAGCCCGTCGCGCCTGGGGCTCCCGAAA
TACAACCAGAGACCTACAGAGGGCAGCACCGAGCCGTAAACGGGTCCTCCGCACTGCAAG
CTTGGGGTCGCCAGACTGCCCAAAGCCAAGTCCCCCTCTTTAGGACAGGGCAGGGTTCGT
GCCCGACCAGTCCCTGGCCTGGATAAAAGTCAGGAAGTGTCTAACCATCACAAGAACCAA
CAGATCCTGGCGGGGACTTAGGACTGACCTAGAACAATCAGGGTTCCGCAATCCAGGTCC
CCAAAGGTGGGATCCTCAACCGCAGGACGGAGGGAATAGCCTTTCGATTCTGGGTGGTGC
ATTGGAAGCCCCAGGCTCTAAAACCCCCAACCTACTGACTGGTGGCCGAGTATGCACCCG
ACTGCTAGCTAGGCAGTGTCCCAAGAACCAGTAGCCAAATGTCTTGGCCTCAGTTTTCCC
GGTGACACCTGGAAAGTGACCCTGCCATTAGTAGAGGCTCAGGTCAGGGCCCCGCCTCTC
CTGGGCGGCCTCTGCCCTAGCCCGCCCTGCCGCTCCTCCTCTCCGCAGGCTCGCTCCCAC
GGTCCCCGAGGTGGGCGGGTGAGCCCAGGATGACGGCTGTAGAACCCCGGCCTGACTCGC
CCTCGCCCCCCGCGCCGGGCCTGGGCTTCCCTAGCCCAGCTCGCACCCGGGGGCCGTCGGA
GCCGCCGCGCGCCCAGCTCTACGCGCCTGGCGCCCTCCCCACGCGGGCGTCCCCGACTCC
CGCGCGCGCTCAGGCTCCCAGTTGGGAACCAAGGAGGGGGAGGATGGGGGGGGGGGGTGT
GCGCCGACCCGGAAACGCCATATAAGGAGCAGGAAGGATCCCCCGCCGGAACAGACCTTA
TTTGGGCAGCGCCTTATATGGAGTGGCCCAATATGGCCCTGCCGCTTCCGGCTCTGGGAG
GAGGGGCGAGCGGGGGTTGGCGCGGGCGCAAGCTGGGAACTCCAGGCGCCTGGCCCGGGA
GGCCACTGCTGCTGTTCCAATACTAGGCTTTCCAGGAGCCTGAGCGCTCGCGATGCCGGA
GCGGGTCGCAGGGTGGAGGTGCCCACCACTCTTGGATGGGAGGGCTTCACGTCACTCCGG
GTCCTCCCGGCCGGTCCTTCCATATTAGGGCTTCCTGCTTCCCATATATGGCCATGTACG
TCACGGCGGAGGCGGGCCCGTGCTGTTCCAGACCCTTGAAATAGAGGCCGATTCGGGGAG
TCGCGAGAGATCCCAGCGCGCAGAACTTGGGGAGCCGCCGCCGCGATTCGCCGCCGCCGC
CAGCTTCCGCCGCCGCAAGATCGGCCCCTGCCCCAGCCTCCGCGGCAGCCCTGCGTCCAC
CACGGGCCGCGGCTACCGCCAGCCTGGGGGCCCACCTACACTCCCCGCAGTGTGCCCCTG
CACCCCGCATGTAACCCGGCCAACCCCCGGCGAGTGTGCCCTCAGTAGCTTCGGCCCCGG
GCTGCGCCCACCACCCAACATCAGTTCTCCAGCTCGCTGGTCCGGGATGGCAGCGGCCAA
GGCCGAGATGCAATTGATGTCTCCGCTGCAGATCTCTGACCCGTTCGGCTCCTTTCCTCA
CTCACCCACCATGGACAACTACCCCAAACTGGAGGAGATGATGCTGCTGAGCAACGGGGC
TCCCCAGTTCCTCGGTGCTGCCGGAACCCCAGAGGGCAGCGGCGGTAATAGCAGCAGCAG
CACCAGCAGCGGGGGGCGGTGGTGGGGGCGGCAGCAACAGCGGCAGCAGCGCCTTCAATCC
TCAAGGGGAGCCGAGCGAACAACCCTATGAGCACCTGACCACAG

Figure 9. Promoter sequence of Mouse *egr1* gene. To distinguish between promoter sequence, and transcriptional and translational start, bases are written in black, orange and red respectively. Consensus specific DNA sequences for transcriptional factors which have at least 90% homology are highlighted in colours: SP1, EGR1, ETS, SRF, CREB and AP2. Overlapping sequences for two transcriptional factors are written in brown.

3.3 EGR-1 AS A PROTEIN: AN ESSENTIAL TRANSCRIPTION FACTOR

EGR-1 is a protein of ~80 kDa which encodes for a modular transcriptional factor. The protein has three different domains: an N-terminus activating domain stretching from amino acids 3-281, a central inhibitory domain and a DNA-binding domain encoded by three zinc fingers (C_2H_2 type) (Figure 10). Russo et al., (1995) identified the central repressive domain as a binding site for two transcriptional co-factors: NAB1 and NAB2 (NGFI-A binding proteins 1 and 2) that block the biological activity of EGR-1. Other members of the EGR family, posses also zinc finger domains, but they differ in the sequences outside of this domain, and therefore they have different biological functions.

EGR-1 binds to specific GC rich DNA sequences such as 5'-GCG(G/C)GGGCG-3' (Lemaire *et al.*, 1990 and Cao *et al.*, 1990) and 5'-TGCGTG/AGGCGGT-3' (Thiel et al., 2002) in a zinc dependent manner. This binding site is highly similar to SP1 binding site, and it has been suggested that both transcriptional factors may compete for the much alike binding site (Huang *et al.*, 1997) as it happens for instance, in the promoter of the *pdgf A* (platelet-derived growth factor A) gene. In this particular case, SP1 is displaced from the binding site when EGR-1 is synthesized upon stimulation of cells with phorbol esters (Kachigian *et al.*, 1995). In contrast, Al-Sarraj et al., (2005) propose that EGR-1 has a distinct sequence requirement to bind and activate EGR-1 responsive target genes and indeed does not compete with SP1 or SP3 during the binding process. However, when the genes contain overlapping SP1/EGR-1 or SP3/EGR-1 binding sites, the actual concentration of those different zinc finger proteins decides which transcription factor binds.

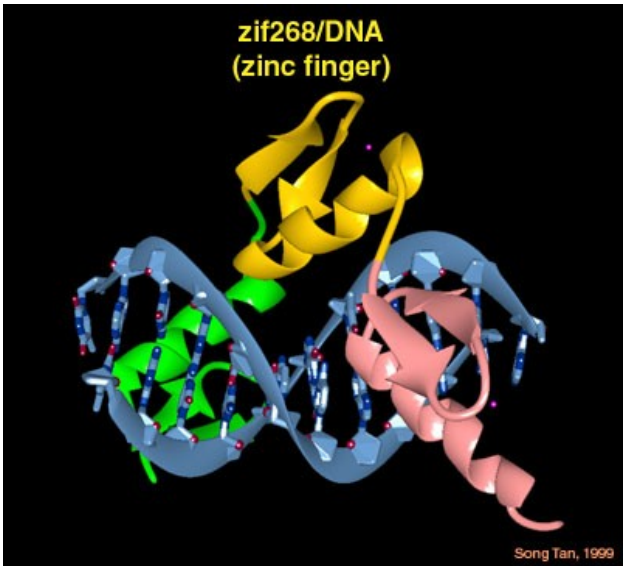


Figure 10. Modular structure of EGR-1 (ZIF268) transcription factor and schematic representation of zinc finger-DNA binding.

EGR-1 protein regulation is achieved by post-translational modifications such as glycosylation (Hedegen *et al.*, 1998), phosphorylation (Huang *et al.*, 1998 and Jain *et al.*, 1996), acetylation (Adamson *et al.*, 2005) and polyadenylation (Simon *et al.*, 2004). It is also probably that Egr-1 can be further modified by SUMOylation (Laura Moro and Elisa Brunelli personal communication). Phosphorylation of EGR-1 is achieved by CK II (Casein Kinase II), which can phosphorylate all the protein domains, and seems to be involved in a decrease in the protein binding affinity, and in the activation of pro-apoptotic genes such as *p53* or *p73* (Mora *et al.*, 2005). Nevertheless, acetylation of EGR-1, which takes place under conditions of modest *egr-1* induction, promotes stabilization of the protein and subsequent induction of proliferative and survival genes. Polyadenylation, on the contrary, seems to be involved in nuclear EGR-1 translocation upon light stimulus.

EGR-1 protein possesses ambiguous effects, as it can stimulate or suppress cell growth depending on the intensity of its induction. For instance, Yu *et al.* (2004), showed that EGR-1 in addition to induce apoptosis can also promote

survival in other cell types by means of activating *p21* instead of *p53* gene. .

In conclusion, the importance of the transcription factor EGR-1 resides in the fact that is responsible for the regulation of multiple cellular processes such as cell growth and cell development (review in Yan *et al*., 2000) and pathologies such as cancer (Lucerna *et al.*, 2006), cardiovascular pathologies (Khachigian, 2006) or neuronal diseases (Awasthi *et al.*, 2003). Consequently, it is very important to elucidate the processes that govern *egr-1* expression and therefore regulate the protein function as a second messenger. Gene expression is achieved by a combination of various chromatin dynamic processes: nucleosome composition, modification and repositioning, and hence the regional promoter properties vary according to those enzymatic activities, that in turn facilitate the ordered access of transcription factors to regulatory regions. The aim of this study was to deepen into the knowledge of *egr-1* expression in a living cell and consequently, in the chromatinic changes that take place in its promoter to achieve a proper induction pattern.

OBJECTIVES

Eukaryotic gene regulation is a highly precise set of mechanisms, each of which has to be tightly regulated in order to ensure the adequate cell response to a cellular stimulus. Understanding the way in which those mechanisms take place is an important goal, both for the basic molecular knowledge and for the therapeutics

of many diseases. The coordinated action of signal transduction cascades, transcription factor binding and chromatin modifying complexes is fundamental for the correct interpretation of the genetic information. So far, there is not a general mechanism for gene regulation, as each gene has both general and unique features.

The OBJECTIVES of the present work were aimed at unravelling some of the epigenetic mechanisms underlying transcriptional activation and repression of an immediate-early gene induced by a cellular stress.

1- The first objective consisted in the selection of an adequate biological system useful for the detailed analysis of the temporal events that take place during the transcriptional regulation.

2- The second objective was to address the nature of the signalling cascades that transduce the signal from the cell surface to the nucleus, signal that in turn regulate the access and binding of the RNA polymerase to the gene/s of interest.

3- Thirdly, we intended to notice which transcriptional factors were involved in the regulation of expression of immediate-early genes, and whether those transcription factors were post-translationally modified in order to exert their function.

4- As DNA in eukaryotic cells is packed with proteins, the access of the transcriptional machinery to it has to overcome this fact, and therefore becomes necessary to study the gene regulation in the context of the chromatin. As a result, our main purpose was to determine which chromatin modifying complexes are needed for the adequate gene regulation. Moreover, we will try to correlate those complexes with the histone modifications that are taking place during the induction process. On the other hand, we are intended to establish the changes in the distribution of positioned nucleosomes during the gene induction and the specific modifications that may determine this nucleosome remodelling.

5- In this objective we pretend to study the effect that the lack of the protein synthesized by the gene of interest produces in the biological system under study.

6- In consequence, by combining the data that we have obtained in the previous objectives we wish to approach towards a model for *egr-1* epigenetic regulation in MLP29 cell line when it is induced by 12-O-tetradecanoylphorbol-13-myristate (TPA).

MATERIALS AND METHODS

1. BIOLOGICAL MATERIALS

All the experiments carried out in this work to achieve a better understanding of gene expression regulation were done with cultured cells. These cells, which are grown *in vitro*, are not longer organized in tissues, so they will grow, survive and maintain its function in suspension or attached to an inert surface such as a plastic plate.

1.1 CELL CULTURE

Routinely, cell lines were grown at 37ºC in an humid atmosphere containing 5% CO_2 and media (see Table 8), which depended on the cell type cultured, were supplemented with 10% FBS[1] (Gibco), 200 mM L-glutamine (Gibco), penicillin 100 U/ml (Sigma), streptomycin 100 µg/ ml (Sigma) and fungizone 2.5 µg/ ml (Gibco). All serum was heat inactivated at 65ºC in a water bath for 30 min. For normal maintenance of cells 100-mm culture dishes were used, subculturing (trypsinizing) them when they were around 70-80% confluence. Cells were then passed to another plate at 1:3-1:5 dilutions, depending again on the cell type. In all the experiments, cells used were not older than 10-12 subcultures, as some of the non-commercial cell lines lose their properties at longer passes.

Table 8. Cell types and their growing medium.

Cell line	Source	Media
Mouse progenitor hepatocyte cell line MLP29	A gift form PhD. E. Medico and PhD. C. Boccacio from the Turin University	DMEM (Sigma)
Mouse fibroblastic cell line C3H/10T1/2	A gift from PhD. Gorospe from the National Institute of Health, Baltimore	MEM (Sigma)
NIH3T3	European Collection of Cell Cultures (E.C.A.C.C.)	DMEM (Sigma)

[1] When starting with a new cell line it is recommended to first test the serum/s and when the result is satisfactory, the same batch should be used in all the experiments that should be compared between them.

1.1.1 Cell Trypsinization

Splitting of cells was done every three days. Media were aspirated and cells were washed twice with PBS, as serum presence inhibits trypsin activity. 100-mm cultured dishes were incubated with 1mL of trypsin solution containing 5.3 mM EDTA (Gibco) for 3 to 5 min, depending on the cell type. Then, cells were pipetted to disaggregate clumps and diluted with 9 volumes of fresh medium containing normal serum concentration to inactivate the trypsin. The wanted dilution was achieved by transferring the appropriate volume of the cell solution to new plate containing culture medium.

1.1.2 Cell Cryopreservation

Cell lines are finally lost after a high number of passages, even though frozen stocks can be obtained by cryopreservation at every passage, and thus the cell line can be restored again after a certain number of subcultures. For the cryopreservation procedure a subconfluent plate was selected and cells were removed by trypsinization and recovered by centrifugation at 1100xg for 5 min at room temperature. The pellet was then resuspended in 1.8 mL of medium containing 20% FBS plus 10% DMSO, to prevent membrane cracking. Cells were then stored in cryovials and placed in a container with isopropanol at -80ºC for 1 day (to cool down slowly the cells temperature), before being stored in a liquid nitrogen tank.

1.1.3 Reconstitution of Frozen Cells

A cryovial was removed from the liquid nitrogen tank and thawed slowly. Cells were then resuspended in 7 mL of tempered growing medium in order to dilute the DMSO as soon as possible and centrifuged at 1100xg for 5 min at room temperature. Afterwards, the cell pellet was resuspended with the desired amount of serum-containing medium and plated in a culture dish.

1.2 CELL TREATMENTS

Cells needed for each experiment were plated in parallel and they were grown until they reached a confluence of 70-80%. Then, all the dishes were kept between 12 and 24 h in serum starvation (0.5% FBS) and after treated with the appropriated reagents for the desired period of time. Controls were done by adding only the correspondent solvent to the cells (vehicle).

1.2.1 Induction of Oxidative Stress to MLP29 Cell Line

To study the effects of oxidative stress in gene expression, serum-starved MLP29 cells were incubated with different agents (see Table 9) in experiments of dose-response. After the oxidative agent was selected, time-dependent assays were done to see the induction profile.

Table 9. Agents used for stressing the cells.

Agent	Source	Solvent	Concentration range	Exposure time
TPA	Sigma	DMSO	25-100 nM	60 min
U.V. light	____	____	65W/m^2	5-15 s
t-BhP	Sigma	____	25-200 nM	30 min
Arsenite	Sigma	H_2O	100-400 µM	60 min

1.2.2 Drug Treatments to MLP29 Mouse Cell Line

Serum-starved MLP29 cells were grown in 6-well plates and after pre-treated with different cell signalling inhibitors for 30 min, except for the experiments with cycloheximide in which the treatment was for 1 h (see Table 10), and for PRMT1 inhibition, in which the three compounds were added to the cells 4 hours before the treatment with the phorbol ester. Then TPA was added to the medium until a 50 nM

concentration was reached and kept for 1 hour also in a cell growth incubator. All the reagents were prepared as a stock solution, filtered through 0.22 μm pore filter and stored at -20ºC until use.

Table 10. Cell signalling inhibition drugs.

Drug	Source	Solvent	[ ] stock	[ ] final
C Vitamin	Sigma	H_2O	0.1 M	1mM
N-Acetyl-Cysteine	Sigma	H_2O	0.1 M	1mM
Trolox	Calbiochem	EtOH	0.1 M	1mM
STAT3 inhibitor	Calbiochem	H_2O	500 μM	100 μM
Hypericin (PKC Inh.)	Sigma	DMSO	2,5 mM	10μM
PD98059 (MEK1/2 Inh.)	Calbiochem	DMSO	25 mM	100 μM
SB203580 (P38 Inh.)	Calbiochem	DMSO	25 mM	100 μM
JNK Inhibitor	Calbiochem	DMSO	25 mM	100 μM
ERKs Inhibitor	Calbiochem	H_2O	500 μM	20 μM
Wortmanin (PI-3K Inh.)	Calbiochem	DMSO	0.25 mM	1 μM
H89 (PKA Inh.)	Calbiochem	DMSO	2.5 mM	10 μM
NFκB inhibitor	Calbiochem	H_2O	0.5 mM	25 μM
Cycloheximide (Protein synthesis Inh.)	Sigma	H_2O	6.25 μg/μL	25μg/mL
Okadaic Acid	Sigma	EtOH	1.25μM	0.5μM
DL- Homocysteine (PRMT1 Inh.)	Sigma	DMSO	0.2 M	2 mM
Adenosine (PRMT1 Inh.)	Sigma	DMSO	0.2 M	2 mM
N-methyl-2-deoxyadenosine (PRMT1 Inh.)	Sigma	DMSO	0.2 M	2 mM

2. RNA EXTRACTION FROM CULTURED CELLS

Total RNA isolation is a method used to analyze global expression profiles or specific gene expression levels. RNA is extracted with a buffer that contains a strong detergent that denatures proteins and thus ensures the rapid inactivation of RNases and allows the extraction of protein-RNA complexes. Denatured proteins are removed by phenol-chloroform extraction and nucleic acids are precipitated with isopropanol.

2.1 TOTAL RNA ISOLATION

Total RNA from 6-well cell culture plate was obtained with the TRIZOL method (Chomzynski *et al.*, 1987). Treated cells were washed three times with cold sterile PBS. When the PBS was totally aspirated, 1ml of TRIZOL was added and cells scraped and transferred to a sterile 2 ml eppendorf tube. After 5 min incubation at room temperature, to let the nucleoproteic complexes dissociate, 0.2 ml of chloroform were added to each tube. Samples were then vigorously shacked and incubated 3 more min at room temperature prior to centrifugation for 15 min at 12000 rpm at 4ºC. The aqueous phase was collected and mixed with 0.5 ml of 2-propanol by gently inversion of the tube. Incubation of the mix for 10 min at room temperature and then centrifugation for 10 min at 12000 rpm at 4ºC leads to RNA precipitation and recovery. Pellets were washed once with 1 ml of ethanol 75%, and after dried in the speed-vac for 5 min and dissolved in 30 µl of RNA sterile H_2O. RNA concentration was estimated by measuring the absorbance at 260 nm and its purity double checked by calculating the ratio A_{260}/A_{280} (RNA was considered pure when the ratio was close to 2) and by RNA visualization on agarose formaldehyde gel electrophoresis.

2.2 SIZE FRACTIONATION OF TOTAL RNA ON AGAROSE FORMALDEHYDE GEL ELECTROPHORESIS

Electrophoretic RNA fractionation was done according to Sambrook *et al.*, 1989. To ensure that the RNA mobility depends only on the size of the fragments it is needed to destroy secondary structures with formaldehyde, and therefore is not possible to use buffers containing amino groups as Tris.

1.2% agarose gel was prepared by melting in water, and after cooling until 50ºC, 1/10 of MOPS buffer (10mM EDTA, 50mM Na-acetate, 200mM MOPS, pH7) and formaldehyde up to 2.2 % were added in a hood.

RNA concentrations were equilibrated by their absorbance at 260 nm, and then from 10 to 30 µg of RNA were mixed with 4 volumes of 1.25X loading buffer (44% deionised formamide, 5.9% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 6.4% glycerol, 0.025%(v/v) of saturated aqueous Bromophenol blue solution and 0.1 µg/µL ethidium bromide) and they were incubated at 65ºC for 10 min. Samples were chilled on ice and after loaded on their respective wells. Electrophoresis was run in MOPS-formaldehyde buffer (1X MOPS, 1.1 M formaldehyde) at 5 V/cm, and it was stopped when the Bromophenol dye had migrated 10 cm from the edge. Then, the gel was exposed to an UV transiluminator and photographed.

2.3 TOTAL RNA RETROTRANSCRIPTION

1 µg of RNA was retrotranscribed to cDNA using Superscript II RNase H$^-$ (Life Technologies) following the manufacturer's instructions. Instead of oligo dT, it was always used random hexamers (Sigma). Reaction was carried out at 42ºC for 50 min and the enzyme was heat inactivated at 70ºC for 15 min.

2.4 cDNA ANALYSIS BY SEMI-QUANTITATIVE POLYMERASE CHAIN REACTION (PCR)

PCR reaction allows the specific amplification of an exact DNA fragment even in the presence of millions of other DNA molecules. The DNA polymerase is able to produce the complementary strand of an already existing DNA molecule with the only requirements of nucleotides (deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) and primers (small DNA molecules that specifically hybridise with the target that is to be amplified).

cDNA from the samples was amplified by PCR using the oligonucleotides from the Table 4 and DNA polymerase Amplitaq (N.E.E.D. S.L.). As an internal control, *rRNA 18S* gene was amplified in the same reaction tube together with the gene of interest. To achieve a proportional amplification between the internal control and the gene to be studied, the signal of the first was reduced by adding oligonucleotides that compete with the ones for *rRNA* 18S. These competitors have the same sequence as the ones that amplify *rRNA* 18S, but they have the 3'-terminus blocked with an amino group, disabling the amplification by the polymerase. The proportion of *rRNA* 18S oligonucleotides and competitors was experimentally determined for each gene. PCR conditions are described in Table 12 and the annealing temperature for each of the primers is shown in Table 11.

Table 11. Oligonucleotides used for the semi-quantitative and real time PCR. The sequence of the oligonucleotides is shown in the Table 12, its position from the starting point and also the annealing temperature specific for each reaction, except for the real-time, in which the temperature is always 60ºC.

Gene	Oligo forwd/rev	Fragment size (bp)	Exon	Annealing (ºC)
Semi-Quantitative PCR				
c-fos	CGAAAGGGCAGCAGCAGC/ GGCCAGATGTGGATGCTTGC	248	4	55.6
***rRNA* 18S**	TGGTTGATCCTGCCAGTAGC/ CTCTCCGGAATCGAA CCCTG	433	1	*Idem* to the gene co-amplified
c-jun	TCCGCGCCAAGAACTCGGAC/ CACTGGGAAGCGTGTTCTGG	231	1	61.0
egr-1	CAGAAGCCCTTCCAGTGTCG/ GATGGGTAGGAGGTAGCCAC	263	2	57.3
pip92	ACAAGTGGGCACGCTTGTG/ GGCAGCAACTACGACCAGC	299	1	61.1
gadd153	GGGCCAACAGAGGTCACACG/ TGGTCTCCACCTCCCTGGTC	259	4	60.0
trx	GGAAGCCTTGGACGCTGC/ AAAACTGGAATGTTGGCGTGC	203	1-3	54.9
nur77	ATCCTCCAGCGGCTCTGAG/ AGAACTCTGGGCCAGGCTG	352	2	60.0
nab2	CACTTGTGGGCATGGCAAGC/ CCCTGACCCAAGCTCTGCAC	291	2	60.9

Table 11. Oligonucleotides used for the semi-quantitative and real time PCR. (Cont.)

Gene	Oligo forwd/rev	Fragment size (bp)	Exon
Real Time PCR			
c-fos	AGAGGAAGAGAAACGGAGAATCC/ AGAGGAGCGAGCGACCAA	76	3
***rRNA* 18S**	CACGGCCGGTACAGTGAAA/ AGAGGAGCGAGCGACCAA	71	1
gadd153	CAACAGAGGTCACACGCACAT/ CCTGGGCCATAGAACTCTGAT	69	4
egr-1	GCCTCGTGAGCATGACCAAT/ ACGATGAAGCAGCTGGAGAAG	66	2
nab2	CACTTGTGGGCATGGCAAGC/ GTCATGATCGTCCATCTCAAA	75	2

Table 12. **Semi-quantitative and real time PCR conditions for the analysis of gene expression.** The annealing temperature for the semi-quantitative reactions is depicted beside the oligonucleotides used in Table 11.

Semi-quantitative			Real Time		
cycles	**Temperature (ºC)**	**Time (min)**	**cycles**	**Temperature (ºC)**	**Time (min)**
1	93	4	1	93	10
30	93	0.5	40	60	1
	annealing	0.5			
	72	0.5			
1	72	7	Melting	95-65	30

2.4.1 DNA Agarose Gel

Products from PCR reaction were visualized on agarose gels. Agarose (Roche) was dissolved in hot TBE 0.5X (45mM basic Tris, 45mM boric acid and 1mM EDTA, pH 8.0) and ethidium bromide was added up to 0.1 µg/ml final concentration. Solution was poured on the casting tank and when the gel was polymerized samples were mixed with the adequate volume of 6X DNA loading buffer (40% (w/v) sucrose, 0.25% bromophenol blue in water) and loaded into the wells. Gels were run in 0.5 X TBE at 10 V/cm for around 30 min and the bands were visualized under UV light. Usually 0.8% and 2% gels were done, and in each case molecular weight markers were use to confirm the right size of the products. Agarose concentration depended on the size of the fragment that was to be visualized; smaller DNA fragments required higher agarose concentrations to increase resolution.

2.5 cDNA ANALYSIS BY REAL TIME PCR

Real time PCR allows the detection in "real time" of the amplification of our gene of study. For this purpose a fluorescent molecule is released from a quencher each time that the polymerase amplifies the fragment of interest, and thus the fluorescence that is emitted at each cycle of PCR will be proportional to the amount of DNA that is being amplified.

Real time PCR reaction were carried out in *ABI GeneAmp 7000 Sequence Detection System*, (Perkin-Elmer, applied Biosystems). cDNA was amplified using the oligonucleotides from the Table 11 and the conditions of Table 12. Each of the reactions was done in triplicate and the fusion curves were done with the software *Dissociation Curves* (Applied Biosystems), to ensure that only one fragment is amplified. As a loading control *rRNA* 18S gene was also analyzed.

3. CHROMATIN IMMUNOPRECIPITATION (ChIP) FROM MOUSE HEPATOCITE CELL LINE MLP29

Chromatin Immunoprecipitation (ChIP) assay is used to evaluate the association of proteins like transcription factors, HATs, HDACs or HMTs complexes, with specific DNA regions. The technique requires a formaldehyde crosslinking of the proteins with DNA, by means of formamide bond formation, and the latter chromatin fragmentation prior to the immunoprecipitation with the desired antibody. The segment of the genome associated with the protein is then identified by PCR amplification of the DNA present in the immunoprecipitates.

3.1 PREPARATION OF FORMALDEHYDE CROSSLINKED CHROMATIN

The assay was carried out according to a protocol described by Wells *et al.*, 2002, optimized for mammalian cells. MLP29 cells were grown in 10 cm Petri dishes ($1X10^7$ cells per dish) until they reached a confluence of approximately 75-80%. Serum starved cells (12-24 h in 0.5% FBS) were treated with 50 nM TPA (Sigma) for the desired times. To fix the samples, growing medium was removed and the dishes were incubated with 10 ml of PBS–1% formaldehyde for 4 min at room temperature with constant shaking. To stop the crosslinking reaction, glycine was added to a final concentration of 0.125 M and cells incubated for 5 min at room temperature on continuous agitation. Then attached cells were washed twice with ice-cold PBS, scraped and recovered by centrifugation at 1500xg at 4ºC for 5 min. The pellet was resuspended in cell lysis buffer (5 mM HEPES, 85 mM KCl, 0.5% NP40 pH 8.0) containing 2 μL/mL of protease inhibitor and phosphatase inhibitor I and II cocktails (Sigma). Samples were then incubated on ice for 15 min and nuclei were recovered by centrifugation at 3500xg for 5 min at 4ºC. The sediment was gently resuspended in nuclear lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS pH 8.1), aliquoted and stored at -20ºC until use.

3.1.1 Formaldehyde Crosslinked Chromatin Fragmentation

To achieve a good immunoprecipitation, soluble chromatin fragments should have an average size around 500 and 600 bp. This population size is obtained by a compromise between the time of formaldehyde crosslinking and the number of sonications (for optimization see *Annex 1*). Chromatin pooled from 6 cell dishes was sonicated 12 times for 10 s at 37% setting ("*Vibra-Cell VCX-500 sonicator"*), and kept in ice for 1 min between each sonication. Insoluble fraction was discarded after centrifuging at 14000xg for 15 min until there was no more pellet. 50 µL aliquot is separated to extract and quantify DNA, while another 5 µL diluted 1/100 in 1% SDS are taken for a direct measure of the chromatin absorbance at 260nm. The remaining sample is then diluted 1:10 with dilution buffer (1.1% Triton X-100, 1.1 mM EDTA, 167 mM NaCl, 2 µL/mL protease inhibitor and phosphatase I and II cocktails (Sigma), 16.7 mM Tris-HCl pH 8.0). These samples can once more be frozen in liquid nitrogen and stored at -20ºC until use.

Annex 1: Optimization of the size of the chromatin

Experimental conditions used for the chromatin fragmentation of murine MLP29 cells were established by varying the time of formaldehyde exposure and also the number of sonications. Excessive formaldehyde crosslinking has the disadvantage of giving long chromatin fragments that are hardly broken by sonication, and has the risk of coimmunoprecipitate more than one gene, and thus give misleading results. It could also have problems for the antibody in recognizing the epitope. On the other hand, small fragments of less than 300 bp diminish the population than can be immunoprecipitated and difficult the following quantification by PCR. As a result a compromise between the fixation time and the number of sonications has to be established for ChIP experiments with different cell lines or tissues.

As it can be seen in Figure 11, long fixation times (6 min) produce longer fragments that can hardly be broken by sonication, whereas shorter incubations with formaldehyde (4 min) produces chromatin fragments in the range of 300-1000 bp, which is the intended size for the following immunoselection.

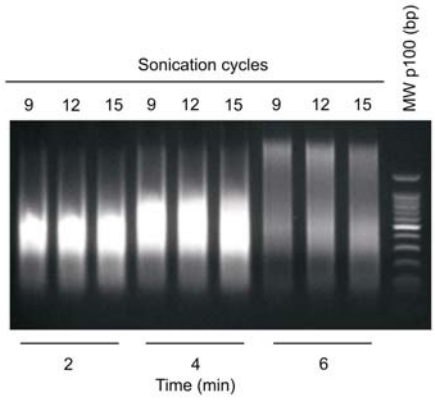


Figure 11. Chromatin fragmentation conditions for MLP29 mouse cell line. Samples are formaldehyde crosslinked for 2, 4 and 6 min and sonicated for 9, 12 or 15 times. P100 molecular weight markers can be seen in the last line of the agarose gel.

3.2 REVERSAL OF FORMALDEHYDE CHROMATIN CROSSLINKING AND DNA EXTRACTION

50 µL aliquot of sonicated chromatin (section 3.1.1) is diluted to 500 µL with TE buffer (1mM EDTA, 10 mM Tris-CL, pH 8). 40 µL/mL of RNase A are added to remove RNA from the sample and it is incubated at 65 ºC overnight to reverse the crosslinking. Then SDS to a final concentration of 1% and proteinase K (100 µg/mL final concentration) is added to the sample in order to deproteinize it for 1 h at 37ºC. DNA is extracted once with phenol, once with phenol/chloroform/isoamyl alcohol (25/24/1) and once with chloroform/isoamyl alcohol (24/1). DNA is then precipitated by addition of 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2 and incubated at -20ºC overnight. The pellet is washed with 70% ethanol and resuspended in 400 µL of TE buffer pH 8.0. DNA quantification is carried out in a 96-well plate using "*Pico Green DNA quantification Kit"* (*Molecular probes*) and following the manufacturer's

instructions. Briefly, a reference curve is obtained by measuring the fluorescence of 100 µL of increasing amounts of a known λ DNA concentration diluted in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) plus 100 µL of Pico Green fluorophor (diluted 1/1000 in the same buffer). Fluorescence is recorded with the instrument *Fujifilm FLA3000* and the results are analyzed with *Image Gauge V3.12*.software. Excitation wave length was fixed at 480 nm and emission wave length at 520 nm. The reference curve that correlates the fluorescence with the λ DNA concentration was used to interpolate the results of the unknown samples. Comparison between the absorbance measurements at 260 nm (of chromatin in 1% SDS) and the results from the picogreen (DNA) showed that one unit of absorbance corresponds approximately to 15 µg/mL of DNA. Then, in order to determine the size of the chromatin fragments, samples were loaded and were run on a 0.8% agarose gel electrophoresis in 0.5X TBE buffer and stained with ethidium bromide (see 2.4.1).

3.3 IMMUNOPRECIPITATION OF CROSSLINKED CHROMATIN

For each immunoprecipitation it was used 3.5 units of absorbance measured at 260 nm, which corresponds approximately to 50 µg of DNA. The size of the chromatin used was previously checked (see 3.2).

Samples containing the soluble chromatin fragments were thaw on ice and then each of them was incubated with 30 µL of protein A/G-sepharose (Amersham Biosciences) that had been previously blocked for 3 h with constant shaking at 4ºC with 100 µg/mL of sonicated λDNA (Sigma), 100 µg/mL of sonicated and denatured λDNA (5 min at 100ºC and then quickly chilled on ice), 1 mg/mL BSA (Sigma) and 250 mg/mL tRNA (Sigma). After 3 h of this preclearing, which serves to avoid the unspecific union of DNA fragments to the sepharose beads, suspensions were centrifuged at 14000xg for 1 min and the supernatants were incubated overnight with the desired antibody in the adequate concentration (see Table 13) at 4ºC with constant shaking. One of the samples, did not contain antibody (No Ab) and serves both the bound fraction as control of unspecific binding of the reaction, and the unbound fraction as a total input of the reaction.

The following day 50 µL of protein A/G-sepharose, previously blocked as before, are added to each of the chromatin samples containing the antibody, and incubated for 4 h at 4 ºC on gently rotation. The complex consisting on chromatin fragments-antibody-protein A/G-sepharose was recovered by centrifugation at 14000xg for 1 min, and the supernatant of the sample that did not contain antibody was also recovered as the total input fraction and kept until used. Samples were then extensively washed with 1 mL of different buffers; twice with Low Salt buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 % Deoxycolic Acid, 0.1% SDS, 1% NP-40, 1 mM EDTA), twice with High Salt buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.5 % deoxycolic acid, 0.1% SDS, 1% NP-40, 1 mM EDTA), twice with LiCl buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40, 0.5 % deoxycolic acid) and finally twice with TE buffer pH 8 (Tris 10 mM, EDTA 0.25 mM). In each of the washes, samples were kept on rotation for 5 min at room temperature and sepharose complexes recovered by centrifugation at 14000xg for 1 min. Chromatin fragments immunoselected were recovered from sepharose beads by two consecutive elutions of 10 and 5 min at 65 ºC with 100 µl of freshly made elution buffer (100 mM $NaHCO_3$, 1% SDS). Eluted fractions and 1/5 of the input fraction, which had SDS added to a final concentration of 1% to correlate with the rest of the samples, were incubated overnight at 65ºC to revert crosslinking between protein complexes and the DNA. The following day, samples were incubated with proteinase K 1µL/100µL for 1 h at 37ºC to deproteinize the samples. DNA was purified by PCR purification Kit (Quiagen) following the manufacturer instructions. DNA eluted in 60 µl is stored at -20ºC until used.

Table 13. ChIP Antibodies. Antibodies used for ChIP experiments and the quantity used for each one.

Antibody	Comercial	Reference	µg used	Antibody	Comercial	Cat. number	µg used
α-SRF	Santa Cruz	(sc-335)	2	**α-H3 K4 Me_2**	Abcam	(ab 7766)	2
α-SP1	Santa Cruz	(sc-59)	2	**α- H3 K4 Me_3**	Abcam	(ab 8580)	0.5
α-Elk	Santa Cruz	(sc-355)	2	**α-H3 K9 Ac**	Abcam	(ab 4441)	2
α-CREB	Santa Cruz	(sc-186)	2	**α-H3 K14 Ac**	Upstate	(07-353)	1
α-EGR-1	Santa Cruz	(sc-110)	2	**α-H3 K18 Ac**	Abcam	(ab 1191)	2
α-CREB-P	Santa Cruz	(sc-7978)	2	**α-H3 K27 Ac**	Upstate	(07-360)	0.5
α-Elk-1-P	Santa Cruz	(sc-8406)	2	**α- H3 S10Ph - K14 Ac**	Upstate	(07-081)	2
α-Nab-1	Santa Cruz	(sc-22813)	2	**α-H4 K5 Ac**	Abcam	(ab 1758)	2
α-Nab-2	Santa Cruz	(sc-22815)	2	**α-H4 K8 Ac**	Abcam	(ab 1760)	2
α-RNAPol II	Santa Cruz	(sc-899)	2	**α-H4hiperAc**	Upstate	(06-598)	2
α-mSin3A	Santa Cruz	(sc-994)	2	**α-H4 K16 Ac**	Upstate	(07-329)	1
α-Brm	Santa Cruz	(sc-28710)	2	**α--PRMT1**	Santa Cruz	(sc-13392)	2
α-Bgr1	Santa Cruz	(sc-10768)	2	**α-GCN5**	Santa Cruz	(sc-6304)	2
α-HDAC3	Santa Cruz	(sc-11417)	2	**α-CBP**	Santa Cruz	(sc-369)	2
α-NCoR	Santa Cruz	(sc-8994)	2				

3.4 PCR ANALYSIS OF ChIP ASSAY

DNA resulting from each of the immunoprecipitated fractions was analyzed by PCR in a Perkin-Elmer 9600 thermocycler (Applied biosystems). Before starting with the analysis of the samples, linearity was determined for each input fraction. All PCR reactions were performed at the conditions indicated in Table 14, with the oligonucleotides from Table 15.

Table 14. PCR conditions for the analysis of the ChIP fractions.

Reagent/ Source	Volume used (µL)	Final concentration	Cycles	Temperature (ºC)	Time (min)
10X Taq Buffer N.E.E.D. S.L	2	1X	1	93	4
$MgCl_2$ N.E.E.D. S.L	0.6	1.5mM	35	93	0.5
25 mM dNTPs/ Invitrogen	0.2	250 µM		Annealing temperature of each primer	0.5
Forward oligo Isogen-lifescience	0.1	0.5µM			
Reverse oligo/Isogen-lifescience	0.1	0.5µM			
Betaine /Sigma	6	1.5M			
H_2O	8				
Taq / N.E.E.D. S.L	1	1U/PCR (20µL)			
Sample	2	Input 1/2500 dilution		72	0.5
	2	Immuno 1/30 dilution	1	72	7

Table 15. Oligonucleotides used for the analysis of ChIP results.

Gene name	Oligo forwd/rev (5'-3')	Fragment size (bp)	Annealing (ºC)
α-actin	CACCTGACCACAGGGCATCC/ AACTGGCTCCAAGGCTCACG	227	58.1
β-actin	TCTGGCTTTCCGGCTATTGC/ AGTTTTGGCGATGGGTGCTG	306	59.1
egr-1	TTCCCTAGCCCAGCTCGCAC/ GAACAGCAGCAGTGGCCTCC	352	62.5
nab2	ACTGCGCTCTCGGCTCACAG/ GAGCCTTGGAGAGGCTGCTG	300	63.4

Products were analyzed in a 1.8% agarose gel, in which the molecular marker *P100 ladder* (*Pharmacia Biotech*) was also loaded to confirm the size of the amplified fragment. The gel is afterwards photographed on an U.V. transiluminator.

Annex 2: Antigen distribution along the promoter

The intensity of the amplifications of the DNA obtained by ChIP experiments depends, among others, on the number of molecules that contain the amplicon used. Consequently, the intensity decreases as the amplicons are further away from the position recognised by the antibody used in the immunoprecipitation.

If the size (L) of the chromatin fragments used in the ChIP was to be constant, the intensity (I) of the amplification, normalized to 1 for the point where the antigen is found, would vary with the distance (d) between the antigen and the amplicon used, according to the following linear equation:

$$I = 1 - \frac{d}{L}$$

In the case of a ChIP experiment, L is a random variable that we assume normally distributed with mean (m) and standard deviation (σ), thus the intensity I, which is function of the random variable L, is also a random variable. If we calculate the probability density function for the intensity ($f_I(i)$) knowing that L is normally distributed, we obtain [2]:

$$f_I(i) = \frac{1}{\sigma\sqrt{2\pi}\frac{(1-i)^2}{d}} e^{-\frac{(\frac{d}{1-i}-m)^2}{2\sigma^2}}$$

This equation, normalized for each value of d, gives the plot in Figure 12, in which the wavelength of the colours represented is proportional to the density of probability (red colour would indicate the highest probability, whereas violet colour would indicate the lowest probability). For this plot, an m of 500 bp and a σ of 167

[2] Mathematical equation kindly developed by Francisco José Rodríguez Fortuño.

bp has been used. Note that the mode of I (the value i at which its probability density function attains its maximum value, and thus the most likely outcome in an experiment) for a fixed d, does not coincide with the mean of I ($\bar{i} = 1 - \frac{d}{m}$).

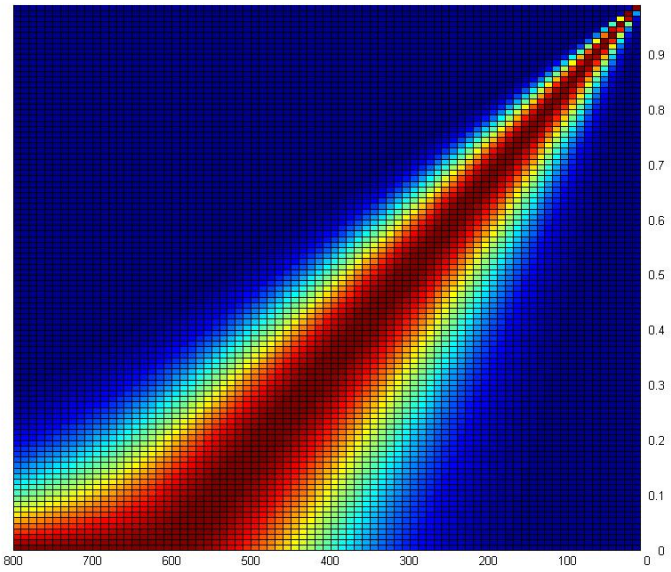


Figure 12. Density function of the intensity probability ($f_I(i)$) for a ChIP experiment in which the mean of the fragments used is 500 bp, and the standard deviation is 167 bp.

4. RNAPol- ChIP

RNAPol-ChIP technique, developed by us (Sandoval *et al.*, 2004), consists in the detection of the presence of the RNAPol II in the coding region of a gene, therefore, allowing knowing the "real time" transcription of a gene. This application of the ChIP technique consists in the immunoprecipitation of the samples with an antibody that recognizes the RNAPol II, and afterwards the immunoprecipitated fragments are amplified in the coding region of the gene (Table 16) and visualized

on 1.8% agarose gels. The only consideration is that the coding sequence that is going to be amplified should reside far enough from the transcription starting point, in order to avoid the immunoselection of the RNAPol II that is paused at the promoter of some genes.

Table 16. Oligonucleotides used for the analysis of the RNAPol-ChIP.

Gene name	Oligo ford/rev (5'-3')	Product extension (bp)	Annealing (ºC)
α-actin	ATCCTGGCCTCGCTGTCCAC/ CACCCTGCAACCACAGCACG	169	59.8
β-actin	ACGCCATCCTGCGTCTGGAC/ ATGACCTGGCCGTCAGGC	226	59.9
egr-1	CAGAAGCCCTTCCAGTGTCG/ GATGGGTAGGAGGTAGCCAC	263	57.3
nab2	CACTTGTGGGCATGGCAAGC/ CCCTGACCCAAGCTCTGCAC	291	60.9

5. NUCLESOME ChIP

This method allows ChIP analysis at nucleosome resolution by taking advantage of the fact that micrococcal nuclease (MNase) can efficiently digest chromatin to mononuclesomes and thus serves us to study histone modifications and factor occupancy on nucleosomes, but not if the factors are recruited to naked DNA sequence. It should be noted however that, if two neighbouring nucleosomes are bridged via intermediary factors, they are not expected to be resolved. In order to investigate individual nucleosomes, the sonication step of the standard protocol is replaced by micrococcal nuclease complete digestion of chromatin, in order to obtain a population highly rich in mononucleosome-size fragments.

5.1 NUCLEI EXTRACTION

Cell culture dishes were washed once with PBS and then cells were scraped with PBS containing 2 µl/ml protease inhibitor cocktail (Sigma), 2 µl/ml phosphatase inhibitor cocktail I and II (Sigma) and 10mM sodium butyrate (Sigma). Afterwards, cells were centrifuged 5 min at 1800xg and the pellet was resuspended in cell lysis buffer, containing also protease and phosphatase inhibitors and sodium butyrate and left 15 min on ice. Nuclei were recovered by centrifuging 5 min at 3000 rpm, and then washed with digestion buffer I (15 mM Tris pH7.5, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 20% glycerol, 5 mM β-mercaptoethanol, 2 µl/ml protease inhibitor cocktail (Sigma), 2 µl/ml phosphatase inhibitor cocktail I and II (Sigma), 10 mM sodium butyrate). Then samples were centrifuged 5 min at 3000 rpm and the pellet was resuspended in digestion buffer I without inhibitors. At this stage the absorbance of the sample was measured, so it could be diluted until a concentration of 0.25 mg/ml approximately.

5.2 MICROCOCCAL NUCLEASE (MNase) DIGESTION

To digest the chromatin, 300 µl aliquots of the nuclei sample were incubated inside a termoblock at 37ºC. For the MNase reaction 3 µl of 0.1 M $CaCl_2$ (1 mM final concentration) and 100 U/ml of MNase were added, and the reaction was left to proceed for 5 min. In these conditions the product is composed mainly of mononucleosomes. Reaction was stopped by addition of EGTA to a final concentration of 1.5 mM and incubation of samples on ice. Finally, the sample was centrifuged at 14000xg 10 min at 4ºC and the supernatant containing the nucleosome fraction was recovered. 10 µl aliquot was used to measure the absorbance of the sample at 260 nm, and another 10 µl aliquot were run on a gel to visualize the percentage of mono-di and tri-nucleosomes. To the rest of the sample it was added 5 mM β-mercaptoethanol, protease and phosphatase I and II inhibitor cocktail (Sigma) and spermidine 0.14 mM), in order to protect it from degradation.

5.3 Formaldehyde Mononuclesomal Crosslinking

Nucleosomes were fixed with formaldehyde at 0.5 % final concentration at room temperature for 3 min on constant shaking (see annex 3 for time conditions optimization). Reaction was stopped by addition of 2.5 M glycine to a final concentration of 0.125 M and left for 5 min also on constant shaking. Samples were then frozen with liquid nitrogen and stored at -20ºC until use.

Annex 3: Formaldehyde crosslinking optimization

Crosslinking conditions were optimized for the mononucleosomal ChIP to ensure that the fixation time was enough to preserve the nucleosomes, but not too much as to produce crosslinking between the different nucleosomes. The conditions were visualized on a 1% agarose gel stained first with Comassie to see the proteins and afterwards with ethidium bromide to see the DNA. As it can be seen in Figure 13 the optimum time was 5 min.

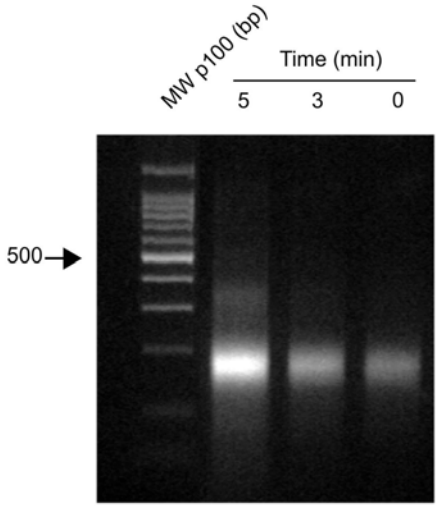


Figure 13. Formaldehyde crosslinking conditions for the nucleosomal ChIP. MLP29 mouse cell line chromatin digested with MNase 100 U/ml was formaldehyde crosslinked for 0, 3 and 5 min and visualized on 2 % agarose gel. P100 molecular weight marker can be seen in the first well.

5.4 IMMUNOPRECIPITATION OF FORMALDEHYDE CROSSLINKED NUCLEOSOMES

The procedure used for the immunoprecipitation of nucleosomes is the same as the one described in section 3.3.

5.5 PCR ANALYSIS OF THE IMMUNOPRECIPITATES

DNA fragments from the immunoprecipitation were analyzed by semi-quantitative PCR and visualized on 2.2% agarose gels run in 0.5X TBE and also by real-time PCR to quantify the differences in histone modifications.

6. NUCLEOSOMAL POSITIONING IN CELL CULTURE

Identification of nucleosomal position in *egr-1* was achieved according to the method described by Steward *et. al.*, (2004) with minor variations (see *annex 4*). This technique not only allowed us to determine the position of the nucleosome but also to detect the changes that may take place in that position during the induction of the gene.

6.1 THEORETICAL PREDICTION OF NUCLEOSOMAL FORMATION SITES

As a preliminary study, *egr-1* sequence was analyzed to investigate whether it contained sequences with a high nucleosome formation potential (NFP). NFP is based on an algorithm of the set of nucleosome positioning sequences found in the nucleosomal DNA database (Ioshikhes *et al.*, 1993). Two programs were used for this purpose: one based on the algorithms derived by Drew and Calladine (Drew *et al.*, 1987 and Estruch *et al.*, 1989) and a second which predicts the nucleosome position according to the thermodynamic possibility of the twists required for the nucleosome formation (Anselmi *et al.*, 2000).

6.2 NUCLEOSOME ISOLATION FROM MLP29 MOUSE CELL LINE

Confluent cells were kept in serum starvation and treated with 50 nM TPA for the desired times (0, 15, 30, 60, 180 min) and harvested to obtain pure preparations of nuclei. The protocol used to obtain fresh nucleus corresponds to the one described in section 6.1, but for this purpose nuclei were resuspended in 5 mL of digestion buffer (15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 20% V/V glycerol, 5 mM β-mercaptoethanol and 10 mM sodium butyrate). 10 µl aliquot of the sample was mixed with 990 µl of 1% SDS and the DNA concentration measured with a spectrophotometer. DNA concentration should be around 0.3 mg/ml with a 260/280 ratio of 1.4-1.6. After the aliquot is taken, 2 µL/mL of phosphatase inhibitor cocktail I and II and protease inhibitor cocktail from Sigma are added to preserve the sample from degradation. These inhibitor cocktails were not added before because their solvent interferes with the absorbance of the sample, and thus could give us misleading results.

6.3 NUCLEI DIGESTION WITH MICROCOCCAL NUCLEASE

Aliquots of 80 µg (measured as naked DNA in 1% SDS) of nuclei suspension (~ 300 µl) were used for each digestion, and $CaCl_2$ was added to each one to reach a final concentration of 1mM prior to the micrococcal nuclease treatment, in order to avoid endogenous digestion as much as possible. Digestions were carried out by adding 100 U of MNase at 37ºC for 5 min. Reaction was stopped by addition of EDTA to a final concentration of 5 mM, SDS at a final concentration of 1% and placing the sample on ice for 5 min. DNA is recovered by sequential treatment with RNase for 1 h at 65ºC and then with 100 µg/mL of proteinase K for 30 min at 37ºC. Nucleosomal DNA is purified by phenolization (phenol-phenol/chloroform/isoamyl alcohol-chloroform/isoamyl alcohol (24:1)) and precipitation with 2.5 vol of ethanol and 1/10 vol of 3 M sodium acetate at -80ºC. The pellet is afterwards dissolved in 30 µL of sterile water. The obtained nucleosomal ladder is visualized by loading 2 DNA µg of each sample onto a 1.8% agarose gel and staining with ethidium bromide. To determine the correct size of the

fragments a molecular marker p100 (Pharmacia Biotech) was used. Finally, fragments of 145-200 bp from each sample were collected by gel purification and quantified by using picogreen.

6.4 NUCLEOSOME MAPPING: PCR AMPLIFICATION AND QUANTIFICATION

To determine the nucleosomal arrangement in *egr-1* promoter, both real-time and semi-quantitative PCR were used with a set of amplicons spanning the region of interest, from the transcription start to -800 (Figure 14 and Table 17). Each amplicon was designed to cover approx 100 bp on the template sequence, so that the regions of the promoter containing nucleosomes can be amplified if the full template is available. As primers of different amplicons do not have similar characteristics, and therefore similar efficiency, a control of total undigested DNA, sonicated to achieve a size of around 200 bp and purified from the gel with a size in between 150 and 200 bp, was used to normalise the efficiency of the reaction.

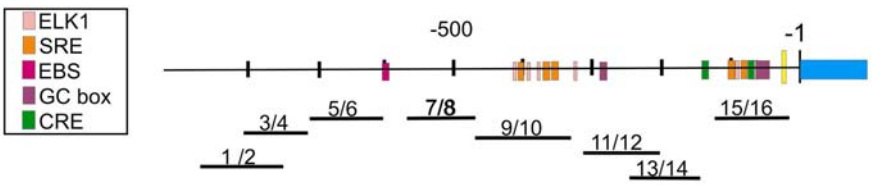


Figure 14. Schematic representation of amplicons along *egr-1* promoter. The cis-sequences of putative transcription factors are also represented in different colours, and TATA box is depicted as a yellow bar.

Table 17. Overlapping nucleotides for the analysis of egr-1 promoter. The oligonucleotides used for real-time PCR are described in the table with its relative position to the starting point.

Name	Oligo forward/reverse (5´-3´)	Position (bp)
Egr-1 1/2	AGGTGGGATCCTCAACCGCA/TGCATACTCGGCCACCAGTC	-1006/-894
Egr-1 3/4	CCAGTAGCCAAATGTCTTGGCC/TGAGCCTCTACTAATGGCAGGG	-763/-695
Egr-1 5/6	GCCATTAGTAGAGGCTCAGGTC/CGAGTCAGGCCAGGGTTCTA	-707/-601
Egr-1 7/8	TTCCCTAGCCCAGCTCGCAC/TCCCAACTGGGAGCCTGAGC	-569/-468
Egr-1 9/10	GACCCGGAAACGCCATATAA/AAGCGGCAGGGCCATATT	-396/-335
Egr-1 11/12	ATATGGCCCTGCCGCTTCCGGCTC/GTATTGGAACAGCAGCAGTGGCCTC	-335/-200
Egr-1 13/14	GGGGCAAGCTGGGAACTCCA/AGAGTGGTGGGCACCTCCAC	-286/-184
Egr-1 15/16	CGTCACTCCGGGTCCTCCCG/AGGGTCTGGAACAGCACGGGCC	-123/-16

Annex 4: Validation of the method

To validate the method used for mapping the nucleosome position in the promoter of *egr-1,* a DNA bearing 12 repetitions of a region (208 bp) of *Lytechinus variegatus 5S* gene[3], whose nucleosomal position is well known (Georgel *et al.*, 1993), was studied. Amplicons utilized for mapping the region are shown on Table 18, and the results obtained by semi-quantitative PCR agree with the known nucleosomal position. As a control, naked DNA, also from the array, has been sonicated and fragments from around 200 bp have been gel purified. As seen in Figure 15, primers 1/2, which are located inside the nucleosome region, give higher signal than the control, showing a probability higher than random of finding the

[3] Kindly provided by Dr. Ramón Sendra

nucleosome located there. On the contrary, primers 3/4 that require the whole internucleosomal region do not give any signal. In the case of primers 5/6, which overlap only in part with the internucleosomal region, a signal is detected but it has lower intensity than the control in both samples, indicating that the probability of finding the nucleosome in that region is lower than hazard.

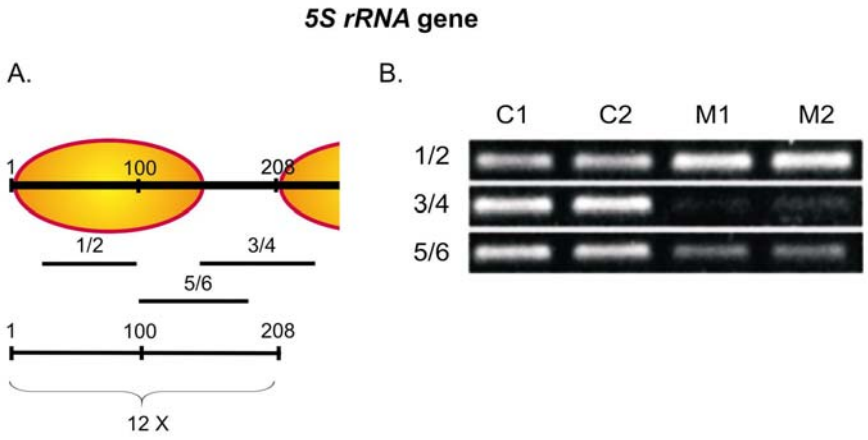


Figure 15. Nucleosomal mapping of the reconstituted 5S *Lytechinus variegatus* (sea urchin) gene. Figure A shows the schematic representation of the reconstituted gene with a scale bar to indicate the oligonucleotides position. Image B shows the result of the semi-quantitative PCR on a 2.0% agarose gel. C1 and C2 correspond to two independent naked DNA samples sonicated and gel purified to have an average size of 200 bp, whereas M1 and M2 correspond to two independent samples of gel purified mononucleosomes obtained by MNase I digestion.

Table 18. Overlapping primers used to map the reconstituted *5S Lytechinus variegatus* (sea urchin) gene with semi-quantitative PCR. Both the sequence and the position related to the starting site are shown in the Table.

Name	Primer up/dn (5´-3´)	Position (pb)	Genic Region
5s 1/2	AAGCCGATGACGTCATAACATCCCTG	+25	Exon 1
	ATTCAGCATGGTATGGTCGTAGGCTC	+98	Exon 1
5s 3/4	GCTCGGTTAGTACTTGGATGGGAGA	+149	Exon 1
	CGGCTTATAAATCCCTGGAAGTTATTCG	+238	Exon 1
5s 5/6	GCTGAATATACCGGTTCTCGTCCGA	+101	Exon 1
	GTATTCCCAGGCGGTCTCCCAT	+187	Exon 1

7. DESIGN OF RNA INTERFERENCE FOR *EGR-1*

RNA interference (RNAi) is a powerful method of knocking down gene activity. In mammalian cells introduction of short sequence-specific RNA duplexes that are 21-23 nt long is able to initiate post-transcriptional gene knockdown and avoid triggering the non-specific effect of the interferon response that may direct the cells to apoptosis. The RNAi process takes place by the cleavage of long double-stranded RNA (dsRNA) into 21-23 nucleotide short (or small) interfering RNA (siRNA) duplexes which is carried out by an enzyme called Dicer. The siRNA associates with an intracellular multi-protein RNA induced silencing complex (RISC), which is responsible for recognizing and cleaving complementary mRNA. This is then targeted for degradation and leads to the knock down of post-transcriptional gene expression in the cell. siRNAs can be transfected into cells (chemically synthesized or in vitro transcribed siRNAs) or endogenously expressed from vectors like pSUPER (transient expression) or viral vectors (stable expression).

7.1 SELECTION OF TARGET SEQUENCES AND OLIGO DESIGN

Selection of target sequences to clone into pSUPER is similar to the method used to select synthetic siRNAs targets. 19 mer oligonucleotides were designed using *Qiagen siRNA design tool* program and sequences were selected based on:

-similar position, of already existing siRNA of the same gene, in different species.
-sequences flanked in the mRNA with AA at the 5' and preferable also TT at the 3'
-the region at the mRNA to select the 19 nt from, should be preferably in the coding region: 100bp from the start or the termination of translation.
-GC richness should be preferably more than 30%.
-The 19nt selected should not contain a stretch of four or more Adenines or Thymines, as this will give premature termination of the transcript.
-The sequence should not match any other gene when blasted against the entire genome.

Table 19. **Target sequences for the siRNA for *egr-1* and oligonucleotides used to clone into pSUPER.** The sequences for the oligonucleotides are in capitals while the sequences for cloning are in lower-case.

Name	Target Sequence	Oligonucleotides forward/reverse (5'-3')
Sh1egr-1	aacgaagaggcataccaa	gatccccGTCGGGCTCCCAGGACTTAttcaagaga TAAGTCCTGGGAGCCCGACtttttggaaa
		agcttttccaaaaaGTCGGGCTCCCAGGACTTAtctcttgaa TAAGTCCTGGGAGCCCGACggg
Sh2egr-1	gtgggagggaagtttgccag	gatccccAACGCAAGAGGCATACCAAttcaagaga TTGGTATGCCTCTTGCGTTtttttggaaa
		agcttttccaaaaaAACGCAAGAGGCATACCAAtctcttgaa TTGGTATGCCTCTTGCGTTggg
Scrbl	ggaattcagcgtagctgact	gatccccAAGCTAGATGATGGGGCGAttcaagaga TCGCCCCATCATCTAGCTTtttttggaaa
		agcttttccaaaaaAAAGCTAGATGATGGGGCGAtctcttgaa TCGCCCCATCATCTAGCTTggg

Once the three target sequences were selected (Table 19) they were used to design the oligonucleotides used to clone into the pSUPER vector (Table 19). As a non-interfering vector was used a non-codifying sequence (scrbl) and as an additional control sh1egr-1 sequence was mutated in three positions to ensure that it did not worked (sh1egr-1mut).

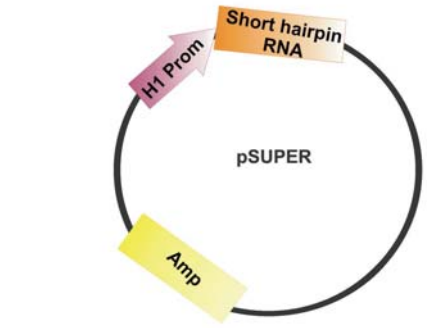
7.2 CLONING THE OLIGOS INTO PSUPER

7.2.1 Insert Preparation

Each pair of oligonucleotides, HPLC purified, was dissolved in the right amount of water to achieve 1 mM scale. 1µl of each one (forward and reverse) was added to 48 µl of freshly made annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Mg-acetate) and incubated for 4 min at 95º C to destroy any secondary structure they may form. Then they were incubated for 10 min at 70ºC to let them hybridize and slowly cooled down to 4 ºC. The annealed oligonucleotides were phosphorylated by the T4 polynucleotide kinase (New England Biolabs), following the manufacturer instructions, and stored at -20ºC until use.

7.2.2 Vector Preparation

pSUPER (Figure 16) was digested with BglII-HindIII and CIPed, using CIAP from Biotools. In doing this, the 5'-P groups from the linear plasmid are removed and therefore the religation with itself becomes more difficult. The sample was purified by gel extraction before to use it the ligation reaction. Ligation was done with 1 µl of ligase (Invitrogen) and leaving the reaction at room temperature overnight.



Piva et al., (Blood 2004)

Forward
5'GATCCCCGTCGGGCTCCCAGGACTTATTCAAGAGATAAGTCCTGGGAGCCCGACT
TTTTGGAAA

Reverse
5'AGCTTTTCCAAAAAGTCGGGCTCCCAGGACTTATCTCTTGAATAAGTCCTGGGAGC
CCGACGGG

Figure 16. pSUPER vector. Schematic representation of pSUPER vector used to transiently transfect cells with RNA interference for EGR-1. Below there is an example of the sequences used in the experiment. Target sequence is depicted in orange, whereas the sequences in black are responsible for the short hairpin formation.

7.2.3 Ligation Reaction

Ligated plasmid was used to transform competent bacteria. Bacteria were grown and use to plate a LBA Petri dish for screening of single colonies. Around 5 of the grown colonies were tested in each case for the correct insert, by double digesting them with EcoRI and HindIII. Positive clones should have inserts around 360 bp, and empty ones should have around 300 bp long fragment.

7.2.4 Bacterial Transformation

Genetic manipulations require large quantities of DNA. One of the easiest ways to get high amounts of DNA is to place the desired DNA into bacteria, allow them to grow and after harvest the bacteria and isolate the DNA. The transformation process consist in the uptake of a plasmid (which contains the desired DNA and a gene that gives resistance to an antibiotic), from a competent bacteria. Bacteria are later selected by plating then on agar plates that contain the antibiotic to which they are resistant.

7.2.4.1 Competent Bacteria Production for Thermal Transformation

100 ml of Dh5α bacteria were grown overnight, and 1 ml of the stationary culture in LB was seeded in 300 ml of LB. The culture was let to grow at 37ºC till it reached an A_{660} between 0.3 and 0.4 (around 2-4 h). Afterwards, culture was cold on ice for a couple of min and then it was centrifuged for 10 min at 3500 rpm. The pellet was resuspended in 20 ml of cold medium RF1 (0.1 M ClRb, 50 mM $Cl_2Mn.2H_2O$, 10 mM $CaCl.2H_2O$, 15% glycerol, 30 mM potassium acetate, pH 5.8 achieved with acetic acid 0.2 M), and the bacteria were left 30 min on ice. Bacterial suspension was centrifuged again for 10 min at 3500 rpm and afterwards the pellet was resuspended in 3 ml of cold RF2 (10 mM ClRb, 75 mM $Cl_2Ca.2H_2O$, 15% glycerol, 10 mM MOPS, pH 6.8 achieved with NaOH). Bacteri were left on ice for 15 min and aliquots of 50 µl were prepared to freeze them quickly in an acetone bath containing dry ice. Stocks were kept at -80ºC.

7.2.4.2 Thermal Bacterial Transformation

Frozen competent bacteria (Dh5α) were thaw in ice, and an amount of DNA between 0.1 to10 ng, in a volume not higher than 5 µl, added and mixed by slow rotation of the tube on the ice. Competent bacteria were left on ice for 30 min, and meanwhile 1 ml of LB was warmed at 37 ºC for each transformation. Then, bacteria were heated at 42ºC for 40 s. After the thermal shock bacteria were left 2 min on ice, and then 1 ml of preheated LB was added and kept for 1 hour at 37ºC in slow agitation. 100 µl from the grown cell culture were plated in a Petri dish with ampicilline (100 µg/ml). It is important to remember to have a negative control (competent bacteria without DNA insert that have followed the same treatment). Plate dish were grown at 37ºC overnight. The following morning there will be only colonies in the transformed plate and no colonies on the negative control.

7.2.4.3 Preparation of Electrocompetent Bacteria

Single *E. coli* colony was selected from a fresh LB plate inoculated the previous night and it was diluted into 10 ml of LB and let to grow overnight at 37ºC on a shaker (250 rpm). This starter culture was used to inoculate 1 L of 2X YT medium and it was incubated at 37ºC in a shaker until the absorbance of the cells at 600 nm was between 0.6 and 0.9 (log phase growth). At this point, cells were placed on ice[4], and the culture was divided into four equal parts and centrifuged at 4000 rpm for 25 min at 4ºC. Supernatant was removed immediately, as cell pellet begins to lift quickly, and the bacteria were gently resuspended in 200 ml of ice-cold distilled water. Cells were once more centrifuged at 4000 rpm for 25 min at 4ºC, and this time they were resuspended in 100ml of ice-cold distilled water, prior to a third centrifugation at 4000 rpm for 25 min at 4ºC. The four bacterial pellets were resuspended in 20 ml of 10% glycerol and transferred to two chilled 50ml conical tubes. Samples were then centrifuged at 4000 rpm for 10 min at 4ºC, and finally resuspended in 1ml of ice-cold 10% glycerol (OD600 of resuspended cells should be around 200-250). Cell suspension was stored in 70 µl aliquots in pre-chilled 1.5 ml eppendorf tubes and frozen in liquid N_2. Cells were kept at -80ºC until use.

7.2.4.4 Electroporation of Competent Bacteria

Frozen competent bacteria (Dh5α) were thaw in ice, and 1 µl of the ligation reaction was added. Mixture was then transferred to the electroporation cuvettes (Bio-Rad) that are kept at 4ºC. The conditions for the electroporation[5] are set at 25 µFD, 200 Ω, and 1.8 kV, with a time constant around 3-4 ms. Once the instrument is set, electroporation cuvettes containing the cells are well dried and placed into the electroporation device. The tone indicates that cells have been successfully electroporated, whereas if there is a sparking coming for the cuvette cells indicates that cells have died and that the procedure should be repeated. When successfully done, add 0.5ml of LB to the cells in the cuvette immediately and transfer them to a

[4] It is very important to keep cells at 4ºC, or on ice, for the remainder of the protocol.
[5] Electroporation conditions vary from cell strain.

new eppendorf tube so they can grow for 1 h at 37ºC, and therefore they can recover from the treatment. After, centrifuge at 500xg for 5 min to pellet the cells and discard all the media but 100 µl to resuspend them. Plate the cells in a Petri dish with ampicilline (100 µg/ml) and remember to have a negative control (competent bacteria without DNA insert that have followed the same treatment). Grow the plate dish at 37ºC overnight. The following morning there will be only colonies in the transformed plate and no colonies on the negative control.

7.2.5 Small-Scale Plasmid DNA Purification Kits

Transformed bacteria producing the desired construction were grown overnight in 5 ml of LB containing 100 µg/ml of ampicilline at 37ºC, and then centrifuged to pellet the bacteria. The plasmid was obtained by alkaline lysis using the QIAprep Spin Miniprep Kit (Quiagen), and following the manufacturer's instructions except for the elution step which was done with sterile water, so the buffer would not interfere in the following treatments. Plasmid DNA concentration was calculated by measuring the absorbance at 260 nm and stored at -20ºC until use.

7.2.6 Glycerol Stocks

Bacteria producing the short interference sequences that proved efficient were stored in glycerol solution to preserve them for one or two years. To do this 750 µl of overnight-grown culture were added to 750 µl of LB media containing 40% glycerol in a screw-cap vial and then kept at -80ºC until use.

To revive the stored stock, streak some of the glycerol stock on a LB plate containing 100 µg/ml of ampicilline, the following day, screen for single colonies.

7.3 TESTING OF shRNA ACTIVITY AGAINST *egr-1* BY CELL TRANSFECTION

Cells used for transfection had been grown without antibiotics until they reached a confluence of around 80%, and then kept for 24 h in serum-starvation. The transfection was carried out using Lipofectamine 2000 (Invitrogen) reagent and following the manufacturer's instructions. Once cells had been transfected for 24 h, *egr-1* presence was tested either by real-time PCR or immunofluorescence.

8. IMMUNOFLUORESCENCE OF MLP29 MOUSE CELL LINE

Immunofluorescence is a technique that allows the visualization of a specific protein or antigen in cells or tissue sections by the binding of an antibody that is chemically conjugated with a fluorescent dye (for example, fluorescein isothiocyanate (FITC)). This is termed direct immunofluorescence in opposition to the indirect immunofluorescence, in which the first antibody is recognized by a secondary antibody labelled with a fluorochrome. Immunofluorescence staining can be done both on cells fixed on slides and tissue sections, and the resulting stained samples are examined under a fluorescence or confocal microscope.

MLP29 cells were plated on sterile coverslips (immersed in 100% ethanol and air dried) 24 h before fixation, at an optimum density to reach 50% confluence. The following day cells were treated with the desired drug (TPA, kinase inhibitors, and so on.) for the appropriate time interval and washed twice with room-temperature[6] PBS prior to be fixed by addition of 10% formaldehyde neutralized and stabilized with methanol (Panreac) during 30 min at 37ºC. Solution was removed by washing the slides with 0.1 M PB-0.05% Triton X-100, and the detergent served also to permeate the cells. To block the unspecific antibody union, coverslips were incubated with 10 % NGS (Normal Goat Serum, Gibco) diluted 1:10 in 0.1 M PB-0.05% Triton X-100, for 1 hour at room temperature. Samples were then incubated overnight with 200 µl of the antibody Egr-1 (Santa Cruz, sc-110, 1:1000 diluted in

[6] It is important to wash the cells with room temperature PBS, otherwise cells may shrink.

10% NGS in 0.1 M PB- 0.05% Triton X-100) in a humid chamber at 4ºC. The unattached antibody was removed by extensively washing the cells three times during 10 min with 0.1 M PB. Addition of the secondary antibody labelled with Anti-Rabbit Ig TRITC (Jackson, dilution 1:200 in 10% NGS in 0.1 M PB-0.05% Triton X-100) was done in the same manner. Cells were incubated for 1h at room temperature on a humid chamber with 200 µL of the antibody dilution. Slides were washed, as before, three times with 0.1 M PB and then mounted with a drop of Fluorsave (Calbiochem) containing DAPI[7] (Sigma, 5mg/ml, water diluted) 1:1000 dilution on the slide. Let the chemical to polymerize for at least 2 h at room temperature and the preparation is ready to be seen on the fluorescence or confocal microscope in the next two days. Samples prepared this way can be stored at 4ºC for up to two months.

Analysis of the samples was done with a Leico TCS-SP2 confocal microscopy equipped with argon and helium-neon lasers, and connected to an inverted Leica DM1RB microscope.

[7] DAPI has to be well shacked, as it has solubility problems. Once diluted can be froze stored at -20ºC, protected from light.

RESULTS AND DISCUSSION

1. SELECTION OF THE SYSTEM OF STUDY

1.1. SELECTION OF THE CELL LINE

The use of cell cultures in the research field has implied a great advantage to study, at a chromatin level, the changes that take place in promoters of mammalian genes, when they are activated by means of different stresses. Firstly, it enables a more accurate control of the experimental conditions and secondly, it ensures a greater reproducibility of the results, as it avoids the problems regarding the animals' genetic background. Moreover, it serves as a simplifying model that can afterwards be corroborated by *in vivo* studies.

In order to find an appropriated biological system to study the changes caused by oxidative stress it was decided to test the response to arsenite of two cell lines: the fibroblastic cell line C3H10T1/2 (ECACC 85012201) and the liver progenitor cell line MLP29 (Medico *et al.*, 1996). Arsenite was added to serum starved cells to a final concentration of 400 μM for 1 h (Li *et al.*, 2001), and the levels of expression of diverse immediate-early genes typically induced during this stimulus, such as *c-fos* (Nriagu *et al.*, 2000; Hu *et al.*, 2002), *c-jun* (Nriagu *et al.*, 2000; Hu *et al.*, 2002) and *gadd153* (Guyton *et al.*, 1996; Tang *et al.*, 2002), were analyzed by RT-PCR.. The results obtained (Figure 17) show a clear increase in the expression of all genes tested, the only difference being a higher increase of *c-fos* in MLP29 than in C3H10T1/2 cell line.

Given the fact that no large differences between the expression patterns of both cell lines exist, the decision to choose any of them was based in the biomedical interest of the study. In this sense, the study in the hepatic cell line seems to be of higher interest since different researchers correlated pathological processes as hepatocellular carcinome development (Bernstam *et al.*, 2000), acute inflammation (Jaeschke, 2000) or problems derived from hepatic transplantation (Cantisani *et al.*, 2004), with the presence of reactive oxygen species (ROS) in this cellular type. Taken this information into account, the liver progenitor cell line MLP29 (Figure 18) was selected to be used in the following experiments.

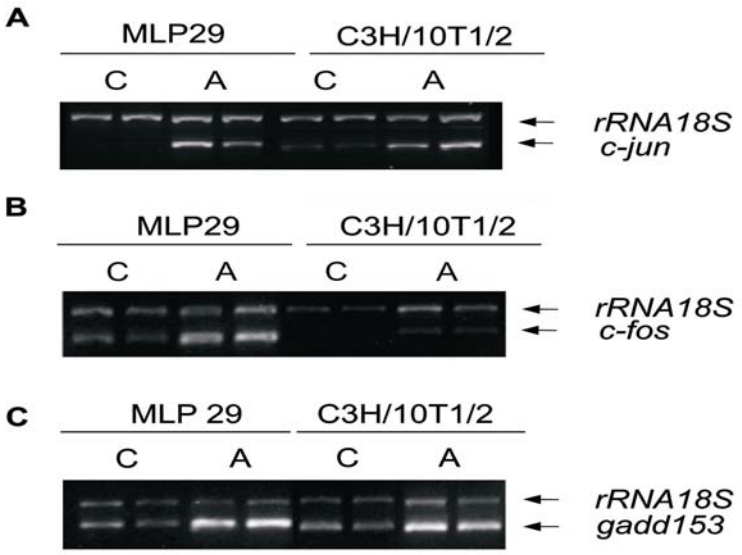


Figure 17. Analysis of *c-jun* (A), *c-fos* (B) and *gadd153* (C) expression in MLP29 and C3H/10T1/2 cell lines stimulated with 400µM arsenite for 1 hour. Total RNA from untreated (C) and arsenite treated (A) cells was purified and analyzed by semi-quantitative RT-PCR. As an internal control *rRNA* 18S gene was co-amplified with the genes of interest. They are shown in duplicates of two independent experimental samples.

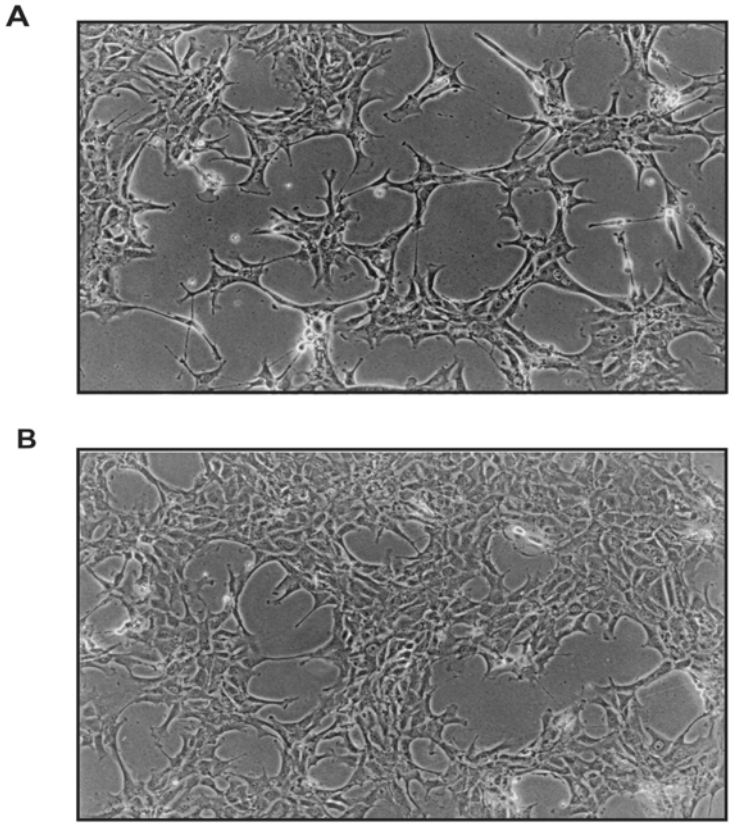


Figure 18. Micrography of the mouse liver progenitor cell line (MLP29). Cells were grown in 100 mm X 20 mm dishes until 40% (**A**) or 60% (**B**) confluence. Pictures were taken with a Nikon FG-20 camera connected to an inverted Nikon DIAPHOT microscopy (100X magnification).

1.2. SELECTION OF THE STRESS CONDITIONS

Following the selection of MLP29 cell line as a biological material, the next step was to select the appropriated conditions to generate an oxidative stress in those cells. From the main drugs used in the literature to produce a robust gene expression pattern, we tested four stress-inducing agents: sodium arsenite, 12-O-tetradecanoylphorbol-13-acetate (TPA), ter-butylhydroperoxide (t-BhP) and UV irradiation. Besides, these agents have biological significance, since, for instance, sodium arsenite is a tumour promoter (Luster *et al*., 2004) involved as well, in many important pathologies as type 2 diabetes (Navas-Acien *et al.*, 2006) or TPA is a carcinogen (Lee *et al.*, 2006). The cells, after serum starvation, were treated in a range of concentrations from 25 to 100 nM for TPA (Zheng *et al.*, 2002) for 1 h, or from 100 to 400 μM for sodium arsenite during 1 h (Li *et al.*, 2001) (Figure 19).

Furthermore, to mimic an oxidative stress caused by production of hydroxyl radicals (Hwang *et al.*, 2002), t-BhP in a concentration range from 25 to 100 mM for 30 min was also used (Figure 19). Finally, cells were, alternatively, irradiated with UV light for time intervals between 5 and 15 s at 65 W/m^2 (Giordani *et al.*, 2000). Total RNA from those treated cells was purified and expression of some sensor genes such as *gadd153*, *trx*, *c-jun* or *c-fos* was analyzed by RT-PCR to select the appropriate treatment conditions (Figure 20 and 21).

The results from semi-quantitative PCR (Figure 19) were normalized against *rRNA 18S* gene and plotted (Figure 20). The data obtained show a strong induction of the genes in response to TPA and arsenite, in contrast to a much weaker stimulation of expression produced when cells are exposed to either t-BhP or UV light. Moreover, in general, TPA induction seems to be more homogeneous and dose-dependent than arsenite for the genes tested.

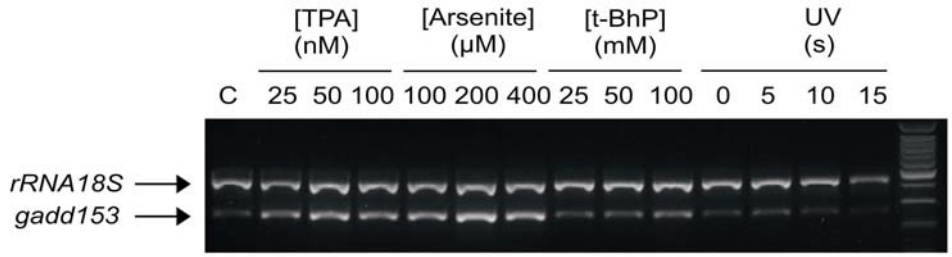


Figure 19. RT-PCR analysis of *gadd153* gene expression induced by different stress-inducing agents. MLP29 cells were either treated with different concentrations of TPA (from 0 to 100nM), arsenite (from 0 to 400μM) and t-BhP (from 0 to 100mM) or UV irradiated (during 0 to 15s at 65 W/m^2). Total RNA was purified and analyzed by semi-quantitative RT-PCR. As an internal control *rRNA* 18S gene was co-amplified with the gene. Results are representative of, at least, three different experiments.

To further check the differences between the treatments with TPA and arsenite with on one part and those with t-BhP and UV light, real-time PCR analysis of *c-fos* and *gadd153* genes was carried out, and the results proved to be comparable to those obtained by semi-quantitative PCR (Figure 21).

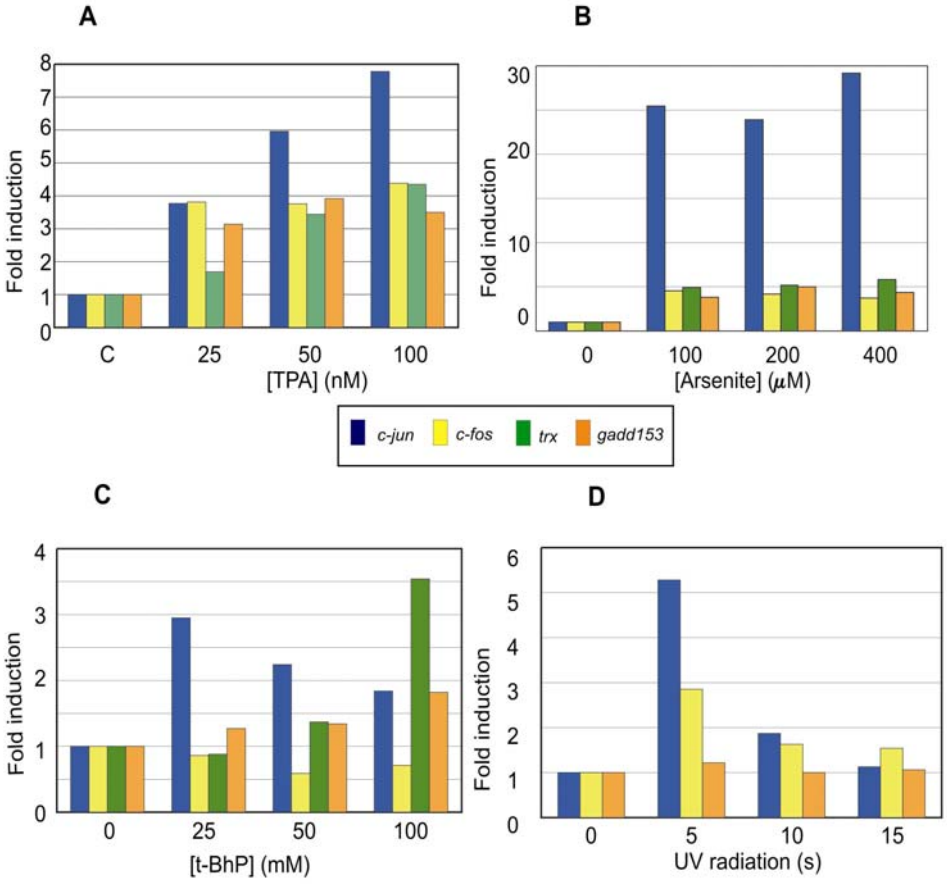


Figure 20. RT-PCR analysis of *c-jun*, *c-fos*, *trx* and *gadd153* gene expression. MLP29 cells were treated with TPA (**A**), arsenite (**B**), t-BhP (**C**) or UV irradiated (**D**), and total RNA was purified and gene expression analyzed by semi-quantitative RT-PCR. Bands were scanned and standardised relative to *rRNA* 18S gene expression. Control, without treatment, was arbitrarily assigned to 1.

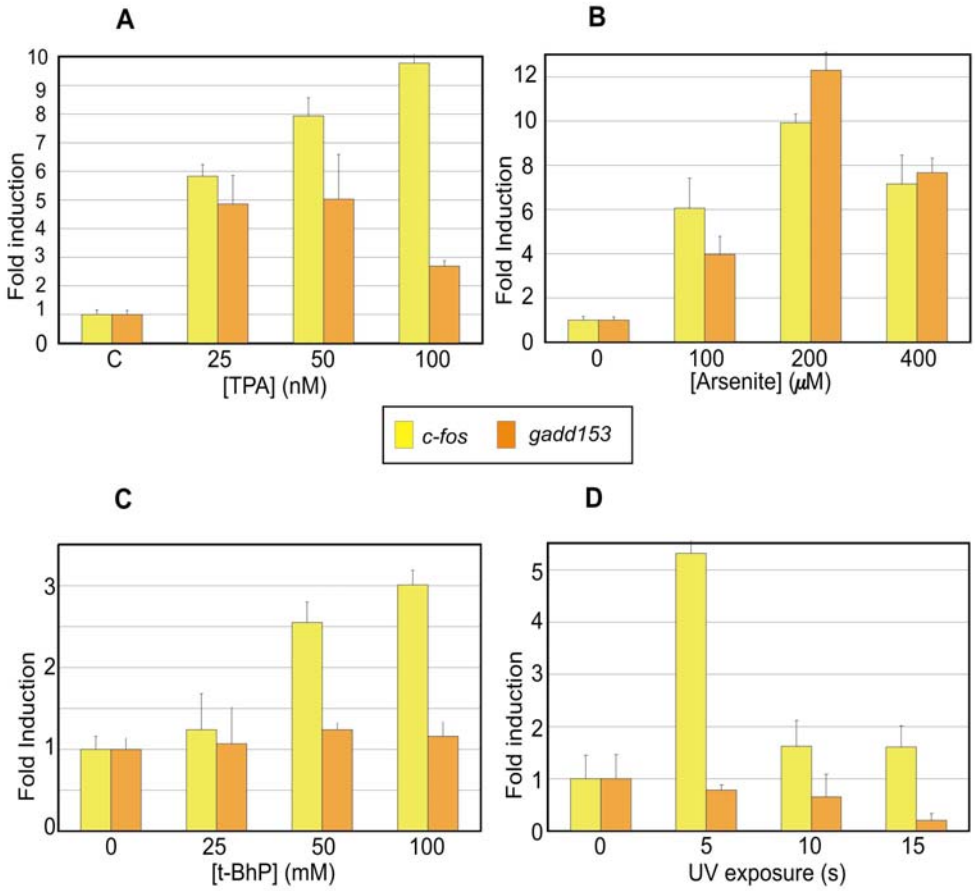


Figure 21. Real-time RT-PCR analysis of *c-fos* and *gadd153* gene expression. MLP29 cells were either treated with TPA (**A**), arsenite (**B**) and t-BhP (**C**) or UV irradiated (**D**). Total RNA was purified and gene expression was analyzed by real time RT-PCR, and normalized relative to *rRNA* 18S gene expression. The control without treatment was arbitrarily assigned to 1.

In conclusion, since TPA shows a more homogenous induction expression pattern than arsenite and, as Berstam *et al.*, (2000) pointed out, the latter produces morphological aberrations in hepatic cell lines that lead the cells to apoptosis, it was decided to use TPA as an induction agent in the following experiments. Moreover, the signalling pathways elicited by TPA seem to be more specific and defined than those started by arsenite, which simultaneously acts upon many different kinases (Tanaka-Kagawa *et al.*, 2003).

Once the agent to be used was selected, the remaining issue was to choose an appropriate concentration, in order to obtain an increment in the gene expression without producing an excessively toxic effect in the cells. For this reason, it was decided to use a 50 nM concentration, which significantly induces all the genes tested in a short period of time without affecting the morphology of the cells.

1.3. SELECTION OF THE GENE TO STUDY

To fulfil a convenient study of the chromatin changes that take place in a gene during its induction, it is necessary to select a gene whose expression pattern is clearly altered in response to a certain stimulus. Consequently, once we had established the biological model to carry out the experiments, that is to say mouse liver progenitor cell line (MLP29) and the concentration and the agent to induce expression (50 nM TPA), we proceed to analyze the expression pattern of a set of genes involved in biological processes such as: transcriptional regulation, immune response, cellular signalling, aminoacid metabolism or apoptosis. This set of genes was partially extracted from a work in which the changes of global expression of mouse cells subjected to oxidative stress with H_2O_2, was analyzed by DNA microarrays. This work was carried out in collaboration with the Physiology Department of the Faculty of Medicine of Valencia.

Among the genes analyzed by semi-quantitative RT-PCR, those of which expression was found to increase with TPA treatment were the following: *trb3*

(tribbles homolog 3), *hif1* (hypoxia inducible factor 1), *atf3* (activating transcription factor 3), *atf4* (activating transcription factor 4), *atf5* (activating transcription factor 5), *C/EBP-γ* (CCAAT/enhancer binding protein gamma), *asns* (asparagine synthetase), *gc/m* (glutamate-cysteine ligase, modifier subunit), *gys1* (glycogen synthase 1, muscle), *psme-1* (proteasome 28 subunit, alpha), *psph* (phosphoserine phosphatase), *sclc1a4* (solute carrier family 1-glutamate/neutral amino acid transporter-member 4), *pycs* (pyrroline-5-carboxylase synthetase: glutamate gamma-semialdehyde synthetase), *pck1* (phophoenolpyruvate carboxykinase), *Ier2* or *pip92* (immediate early response 2), *egr-1* (early growth response 1) and *nur77* or *Nr4a1* (nuclear receptor subfamily 4, group A, member1).

As an example of the genes analyzed, Figure 22 shows the induction of *egr-1*, *pip92* and *nur 77* in cells stimulated by TPA. The three of them encode for transcriptional factors involved in different gene activation process, and therefore, they are interesting for our study as they have to act in a chromatin environment. As seen in Figure 22, *egr-1* and, in less extension, *pip92* have a significant increase in their expression at a relatively short time from the treatment, whereas the expression of *nur77* is somewhat delayed on time and its increase is more subtle.

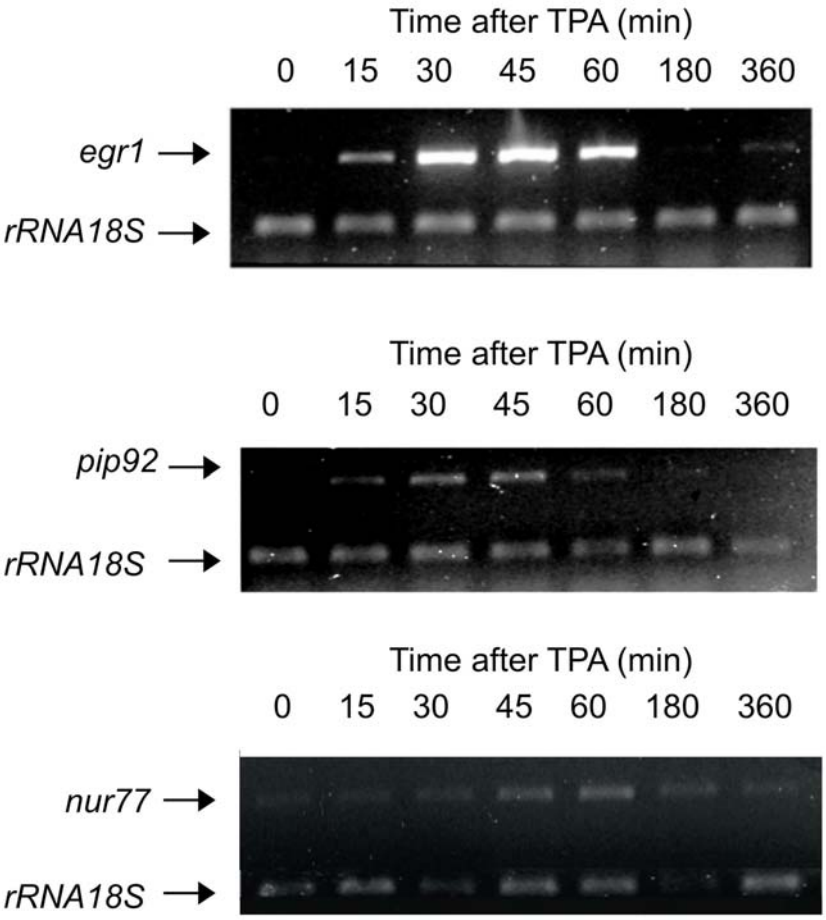


Figure 22. RT-PCR analysis of *egr-1*, *pip92* and *nur77* expression. Serum-starved MLP29 cells were treated with 50 nM TPA, and total RNA was purified and analyzed by semi-quantitative RT-PCR. As an internal control *rRNA* 18S gene was coamplified in each case.

In view of the above reasons, we decided to make *egr-1* the gene of choice to study the structural and functional changes in its chromatin, since it encodes for a transcriptional factor that exerts important roles in regulating cell proliferation (Wada *et al.*, 2003), apoptosis (reviewed by Ahmed, 2004) or tumour suppression (reviewed by Liu C. *et al.*, 1996) by activating secondary-response genes.

2. ANALYSIS OF *egr-1* EXPRESSION

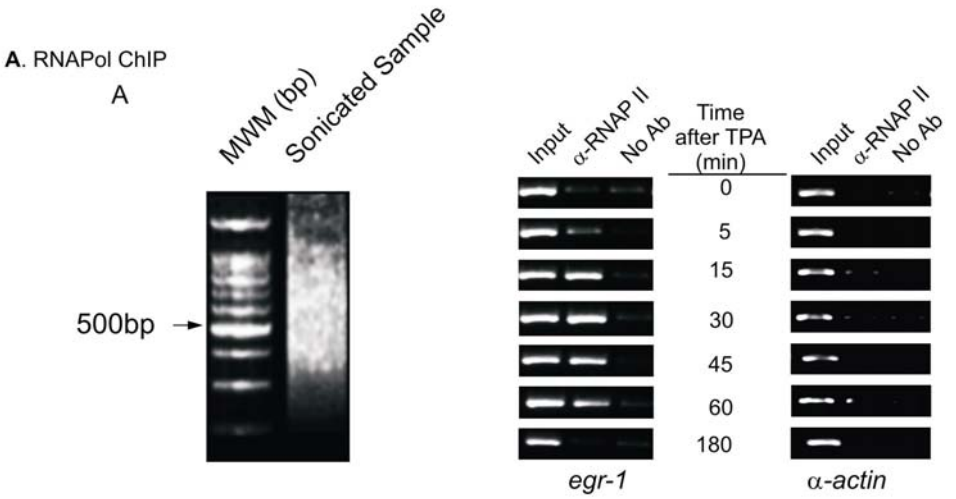
The study of the events that take place in the chromatin of the promoter of a gene during its induction require an accurate analysis of its expression, in order to correlate those events with the transcription process. Among the most common methods used to quantify transcriptional rate, the more useful and powerful is RT-PCR. This technique allows a sensitive detection of mRNA with a relative small amount of starting sample although all the variables that influence the amplification reaction should be tightly controlled. Its main disadvantage is that it only measures steady-state levels of mRNA, and not the actual transcriptional rate.

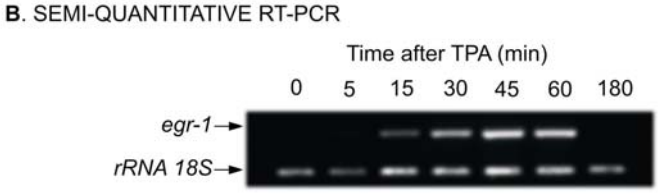
Chromatin immunoprecipitation (ChIP) is a powerful approach that allows the analysis of the interaction of transcriptional factors with DNA, thereby providing a live cell picture of the native chromatin structure. A novel application of this methodology termed RNAPol ChIP and developed by us (Sandoval *et al.*, 2004), consists in detecting the RNA polymerase II within the coding region of the gene under study. Using this technique, in combination with the standard RT-PCR, we have established a precise pattern of the transcriptional rate of *egr-1* in MLP29 cells treated with TPA. For these experiments we used oligonucleotides that specifically amplify a region within exon 2 of the coding region (Figure 23).

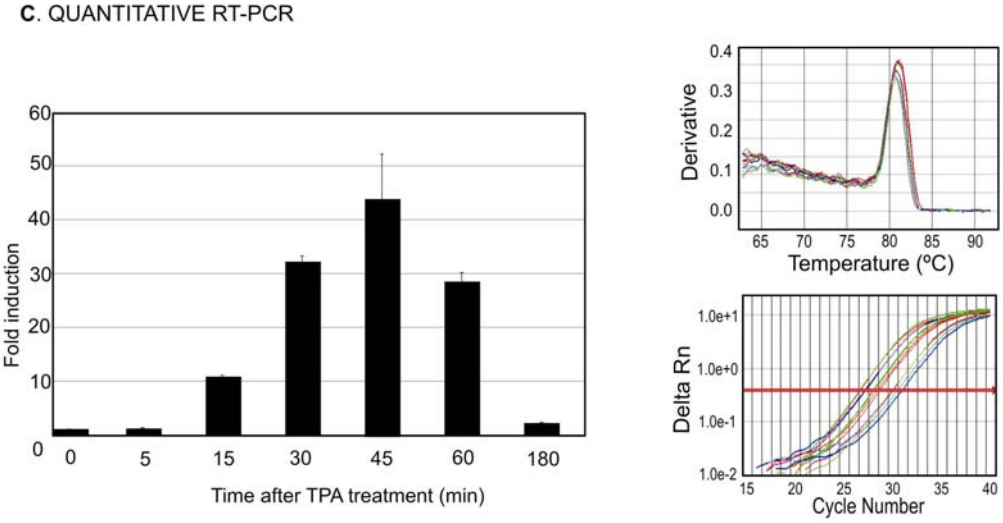
The results obtained with RNAPol-ChIP technique show that it is possible to detect *egr-1* transcription as soon as 5 min after TPA treatment. This transcriptional rate increases until it reaches a maximum around 30 min, and then decreases steadily, being no longer detectable after 180 min of the induction. Some differences can be observed between the results obtained by RNAPol-ChIP and by mRNA analysis by RT-PCR. The early RNA pol II activity at 5 min, cannot be detected by mRNA analysis in which the induction of *egr-1* is first noticeable at 15 min after the treatment. Moreover, the maximum level of mRNA is observable at 45 min, when measuring it by both, semi-quantitative and real-time RT-PCR. These minor differences in the expression timing of RNAPol-ChIP and RT-PCR analysis can be due to the fact that the first is able to detect real-time transcription, whereas the latter

detect steady-state levels of mRNA that may be influenced by the messenger metabolism.

Figure 23. Transcriptional analysis of *egr-1* in MLP29 cell line after TPA treatment. (**A**) RNAPol-ChIP assay of *egr-1* transcription. The panels in the top show PCR analysis of the DNA extracted from: input samples, Immunoprecipitated with RNA pol II antibody (α-RNAP II) and as a specificity control, without antibody (No Ab). The left panel shows an electrophoresis of the sonicated samples to estimate the size of the chromatin fragments. (**B**) RT-PCR analysis of *egr-1* transcription. Total RNA was purified and analyzed by semi-quantitative RT-PCR. As an internal control *rRNA* 18S was used. (**C**) Quantitative analysis of real-time RT-PCR of *egr-1* expression. Samples were analyzed in triplets and normalized *versus rRNA* 18S. The sample untreated with TPA was arbitrarily assigned to value 1. The melting curves of the amplicons and the plot of amplicon production *versus* number of cycles are given as PCR product verification.


A. RNAPol ChIP
A
MWM (bp)
Sonicated Sample
500bp
Input
α-RNAP II
No Ab
Time after TPA (min)
0
5
15
30
45
60
180
egr-1
α-actin


B. SEMI-QUANTITATIVE RT-PCR
Time after TPA (min)
0 5 15 30 45 60 180
egr-1
rRNA 18S


C. QUANTITATIVE RT-PCR
Fold induction
Time after TPA treatment (min)
Derivative
Temperature (°C)
Delta Rn
Cycle Number

3. SIGNALLING PATHWAYS INVOLVED IN *egr-1* ACTIVATION

Once the pattern of *egr-1* expression was established, we were interested in knowing which signalling pathway drives TPA induction of the cells towards the promoter of the *egr1* gene. To answer this question, serum-starved cells were pre-treated during 30 min with inhibitors of the main kinase cascades (i.e. ERK, MEK 1/2, p38, PKA, PKC and PI3K) prior to the incubation of the cells with TPA.

Figure 24 shows the expression level of *egr-1* analyzed by real-time RT-PCR after using each of the inhibitors tested. Results show that both MEK 1/2 (inhibitor PD98059) and p38 (SB203580) kinase cascades are involved in the transduction of the signal from TPA to *egr-1* promoter, as gene expression is greatly reduced. On the contrary, the remaining cascades tested: JNK (inhibitor peptide i-JNK), PKC (inhibitor hypericin), PI3K (inhibitor wortmanin) or ERK1 /2 (inhibitor peptide i-ERK 1/2) do not seem to have any control over *egr-1* expression induced by TPA. All the same, the use of inhibitors specific for transcription factors, such as NFκB or STAT3, shows that none of the signalling pathways in which those factors are involved regulates the expression of the gene.

These results prompted us to study weather TPA induction of *egr-1* is caused by means of oxidative stress, due to the presence of free radicals. To address this issue, we analyzed the effect of diverse antioxidants, like ascorbate, Vitamine C, Trolox or N-acetyl-cysteine, in the activation of the gene by phorbol esters. The results shown in Figure 24 indicate that only Trolox reduces the induction to less than 50% the induction, whereas the two other antioxidants have only minor effects.

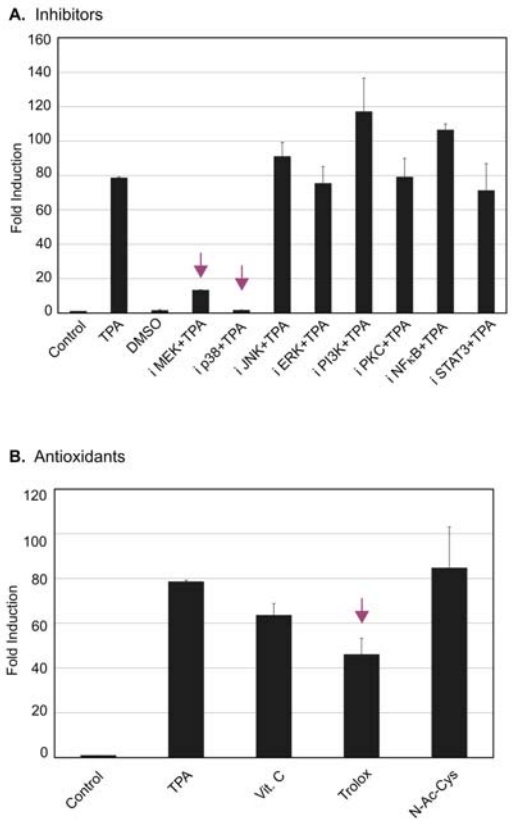


Figure 24. Effect of the protein kinase inhibitors and of the antioxidants over *egr-1* expression in TPA treated MLP29 cells. (A) Serum-starved cells were or were not pre-incubated for 30 min with MEK1/2 inhibitor (PD98059), p38 inhibitor (SB203580), JNK1/2 inhibitor (peptide i-JNK), PI3K inhibitor (Wortmanin), PKA inhibitor (H89), PKC inhibitor (hypericin), ERK1/2 inhibitor (peptide i-ERK 1/2), NFkB inhibitor (peptide i-NFkB) and STAT3 inhibitor (peptide i-STAT3) at the indicated doses (section 1.2.2 from *Materials and Methods*). Then, cells were or were not treated with TPA during 1 h and then harvested. Total RNA from those cells was purified and analyzed by real-time RT-PCR using oligonucleotides that spans *egr-1* coding region (Table 11) and the results were normalized relative to *rRNA* 18S gene. Control sample, TPA untreated cells, was arbitrarily assigned to a value of 1. Data are representative of three independent experiments. **(B)** Serum-starved cells were pre-incubated or not for 30 min with different antioxidants such as vitamin C, Trolox or N-acetyl-cysteine prior to 1 h TPA treatment, and *egr-1* expression was analyzed as in panel A.

These experiments indicate that the signalling cascade that connects *egr-1* expression to TPA treatment is not mainly caused by the action of free radicals but through MEK 1/2 and p38 cascades. Bauer *et al.*, (2005) correlate *egr-1* induction upon TPA treatment with activation of the MEK-ERK pathway. Our results do show the importance of MEK 1/2 in the activation, but nevertheless they do not seem to give the same importance to the ERK pathway. The reasons for this discrepancy are not clear, and therefore the results are misleading, but it could be due to the existence of a different MEK 1/2 signalling pathway that bypasses ERK 1/2 for *egr-1* activation. It is also important to note that TPA strongly activates p38, in agreement with previous data showing that the phorbol ester can phosphorylate p38 (Guo *et al.*, 2005), but to date, no report has implicated both signalling cascades in the up-regulation of *egr-1*.

Finally, it has to be remarked that interestingly in our system, TPA does not act through PKC to activate *egr-1*, despite the commonly accepted fact that phorbol esters act mainly through this particular protein kinase (Brose *et al.*, 2002).

4. TRANSCRIPTIONAL FACTORS ASSOCIATED TO *egr-1* PROMOTER DURING ITS INDUCTION

Data presented until this point have enabled us to know that TPA activates the MEK 1/2 and p38 kinase cascades, which in turn must have an effect over the chromatin of *egr-1* and therefore dictate its robust and quick induction. The question to be addressed now is how this effect takes place over the promoter of the gene, which kind of histone modification, remodelling complexes and transcriptional factors are needed to regulate the expression of the gene by TPA. To answer it, we have used the chromatin immunoprecipitation technique, ChIP, using antibodies against the different factors. As previously mentioned, the method allows the *in vivo* detection of proteins bound to the chromatin, and as a result, it enables us to study which proteins and at what time are they bound to *egr-1* promoter. This, in turn, may give us a clue as to what factors have a role in the regulation of the gene.

First, we analyzed which transcriptional factors are recruited to *egr-1* promoter during its activation. To this purpose, we performed ChIP studies of MLP29 cells at different times after TPA treatment. Crosslinked chromatin obtained at different times after induction was subsequently immunoprecipitated with antibodies against: SRF, ELK1, SP1, EGR1, CREB, NAB1 and NAB2. All these transcription factors have putative binding sites in the promoter of *egr-1* (see point 2.2 from *Introduction* section), as was established by TRANSFAC program analysis, except for NAB1 and NAB2 that have been described to bind to EGR1 in order to stimulate its repressing activity (Thiel *et al.*, 2002).

Serum response factor, SRF, is a transcriptional factor whose functionality depends on the formation of a ternary complex with the DNA and an Ets element (Shaw *et al.*, 1989). Its binding sequence can be found in many genes, and it has been related to biological events such as neuronal transmission or cellular growth (Chai *et al.*, 2002). In our results, (Figure 25 B), SRF is intensively and constitutively bound to *egr-1* promoter. The high signal obtained could be attributable on one hand, to the high affinity of the antibody, but one the other hand,

there are five putative binding sites for SRF and this factor is able to bind as a dimmer, which no doubt could produce a signal for SRF higher than for the other transcription factors that are less represented. Furthermore, ELK1, one of the Ets domain transcription factors, involved in cellular functions like cell differentiation or proliferation upon ternary complex formation with DNA and SRF (reviewed in Buchwalter *et al.*, 2004) is also constitutively bound. This could lead us to hypothesize that this ternary complex is permanently present all the time in the chromatin of *egr-1* promoter, but it is not always active, possibly because it needs to be further modified by any of the kinase cascades activated by TPA.

CREB (cAMP response element binding) is a transcriptional factor which binds to specific sequences in the DNA to increase or to decrease the transcription of certain genes. CREB can be bound by CBP (CREB binding protein) in a process that enhances its activity (reviewed in Johannessen *et al.*, 2004). This transcriptional factor has many functions in different organs such as long term memory in the neurons (Barco *et al.*, 2006). Our results show (Figure 25 B) that CREB is also constitutively bound at the promoter, though it seems to decrease slightly during the peak of transcription, between 15 and 30 min after TPA induction. There could be various reasons for this: firstly, it could be that CRE, which is located near the transcription starting point, has only a weak effect in the *egr-1* induction in TPA-induced expression. Secondly, it could be that the epitope recognized by the antibody against CREB, due to the localization of the factor near the starting point, is somewhat inaccessible to the antibody because it may be hidden by the PIC and/or mediator machineries. The importance of CREB in TPA-induced *egr-1* expression is still controversial, as some authors gave a marginal role to CREB (Bauer *et al.*, 2005), whereas other authors show that *egr-1* expression was up-regulated in constitutively active CREB mutants, indicating an important role for this transcription factor in the induction (Raychowdhury *et al.*, 2002). These results, reveal that CREB is always bound to its binding site, but do not let us, so far, to decide its role in controlling gene expression because a previous phosphorylation to become functional might be required. This assumption was afterwards checked, as shown below.

Among the transcriptional factors analyzed, stimulating protein factor, SP1, belongs to a family of transcription factors which bind GC/CT- rich elements found in the promoter of multiple genes (reviewed in Safe *et al.*, 2005). In our studies, SP1 has been the only one whose significant decrease has been detected at the peak of transcription (Figure 25 B). These results are in agreement with those of Cao *et al.*, (1993), in which they demonstrate a drop in SP1 binding abilities upon TPA-induction of *egr-1*. A feasible reason for this could be that, since SP1 and EGR1 have partially overlapping binding sites (Silverman *et al.*, 1998), both factors may compete for binding *egr-1* promoter, although the presence of both proteins at 60 min post-induction does not seem to agree with such possibility. In any case, SP1 would have a repressive role. For instance, SP1 can be deacetylated by HDAC1 and so mediate transcriptional repression (Bouwman *et al.*, 2002). Inocente *et al.*, (2005) also related SP1 activity to the repression of cyclin B1 transcription.

Finally, EGR1 factor, which is able to bind to specific GC-rich sequences, and which has been related to the regulation of tumour suppressor (Baron *et al.*, 2006), has been shown to bind to its own promoter at 15 min after the gene induction. EGR1, remains bound until 3h, when is not longer detectable by ChIP. This protein, as mentioned in the *Introduction*, has an activating and a repressing domain. The repressive domain becomes active upon binding two proteins: NAB1 and NAB2 (Thiel *et al.*, 2000). Our results show that both NAB1 and NAB2 are not recruited to the promoter until 30 min after the treatment. Therefore, it seems reasonable to argue that EGR1 may bind first to its promoter to enhance its own transcription and subsequently, the union of the co-repressors activates its repressive domain. This mechanism serves as an autoregulatory loop, in which EGR1 is implicated in the inhibition of its own expression. The situation, indeed, is more complex, because EGR1 is also responsible for activating NAB2 transcription, albeit NAB1 is constitutively expressed (Swirnoff *et al.*, 1998; Lucerna *et al.*, 2003).

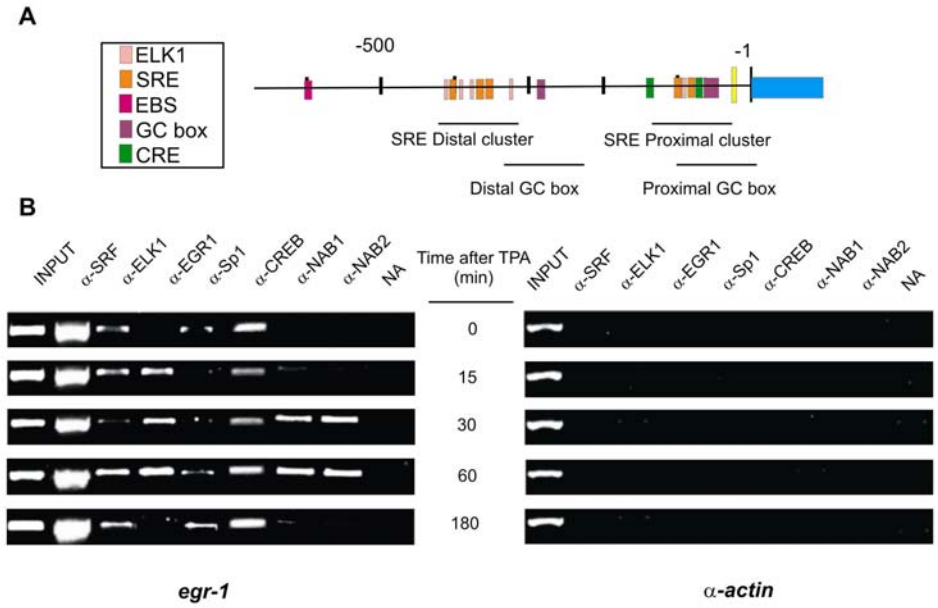


Figure 25. Recruitment of transcription factors to *egr-1* promoter. (**A**) Map of *egr-1* promoter with coloured boxes representing the binding sites for the different factors: ELK1 (ELK1 binding site), SRE (SRF binding site), EBS (EGR1 binding site), GC box (SP1 binding site) and CRE (CREB binding site). (**B**) Time-dependent ChIP analysis of MLP29 cells incubated or not with TPA. Crosslinked chromatin was immunoprecipitated with antibodies against SRF, ELK1, SP1, EGR1, NAB1, NAB2 and CREB. Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*. As a negative control a fragment of the promoter of α-*actin* gene (right panel) was amplified. Figures are representative of at least three different experiments.

ChIP analysis enables a reliable image of the proteins bound to the chromatin, but the sensitivity of the method is given by the average size of the fragments analyzed (normally around 500 bp), and therefore is not accurate enough to distinguish between the occupancy of neighbour sites for the same protein. The analysis of the putative binding sites for *egr-1* promoter (see the map in Figure 25) reveals the presence of two different CG boxes were SP1 can bind, as well as two separated groups of SREs, the proximal cluster containing two binding sites and the distal cluster containing three binding elements for SRF. To obtain more precise

data on the occupancy of these sites, we analysed the immunoprecipitated DNA by real-time PCR with the appropriate oligonucleotides to obtain overlapping amplicons spanning the *egr-1* promoter (Figure 26). The analysis of the probability of SP1 occupancy within the two GC boxes (proximal GC box and distal GC box) indicate that when the gene is inactive SP1 is located at the more proximal GC box (GC box, within amplicon 15/16 in the Figure 26), since a linear adjustment shows that the probability of finding SP1 is inversely proportional to the distance (see *Annex 2* from *Materials and Methods* section). On the contrary, the distal GC box (GC box within amplicon 11/12 in the Figure 26) does not seem to be occupied by the transcription factor.

In the case of SRF occupancy, the results are noticeably different (Figure 27). The peaks of probability show that, both the proximal (SRE, within amplicon 15/16 in the Figure 27) and the distal (SRE, within amplicon 9/10 in the Figure 27) clusters, seem to be occupied by the transcriptional factor. The importance of one or the other SRE clusters in *egr-1* expression is not yet clear since Bauer *et al.*, (2005) assign a predominant role to the distal SRF. Promoter deletion studies give a predominant role to the distal SRF cluster in TPA-induced *egr-1* expression in human hepatoma cells (Bauer *et al.*, 2005), whereas Grundker *et al.*, (2004) suggest that during GnRH stimulation of *egr1* expression the predominant role corresponds to the SREs present in the proximal cluster. Our studies are not able to rule out any of the possibilities, but rather show that *in vivo* both clusters are occupied in MLP29 cells, and it makes sense to think that both may contribute to the final output.

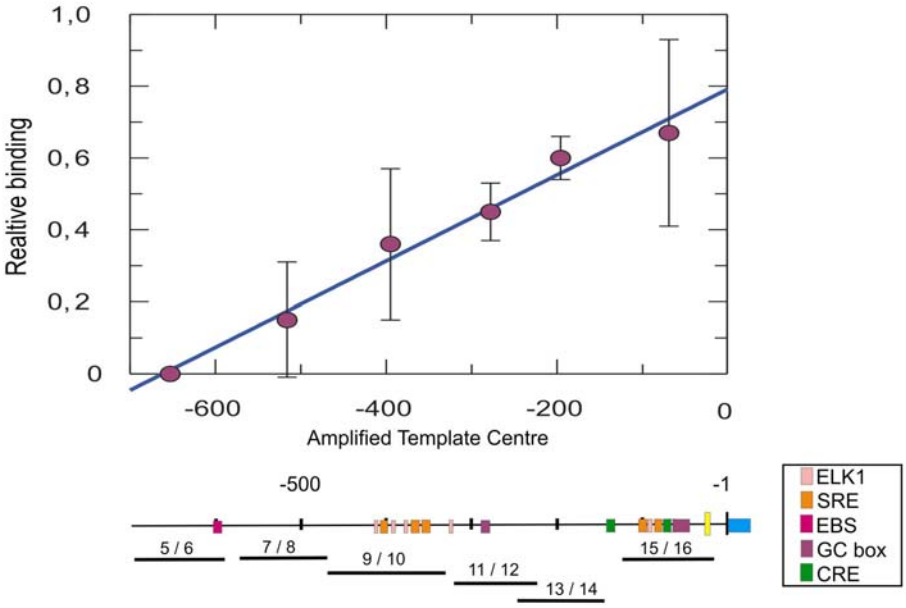


Figure 26. SP1 occupancy of the GC box along *egr-1* promoter. Crosslinked chromatin of MLP29 control cells with an average size of 500 bp was immunoprecipitated using an antibody against SP1, and the purified DNA was amplified by real-time PCR using the oligonucleotides depicted in the gene scheme (Table 17). The plot shows the probability of finding SP1 along *egr-1* promoter at the centre of the amplicons along *egr-1* promoter. Coloured boxes in the map represent the binding sites for the different factors: ELK1 (ELK1 binding site), SRE (SRF binding site), EBS (EGR1 binding site), GC box (SP1 binding site) and CRE (CREB binding site). A linear fit with a correlation coefficient (r)= 0,9913 is also shown (*Annex 2*).

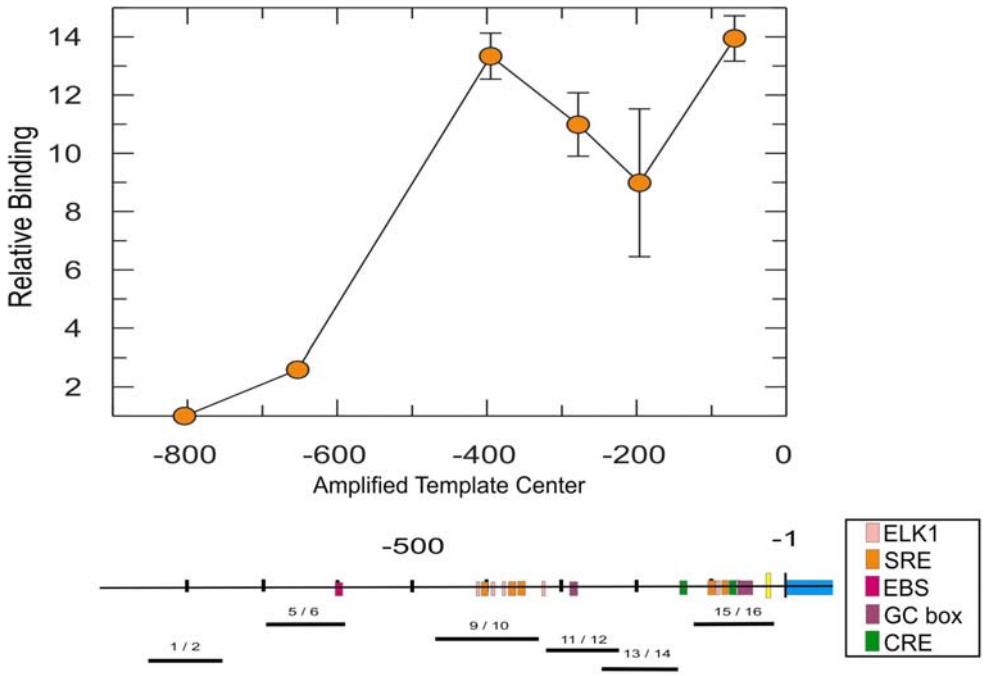


Figure 27. SRF occupancy along *egr-1* promoter. Crosslinked chromatin of MLP29 control cells, with an average size of 500 bp, was immunoprecipitated using an antibody against SRF, and the purified DNA was amplified by real-time PCR using the oligonucleotides (Table 17) for the amplicons shown in the promoter map. The plot shows the probability of SRF binding at the centre of the amplicons as in figure 26.

The results shown until now indicate that all transcriptional factors studied are present during the inactive state of the gene. Therefore, it seems that their binding is not part of an activating mechanism to induce *egr-1* expression. There are abundant data in the literature (reviewed in Gardiner, 2006) that several of those factors are activated by post-translational modifications (for instance, phosphorylation) to be able to switch on gene transcription. To test this possibility we studied the phosphorylation state of the constitutively present factors during *egr-1* transcriptional activation by TPA, and specifically of CREB and ELK1, as we considered these two factors to be more interesting than the rest.

Our results point out that both ELK1 and CREB become phosphorylated when the gene is being actively expressed (Figure 28). Nevertheless, the phosphorylation of ELK1 seems to be more stable than that of CREB, since the latter is only visible at 15 min whereas ELK1 phosphorylation is also observable at 30 min after TPA treatment. To sum up, these results suggest that phosphorylation of CREB and ELK1 is a feasible mechanism by which those transcriptional factor may act to trigger *egr-1* expression.

Results shown in Figure 28 are in agreement with the fact that these proteins can be phosphorylated by MEK 1/2 and p38 kinase cascades (Shaw *et al.*, 2002 and Patak *et al.*, 2004), which, as previously shown, are the two signalling networks involved in coupling *egr-1* expression to TPA stimulus. To analyze if any of those kinase cascades is indeed involved in the phosphorylation of those transcriptional factors, we decided to do a ChIP experiment with cells treated with MEK 1/2 and p38 inhibitors. For these assays we used cells treated with TPA for 15 min, a time in which both CREB and ELK1 are phosphorylated (Figure 28). Figure 29 illustrates the results of this experiment, which indicate that both MEK 1/2 and p38 kinase cascades are responsible for CREB and ELK1 phosphorylation. It has also to be noted that RNApol II from Figure 29, although amplified from the promoter region reflects quite well the transcriptional state of *egr-1*, that is to say the polymerase is more abundantly found in TPA-treated cells, that are actively transcribing *egr-1* gene, than in the cells treated with MEK 1/2 and p38 inhibitors.

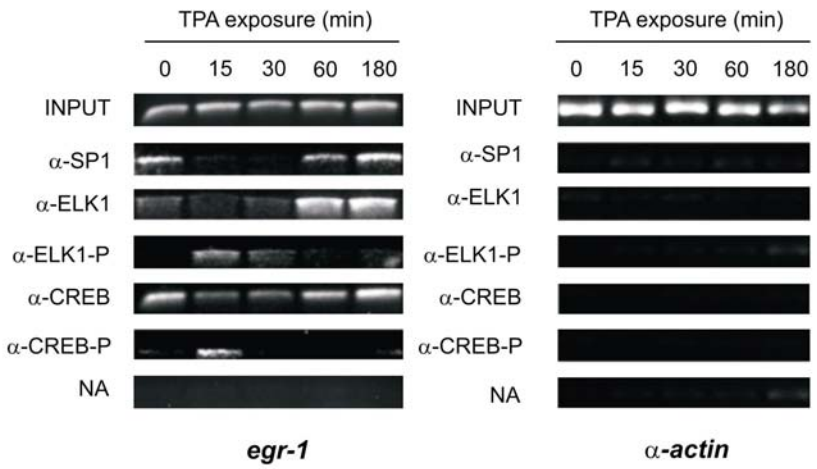


Figure 28. Recruitment of transcription factors to *egr-1* promoter. Time-dependent ChIP analysis of MLP29 cells incubated or not with TPA. Crosslinked chromatin was immunoprecipitated with antibodies against SP1, ELK1, ELK1-P, CREB and CREB-P or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*. As a negative control *α-actin* promoter region (right panel) was amplified. Figures are representative of, at least, three different experiments.

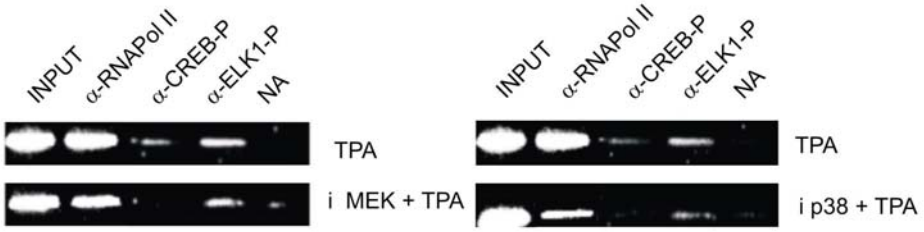


Figure 29. Recruitment of phosphorylated CREB and ELK1 transcription factors to *egr-1* promoter in cells treated with MEK 1/2 and p38 inhibitors. ChIP analysis of MLP29 cells pre-incubated or not with MEK 1/2 (PD98059) or p38 (SB203580) inhibitors and treated with TPA for 15 min. Crosslinked chromatin was immunoprecipitated with antibodies against RNAPol II, ELK1-P and CREB-P or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*.

All these results suggest that CREB and ELK1, the latter probably forming part of the TCF (DNA and SRF), are implicated, via phosphorylation of the factors, in the transduction of the signal produced by TPA that activates *egr-1* expression.

5. CHROMATIN MODIFYING ENZYMES ASSOCIATED TO *egr-1* PROMOTER DURING ITS INDUCTION

Post-translational modifications of histone tails are among the most important mechanisms which serve to regulate the chromatin state of a gene and consequently to direct its expression or repression in the cell. Rice *et al.*, (2001) reviewed the cross-talk between these complex modifications and deepened into the *"histone code"*, which would be read by proteins bearing characteristic binding domains that recognize specific modifications (for instance bromodomains recognize and bind acetylated lysines and chromodomains recognize methylated lysines and arginines).

5.1 CYCLOHEXIMIDE EFFECT

Cycloheximide is an inhibitor of the protein synthesis in eukaryotic organisms that acts suppressing the elongation of translation, and therefore it is widely used in *in vitro* experiments to test the importance of newly synthesized proteins in gene expression mechanisms.

To test the effect of this compound in our model system, we treated MLP29 cells with cycloheximide for 1 h at 25mg/mL, and then total RNA was purified and analyzed to determine *egr-1* transcriptional rate. Results from Figure 30 show that cycloheximide on its own is able to stimulate intensely the expression of the gene under study, suggesting the existence of a repressor that is being continuously synthesized and which is able to silence the gene. There are various possibilities to explain the effect of the cycloheximide. On one side, we have previously suggested a repressive role for SP1 and, since this transcription factor is constitutively expressed in our system (data not shown), it could make sense to consider that cycloheximide-induced *egr-1* activation could be due to the inhibition of SP1 synthesis, and therefore could be affecting the repressive co-factors that it may be recruiting such as histone deacetylases. On the other side, it could be also feasible that cycloheximide would be affecting part of the mediator or elongation machinery

needed to express the gene. Similar consequences of cycloheximide treatment on *egr-1* super induction were also reported by Chauhan *et al.*, (1994) in Jurkat cells as well as in different experiments carried out by Frick *et al.*, (1997) in mouse calvarian cells.

Okadaic acid (inhibitor of PP1 and PP2A) has also been reported by Chauan *et al.*, (1994) as an inducer of *egr-1* transcription. Therefore we next studied if this stimulation could increase the cycloheximide effect and whether it could be possible to correlate these two effects. Our results, in spite of showing an increase in the stimulation with the combined treatment are not significantly different from the results obtained with cycloheximide alone to establish a synergistic effect of these two substances (Figure 30).

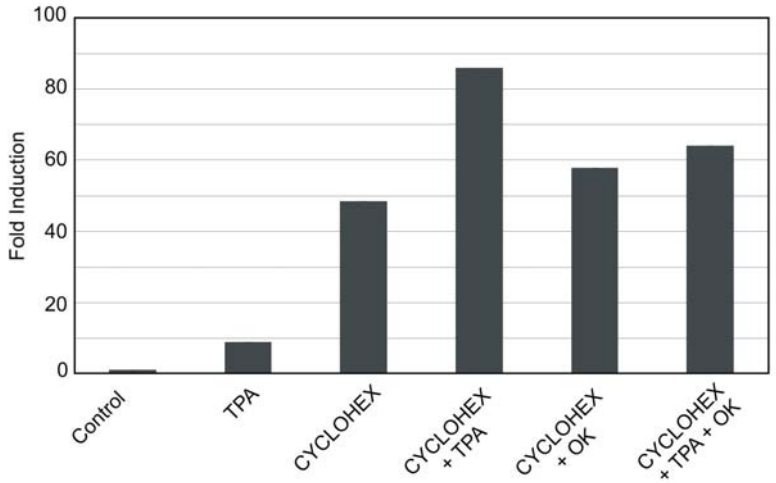


Figure 30. Effect of cycloheximide over *egr-1* expression in MLP29 cells. Serum-starved cells were incubated or not with cycloheximide (protein synthesis inhibitor) at the indicated doses (section 1.2.2 from *Materials and Methods*) and then were or were not treated with TPA and/or okadaic acid (OK) and harvested 1 h following the drug treatment. Total RNA was purified and analyzed by real-time RT-PCR and the results were normalized against *rRNA* 18S gene. Control sample, TPA untreated cells, was arbitrarily assigned to a value of 1. Results are representative of, at least, three different experiments.

5.2 HISTONE DEACETYLASE AND HISTONE ACETYLTRANSFERASE COMPLEXES INVOLVED IN *egr-1* REGULATION

Nucleosome core particles with their conservation through evolution serve primarily to package DNA. Nevertheless, they possess a second important function, namely, their ability to carry epigenetic information through post-translational histone modifications. These modifications are performed by specific enzymes such as histone acetyltransferases, deacetylases, methylases, kinases, etc.

To study the epigenetic mechanism involved in the regulation of *egr-1*, we proceed to analyze the HDACs and HATs that are present in the promoter of the gene, and, therefore, are potentially able to imprint specific signals that regulate its transcription. Traditionally, histone acetylation has been linked to gene expression, assuming that it neutralizes the basic charge of the histones, weakening its interaction with DNA and rendering it more accessible to the transcriptional machinery. For instance, Wallberg *et al.*, (1999) demonstrated that SAGA complex was needed to mediate transcriptional activation of chromatin templates on yeast. On the contrary, histone deacetylation has been related with transcriptional repression (Laherty *et al.*, 1997). Therefore, HATs are in general considered activators and HDACs have been ascribed to the role of gene expression repressors. While the relationships between histone acetylation and transcriptional activity are, generally speaking, firmly established, the ultimate reasons seem to be much more complex than a simple charge neutralization, and the participation of the signalling implied by the histone code hypothesis has to be considered.

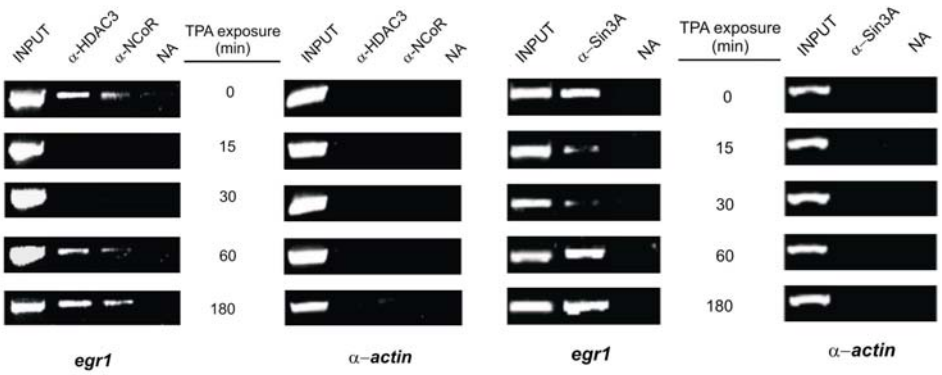


Figure 31. Recruitment of HDAC complexes to *egr-1* promoter. Time-dependent ChIP analysis of MLP29 cells incubated or not with TPA. Crosslinked chromatin was immunoprecipitated with antibodies against HDAC3, NCoR and mSIN3A or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (see Table 15) specific for the promoter region of *egr-1*. As a negative control α-*actin* promoter region was also amplified. Results are representative of at least three different experiments.

The analysis by ChIP assay of the presence of HDAC and HAT complexes in the promoter of *egr-1*, detects the presence of at least, two different HDAC complexes (Figure 31). One of them is the mSIN3A, complex that is usually associated to HDAC1 and HDAC2. In our case, it is probable that this two deacetylase activities form part of the complex, but the results obtained (data not shown) are not completely clear, probably due to the fact that the antibodies do not work properly for ChIP experiments. The second one, represented by NCoR, contains also HDAC3 activity (Figure 31). Association of NCoR and HDAC3 was first described by Wen *et al.*, (2000) in pull-down assays in HeLa cells. Our results show a clear correspondence for the binding of both proteins in the promoter of *egr-1*. mSIN3A and NCoR complexes are present when the gene is inactive, and leave the promoter during induction, around 15 to 30 min. Later, when the gene is switched off at 60 and 180 min, the complexes become again detectable in the promoter. The significance of the presence of at least two HDAC complexes

implicated in *egr-1* repression is not yet clear, but maybe it is needed for a strict chromatin regulation of this immediate-early gene expression.

Next, we studied the recruitment of HAT complexes to the chromatin of *egr-1*, and for this purpose, we selected among the different possible HATs, CBP and GCN5 for different reasons. CBP and p300 are highly homologous general transcriptional coactivators usually found together (Grant *et al.*, 1999), and therefore we decided to look only for one of them. ChIP analysis presented in Figure 32, shows that CBP complex is bound at the promoter during the basal state of the gene, and that it is released when the gene is active to return again when the gene expression decreases. The presence of the HAT complex in *egr-1* promoter during the repression of the gene may mean that CBP is implicated in maintaining a basal state of histone acetylation. The basal state of acetylation may keep the immediate-early gene in a potentially active transcriptional state ready to answer to the appropriate cellular stimulus. This hypothesis is sustained by the fact that the gene already has all the transcriptional factors required for its induction, and that even the RNApol II is paused in its promoter. These results are in agreement with those of Chen *et al.*, (2001), who showed that a diminution in CBP activity occurs when it was incubated with phosphorylated CREB in *in vitro* acetylation experiments with HeLa nuclear extracts, and this may also occur in *egr-1* transcriptional activation. Nevertheless, since histone acetylation is mainly related to gene expression, the complex of GCN5 would be, according to our results, the enzyme responsible for the specific acetylations that would direct *egr-1* to its expression. ChIP analysis demonstrates that GCN5 is specifically recruited as early as 15 min and leaves the promoter again at 60 min after TPA treatment (Figure 32). We also tried to determine whether PCAF, a HAT from another complex, was also present in the gene, but we were not able to detect its presence in *egr-1* promoter (data not shown). Nevertheless, these data can not rule out completely its presence because it could be possible that the antibody is not able to recognize the protein in the complex containing PCAF within *egr-1* promoter.

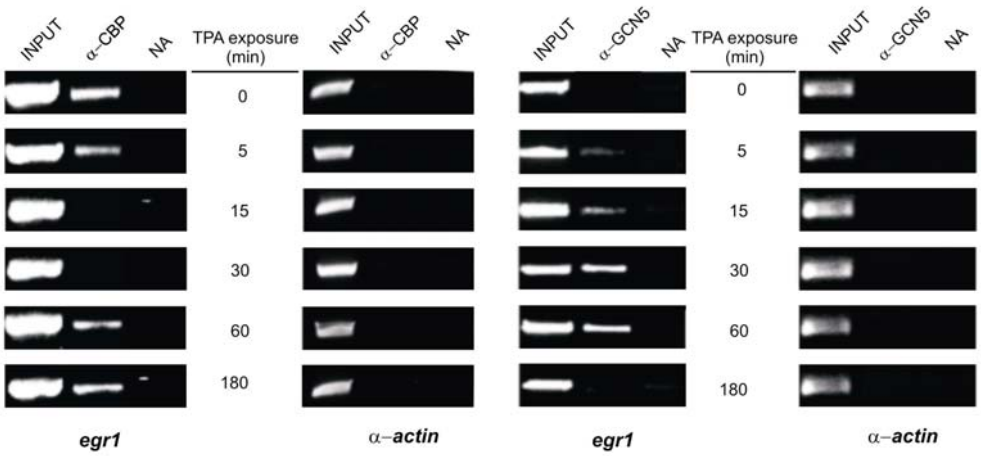


Figure 32. Recruitment of HAT complexes to *egr-1* promoter. Time-dependent ChIP analysis of MLP29 cells incubated or not with TPA. Crosslinked chromatin was immunoprecipitated with antibodies against CBP and GCN5 or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*. As a negative control *α-actin* promoter region was amplified. Results are representative of, at least, three different experiments.

So far, we have been able to correlate *egr-1* regulation with three different chromatin modifying complexes. At this point, we asked ourselves if there may be a non random distribution of these enzymes along the promoter. To respond to this question, we decided to analyze the intensity of the signal by using overlapping amplicons of around 100 bp along the promoter. Basing ourselves in previous results, (Figures 31 and 32), we used cells untreated with TPA to analyze HDACs (HDAC3 and mSIN3A), and cells incubated with TPA for 30 min to analyze GCN5. We did not study CBP distribution due to its presence prior to the induction, which probably indicates a less important role for the enzyme in the induction process. Data displayed on Figures 33 and 34 show that despite the difference in intensity, the three enzymes seem to have a highly similar distribution along the promoter. In view of these results, we wonder whether these three more probable sites could correspond to nucleosomes located at the gene, and as a result we decided to deepen later in the histone octamer position along *egr-1* promoter.

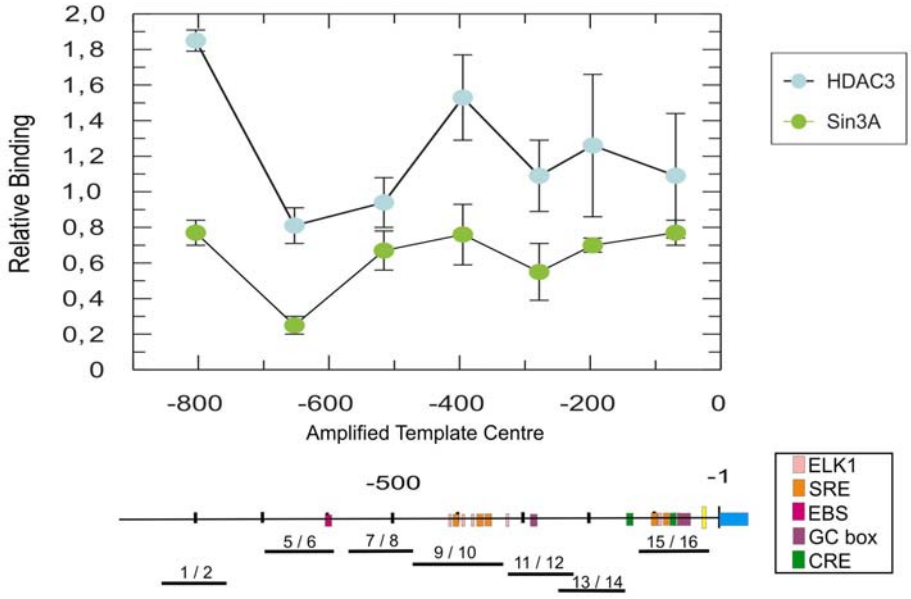


Figure 33. HDACs distribution along *egr-1* promoter. Crosslinked chromatin of MLP29 cells, untreated with TPA, with an average size of 500 bp was immunoprecipitated using antibodies against HDAC3 and mSIN3A, and the purified DNA was amplified by real-time PCR using the oligonucleotides (Table 17) for the amplicons shown in the promoter map. The plot shows the probability of HDAC3 and mSIN3A binding at the centre of the amplicons as in figure 26.

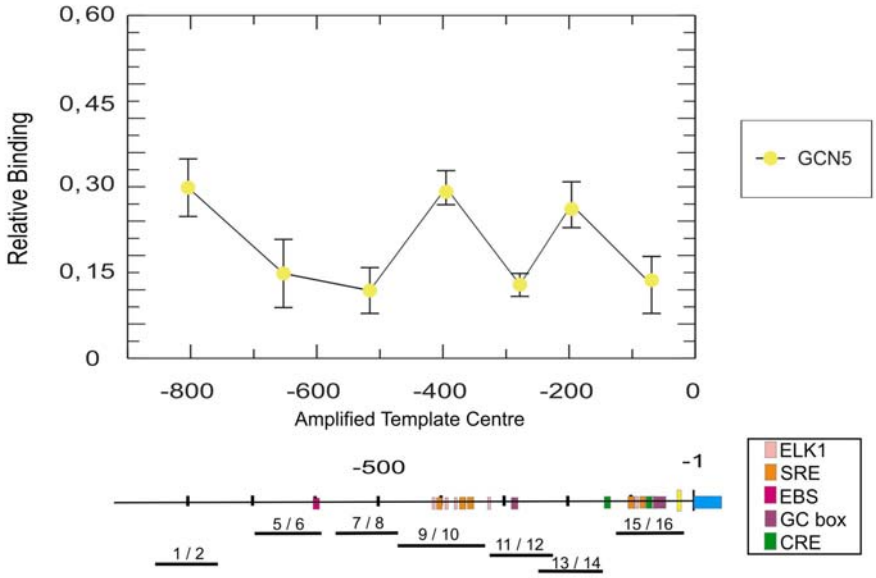


Figure 34. HAT distribution along *egr-1* promoter. Crosslinked chromatin of MLP29 cells, treated with TPA for 30 min, with an average size of 500 bp was immunoprecipitated using antibodies against GCN5 and the purified DNA was amplified by real-time PCR using the oligonucleotides (Table 17) for the amplicons shown in the promoter map. The plot shows the probability of GCN5 binding at the centre of the amplicons as in figure 26.

Next we asked ourselves if the presence of HDAC complexes was affected by treatment of the cells with inhibitors of the signalling cascades responsible for *egr-1* induction (Figure 35) or by treatment of the cells with cycloheximide (Figure 36). For this reason we carried out ChIP experiments in which the cells were previously treated with the corresponding kinase inhibitors. Results shown in Figure 35 indicate that N-CoR needs a phosphorylation to be released from the gene. This phosphorylation can be carried out by MEK, as treating the cells with the inhibitor of this kinase hinders NCoR release from the promoter, and although the signal is less clear, p38 should also be capable to phosphorylate the factor. Therefore our results do not allow us to rule out any of the kinase cascades. On the contrary, mSIN3A appears to be less represented when the cells are treated with the p38 inhibitor prior to their induction with TPA, and consequently it would imply that the complex requires a phosphorylation to remain bound to the promoter. On the contrary, Figure 36 illustrates that the protein synthesis inhibitor does not seem to affect any of the co-repressor complexes and as a result there must be, at least, a third one that is holding back *egr-1* expression. Finally, looking at the results obtained for RNAPol II, we have to say that its presence in the promoter of *egr-1* correlates accurately with the events that are taking place at the chromatin of the gene. In other words, when cells are treated with cycloheximide and the gene is being actively expressed, the polymerase is more tightly bound to the promoter, whereas when the cells are pre-incubated with inhibitors of the kinase cascades the amount of RNA Pol II detected at *egr-1* promoter is less than the that detected in the control sample, that is to say, cells treated with TPA (Figures 35 and 36). Despite this fact, in considering these data it should be kept in mind that the region amplified corresponds to the promoter and not to the coding region of the gene.

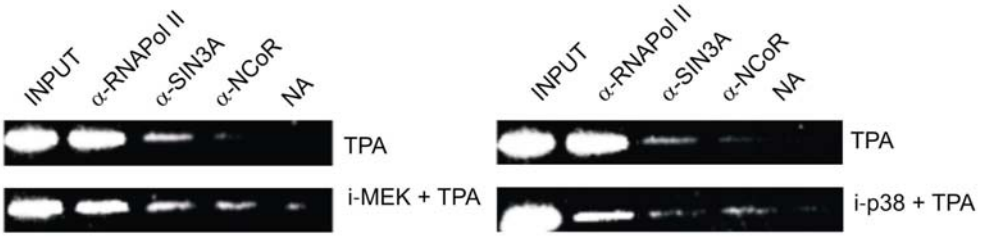


Figure 35. Recruitment of repressors and transcription factors to *egr-1* promoter. ChIP analysis of MLP29 cells incubated or not with: MEK inhibitor (i-MEK, PD98059) (left panel) and p38 inhibitor (i-p38, SB203580) (right panel), and treated with TPA (15 min) and. Crosslinked chromatin was immunoprecipitated with antibodies against RNAPol II, mSIN3A and NCoR or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*. Results are representative of, at least, three different experiments.

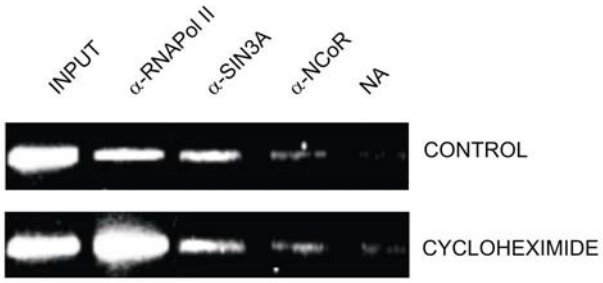


Figure 36. Recruitment of repressors and transcription factors to *egr-1* promoter. ChIP analysis of MLP29 cells untreated and cells incubated with cycloheximide. Crosslinked chromatin was immuno-precipitated with antibodies against RNAPol II, mSIN3A and NCoR or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (Table 15) specific for the promoter region of *egr-1*. Results are representative of, at least, three different experiments.

5.3 HISTONE MODIFICATIONS OF *egr-1* PROMOTER

As previously mentioned, the mechanism for storing information and regulating genomic function relies within a variety of post-translational modifications of histones (i.es phosphorylation, acetylation, methylation, ADP-ribosylation and mono-ubiquitination). Consequently, the *"histone code"* hypothesis has assigned crucial regulatory roles to those modifications that govern the accessibility and function of the genome. In our model, we intended to determine the pattern of histone modifications produced by the HDAC and HAT complexes found in *egr-1* promoter that may shift the gene to a transcriptionally competent state.

Figure 37 shows the pattern of histone modifications obtained by ChIP experiments with antibodies recognizing specific histone modifications either with inactive (cells not treated with TPA) or active (cells treated with TPA for 30 min) *egr-1*. At first glance, one can conclude that our gene of study has a highly level of basal acetylation, result that could correlate nicely with the presence of CBP at time 0 min, despite the presence of the HDACs. It is also noticeable that the level of basal acetylation is reduced in the coding, implying a differential pattern of modifications in those regions. This result is not surprising, as the RNApol II machinery has to be recruited by a tightly regulated mechanism, whereas the coding region has only to recruit those factors that facilitate the passing of the polymerase through the chromatin template. As a control, *α-actin* was analyzed as an inactive gene and, consequently, devoid of modifications related to gene transcription, *β-actin* was also analyzed as representative of a gene which is being continuously expressed and possibly does not possess specific modifications that may be related to immediate-early gene transcription.

Among the different posttranslational modifications of the histones, lysine acetylation is the most extensively modification studied. For instance, Brownell *et al.* (1996) linked H3 K14 acetylation to gene activation. Grant *et al.* (1999) did the same three years later with H3 K9 and H3 K18 modifications, whereas Schiltz *et al.* (1999) established the importance of H4 K5 and K8 acetylations in gene activation.

Later on, H4 K16 (Kawasaki *et al.*, 2000) and H3 K23 acetylation (Daujat *et al.*, 2002) were also correlated with gene expression.

When studying the specific pattern of acetylations in *egr-1* promoter, we noticed that histones were already acetylated even in its inactive state, mainly at H3 (in H3K9Ac, H3K14Ac and H3K18Ac), whereas histone H4 had only lysine K5 acetylated. Nevertheless, the acetylation level of H3 increased during the expression of the gene, specially in the lysines K18 and K27, as the latter is the only one studied in H3 that is absent in the basal state. It is also important to note that H3K18 signal is also present in the *β-actin* gene, which would indicate that such modification may not be specific for immediate-early gene activation.

The case of histone H4 acetylation seems to be even more interesting, as there is an increase in the level of all the acetylations studied (H4K5Ac, H4K8Ac and H4K16Ac), and except for H4K5Ac, the others are restricted to the promoter and not to the coding region. This combination of histone modifications appear to be related to immediate-early gene transcription as they are absent from the active *β-actin* gene.

Regarding to histone methylation we centred our studies in the modification of lysine 4 of H3 (H3K3Me), and its differential state of di- or tri-methylation ($H3K4Me_2$ *vs.* $H3K4Me_3$). Bernstein *et al.* (2005) have correlated lysine trimethylation as well as H3K9/14Ac with sites near the transcription, whereas di-methylation was mainly found in the vicinity of active genes in both human and mouse samples. Our results show that both $H3K4Me_2$ and $H3K4Me_3$ seem to be present in *egr-1* promoter independently of the transcriptional state of the gene. These results suggest that methylation of lysine 4 from H3 would not be a universal specific signal for triggering gene transcription. It is also noticeable that the abundance of di- and tri-methylation seems to differ in the promoter and in the coding region, because $H3K4Me_3$ is more represented in the promoter and $H3K4Me_2$ is more abundant in the coding region (Figure 37). On the other hand,

methylation seems to be a mark for active genes (such as *β-actin*) or for potential active genes (such as *egr-1*).

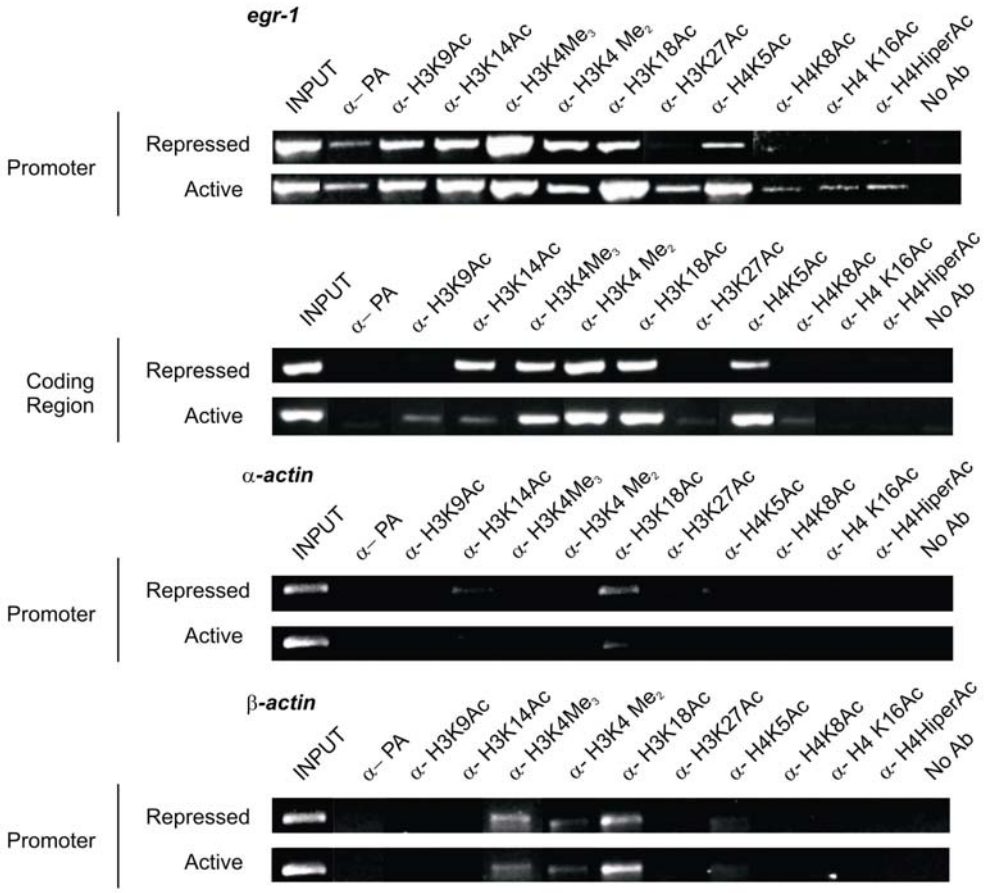


Figure 37. Post-transductional histone modifications in *egr-1* promoter. ChIP analysis of MLP29 cells non-treated (repressed) or treated (active) with TPA for 30 min. Crosslinked chromatin was immunoprecipitated with antibodies against H3K9AcS10P (PA), H3K9Ac, H3K14Ac, $H3K4Me_3$, $H3K4Me_2$, H3K18Ac, H3K27Ac, H4K5Ac, H4K8Ac, H4K16Ac and H4HiperAc or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers specific for the promoter (see Table 15) and coding region (see Table 16) of *egr-1*. *β-actin* and α-*actin* genes were also amplified as positive and negative controls. Results are representative of, at least, three different experiments.

Deepening into the specific modifications of *egr-1* promoter, it has to be noted that there is an increment in phosphorylation of serine 10 in histone H3, as shown by the increase in the signal obtained with the antibody PA that specifically recognizes the modification of H3 lysine 9 acetylation and serine 10 phosphorylation (H3K9AcS10P). This combination has been related to immediate-early gene activation by Cheung *et al.* (2000), and accordingly it appears in the induction of our gene, but not in the constitutively expressed *β-actin* gene. However, this modification is specific for the promoter, as it is lacking in the coding region amplified. Phosphorylation of serine 10 is usually carried out by kinases downstream MEK, one of the signalling networks implicated in the *egr-1* activation, as it is the case for MSK1 and RSKs (Chee *et al.*, 2004).

So far, we have been able to determine some of the modifications that are needed for the activation of *egr-1* transcription by TPA, as well as the enzymes that seem responsible for those modifications. Although we cannot establish a direct correlation between enzymes and modifications, we may suggest that GCN5, detected by ChIP at 15 min after TPA treatment, can account for the acetylations at H3K9, H3K14, H4K18 and H4K16 acetylations, as all of them increase in the promoter upon gene induction. In the same manner, CBP which attributable function may be to maintain the basal level of acetylation, could be responsible to acetylate H3K14 and H3K18 residues, that are substrates described in the literature for this HDAC activity. Anyway, it can not be ruled out that any of the modifications can be carried out by different HATs, whose presence we have not yet detected, as reviewed in the introduction (see section 1.2.2 from *Introduction*).

6. NUCLEOSOMAL POSITIONING AT *egr-1* PROMOTER

Nucleosome positioning has an essential role in facilitating the regulated transcription of eukaryotic genes, and therefore it is not possible to study the mechanism of *egr-1* transcriptional regulation without examining at the chromatin structure of the promoter. As previously described, some transcription factors are able to bind to DNA only when it is appropriately wrapped around the histone core, in contrast to those whose binding ability is impeded by the severe deformation of the DNA structure. Furthermore, positioned nucleosomes can facilitate the transcription process by providing the appropriate scaffold to allow the interaction of regulatory factors bound at distant sites.

To map nucleosomes on the *egr-1* promoter, we searched for sequences in the promoter which had high nucleosomal formation potential, by means of studying the gene sequence with two programs. One that predicts the nucleosome position basing its algorithms in the analysis of the frequency of distribution of di- and tri-nucleotides in the minor groove when it points out or when it faces the histone octamer, in 177 different nucleosome cores of chicken erythrocyte (Drew *et al.*, 1987 and Estruch *et al.*, 1989) and the other, that predicts nucleosome position basing its algorithms on thermodynamic criteria (Anselmi *et al.*, 2000). As shown in Figure 38, both programs predict the probability of finding two positioned nucleosomes. The first program (Figure 38A) locates a nucleosome around position -230 bp from the starting point and another one around -820 bp from the starting point. The second program used (Figure 38B) predicts almost the same positions with minor differences. The first histone octamer would be centred at about -200 bp, whereas the second would be around -800 bp. In conclusion, both regions are highly favourable for the localization of nucleosomes, as they have a region that spans approximately 100 bp with a predicted high potential for nucleosome formation.

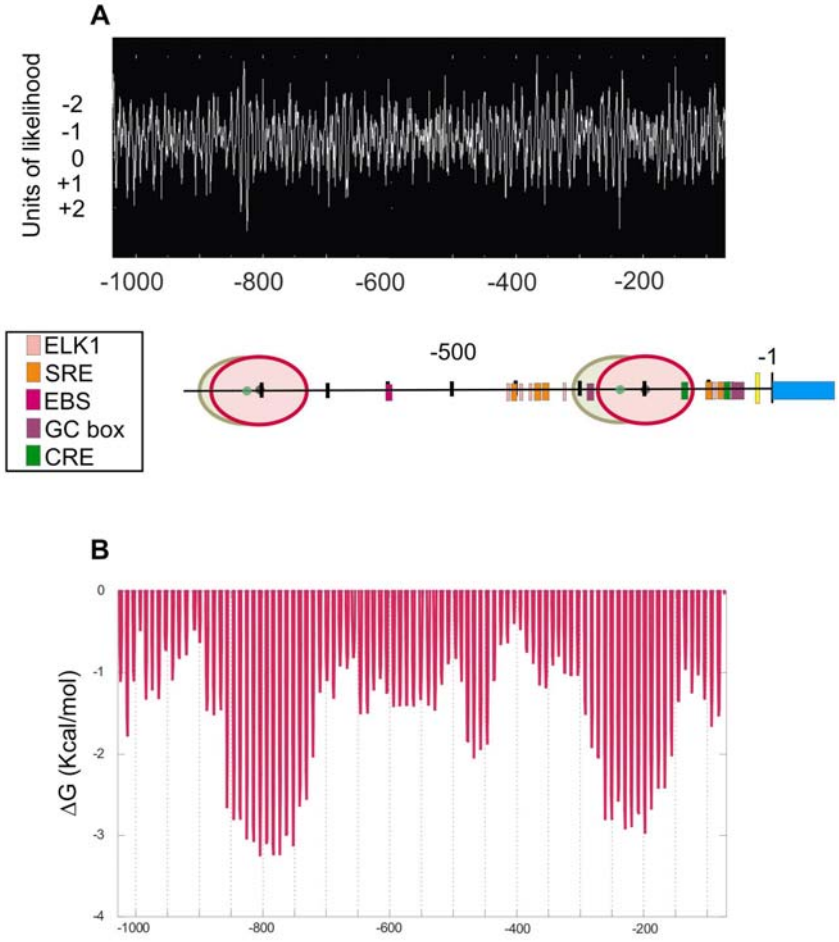


Figure 38. Theoretical analysis of the nucleosomal rotational formation potential at the *egr-1* promoter sequence. (**A**) Prediction based on known DNA sequences which are found in histone octamers. Lower peaks show the higher probability of finding the dyad axis of a nucleosome. Panel (**B**) shows the nucleosome prediction pattern based on energy minimization criteria. In between both Figures there is a schematic representation of *egr-1* promoter containing the binding sites for different transcription factors and also the predicted centres of the nucleosomes according to both predictions: green stands for the prediction in A and black for that of B.

The experimental mapping of nucleosomes within *egr-1* promoter was done by micrococcal nuclease treatment of MLP29 cells, following a modification of a method previously described by Steward and Sano (2004), (see Section 7 of *Materials and Methods*). As already commented, this technique allows us to determine nucleosomal positions due to their protection against micrococcal nuclease digestion.

The results obtained (Figure 39) suggest the existence of three putative nucleosomes: N3 (dyad axis located around -800 bp), N2 (placed between -435 and -585 bp) and N1 (around -200 bp). Both N3 and N1 would be positioned by their sequence, as they correlate with the high probability of nucleosome positioning predicted by the two theoretical programmes used to analyze the sequence (Figure 39B). On the contrary, position of N2 nucleosome would be established indirectly by the existence of N1 and N3 or other factors bound in the promoter, such as the SRF clusters. Moreover, it is a general feature that between two sites of high probability of nucleosome formation potential may be indirectly positioned another nucleosome if there is enough space, as it happens at *egr-1* promoter.

The presence of positioned nucleosomes encouraged us to search for ATP-dependent remodelling enzymes that could be bound to the promoter and as a result act over the found nucleosomes. ChIP analysis of the presence of these enzymes shows that two different ATPases, BRG1 and BRM, (which belong to the SWI/SNF family) are bound to *egr-1* promoter (Figure 40). According to their binding pattern, both seem to be present when the gene is not being expressed, that is to say in the basal state (0 to 5 min after TPA treatment) and when the repression of the gene is resumed (60 to 180 min after TPA treatment). On the contrary, when the gene is active (15 to 30 min after TPA treatment) none of those remodelling complexes is bound to the promoter. This unexpected binding profile could be explained through different hypothesis. Firstly, it could be that the remodelling activity was as quick as 5 min after TPA induction and then the enzymes were to leave the promoter to allow the rest of co-activators to bind. Secondly, it could be that BRG1 and BRM were forming part of some complex, as for instance of mSIN3A complex and therefore its

function was mainly related to this complex activity. In this case, remodelling at *egr-1* promoter would be carried out by another undetected remodelling machine. Pal *et al.*, (2003) co-purified a complex formed by mSIN3A, HDAC2, PRMT5 and BRG1, and linked this complex to the *cad* gene repression process. In our results (Figures 31 and 40) the timing of detection of mSIN3A, BRG1 and BRM by ChIP analysis in *egr-1* promoter correlates quite well, so we can not rule out a similar possibility. A third hypothesis would be to link the function of BRG1 and BRM with *egr-1* repression; this would imply that the remodelling activities are required to keep the nucleosomes at their basal position so avoiding gene expression. Remodelling activities, although traditionally related to gene activation, have also been linked to gene repression. Wang *et al.*, (2004) linked BRG1 and BRM chromatin modifiers with estrogen antagonist-mediated growth suppression through the estrogen receptor, whereas Strobeck *et al.*, (2000) showed in C33A cells that the lack of BRG1 inhibited Rb cell cycle arrest, deficiency that was rescued through ectopic expression of BRG1.

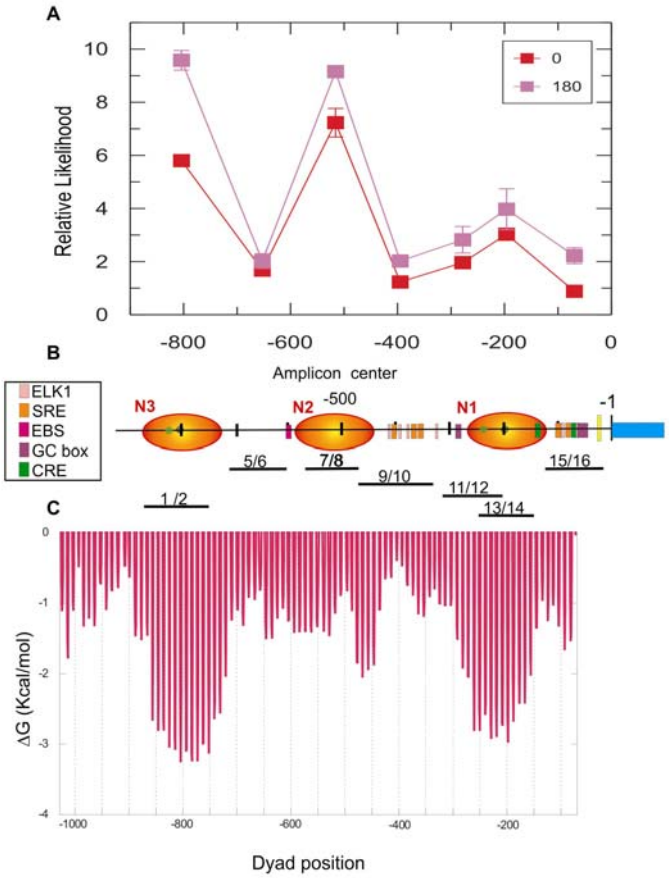


Figure 39. Study of the nucleosomal probability pattern in *egr-1* promoter of MLP29 cells. Chromatin was digested to mononucleosomal size with MNase, and DNA was purified and analyzed by real-time PCR using various sets of oligonucleotides, so the amplicon spans the promoter region. Each amplicon was designed to cover around 100 bp on the template sequence, so that the regions of the promoter containing nucleosomes could be amplified if the full template was available. **(A)** Plot shows the nucleosomal probability of samples treated with 50 nM TPA at different time intervals (0 and 180 min), represented according to the centre of the template amplified, and normalized *versus* random DNA of around 150 bp. **(B)** Diagram that illustrates the binding sites for the transcriptional factors found at *egr-1* promoter. The putative position of the "inert" nucleosomes is depicted in light orange, in accordance with the centre of the template amplified. It can also be seen the position of the amplicons used during the experiment. **(C)** Nucleosome prediction pattern based on energy minimization.

The fact of finding two remodelling complexes bounded to *egr-1* promoter prompted us to study the nucleosomal pattern in *egr-1* during the activation process, searching for a feasible movement of the octamers due to the remodelling activities found.

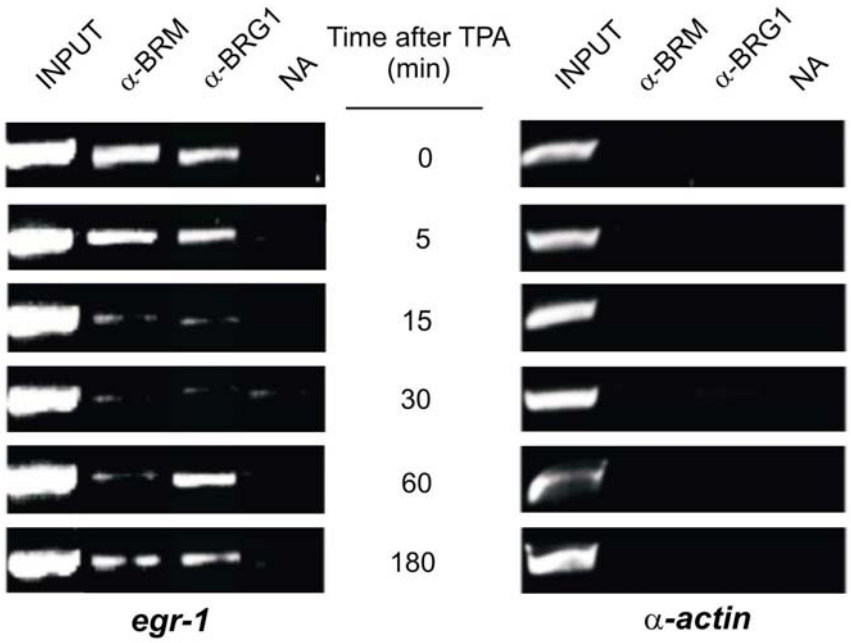


Figure 40. Recruitment of ATP-dependent chromatin remodelling enzymes to *egr-1* promoter. Time-dependent ChIP analysis of MLP29 cells incubated or not with TPA during the indicated times. Crosslinked chromatin was immunoprecipitated with antibodies against BRM and BRG1 or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers (see Table 15) specific for the promoter region of *egr-1*. As a negative control *α-actin* was amplified. Results are representative of at least three different experiments.

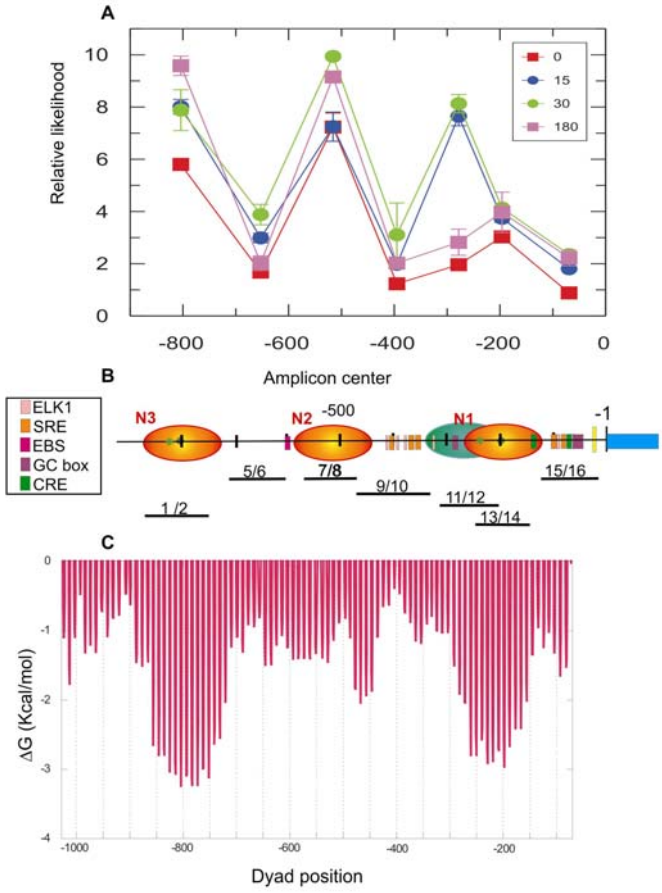


Figure 41. Study of the nucleosomal remodelling pattern in *egr-1* promoter of MLP29 cells during its induction by TPA. Chromatin was digested to mononucleosomal size with micrococcal nuclease and DNA was purified and analyzed as in Figure 39. **(A)** Plot shows the nucleosomal probability of samples treated with 50 nM TPA for different time intervals (0, 15, 30 and 180 min), represented as in Figure 39. **(B)** Diagram that illustrates the binding sites for the transcriptional factors found at *egr-1* promoter. The putative position of the "inert" nucleosomes is depicted in orange according to the centre of the template amplified, whereas the nucleosome that moves is coloured in green at the new position, also according to the centre of the template amplified. It can also be seen the position of the amplicons used during the experiment. **(C)** Nucleosome prediction pattern as in Figure 39. Results are representative of, at least, three different experiments.

Figure 41 shows the putative gene induction-dependent changes in the positioning of the three nucleosomes detected in the basal state. Our results indicate a movement of N1, during *egr-1* expression, but the other two nucleosomes remain static. Furthermore, when the gene is not active, nucleosome N1 protection to micrococcal nuclease is not as significant as in the case of N3 and N2, suggesting that N1 is not as strictly positioned as N2 and N3. In other words, it might be sliding to and fro along the DNA. On the contrary, when the gene is induced N1 seems to be forced to a new position further upstream (around -260 bp location for the dyad axis) in which, the protection probability satisfactorily correlates with the probability found for N3 and N2. Nevertheless, we have to bear in mind that we have represented the nucleosomes at the centres of the amplicons and that these positions should be precisely defined to exactly locate the nucleosome.

Classically, the nucleosomal organization of chromatin has been considered as a constraint for gene transcription, and therefore it would be necessary to reorganize their position. We have empirically corroborated the existence of three nucleosomes: N1, N2 (positioned by the boundary effect of N3) and N3. Our results suggest that the nucleosomal positioning probability of N1 is about half of that for N3 and N2, and just this nucleosome becomes displaced during *egr-1* activation. We hypothesize that this movement could be caused by BRM, an ATPase subunit of the SWI/SNF family of ATP-dependent chromatin remodelling complexes, and therefore that would imply that the cell is using energy to repress an important gene as *egr-1*, which encodes a transcriptional factor. It is important to remember that the promoter of the gene also contains at least two more co-repressors: mSIN3A complex and NCoR/HDAC3 (Figure 31).

This N1 "*nucleosomal oscilation*" would correlate to the need of the transcriptional machinery to recruit other factors that are also required for the transcription, as for instance, the mediator complex, which will probably facilitate the successive rounds of initiation, or elongation factors as P-TEFb (Positive Transcription Elongation Factor b) that is needed to phosphorylate serine 2 of RNApol II CTD (C-terminal Domain). Some authors have suggested that the

mediator could be the responsible for recruiting the RNA pol II to the promoter, but this possibility can be ruled out for our gene, as the polymerase is paused in the promoter. Nevertheless, the movement of the nucleosome will be required to guarantee that components of the PIC and of the elongation complex have enough space to bind and act.

So far, it has not been described a gene in which a remodelling complex is impeding the accurate nucleosomal position for transcription. On the contrary, the usual model is that remodelation is required to allow transcriptional factors to gain access to the template which is being occupied by the nucleosome. Despite this fact, BRM and its counterpart BRG1, have been widely implicated in the literature in transcriptional repression. As it has been previously said, Wang *et al.* (2004) proposed a mechanism in which JNK1 pathway induces their recruitment to prevent estrogen-induced growth. In the same way, Kemper *et al.*, (2004), described the arrest of cholesterol 7α hydroxylase gene (*cyp7a1*) gene by the recruitment of a complex formed by mSIN3A and SWI/SNF. Furthermore, the fact that BRM seems to leave the promoter upon gene induction and returns to it when *egr-1* is again repressed, supports our hypothesis.

To this point, we have been able to describe the movement of the nucleosome but the actual remodelling mechanism remains obscure. As commented above, Pal *et al.* (2003) described the need of HDAC2/mSIN3A/PRMT5 for the BRG1 complex to repress the MYC target gene *cad*. Having these results in mind, we tried to know whether any histone methyltransferase, as PRMT5, was present in *egr-1* promoter by using ChIP assay at nucleosomal resolution (see section 6 from *Materials and Methods*). Mononucleosomal resolution provides a significant advantage over classical ChIP results, despite the fact that the amount of sample required is noteworthy higher, this limiting the number of experiments that can be done. Figure 42, shows that the histone methyltransferase PRMT1 (the main responsible to methylate H4R3) is differentially recruited to the nucleosomes and that the histone modification associated to this enzyme, H4Arg3Me_2a, also occurs there. Asymmetric methylation seems to increase only in N2, and remains more or

less constant in the other two nucleosomes. This could be in agreement with the fact that PRMT1 is detected upon nucleosome N2 only in the activated state and not in the repressed one. The objective of this experiment was to determine if the methylase was specifically bound to N1 nucleosome. Our results, as previously mentioned only show an increase in the methylation state of nucleosome N2. Therefore, to search for other possible specific causes for the movement of N1 nucleosome, we next examined the phosphorylation of the enzymes involved in the process.

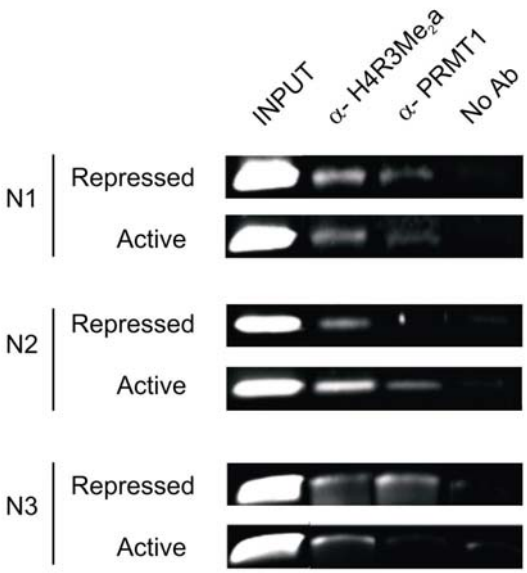


Figure 42. Recruitment of PRMT1 HMTs to specific nucleosomes at the *egr-1* promoter and analysis of histone methylation. ChIP analysis of MLP29 cells expressing or not *egr-1* mRNA. For the active sample, cells were treated with TPA for 15 min. Crosslinked mononucleosomes were immunoprecipitated with antibodies against $H4R3Me_2a$ and PRMT1 or in absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers 1/ 2, 7/ 8 and 13/ 14 (Table 17) contained into nucleosomes N1, N2 and N3 of *egr-1*.

We further decided to investigate the mechanism to understand the specific movement of N1 nucleosome during activation. For this purpose, N1 position was analyzed in samples treated with okadaic acid (an inhibitor of protein phosphatases PP1 and PP2A) and with D-L-homocysteine, adenosine and N-methyl-2-

deoxyadenosine (inhibitors of PRMT1; Smith *et al.*, 2004) (Figure 43). Our results, analysing the amplicons 11/12 and 13/14 that cover the positions of nucleosome N1 under repression and activation of *egr-1* respectively, indicated that histone methylation of N1 by PRMT1 did not seem to have an effect on remodelling activity. On the contrary, inhibition of the phosphatases did result in an increase in the remodelling abilities of our system, suggesting that the level of phosphorylation of certain proteins has an important role in the overall remodelling process.

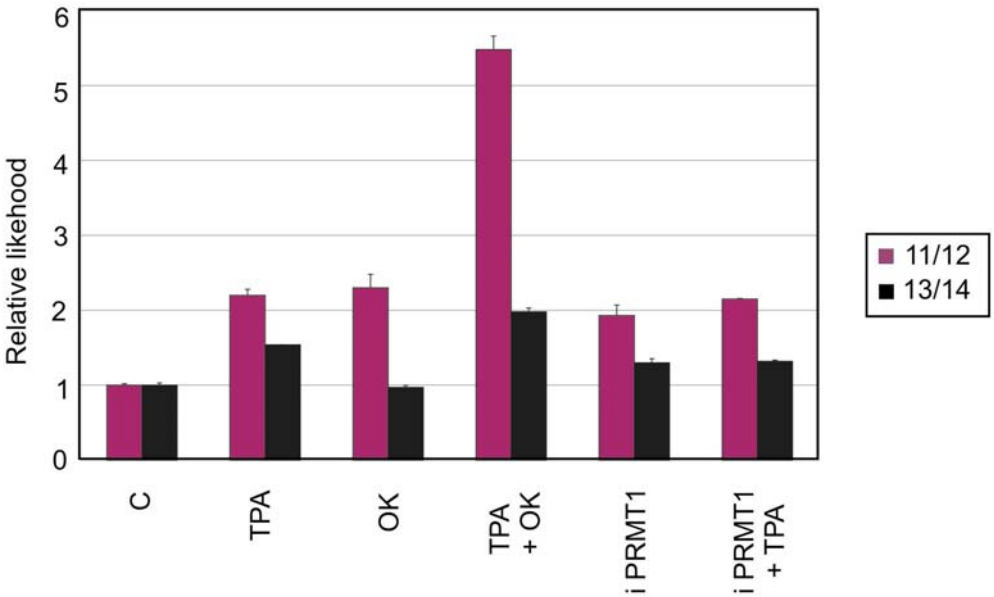


Figure 43. Effect of phosphatase and methylase inhibitors in the nucleosomal remodelling induced by TPA in MLP29 mouse cell line. Serum-starved cells were treated or not with okadaic acid (OK, PP1 and PP2A inhibitor), S-L-homocysteine, adenosine and N-methyl-2adenosine (PRMT1 inhibitors, i PRMT1) for 1 h prior to 30 min treatment with TPA. Mononucleosomal chromatin fragments were purified and analyzed by real-time PCR. The control was arbitrarily set to 1. Results show the average $\pm$ S.D. of, at least, three independent experiments.

Finally, we decided to check the importance of phosphorylation in the remodelling mechanism by analysing *egr-1* expression in cells treated with okadaic acid. As seen in Figure 44, okadaic acid by itself is able to induce *egr-1* expression, despite the fact that it is not as pronounced as when the cells are treated with TPA. Cao *et al.* (1991) described a sustained induction of *egr-1* upon okadaic acid treatment, as a consequence of the multiple phosphorylation of EGR1 at multiple sites. The phosphorylated isoforms are more stable than the usual unphosphorylated form that is produced in cells not treated with serine/threonine phosphatase inhibitors. Nevertheless, our results suggest that the induction could be related to the remodelling that takes place in N1, despite the fact that the other transcription factors are not bound and therefore the induction is not as high as with TPA treatment. Even though, this explanation does not exclude that there is a sustained *egr-1* expression due to the phosphorylation of the gene product.

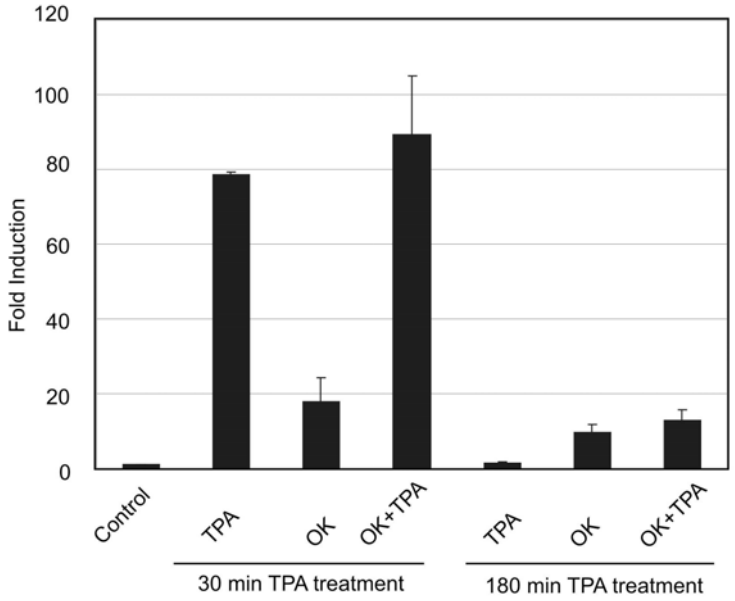


Figure 44. Effect of okadaic acid (PP1 and PP2A phosphatase inhibitor) in *egr-1* expression in MLP29 mouse cell line. Serum-starved cells were treated or not with okadaic acid for 1 h prior to 30 and 180 min treatment with TPA. Total RNA was extracted and analyzed by real-time PCR. The control used was arbitrarily assigned to 1. Results show the average $\pm$ S.D. of, at least, three independent experiments.

7. HISTONE MODIFICATIONS AT MONONUCLEOSOMAL LEVEL DURING *egr-1* INDUCTION

Nucleosomal histones are the target of covalent modifications and, therefore, they play an intrinsic role in the regulation of transcription (Turner *et al.*, 2002). Nevertheless, there is only limited information regarding the modifications found at specific nucleosomes at a given gene after the activating signals. To know if there are specific modifications over defined nucleosomes in our model system, we prepared chromatin of mononucleosomal size and immunoprecipitated it with some of the antibodies that we had previously analyzed: H3K9AcS10P (PA), H3K4Me_3, H3K9Ac, H3K14Ac, H3K18Ac, H3K27Ac, H4K5Ac and H4HiperAc. It is important to note that in the case of N1, the nucleosome displaced during the induction process, the oligonucleotides used to amplify the immunoprecipitated fragments from the repressed and activated state are different. This fact may difficult the comparison between the results, due to the potential differences in the quality of the oligonucleotides used. Nevertheless, it was the only possible way to perform the experiment, as using the same set of oligonucleotides would not have allowed us to amplify the signal in one of the gene states.

The analysis of the modifications present in the three nucleosomes detected in *egr-1* promoter can be seen in Figure 45. The results show a similar pattern for the three positioned nucleosomes analyzed, although there are subtle differences among them in the activated state. For instance, H3K4Me_3, H3K9Ac and H4K5Ac increase in all the cases relative to the control cells. On the contrary, phosphoacetylation of H3 increases in the two nucleosomes that are nearer the transcriptional start, and remains unchanged in N3. In the case of H4, the hyperacetylation shows an opposite pattern, that is to say, increases greatly at nucleosome N3, only a little in nucleosome N2 and it remains unchanged at nucleosome N1. There are also certain modifications that seem to decrease, as H3K27Ac, which could be explained because, as mentioned above, in the mononucleosomal ChIP we have used two different sets of oligonucleotides to amplify the samples from the repressed (0 min TPA) and the activated (15 min) states, and therefore the quality of the

oligonucleotides may differ. Another possible reason could be the fact that we have two molecules of each histone in each of the nucleosomes, and maybe the modification of only one of them is enough to account for the induction process. Therefore, these results would suggest a more precise print of histone changes in the nucleosomes nearer the transcriptional start, probably due to the need of a highly specific signal to dock the proteins necessaries for transcription.

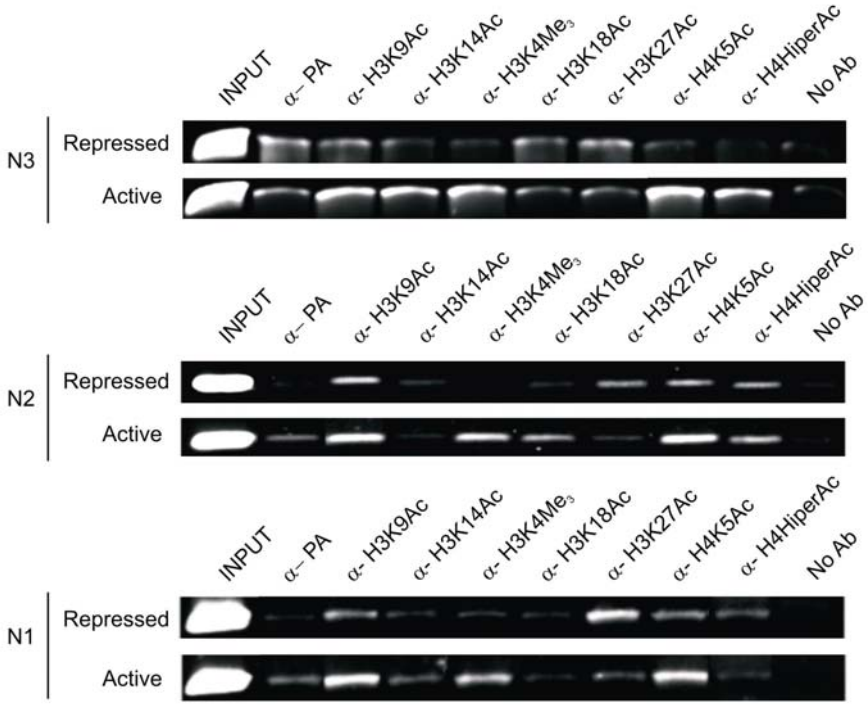


Figure 45. Post-translational histone modifications of the nucleosomes located at *egr-1* promoter. ChIP analysis of MLP29 cells expressing or not *egr-1* mRNA. For the active sample, cells were treated with TPA for 15 min. Crosslinked mononucleosomes were immunoprecipitated with antibodies against H3K9AcS10P (PA), H3K9Ac, H3K14Ac, H3K4Me$_3$, H3K18Ac, H3K27Ac, H4K5Ac, and H4HiperAc or in the absence of antibody (NA). Immunoprecipitated DNA was assayed by PCR with primers with primers 1/ 2, 7/ 8 and 13/ 14 (Table 17) that fall, respectively, within nucleosomes N1, N2 and N3 of *egr-1*.

In conclusion, there are differences between the histone modifications of the three nucleosomes found at *egr-1* promoter, probably because they have diverse roles in the induction process serving to recruit distinct protein complexes. Furthermore, it has to be kept in mind that nucleosome N1 modifies its position during the expression of the gene and there must be specific modifications, not yet determined, that target the nucleosome that has to be displaced.

8. BIOLOGICAL ROLE OF THE TRANSCRIPTIONAL FACTOR EGR1

8.1. EGR1 ACTIVITY ON OTHER TARGETS

Egr-1 activity is partly modulated by the binding of two corepressors named NAB1 and NAB2 (NGFI-A-Binding Protein 1 and 2) (Miano *et al.*, 1999; Srnivasan *et al.*, 2006). NAB1 was first identified in a yeast two-hybrid experiment as interacting with the repression domain, subunit R1, of EGR1 and later, NAB2 was identified by homology with the previously-found protein (Svaren *et al.*, 1996). Both proteins interact with EGR1 through its NCD1 domain (NAB conserved domain 1) and stimulate the repression activity of the transcriptional factor. Therefore, to deepen into the study of *egr-1* regulation we decided to analyze *nab1* and *nab2* genes.

The measurement of the expression of these genes shows that *nab1* is constitutively expressed in MLP29 (data not shown), whereas *nab2* is induced by TPA (Figure 23 and Figure 46A). These results are in agreement with those described by Miano *et al.* (1999), in which they demonstrate that *nab2* expression is also activated by the same stimulus that *egr-1* expression. Moreover, since these three proteins EGR1, NAB1 and NAB2, are implicated together in the control of gene expression, we decided to analyze if there is any auto regulation in the expression of them. To do this, we performed a ChIP assay to study the binding of these factors to *nab2* promoter (Figure 46B). The results demonstrate that EGR1, in conjunction with NAB1 and NAB2 is recruited to the *nab2* promoter at 30 and 60 min after TPA treatment and they are barely seen at 180 min post-induction. These results confirm that in our model system there seems to be a negative feed-back loop between *egr1* and *nab2* regulation.

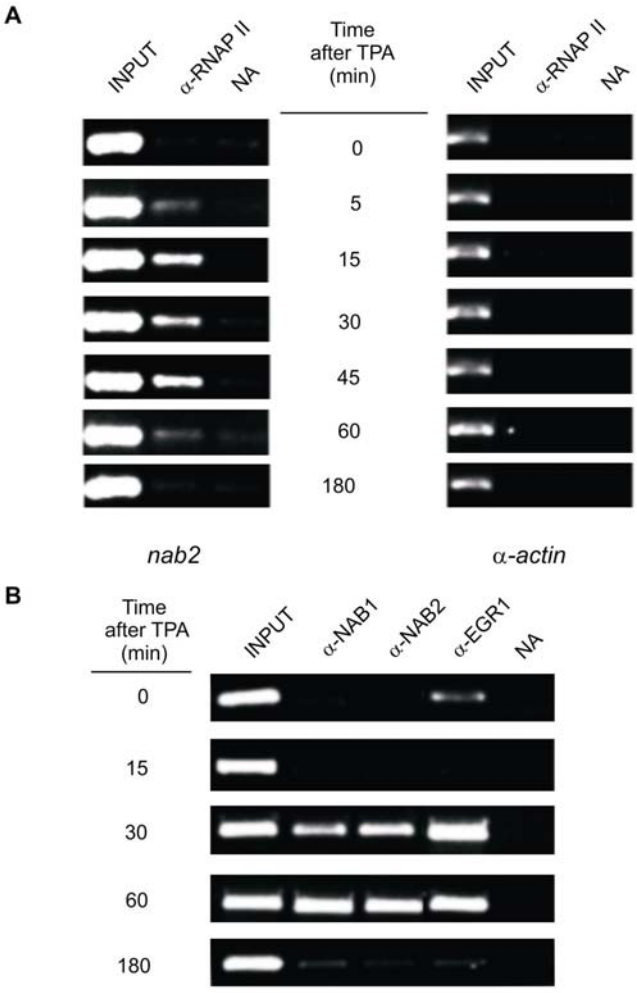


Figure 46. Analysis of *nab2* gene. (**A**) analysis of *nab2* expression by RNAPol-ChIP technique (**B**) Time-dependent ChIP analysis of EGR1, NAB1 and NAB2 binding to *nab2* promoter upon TPA treatment of MLP29 cells.

8.2. EGR1 SHORT INTERFERENCE RNA

RNA interference is a powerful tool that simulates the cellular mechanism for the targeted destruction of gene specific RNA molecules, proving to be very useful to study the function of a protein. This technique is referred by many authors to as "knockdown", as it may not totally abolish the expression of the gene, in contrast to the "knockout" procedures, in which gene expression is entirely eliminated by removing or destroying its DNA sequence.

To gain more knowledge on the role of EGR1 as a transcriptional regulator, we produced *egr-1* short-interfering RNAs and examined weather depletion of the protein had any effect over a functional target such as *nab2* gene. Firstly, we selected three different target sequences (see Section 9.1 from *Materials and Methods*) and cloned them into a pSUPER vector and transfected NIH3T3 mouse fibroblasts. We also tried to transfect MLP29 cells but unfortunately the efficiency of the transfection measured by the green fluorescence of the GFP associated to the vector was very low (results not shown). The effectiveness of the ShRNA used to *knockdown egr-1* is shown in Figure 47A. The expression of *egr-1* is reduced to 30% with Sh1*egr-1*, to 35% with Sh2*egr-1* and just to 50% for Sh3*egr-1*, in comparison to the transfected cells with the vector containing the scrambled sequence (Sh*scrbl*). Furthermore, the effect of Sh1*egr-1* over *egr-1* expression was further confirmed by confocal analysis of samples of cells that have been immunostained with the antibody against EGR1. Figure 47B shows, that the cells co-transfected with the interfence vector for EGR1 (red fluorescence) and GFP expressing vector (green fluorescence) contain a significantly reduced red fluorescence in comparison with the ones that have not been successfully transfected. To have an idea of the importance of these results we decided to quantify the fluorescent red signal, in the untransfected and in the transfected cells, by using ImageJ program (http://rsb.info.nih.gov/ij/). The results obtained assigning a 100% of EGR1 presence in the untransfected cells, showed that in the case of the cells co-transfected with the interference vector for EGR1 and GFP expressing vector, EGR1 presence was as low as 28,3%. This data is totally in

agreement with the decrease detected by *egr-1* mRNA analysis by real-time RT-PCR (see Figure 47A).

Another important conclusion that could be extracted from the confocal analysis resides in the fact that EGR1 transcription factor seems to be mainly localized to the nucleus of the TPA stimulated cells, in contrast to the green fluorescent protein, which is unspecifically localized.

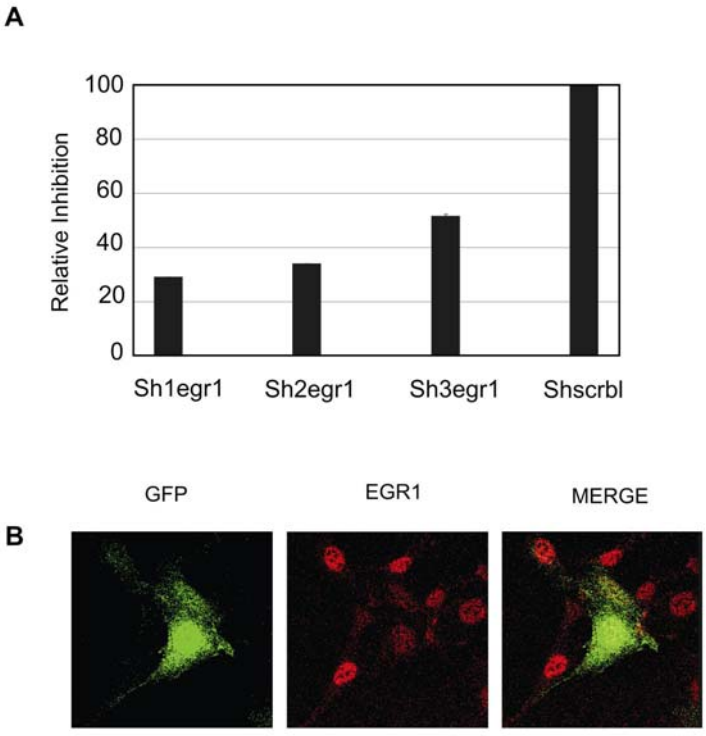


Figure 47. Validation of the efficiency of *egr-1* short interference RNA. (**A**) Cells co-transfected with GFP and pSUPER were sorted with a FACS and total RNA was extracted and *egr-1* expression analyzed by real-time RT-PCR. Cells containing the vector with the scrambled (Shscrbl) sequence were arbitrarily assigned to 100 % expression. (**B**) Confocal image of NIH3T3 mouse fibroblast cells co-transfected with a vector containing GFP (Green Fluorescent Protein) and with Sh1egr-1 pSUPER vector. Cells were immunostainned against EGR1.

To validate the biological activity of *egr-1* short interference RNA, as well as to confirm the importance of EGR1 as a key regulator of *nab2*, we analyzed *nab2* expression by real-time PCR in cells co-transfected with the GFP expressing vector and the sh1*egr1* or sh2*egr1* RNA expressing vector for EGR1. The histogram shown in Figure 48 confirms a decrease in the *nab2* mRNA when cells bear sh*egr1* RNAs. This decrease is more pronounced in the case of sequence 1 than in the case of sequence 2. As a result, our experiments provide evidence that EGR1 is an important regulator of *nab2* expression. This interrelation between *egr-1* and *nab2* was also stated by Kumbrink *et al.* (2005) in their model system, despite the fact that the triggering signal in their cells acted via PKC cascade.

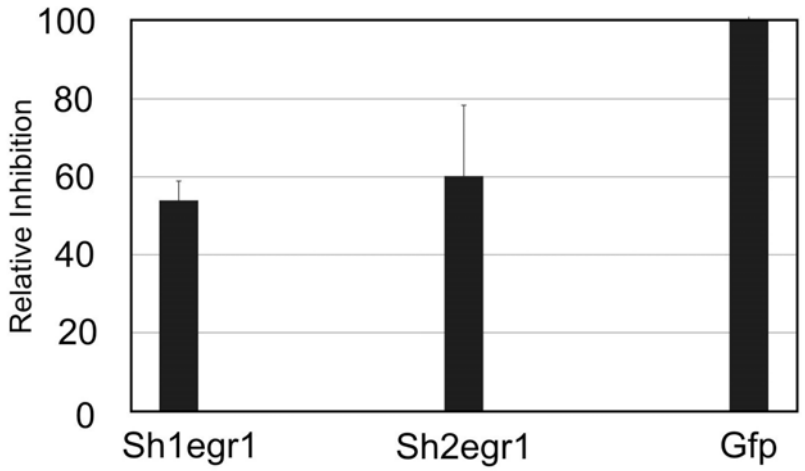


Figure 48. Validation of the biological activity of *egr-1* short interference RNA. Cells co-transfected with GFP and pSUPER were sorted with a FACS and total RNA was extracted and analyzed by real-time RT-PCR. Cells containing only empty vector with GFP were arbitrarily set to 100 % expression.

9. TOWARDS A MODEL FOR *egr-1* INDUCTION

The whole of results obtained so far indicates that *egr-1* gene expression is achieved by an ordered program of recruitment of activators, chromatin-remodelling complexes and coactivators to the promoter. The formation of the transcriptional activating complex in TPA-stimulated MLP29 cells is dependent on stimulation of MEK and p38 signalling pathways. In contrast, co repressor complexes are released from the promoter during transcriptional activation, driving specific histone modifications that allow a shift from repressive to permissive transcriptional state of *egr-1*. Chromatin remodelling, induced by BRM and/or BRG1, is also favouring this process. Therefore, we propose a hypothetical model that attempts to explain the mechanism of *egr-1* transcriptional regulation (Figure 49).

The model is based on a *"preassembled promoter"*, in which transcriptional factors, such as SRF, ELK1, CREB and SP1 are bound to their cognate *trans*-sequences in *egr-1* promoter. These preassembled factors would be in charge of keeping co repressor complexes bound in the gene. Our time-dependent ChIP analyses at different times show, at least, two independent HDACs complexes, one represented by mSIN3A and another represented by NCoR-HDAC3 complex. According to Hong *et al.* (2000), NCoR is able to interact with mSIN3A complex, but our results do not confirm this contact, not only because mSIN3A is usually accompanied by HDAC1 and HDAC2 instead of HDAC3, but also because the recruiting time of both enzymes is slightly different. On the other hand, a complex formed by HDAC3, NCoR and enzymes like BRG1 has been effectively purified in HeLa cells (Underhill *et al.*, 2000, Wen *et al.*, 2000 and Guenter *et al.*, 2001). Unexpectedly, a HAT complex, represented by CBP would also be attached to *egr-1* promoter under repression conditions (represented by the red traffic light in Figure 49). It is usually accepted that HDACs are in charge of maintaining a hypoacetylated transcriptional repressive state of chromatin, but this acetylation level must be achieved by a subtle balance between HDACs and HATs, usually considered as activators. We suggest that CBP found in *egr-1* promoter before its

induction would be responsible of maintaining the basal level of acetylated lysines 9, 14 and 18 from H3 and lysine 5 from H4 found in the promoter.

All the complexes that are present in the *egr-1* promoter are able to be recruited by the bound transcriptional factors, as it has been demonstrated by pull-down or two-hybrid analyses. For instance, ELK1 is able to recruit mSIN3A complex (Yang *et al*., 2001) and both, SRF and SP1 are able to interact with NCoR (Lee *et al.*, 2000; Lee *et al.*, 2005). Furthermore, SP1 and CREB can target CBP to the promoter of some genes (Deng *et al.*, 2003). In conclusion, our model proposes that the bound transcriptional factors are responsible for the ordered recruitment and for the maintenance of co-repressors to the promoter. Anyway, further experiments would be necessary to corroborate the specific interactions that may drive such recruitments.

The ChIP assays also demonstrated the presence of BRM and BRG1 remodelling complexes in the *egr-1* promoter prior to induction, as if they were somehow involved in the N1 nucleosome *"oscillation"* along the region next to the transcriptional start. This hypothesis would imply a permanent energy-consuming process while *egr-1* is repressed, but a possible explanation for this energy waste might be that the importance of *egr-1* regulation resides in its repression more than in its activation. For instance, the nucleosomal sliding would prevent the binding of some necessary components of the pre-initiation complex or components from the elongation complex needed by the *egr-1* gene to be activated.

Upon TPA treatment, the gene is induced (represented by the green traffic light in Figure 49), and p38 and MEK1 drive the signal to *egr-1* promoter that leads to the phosphorylation of ELK1 and CREB. ELK1 phosphorylation would direct the formation to the ternary complex with SRF and DNA (TCF) (Chai *et al.*, 2002, Lindecke *et al.*, 2006), and to the release of mSIN3A. In the same manner, CREB phosphorylation is able to diminish CBP activity, as it was pointed out in the experiments of Chen *et al.*, (2001) and, as a consequence it may leave the promoter. However, there are other experiments in which CREB results phosphorylated by

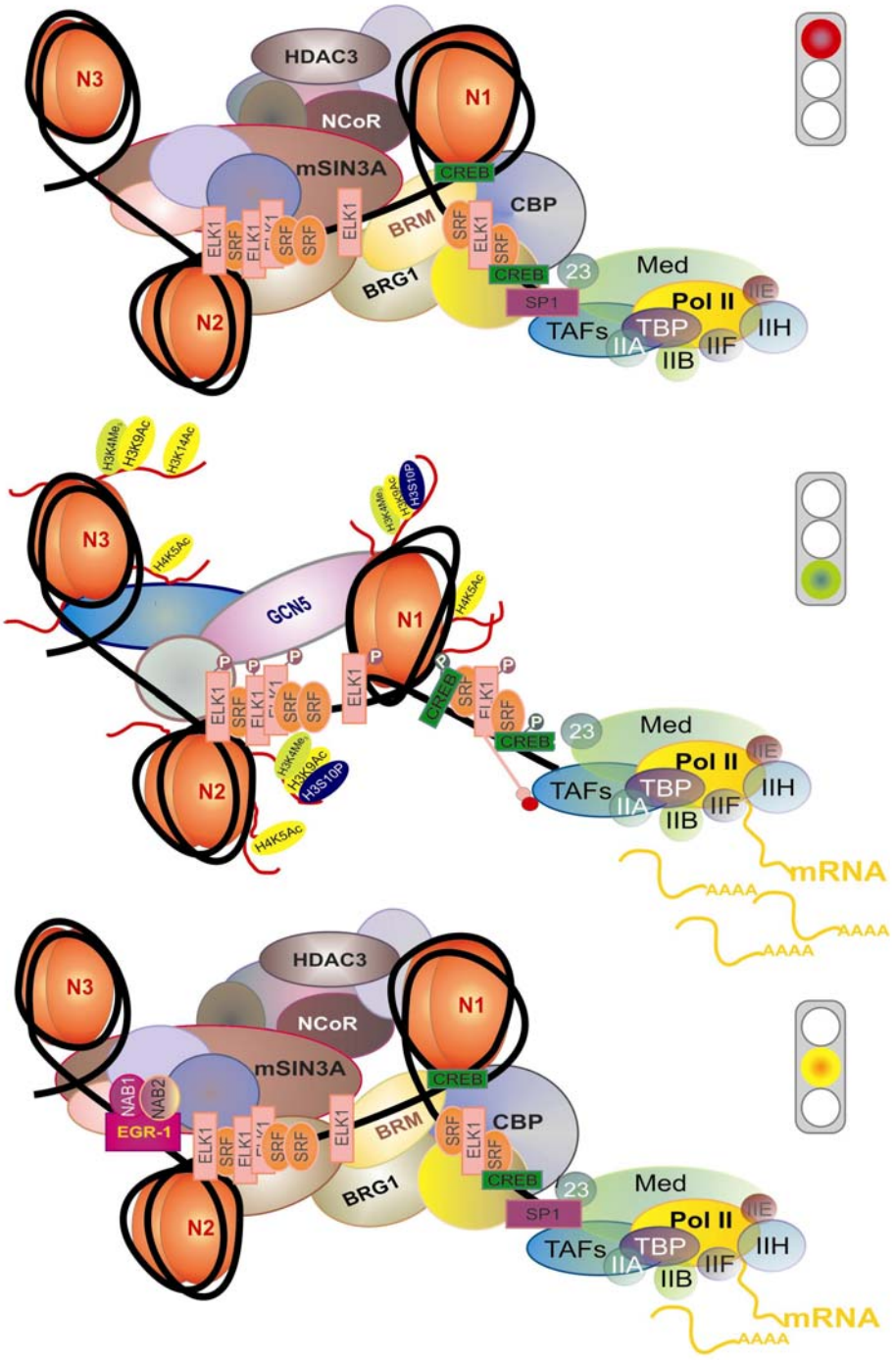
PKC and then it recruits and activates CBP (Molstad *et al.*, 2004). Probably the function of CREB will depend on the characteristics of the kinase cascade and, by extension, on the type of cellular stimulus. When the ternary complex is formed, it would become an activator and, in turn, induce *egr-1* transcription. This activation would be helped by the release of SP1 which would be acting as a gene repressor being responsible for the recruitment of NCoR-HDAC3 complex. The union between SP1 and NCoR complex would be broken by the phosphorylation of NCoR, as ChIP assays with pre-treated cells with MEK or p38 inhibitors show the permanent presence of the NCoR complex, absent in untreated cells. Lee *et al.*, (2005) demonstrated, by co-immunoprecipitation assays, that SP1 and NCoR interaction is regulated by MEK signalling.

In the context of chromatin remodelling, the ATP-dependent remodelling complexes leave the promoter possibly permitting the proximal nucleosome N1 to relocate itself and allowing the binding of new components to the transcriptional machinery. It could also be probable that phosphorylated ELK1 interacts with the mediator components to recruit lacking subunits or to enhance *egr-1* transcriptional rate. It has been shown that phosphorylated ELK1 is able to interact with SUR2 (Cantin *et al.*, 2003) and with MED23 (Wang *et al.*, 2005), two subunits of the mediator complex, and subsequently stimulate RNA Pol II initiation.

All these changes induced in the promoter would recruit GCN5 that changes the level of acetylation of specific sites over the histone tails, creating another point of expression control. In addition to general increased acetylation levels, such as of lysines 9, 14 and 18 of H3 and of lysine 5 in H4, we found that there are subtle differences in each of the three nucleosomes arranged in *egr-1* promoter. We observed an increment of levels of phosphoacetylation (H3S10-PhK9Ac) in nucleosomes N1 and N2, whereas these levels remain constant in nucleosome N3. This modification may be responsible for the relaxed chromatin structure, necessary for *egr-1* expression, and would also be functionally linked to GCN5, as a first step for the enzyme-mediated acetylation of H3K14Ac (Cheung *et al.*, 2000). It is noteworthy that H3K14Ac increases only in N3 remaining almost unchanged in N1

and N2, implying that the acetylation mediated by GCN5 would be the consequence of a general increase but it seems not to be nucleosome-specific. $H3K4Me_3$ and H4K5Ac are also generally increased all over *egr-1* promoter. As previously mentioned, K4 methylation of H3 is a general mark for active gene transcription, and therefore is reasonable to find it increased in the nucleosomes of *egr-1*. All these modifications would help to recruit co-factors and polymerase II following the instructions given by the histone code and after an appropriated remodelling of the nucleosomes.

Figure 49. Hypothetical mechanism of *egr-1* induction upon TPA treatment in MLP29 mouse cells. In the arrested gene (red traffic light), SP1, ELK1, SRF and CREB transcription factors are bound to their recognition sequences in the promoter. The gene is kept repressed by two different HDAC complexes, one containing mSIN3A and the other containing HDAC3 and NCoR. Nevertheless there is a HAT complex, CBP, which is in charge of the basal acetylated state of the gene. Furthermore, there are two ATP-dependent remodelling enzymes: BRM and BRG1. Upon TPA treatment (green traffic light), p38 and MEK phosphorylate ELK1, which is able to form and activate ternary complex with SRF and CREB, interacting with the mediator and facilitating *egr-1* transcription. At the same time, the remodelling and HDAC complexes leave the promoter, facilitating nucleosome N1 displacement, and GCN5 containing complex is recruited. To simplify the model, the modifications that take place in the tails of the histones H3 and H4 of the nucleosomes are only depicted in the activated state, and therefore only those that are increased in each of them are represented. Finally, in the repression process of the gene (yellow traffic light), EGR1 binds to its own promoter and recruits NAB1 and NAB2, which in turn activate the repression domain of EGR1. GCN5 complex leaves the promoter and the HDAC and remodelling complexes are able to return. In the same manner, N1 returns to its initial repressive position.


HDAC3
N3
N1
NCoR
mSIN3A
CREB
CBP
ELK1
SRF
BRM
BRG1
N2
CREB
23
Med
SP1
Pol II
IIE
TAFs
TBP
IIA
IIF
IIH
IIB
H3K4Me3
H3K9Ac
H3K14Ac
H4K5Ac
GCN5
H3S10P
P
mRNA
AAAA
NAB1
NAB2
EGR-1

Once the roles of EGR1 have been fulfilled, the protein itself binds to its own promoter creating a negative autoregulatory loop (Figure 49, yellow traffic light), the protein itself binds to its own promoter creating an autoregulatory loop. Upon EGR1 binding to the promoter, the transcriptional factors NAB1 and NAB2 bind to EGR1 and activate its repression domain. It has been demonstrated that NAB1 is able on its own to repress gene expression (Thiel *et al.*, 2000) and also that both NAB1 and NAB2 are capable of interacting with subunits of the repressor NuRD complex (Srinivasan *et al.*, 2006). Nevertheless, in our model system, as both NAB1 and NAB2 are bound to *egr-1* promoter, it makes sense to think that both of them act synergistically to activate EGR1 repression domain. It could also be possible that only one of the co-factors would be enough to active EGR1 repressive properties, and therefore the coupling of them acts as a security brake.

Consequetly, our results suggest a model in which EGR1 and SP1 would be responsible to return the gene to its basal repressive state, by recruiting again all the co-repressors found in the basal state (Figure 49, red traffic light). In addition, N1 will be again *"oscillating"* along the promoter, maybe preventing the recruitment of components of the basal transcriptional machinery, or avoiding further transcription factors, not yet identified, to interact with its DNA-binding sequence. Therefore, cell-specific factors and signalling pathways may regulate transcriptional activation of the murine *egr-1* gene by modulating its gene-specific transcriptional program under the mitogenic signal of phorbol esters.

CONCLUSIONS

1- The gene *egr-1* (early growth response-1) is highly expressed upon TPA treatment of different cell lines. RNAPol-ChIP experiments show a pattern of expression that begins as early as 5 min, with a peak around 30 min to decrease subsequently and be again undetectable at 3 h after the treatment.

2- *Egr-1* may be considered as a "potentially active gene" as it seems to be "ready" to be expressed after the appropriated stimulus, seeing that the RNApol II and all the necessary transcription factors are bound to the promoter in the basal state. The two SRE clusters seem to be occupied and, by contrast, only the more proximal SP1 binding site has the transcription factor attached. The existent transcriptional factors in the promoter (CREB and ELK1) need only to be phosphorylated by MEK and/or p38 network to induce expression. In concrete, ELK1 becomes phosphorylated to render active the ternary complex (TFC) with the pre-bound SRF. These results are in accordance with the lack of gene expression when MLP29 cells are treated with p38 and MEK kinases inhibitors.

3- SP1, is the unique transcriptional repressor found in our studies which seems to leave the promoter during *egr-1* induction to return subsequently when the gene starts to be repressed.

4- The transcriptional factor EGR1 binds to its own promoter 15 min after TPA administration and therefore, it seems to operate as an enhancer of its own transcription. Afterwards, at 30 min of TPA induction, the factors NAB1 and NAB2 interact with EGR1, activating its repression domain and hence repressing *egr-1* expression.

5- There are at least two histone deacetylase repressor complexes in *egr-1* transcriptional basal state: mSIN3A and HDAC3-NCoR. Those complexes leave the promoter during transcriptional activation and return afterwards when *egr-1* is repressed. Furthermore, two histone acetyltransferases complexes have been involved in *egr-1* gene expression. CBP, which would be in charge of the basal acetylated state of the gene, and GCN5, which activity would be specific for the gene induction by TPA. When histone

modifications in *egr-1* promoter are studied, we detect an increase in the H3K9AcS10P, which is a transcriptional mark for immediate-early genes. Furthermore, there is a significant increase in the acetylation of H3 and H4, in particularly H4, which only has K5 modified when the gene is not being expressed and, upon induction, K8 and K16 are also subject to acetylation. These modifications seem to be particular for immediate-early genes, as they are absent in the promoter of the constitutively active *β-actin* gene.

6- We have also found that di- and tri-methylated K4 of H3 are present along the promoter and coding region of the gene, but they are also present in the *β-actin* promoter, implying that they may be a general epigenetic signal for genes that are transcriptionally active.

7- There are three regularly positioned nucleosomes along *egr-1* promoter. Nucleosome N1 has its putative dyad axis position at -200 bp, nucleosome N2 at -510 bp and nucleosome N3 at -800 bp from transcriptional nucleotide initiation. Nucleosomes N1 and N3 would be positioned by its sequence, whereas N2 would be located indirectly by the boundary action of the other two. The nucleosome N1 moves around 50 bp upstream upon gene TPA induction, and it returns to the previous position during the subsequent gene repression.

8- BRG1 and BRM remodelling complexes have also been found in the promoter when the gene is inactive, probably facilitating the nucleosome N1 sliding.

9- The phosphorylation of specific transcriptional factors or histones seems to be important for the remodelling process as phosphatase inhibitors alter the remodelers' nucleosomal affinity. On the contrary, methylation of histones seem no to have any effect, as PRMT1 inhibitors or the lack of H4R3Me_2a do not alter the nucleosomal positioning.

10- Nucleosomal-resolution ChIP analysis of N1, N2 and N3 revealed general modifications in the three nucleosomes, as an augment in H3K4Me_3, H3K9Ac and H4K5Ac. On the contrary, nucleosomes N1 and N2 increase its modification H3K9AcS10P, that remains unchanged for nucleosome N3,

but the later is the one that has a considerable increase in H3K14Ac and H4hiperAc modifications.

11- The transcriptional factor EGR1 regulates *nab2* expression, and the incubation of cells with short interference for EGR1 synthesis produces a noteworthy diminishing both in *egr1* and *nab2* expression.

12- Sequence 1 of short interference for EGR1 on its own, is able to reduce *egr-1* expression 70%, as it was demonstrated both by real-time analysis of the gene expression and, by confocal analysis of co-transfected cells with GPF expressing vector and EGR1 short interference expressing vector, analyzed with ImageJ program.

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SPANISH SUMMARY

I. INTRODUCCIÓN

1. PAPEL DE LA CROMATINA EN LA TRANSCRIPCIÓN

Las células eucariotas han resuelto el problema de adaptar su largo genoma (~ 2 metros) al pequeño espacio del núcleo (~ 10 μm de diámetro), mediante la formación de una estructura compacta básica, formada por DNA y proteínas, llamada nucleosoma, que es además capaz de plegarse formando estructuras más complejas (Figure 2). La unión del DNA con proteínas histona y no histona, es decir, la cromatina (Fleming, 1882), a pesar de ser una estructura dinámica crea un obstáculo para la maquinaria transcripcional puesto que dificulta la unión de los factores transcripcionales al DNA. Para superar estas restricciones, la célula ha organizado el reclutamiento de enzimas modificadores de la cromatina, que son capaces de producir cambios en los promotores de los genes, alterando así la accesibilidad de los factores necesarios para la transcripción. Estas enzimas pueden ser clasificadas en dos grandes grupos: enzimas modificadoras de la cromatina y complejos remodeladores de la cromatina dependientes de ATP. Las enzimas del primer grupo son las encargadas de modificar covalentemente las histonas, alterando con ello la carga generalmente positiva de las mismas y perturbando la estructura de la cromatina. Por el contrario, los complejos del segundo grupo utilizan la energía procedente de la hidrólisis del ATP para modificar las interacciones histona-DNA, incrementando la accesibilidad al DNA nucleosomal tanto de los factores transcripcionales como de la maquinaria transcripcional. Además, la transcripción en eucariotas puede también ser facilitada por las alteraciones que tienen lugar en el "*core*" del nucleosoma (Becker *et al.*, 2002).

1.1 Estructura de la cromatina e hipótesis del código de histonas

El nucleosoma está formado por unos 200 pb (dependiendo del organismo), un octámero de histonas, formado por un tetrámero de H3/H4 y dos dímeros de H2A/H2B, y una copia de la histona H1. El "core" nucleosomal, por su parte tiene

147 pb y le falta la histona H1, que se sitúa en el DNA espaciador entre dos nucleosomas consecutivos (Thomas *et al.*, 1975 y Richmond, 1985).

Las histonas son proteínas básicas altamente conservadas a lo largo de la evolución y que se caracterizan por poseer un dominio estructural denominado "histone fold". Este dominio se compone de una hélice α central, separada mediante dos giros de hojas β de dos hélices α en los extremos, y es el responsable de la dimerización de histonas (Arents *et al.*, 1991 y Richmond *et al.*, 2003). Las colas N-terminal de las histonas sobresalen del "core" nucleosomal y pueden ser modificadas covalentemente por reacciones de acetilación, fosforilación, metilación y otras (Figure 3 y Table 1). Estas modificaciones, tienen una doble importancia: por un lado, son importantes para la formación de estructuras de orden superior y por otro, sirven para reclutar cofactores (Cosgrove *et al.*, 2004) y transmitir la información epigenética contenida en el DNA (López-Rodas *et al.*, 1993). El hecho de que combinaciones específicas de modificaciones de histonas tengan lugar durante determinadas funciones biológicas ha llevado a proponer la hipótesis del "código de modificación de histonas" (Strahl *et al.*, 2000). Sin embargo, hay que tener en cuenta que una modificación determinada puede tener, en contextos celulares diversos, diferentes funciones. Por ejemplo, la metilación de la lisina 9 de H3, generalmente asociada a represión transcripcional, puede asociarse en determinadas circunstancias con genes que están siendo activamente transcritos (Beisel *et al.*, 2002).

La hipótesis del código de histonas se apoya en varios hechos. Por un lado, la existencia de enzimas modificadoras de la cromatina, como son los complejos enzimáticos histona acetiltransferasa o histona desacetilasa (Tables 2, 3 y 4), que son reclutados específicamente a determinados residuos. Por otro lado, en el hecho de que estas modificaciones específicas pueden ser reconocidas por dominios concretos de diversos cofactores necesarios para el proceso transcripcional, permitiendo así la lectura del código. Por ejemplo, los bromodominios, generalmente presentes en complejos histona acetiltransferasa, son capaces de reconocer lisinas acetiladas de las colas N-terminales de las histonas, o los cromodominios, encontrados generalmente en complejos remodeladores y que reconocen específicamente lisinas metiladas.

1.2 Enzimas modificadoras de la cromatina

Como se ha comentado anteriormente, las colas N-terminales de las histonas son susceptibles de ser acetiladas, metiladas y fosforiladas en residuos específicos. Estos procesos son llevados a cabo por diferentes complejos enzimáticos: histona acetiltransferasas, histona desacetilasas, histona metiltransferasas e histona quinasas.

Las histona acetiltransferasas (HATs) catalizan la transferencia de un grupo acetilo del acetil-coenzima A al grupo ε-amino de los residuos de lisina presentes en las colas de las histonas (Figure 4). Este proceso está generalmente relacionado con la activación transcripcional (Kouzarides, 1999). Las HATs pueden ser clasificadas en 3 superfamilias: la superfamilia GNAT, que incluye enzimas como GCN5, PCAF, ELP3 o HAT1; la superfamilia MYST, dentro de la se encuentran MOZ y ESA1 y la superfamilia p300/CBP (Table 2).

Las histona desacetilasas (HDACs) por el contrario catalizan la eliminación de los grupos acetilo de los residuos de lisina, revirtiendo la modificación de las HATs, y por tanto su actividad ha sido relacionada con la represión y el silenciamiento génicos. Estas enzimas se clasifican en tres subfamilias (I, II y III). Las subfamilias I y II contienen un catión de zinc en su sitio catalítico y se inhiben con la tricostatina A (TSA), mientras que la subfamilia III requieren el coenzima NAD^+ como cofactor y son insensibles al tratamiento con TSA (Sengupta *et al.*, 2004) (Table 3).

Los residuos de lisina y arginina de las colas N-terminales pueden ser asimismo metilados, en un proceso llevado a cabo por los complejos histona metiltransferasa (HMTs), consistente en la transferencia de un grupo metilo de la S-adenosil-L-metionina al residuo receptor. Esta reacción incrementa la hidrofobicidad de la molécula pero no altera la carga global de las histonas como ocurre en el caso de la acetilación. Existen dos familias principales de HMTs clasificadas atendiendo a si modifican lisinas (HMTs) o argininas (PRMTs) (Table 5). La metilación de

histonas se ha asociado tanto con procesos de activación (Castellano *et al.*, 2006) como de represión transcripcional (Mal, 2006).

Recientemente se han aislado enzimas capaces de revertir la reacción de metilación (Steward *et al.*, 2007), pero además se han propuesto varios mecanismos capaces de eliminar el grupo metilo como son la oxidación, el recambio de histonas o la eliminación de la cola N-terminal (Jenuwein *et al.*, 2001, Ahmad, 2002 y Benister 2002) (Figure 5).

Otra modificación que afecta a las histonas es la fosforilación de residuos de serina y treonina (Davie *et al.*, 1999). De todas las fosforilaciones posibles, la más estudiada ha sido la fosforilación de la serina 10 de la H3, que puede ser llevada a cabo por enzimas como RSK2 y MSK1 y que suele estar relacionada con la activación transcripcional de genes inmediato-tempranos. La reacción de eliminación del grupo fosfato es generalmente llevada a cabo por la fosfoproteína fosfatasa 1 (PP1).

1.3 Remodelación de la Cromatina

Una de las barreras a las que se enfrenta la maquinaria transcripcional durante el ensamblaje de la RNA polimerasa II es el acceso al DNA dificultado en la estructura de la cromatina. Para salvar este obstáculo, la célula posee familias de enzimas remodeladoras de la cromatina, que pueden alterar las interacciones DNA-histona usando la energía liberada por la hidrólisis del ATP, y facilitar de este modo las interacciones necesarias.

Todos los complejos remodeladores, poseen una subunidad ATPasa y pueden clasificarse, atendiendo al tipo de ésta, en tres subfamilias diferentes: ISWI, SWI/SNF y CHD (Table 6). Estos complejos enzimáticos pueden estar además asociados a complejos modificadores de la cromatina, como por ejemplo HDACs en el caso del complejo remodelador NuRD (Serna *et al.*, 2006).

La regulación de los complejos remodeladores de la cromatina no es todavía completamente conocida, pero modificaciones de alguna de sus subunidades, por ejemplo por fosforilación, producen cambios en las propiedades del complejo y por tanto en su actividad (Becker, 2002).

2. EL GEN *egr-1*.

El gen *egr-1* (early growth response-1), también llamado *tis8*, *zif268*, *ngfi-A* o *krox-24*, pertenece a una familia génica que codifica factores transcripcionales con motivos de dedos de zinc. La inducción de *egr-1* se produce rápidamente en repuesta a una gran variedad de estímulos como son: factores de crecimiento, citoquinas, hormonas, ésteres de forbol o radiación ultravioleta. La expresión de *egr-1* se ha relacionado con diferentes procesos biológicos como son el crecimiento, la diferenciación celular y la apoptosis (Thiel *et al.*, 2002). Ahí radica justamente su importancia como puente entre las señales extracelulares que afectan a la célula y el mantenimiento de la homeostasis de la misma mediante la alteración de genes de respuesta secundaria.

El promotor de *egr-1* contiene sitios de unión para factores transcripcionales como: SRE (elemento de respuesta al suero), ETS (elemento de respuesta a factores Ets, como ELK1), CRE (elemento de respuesta al AMP cíclico), caja GC (sitio de unión del factor SP1) o EBS (elemento de respuesta a EGR1) (Figure 9). El factor de respuesta al suero SRE es un factor transcripcional que se une como dímero a la secuencia $CC(A/T)_6GG$ y que necesita formar un complejo ternario con el DNA y un elemento Ets adyacente para ser activo. Igualmente, el factor CREB es capaz de formar dímeros, y dependiendo de la naturaleza de los mismos puede tener función activadora o represora. Por ejemplo, cuando dimeriza con CREM actúa como represor transcripcional mientras que su interacción con ATF2 o con si mismo le otorga un papel como activador (Carlezon *et al.*, 2005). Además, estos factores transcripcionales pueden ser modificados mediante acetilaciones y/o fosforilaciones, como otro modo de regular sus propiedades de unión al DNA y su actividad.

2.1 EGR1 como factor transcripcional

EGR1 es una proteína modular de aproximadamente 80 kDa, que tiene un dominio activador N-terminal, un dominio inhibidor central y un dominio de unión al DNA formado por tres dedos de zinc (Figure 10). El dominio central es capaz de unir dos co-factores transcripcionales, NAB1 y NAB2, que modifican la actividad biológica de EGR1 transformándolo de activador en represor. Otros miembros de esta familia de factores transcripcionales contienen también motivos de dedo de zinc, pero difieren en el resto de dominios y, por tanto, ejercen funciones biológicas diferentes.

EGR1 se une a secuencias específicas de DNA, ricas en GC, como son los casos 5'-GCG(G/C)GGGCG-3' (Lemaire *et al.*, 1990 y Cao *et al.*, 1990) o 5'-TGCGTG/AGGCGGT-3' (Thiel *et al.*, 2002). Estos sitios de unión son altamente similares a aquellos que reconoce el factor SP1 y se han descrito casos en que se estable una competencia entre ambos factores por el sitio de unión común (Kachigian *et al.*, 1995).

La regulación de la proteína EGR1 se consigue mediante modificaciones post-traduccionales como glicosilación (Hedegen *et al.*, 1998), fosforilación (Huang *et al.*, 1998), acetilación (Adamson *et al.*, 2005) y poliadenilación (Simon *et al.*, 2004). La fosforilación de EGR1 llevada a cabo por la quinasa CKII, parece disminuir las propiedades de unión de la proteína y se ha relacionado con la activación de genes pro-apoptóticos como *p53* (Mora *et al.*, 2005). Sin embargo, la acetilación de EGR1 mejora la estabilidad de la proteína y se ha relacionado con la activación de genes de proliferación celular.

En general la proteína EGR1 tiene efectos ambiguos, ya que puede estimular o suprimir el crecimiento celular dependiendo de la intensidad de la inducción de *egr-1* y del gen diana sobre el que vaya a actuar. Por ejemplo, Yu *et al.*, (2004) demostraron que EGR1 puede inducir además de apoptosis así como la supervivencia celular por medio de la activación del gen *p21*, en lugar del gen *p53*.

II. OBJETIVOS

La regulación génica en eucariotas requiere de múltiples pasos que deben ser altamente regulados para asegurar una adecuada respuesta celular a los diferentes estímulos que sufre la célula. La comprensión del modo en que dichos mecanismos tienen lugar es de gran importancia tanto para el conocimiento básico molecular como para la comprensión de diferentes patologías. La acción coordinada de las diferentes cascadas de señalización y la unión de factores transcripcionales y de complejos enzimáticos modificadores a la cromatina es fundamental para la correcta interpretación del código genético. Actualmente, no existe un mecanismo general para la regulación génica, puesto que cada gen tiene tanto características independientes como generales.

Los objetivos del presente trabajo se centran en el estudio de algunos de los mecanismos epigenéticos que subyacen a la activación y represión transcripcional de genes inmediato-tempranos.

1- El primer objetivo consistió en la selección de un sistema biológico adecuado para el análisis temporal de los diferentes eventos que tienen lugar durante la regulación transcripcional.

2- El segundo objetivo fue el estudio de las diferentes cascadas de señalización implicadas en la transducción de la señal desde la superficie celular hasta el núcleo regulando el acceso y la unión de factores transcripcionales y de la RNA polimerasa al gen o genes de interés.

3- Del mismo modo, se quiso averiguar qué factores transcripcionales están implicados en la expresión de genes inmediato-tempranos, y si esos factores están o no sujetos a modificaciones post-traduccionales durante el proceso de activación.

4- Como el DNA eucariótico se encuentra empaquetado con proteínas histonas, el estudio de la regulación transcripcional tiene que realizarse

teniendo en cuenta el contexto cromatínico en que se encuentran los promotores de los diversos genes. Consecuentemente, nuestro principal propósito fue determinar qué complejos modificadores de la cromatina eran necesarios para la adecuada regulación de la expresión e intentar correlacionar la existencia de dichos complejos con las modificaciones en las histonas que tienen lugar durante la inducción de *egr-1*. Igualmente, se intentó establecer la distribución nucleosomal durante la inducción del gen para determinar la posible existencia de remodelación nucleosomal.

5- Puesto que la traducción del gen bajo estudio tiene importantes consecuencias se consideró conveniente estudiar alguno de los efectos que tendría la falta de su traducción en el sistema biológico de estudio.

6- Finalmente, mediante la combinación de los resultados obtenidos en los objetivos previos, se pretendió postular un modelo de regulación epigenética para el gen *egr-1* en células MLP29 inducidas con miristato de 12-O-tetradecanoilforbol (TPA).

III. RESULTADOS Y DISCUSIÓN

1. SELECCIÓN DEL SISTEMA DE ESTUDIO.

Para la selección del sistema de estudio se analizó mediante RT-PCR la expresión de genes sensores de estrés (*gadd 153* y *c-fos* entre otros) en dos líneas celulares: C3H10T1/2 y MLP29 tratadas con arsenito 400 μM (Figure 17). Del mismo modo, se probó el nivel de inducción de dichos genes producido por diferentes agentes como: el arsenito, el tert-butilhidroperóxido, el TPA o la radiación ultravioleta (Figures 19, 20 y 21). Con los resultados obtenidos se eligió como sistema biológico más apropiado para los estudios la línea celular progenitora de hepatocitos MLP29, como agente inductor el TPA a una concentración 50 nM y, tras un rastreo en que se analizó la inducción de diferentes genes (Figure 22), se eligió el gen *egr-1* para el estudio detallado de su proceso de inducción.

2. ANÁLISIS DE LA EXPRESIÓN DE *egr-1*.

El análisis de la expresión de *egr-1* en células MLP29 tratadas con TPA a diferentes tiempos se realizó mediante dos técnicas: la extracción del RNA total y su análisis mediante RT-PCR semi-cuantitativa y a tiempo real, y mediante la técnica de la RNAPol-ChIP consistente en la inmunoprecipitación de la cromatina a la que está unida la RNA pol II y la amplificación a partir del DNA inmunoprecipitado de la región codificante del gen bajo estudio.

Los resultados obtenidos mediante la RNAPol-ChIP (Figure 23A) demuestran que *egr-1* comienza a expresarse a los 5 min. del tratamiento, alcanza un máximo a los 30 min. y a partir de este momento comienza a decrecer hasta que ya no se detecta a los 180 min. de la inducción. Por su parte, mediante RT-PCR (Figure 23B y C) se detecta expresión a los 15 min., con un máximo a los 45 min. Estas pequeñas variaciones con respecto a la otra técnica son debidas a que la RNAPol-ChIP es capaz de detectar la transcripción en tiempo real, mientras que la RT-PCR analiza los niveles estacionarios de mensajero, que pueden estar influenciados por el metabolismo del mismo.

3. CASCADAS DE SEÑALIZACIÓN IMPLICADAS EN LA ACTIVACIÓN DE *egr-1*.

Con objeto de estudiar las cascadas de señalización implicadas en la activación del gen *egr-1* por TPA se trataron las células MLP29 con inhibidores de las diferentes rutas de señalización (MEK1/2, p38, JNK, ERK, PI3K, PKC, NFκB y STAT3) durante 1 h y posteriormente se estimularon con TPA durante 30 min. El análisis de la expresión de *egr-1* mediante RT-PCR y su comparación con las células control no tratadas, han permitido implicar a las rutas reguladas por las quinasas MEK1/2 y p38 en el proceso de inducción del gen bajo estudio (Figure 24).

4. FACTORES TRANSCRIPCIONALES ASOCIADOS AL PROMOTOR DE *egr-1* DURANTE SU INDUCCIÓN.

El estudio de los factores transcripcionales asociados al promotor de *egr-1*, requiere como paso previo el análisis bioinformático de los promotores para identificar los potenciales sitios de unión de dichos factores a la cromatina. Una vez identificados, utilizando el programa TRANSFAC (Figure 25A), se comprobó mediante inmunoprecipitación de cromatina (ChIP) la presencia o no de los mismos en el promotor de *egr-1 in vivo*. Los resultados de los experimentos de ChIP realizados a diferentes intervalos de tiempo tras la administración del TPA muestran que los factores SRF, ELK1 y CREB se encuentran presentes constitutivamente en el promotor del gen (Figure 25B). Por el contrario, el factor SP1 está unido a tiempo 0, se libera a los 15 min y vuelve a ser detectado en el promotor a los 60 min (Figure 25B y 28), lo cual le atribuiría un posible papel represor en la inducción de *egr-1*. El análisis de la ocupación de los diferentes sitios de unión de los factores SRF y SP1, demuestra que las dos agrupaciones de sitios de unión de SRF (Figure 25A) están ocupadas, mientras que para el caso de SP1, únicamente el sitio de unión más próximo al inicio de la transcripción estaría ocupado a tiempo 0 (Figures 26 y27).

Por otra parte, el producto del gen, la proteína EGR1, se une a su propio promotor a los 15 min del tratamiento con TPA, para abandonarlo posteriormente a los 60 min (Figure 25B). Otros dos cofactores que se unen al promotor a los 30 min de tratamiento son: NAB1 y NAB2 (Figure 25B), que probablemente interactúan con EGR1 para activar su dominio represor. Estos resultados sugieren que EGR1 se une a su promotor activando la transcripción a los 15 min, para después regular su represión a los 30 min con la ayuda de NAB1 y NAB2.

Debido al hecho de que todos los factores encontrados se encuentran presentes en el promotor de *egr-1* antes de la inducción, se decidió estudiar si dichos factores eran susceptibles de ser modificados por fosforilación, de tal forma que se modificase su función y activar en esta forma al gen *egr1*. Los resultados obtenidos

mediante ChIP muestran que ELK1 se encuentra fosforilado a los 15 y 30 min tras la inducción, mientras que CREB sólo aparece con la forma fosforilada a los 15 min de la inducción (Figure 28). En ambos casos, cuando se tratan previamente a las células con inhibidores de las rutas MEK1/2 y p38, la forma fosforilada de CREB es detectada con menor intensidad en el promotor del gen (Figure 29). Con estos resultados parece lógico pensar que el TPA induce *egr-1* a través de la activación de las rutas de quinasas MEK1/2 y p38, las cuales encargarían de fosforilar, al menos, los factores CREB y ELK1 para que puedan actuar como activadores de la transcripción del gen.

5. ENZIMAS MODIFICADORES DE LA CROMATINA ASOCIADOS AL PROMOTOR DE *egr-1* DURANTE SU INDUCCIÓN.

Debido a la importancia de las modificaciones de las histonas como mecanismo de regulación de la expresión génica se decidió estudiar, por un lado, si existía algún represor que debiera ser sintetizado continuamente y, por otro, qué complejos HDAC y HAT se encontraban presentes en el promotor de *egr-1*. Para abordar la primera cuestión, las células con cicloheximida, un inhibidor de la síntesis de proteínas, previo a la estimulación de la células con TPA. El análisis del RNA mediante RT-PCR a tiempo real determinó que las células tratadas con cicloheximida tenían una mayor grado de expresión de *egr-1* (Figure 30) lo cual indicaba la necesidad de síntesis de algún componente para la represión del gen.

Por otro lado, para detectar la presencia de enzimas modificadores de la cromatina, realizamos estudios de ChIP a diferentes tiempos tras el tratamiento con TPA. Dichos experimentos revelaron la presencia de dos complejos represores HDAC (mSIN3A y HDAC3-NCoR), unidos a tiempo 0 min, que se liberaban durante la inducción del gen, para volver al promotor a los 60 min de tratamiento (Figure 31). Igualmente, se observó la presencia de dos complejos HAT: CBP y GCN5 (Figure 32). CBP se libera del promotor durante la inducción, lo cual le otorgaría un papel como posible encargado de mantener el nivel basal de acetilación del gen. Por el contrario, GCN5 entra en el promotor entre los 5 y los 15 min, con lo

cual las acetilaciones de las que es responsable podrían tener una función más relevante en el proceso de inducción de *egr-1*.

En un intento de correlacionar la presencia de estos complejos con las diferentes modificaciones que tienen lugar en *egr-1* durante la inducción, se realizaron experimentos de inmunoprecipitación utilizando anticuerpos específicos frente a modificaciones concretas de las histonas. En general, los resultados muestran un alto nivel de acetilación basal cuando el gen no se está transcribiendo y un claro aumento de las modificaciones H3K9Ac, H3K14Ac, H3K18Ac, H3K27Ac, H4K5Ac, H4K8Ac, H4K16Ac y H3K9AcS10P durante la inducción (Figure 37).

6. POSICIONAMIENTO NUCLEOSOMAL DEL PROMOTOR DE *egr-1*.

Uno de los factores a tener en cuenta durante la inducción de *egr-1* es el posicionamiento de los nucleosomas que se encuentran presentes en el promotor y que pueden facilitar o dificultar la interacción con factores y cofactores necesarios para el proceso de expresión del gen. Por este motivo, se analizó la secuencia de DNA del promotor de *egr-1* con dos programas informáticos diferentes para determinar el posible posicionamiento. En ambos casos, es posible detectar una alta probabilidad de formación nucleosomal alrededor de las posiciones -200 pb y de -800 pb en el promotor (Figure 38). Para comprobar experimentalmente estos resultados, se utilizó una variación de la técnica descrita por Steward y Sano (2004) que se basa en la protección del nucleosoma frente a la digestión por MNasa y en la posterior amplificación de los fragmentos protegidos mediante PCR a tiempo real. Para la amplificación se utilizan oligonucleótidos cuyos amplicones están solapados a lo largo del promotor del gen. De este modo, sólo si el amplicón se encuentra dentro del nucleosoma podrá ser amplificado.

Los resultados obtenidos con los experimentos de protección frente a la nucleasa de micrococo apuntan hacía la existencia de 3 nucleosomas posicionados, dos de ellos cuya posición coincide con la predicción teórica, y un tercero entre ambos, que se encontraría posicionado por el efecto barrera que producirían los otros dos nucleosomas posicionados por secuencia (Figure 39). En el caso del nucleosoma

más próximo al inicio de la transcripción, N1, se observa un desplazamiento hacía la izquierda durante la inducción de *egr-1*, es decir a los 15 y 30 min tras el tratamiento con TPA, para posteriormente volver a ocupar su posición inicial a los 180 min del estímulo (Figure 41).

En vista de los resultados que argumentan una remodelación del nucleosoma N1 durante la inducción, se procedió al estudio mediante la técnica de ChIP de la presencia de enzimas remodelantes en el promotor de *egr-1*. Los resultados obtenidos muestran que tanto BRG1 como BRM se encuentran unidas al promotor a 0 y 5 min tras el tratamiento con TPA, luego se altera su estructura o desaparecen del promotor, y finalmente regresan a los 60 min (Figure 40). Estos resultados, podrían otorgar un papel represor a estos complejos impidiendo la correcta posición nucleosomal para la expresión de *egr-1*. A pesar de que hasta la fecha no se ha establecido que una remodelación nucleosomal esté dificultando la transcripción, sí que se han descrito casos en los que BRG1 y BRM forman parte del mecanismo de represión de la inducción (Wang *et al.*, 2004).

7. MODIFICACIONES EN LAS COLAS DE LAS HISTONAS DE LOS NUCLEOSOMAS DEL PROMOTOR DE *egr-1*.

Para estudiar las modificaciones específicas en cada nucleosoma presente en el promotor de *egr-1*, se realizaron experimentos de ChIP con resolución mononucleosomal, en los que se utilizaron anticuerpos frente a modificaciones específicas de las histonas H3 y H4. Los resultados obtenidos indican un aumento de las modificaciones H3K9Ac, H4K5Ac y $H3K4Me_3$ en los tres nucleosomas estudiados, mientras que la H3K9AcS10P parece constante en el nucleosoma N3 y aumenta claramente en los nucleosomas N2 y N1. Por el contrario, la hiperacetilación de la H4 incrementa en mayor medida en el caso del nucleosoma N3 que en el de los otros dos (Figure 45).

8. PAPEL BIOLÓGICO DEL FACTOR TRANSCRIPCIONAL EGR1.

En el sistema biológico estudiado, el gen *nab2* se expresa entre los 5 y 60 min de tratamiento con TPA, como demuestran los resultados obtenidos por RNAPol-ChIP (Figure 46A). Por su parte, EGR1 se une, junto con NAB1 y el propio NAB2, al promotor del gen *egr1* para frenar su inducción a partir de los 30 min del tratamiento (Figure 46B).

Para estudiar con mayor profundidad el papel de EGR1, diseñamos una secuencia para RNA de interferencia la cual es capaz de reducir la expresión de *egr-1* hasta un 70%. Los resultados obtenidos coinciden al analizar la expresión del gen mediante RT-PCR a tiempo real (Figure 47A) y la intensidad de fluorescencia, con el programa ImageJ, de las imágenes de microscopio confocal obtenidas al co-transfectar células con un plásmido que expresa la proteína GFP y un plásmido que expresa el RNA de interferencia. La acción del RNA de interferencia de egr1 es también capaz de reducir la expresión del gen *nab2* (Figure 48) mostrando un efecto *in vivo* de la inhibición de la síntesis del factor EGR1.

9. HACIA UN MODELO DE INDUCCIÓN DE *egr-1*.

Con el conjunto de resultados anteriores, intentamos proponer un modelo de los acontecimientos que tienen lugar en el promotor de *egr-1* durante su inducción por TPA en células MLP29. El modelo sugiere un promotor "preparado" para expresarse, en el que todos los componentes se encuentran presentes y que deben ser posiblemente modificados, por fosforilación, para ser activos. Además existirían dos complejos HDACs co-represores (mSIN3A y HDAC3-NCoR) a tiempo 0 min, , un complejo HAT (CBP) que estaría manteniendo el nivel de acetilación basal del promotor y dos complejos remodeladores, BRM y BRG1. Una vez las células se estimulan con TPA, SP1 se libera del promotor, CREB y ELK1 se fosforilan, el nucleosoma N1 se remodela y los complejos mSIN3A, HDAC3-NCoR, CBP, BRM y BRG1 abandonan el promotor. En este momento entra otro complejo HAT que

contiene a GCN5 y que se encargaría de acetilar específicamente determinadas posiciones de histonas que conllevarían a la inducción del gen. El proceso de parada de la expresión de *egr-1* estaría regulado por la unión de EGR1 y los dos cofactores NAB1 y NAB2 encargados éstos de activar el dominio represor de EGR1. Igualmente, a lo largo de este proceso volverían todos los factores y/o complejos presentes a tiempo 0 min (Figure 49).

IV. CONCLUSIONES

1- El gen *egr-1* (early growth response-1) se expresa activamente, en diferentes líneas celulares como respuesta al tratamiento con TPA. Su patrón de expresión comienza a los 5 min,, tiene un pico alrededor de los 30 min, y después comienza a decrecer hasta dejar de ser detectable a los 180 min.

2- *Egr-1* puede ser considerado como un gen "potencialmente transcribible", ya que parece estar "preparado" para ser expresado en respuesta al estimulo adecuado, como se demuestra porque tanto la RNA pol II como diversos factores transcripcionales se encuentran presentes en el promotor en el estado basal del gen. Existen dos grupos de elementos de respuesta al suero, presentes en el promotor de *egr-1*, y nuestros experimentos indican que ambos están ocupados por SRF. Del mismo modo existen también dos sitios de unión para el factor transcripcional SP1,aunque sólo el más cercano al inicio de la transcripción parece estar ocupado en nuestro sistema. El resto de factores transcripcionales presentes en el promotor (CREB y ELK1) deben sen fosforilados para la inducción del gen. Puesto que esta fosforilación es eliminada, o al menos disminuida, por los inhibidores de las quinasas MEK1/2 y/o p38, capaces de inhibir la expresión del gen, los resultados son compatibles con asignar un papel importante a CREB y a la formación del complejo ternario (DNA, SRF y ELK1) en la inducción de *egr-1* en células MLP29 estimuladas con TPA.

3- El factor transcripcional SP1 parece ser el único encontrado en nuestro sistema que es capaz de actuar como represor de la transcripción de *egr-1*, ya que durante el proceso de inducción abandona el promotor, como demuestran los experimentos de ChIP realizados, y posteriormente, cuando comienza el proceso de represión, vuelve a detectarse su presencia en el promotor.

4- El factor EGR1 se une a su propio promotor tras 15 min de tratamiento con TPA, lo cual le atribuiría un papel como activador de su propia transcripción. Posteriormente, a los 30 min de la estimulación, los co-represores NAB1 y NAB2 se unen al promotor, interaccionando probablemente con EGR1, y activando su dominio represor.

5- Hemos detectado, al menos, dos complejos histona desacetilasa implicados en la represión de *egr-1*: mSIN3A y HDAC3-NCoR. Ambos complejos se liberan del promotor durante el proceso de activación del gen y vuelven durante el proceso de represión. Hay también al menos dos complejos histona acetiltransferasa implicados en la regulación de *egr-1*. CBP, cuya actividad podría consistir en mantener el nivel basal de acetilación en las histonas del promotor del gen, y GCN5, cuya actividad sería más especifica del proceso de inducción del gen por TPA.

6- Cuando se estudian las modificaciones presentes en las histonas del promotor de *egr-1*, se detecta un incremento en H3K9AcS10P, una modificación tradicionalmente asociada con la activación de genes inmediato-tempranos. Existe además un incremento significativo en los niveles de acetilación de las histonas H3 y H4, en particular de ésta última, que sólo tiene modificada la lisina 5 en el estado inactivo del gen y que durante la inducción se acetilan además las lisinas 8 y 16. Estas modificaciones parecen ser específicas de genes inmediato-tempranos, ya que están ausentes del promotor de la *β-actina*, un gen que se expresa de forma constitutiva.

7- Hemos encontrado que la di- y tri-metilación de la lisina 4 de la histona H3 está presente a lo largo del promotor y de la región codificante tanto de *egr-*

1 como de la *β-actina*. Este hecho otorgaría una posible función como señal epigenética general de genes transcripcionalmente activos a esta modificación post-traduccional.

8- Existen tres nucleosomas posicionados a lo largo del promotor de *egr-1*. El nucleosoma N1 tiene su centro alrededor de -200 pb, el nucleosoma N2 a -510 pb y el nucleosoma N3 sobre los -800 pb. Los nucleosomas N1 y N3 se encontrarían posicionados en base a su secuencia, mientras que N2 estaría posicionado indirectamente por la acción barrera de los otros dos. El nucleosoma N1 se mueve en dirección 5' durante la inducción del gen por TPA y posteriormente vuelve a su posición inicial durante el proceso de represión del gen.

9- Los complejos remodeladores que contienen a BRG1 y BRM han sido también identificados en el promotor de *egr-1*. Su presencia coincide con el estado reprimido del gen por lo que se les podría asignar una función represora.

10- La fosforilación específica de factores transcripcionales o de histonas, parece ser importante para el proceso de remodelación de los nucleosomas, puesto que la inhibición de fosfatasas altera el proceso de remodelación. Por el contrario, la metilación de histonas no parece ser indispensable en el proceso de remodelación, como demuestra el hecho de que la inhibición de PRMT1 y de la modificación que produce, H4R3Me_2a, no afecta de manera significativamente el posicionamiento nucleosomal.

11- El análisis mediante ChIP con resolución nucleosomal de N1, N2 y N3, revela modificaciones generales en los tres nucleosomas, como son el aumento de H3K4Me_3, H3K9Ac y H4K5Ac. Por el contrario, la modificación H3K9Ac aumenta principalmente en los nucleosomas N1 y N2, mientras que el nucleosoma N3 posee el mayor incremento en el nivel general de acetilación de la histona H4 y en la acetilación de la lisina 14 de la H3.

12- El factor transcripcional EGR1 regula la expresión de *nab2,* ya que la incubación de células con RNA de interferencia para EGR1 produce una

considerable disminución en la expresión tanto de *egr-1* como de *nab2*. La secuencia 1 utilizada en la construcción del RNA de interferencia para *egr-1* es capaz, por si sola, de disminuir la expresión de *egr-1* hasta un 70%, como se comprueba mediante experimentos de RT-PCR a tiempo real y por análisis mediante el programa Image J de los resultados de confocal de células co-transfectadas con un vector que expresa la proteína GFP y un vector que expresa el RNA de interferencia para *egr-1*.


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