Mechanistical studies of two functions of HMGB1 protein : facilitation of nucleosome sliding and translocation from the nucleus to the extracellular medium

Thesis

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MECHANISTICAL STUDIES OF TWO FUNCTIONS OF HMGB1 PROTEIN: FACILITATION OF NUCLEOSOME SLIDING AND TRANSLOCATION FROM THE NUCLEUS TO THE EXTRACELLULAR MEDIUM

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------Aknowlegments

In the first page of this thesis I want to express my deepest thanks to all the people that supported me during the past four years of PhD.

First and foremost there is my boss, mentor and supervisor, Prof. M. Bianchi, who followed me since I took my first step into science. Thanks because in these years he offered a precious example of how to make good science and for his patience and self control.

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At this point of my studies I can't forget two special people who, in different ways, passed to me their thirst of knowledge: my teacher of philosophy, Piero Carelli and Don Lino, that we miss so much.

Finally, the deepest thanks go to my family and to my boyfriend: nothing would have been gained without their constant and unconditional support, love and confidence.

All the efforts, suffers and joys held in this pages were shared day by day with them and are entirely dedicated to them.

In memory of Don Lino

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LIST OF ABBREVIATIONS

ACF: ATP-utilising chromatin assembly and remodelling factor

BBP: bromophenol blue

BSA: bovine serum albumin

CBP: CREB - binding protein

CEA: chicken egg albumin

CHRAC: chromatin accessibility complex

DAPI: 4', 6-diamidino-2-phenylindole

DCs: dendritic cells

DMEM: Dulbecco's modified Eagle's medium

DTE: dithioerythritol

DTT: dithiothreitol

ELISA: enzyme linked immunosorbent assay

ER: endoplasmic reticulum

ERK: extracellular-signal Regulated Kinase

ESI: Electro –Spray Ionisation

FA: Formic Acid

FBS: foetal bovine serum

FITC: fluorescein isothiocyanate

FPLC: fast phase liquid chromatography

FRAP: fluorescence recovery after photobleaching

GFP: green fluorescent protein

GR: glucocorticoid receptor

HATs: histone acetyl transferases

HDACs: histone deacetylases

HMGB: high mobility group box

HOX: homeobox genes

H1: histone 1

IAA: iodoacetamide

IEF: isoelectrofocusing

IgG: immunoglobulin G

IL-1: interleukin 1

IPTG: isopropil-B-D-thiogalactopyranoside

ISWI: imitation of switch

LB: Luria-Bertani

LEF-1: lymphocyte enhancer factor 1

LMB: Leptomicyn B

LPC: lysophosphatidylcholine

LPS: lipopolysaccaride

MALDI: Matrix-Assisted Laser Desorption Ionisation

MAPK: mitogen activated protein kinase

MEL: murine erythroleukemia cells

MS: mass spectrometry

NES: nuclear export signal

NF1: nuclear factor 1

NF-kB: nuclear factor-kB

NHP6: non-histone proteins 6

NLS: nuclear localisation singal

NMR: nuclear magnetic resonance

NP-40: nonidet P-40

OCT: octamer binding protein

PBS: phosphate-buffer saline

PCA: precloric acid

PCR: polymerase chain reaction

PFA: paraformaldehyde

PGA: polyglutammic acid

PKC: protein kinase C

PLC: PhosphoLipase C

PMSF: phenylmethylsulphonylfluorideliuos

RAG: recombination activating genes

RAGE: receptor for advanced glycation end-products

RSMC: rat smooth muscle cells

RSS: recombination Signal Sequences

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS polyacrylamide gel electrophoresis

Sox: SRY-related homeobox

SRY: sex-determining region Y

TBP: TATA-box binding protein

TBS: Tris-Buffer Saline

TBE: Tris- Borate EDTA

TCA: Trichloroacetic Acid

TFA: Trifluoro Acetic Acid

TFII: transcription factor II

TNF-α: tumor necrosis factor-α

TOF: Time-of-Flight

TSA: trichostatin A

Wt: wild type

ABSTRACT

This thesis develops two lines of investigation that focus on HMGB1 protein from different points of view. In **Section 3**, acetylation of lysine is identified as the molecular switch that controls movement of HMGB1 from the nucleus to the cytoplasm and **Section 4** investigates its role in chromatin remodelling. High Mobility Group protein HMGB1 is a chromatin component that, when leaked out by necrotic cells, triggers inflammation. HMGB1 can also be secreted by activated monocytes and macrophages and functions as a late mediator of inflammation. Secretion of a nuclear protein must require a tightly controlled relocation programme. It was found that in all cells HMGB1 shuttles actively between nucleus and cytoplasm due to two nuclear localisation signals (NLSs) and two nuclear export signals (NESs) within its sequence. Analysis of HMGB1 samples, extracted under specific conditions, using iso-electric focussing/SDS 2-dimensional gels, followed by mass spectrometry, demonstrated that HMGB1 is extensively acetylated (up to 10 modification sites per molecule) and that acetylation of the two NLSs inhibits nuclear import, causing cytoplasmic accumulation of the protein. This provides the first step in directing the protein to the secretion pathway, since cytosolic HMGB1 is then concentrated by default into secretory lysosomes and finally secreted when monocytic cells receive an appropriate second signal. Multiple acetylation of lysine sidechains was thereby defined as the molecular switch redirecting the nuclear protein HMGB1 to the cytoplasm and subsequently to secretion.

The second project investigated the potential role of HMGB1 in nucleosome sliding. Nucleosome remodelling complexes containing the ATP-ase ISWI, such as ACF, contribute to chromatin remodelling by converting chemical energy into the sliding of nucleosomes on DNA. ISWI interacts with DNA at the sites of its entry into the nucleosome, where it alters histone/DNA interactions that may lead to the relocation of DNA relative to the associated histone octamer. This work elaborated on this concept: if the rate-limiting step in nucleosome sliding is the distortion of linker DNA, a protein that can generate and/or stabilise such distortions might facilitate sliding. HMGB1 is able to transiently bend DNA, so is a good candidate to help "lubricate" nucleosome sliding. Using gel-shift assays, it was found that transient interaction of HMGB1 with nucleosomal DNA, at sites overlapping with ISWI binding sites, enhanced ACF-induced sliding. The ACF1 subunit of ACF was required to render the remodelling process

sensitive to HMGB1 action since no enhancement was detected when the sliding assay was performed using ISWI alone. In contrast, an HMGB1 derivative lacking the acidic tail that interacts with chromatin in a more dynamic way, had a strong inhibitory effect on sliding. These data suggest that HMGB1 is able to increase chromatin 'fluidity' by generating strategic DNA bends, or 'bulges', which are profitably used by ACF to induce nucleosome sliding. Moreover they support a "local loop" model of sliding and identify HMGB1 as a potential regulator of ATP-dependent nucleosome remodelling processes. This work was published in the *EMBO Journal* on 16th December 2002.

CHAPTER 1. INTRODUCTION

1.1 Literature review: HMGB1 and HMG-box family

1.1.1 HMGB proteins (High Mobility Group Box)

The HMGB family comprises in mammals the three proteins HMGB1 (previously, HMG1), HMGB2 (previously, HMG2) and HMGB3 (previously, HMG4 or HMG2b) (Bustin, 2001). HMGB proteins are very abundant, non-histone chromatin proteins present in the nucleus of all eukaryotic cells. They are small proteins (~ 25 KDa), characterised by high mobility in denaturing SDS polyacrylamide gels, hence the acronym High Mobility Group (Bustin et al., 1990).

HMGB1 is the archetypal member of the group and gives the name to the family. It is ubiquitous and only 10 times less abundant than core histones, counting about one million molecules per typical mammalian cell. The expression of the other two family members is more restricted: HMGB3 is only expressed in a significant amount during embryogenesis (Vaccari et al., 1998); HMGB2 is widely expressed during embryonic development, but restricted mainly to lymphoid organs and testis in the adult mouse (Ronfani et al., 2001).

The structure of these three proteins is highly conserved (**Fig. 1**) (more than 80% amino acid identity), and their biochemical properties are so far indistinguishable. HMGBs are composed of three different domains. The two homologous DNA binding domains, HMG box A and B, are each around 75 amino acids in length. The C-terminal domain is highly negatively charged, consisting of a continuous stretch of glutamate or aspartate residues, and is longest in HMGB1 and shortest in HMGB3 (reviewed in (Bianchi and Beltrame, 2000); (Bustin, 1999)).

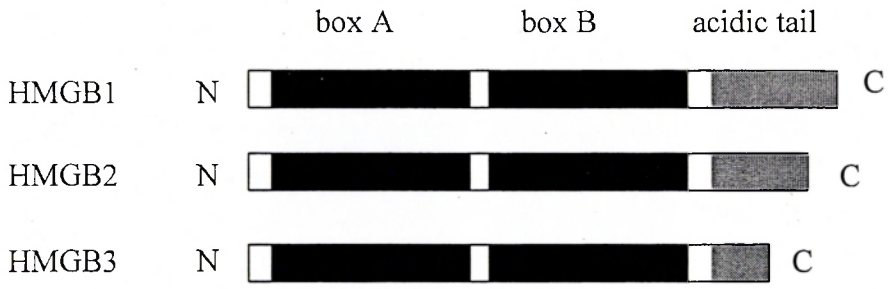


Fig. 1 Structure of HMGB proteins

All HMGBs possess two DNA binding domains, HMG box A and B (black boxes), and an unstructured acidic tail of variable length at the C-terminus (grey box).

Putative counterparts of HMGBs are present in *Saccharomyces cerevisiae* (non-histone proteins 6A and 6B, NHP6A and NHP6B) (Kolodrubetz and Burgum, 1990) and *Drosophila melanogaster* (HMG-D and HMG-Z) (Wagner et al., 1992), as well as in plants (**Fig. 2A**). Single HMG-box domains, with no acidic tail, characterize also an increasing number of mammalian sequence-specific transcription factors, such as the sex determining factor SRY (sex-determining region Y) (Goodfellow and Lovell-Badge, 1993) and the lymphocyte enhancer factor 1 (LEF-1).

1.1.2 The HMG-box domain

All the known biochemical and functional properties of HMGBs are specified by their HMG-boxes. The HMG-box motif is a stretch of about 75 residues, with a net positive charge and rich in aromatic residues and prolines (Bianchi et al., 1992). No amino acid is absolutely conserved in all known HMG-boxes, and only three residues have conservative substitutions.

The three-dimensional structures of several HMG-boxes have been resolved by means of either NMR or crystallography, showing that the global fold of the domain is well conserved (Read et al., 1993); (Hardman et al., 1995); (Weir et al., 1993); (Jones et

al., 1994); (Werner et al., 1995)). The HMG-box of HMGB1 consists of three α-helical segments forming an L-shaped structure stabilised by a hydrophobic core. The shorter arm of the "L" consists of helices I and II and the longer arm is composed of the extended N-terminal section packed against the C-terminal region, helix III (**Fig. 2B, C**).

Despite having a broadly similar fold, the structures of the A and B domains of HMGB1 differ significantly in the relative disposition of helices I and II and in the trajectory of the helix I–II loop. In box A, helix I is essentially straight whereas in box B it is bent, and the loop between helices I and II is longer in box A than in box B.

The HMG boxes of HMGB1 are highly similar to the HMG domains of *S. cerevisiae* NHP6A and *Drosophila* HMG-D and, to a lesser extent, to the sequence-specific (e.g. SRY and LEF-1) HMG-boxes.

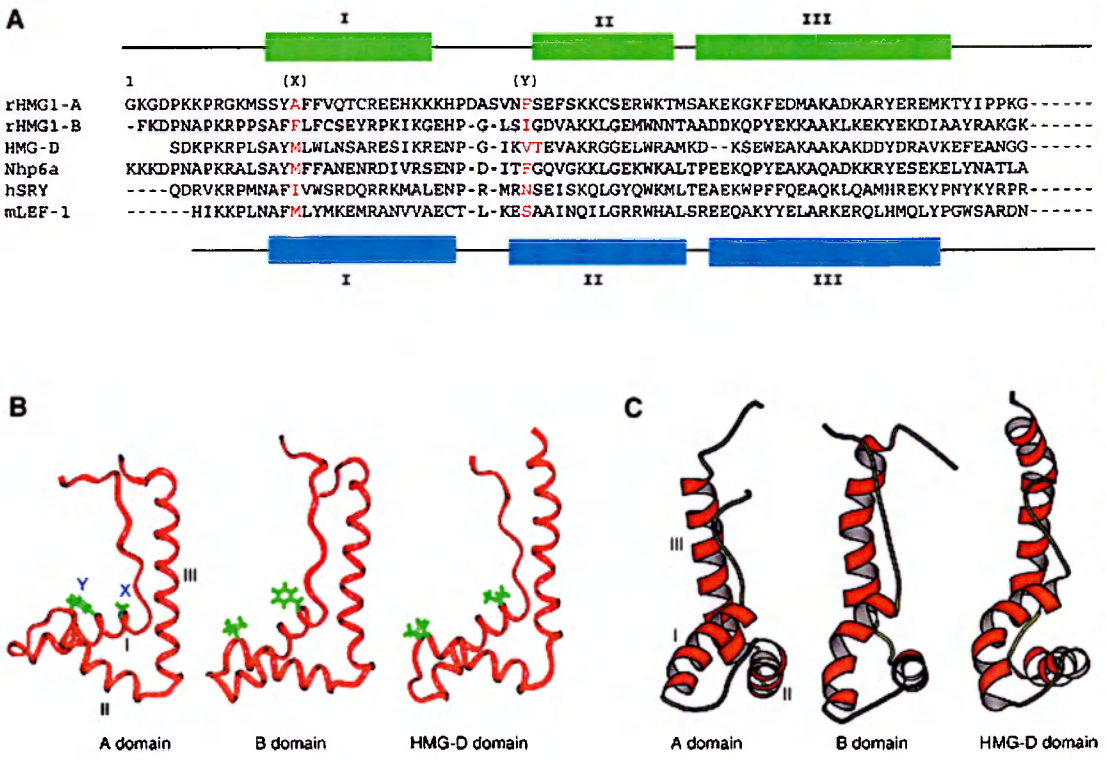


Fig. 2 Comparison between aminoacid sequences and folds of different HMG boxes

A) Residues of non-sequence-specific and sequence-specific HMG boxes intercalating into the DNA minor groove (marked in red and indicated as X and Y) are localised with respect to the α-helices determined by NMR spectroscopy for HMGB1 box A (shown in green, above the sequence) and the sequence-specific LEF-1 HMG box (shown in blue below the sequence). Prefixes r, h and m signify rat, human and mouse, respectively.

B) Location of residues at X and Y in the 3D structures of HMGB1 box A, HMGB1 box B (inferred intercalations) and the HMG-box of HMG-D.

C) Solution structures of the A and B HMG boxes of HMGB1 and of the HMG box of HMG-D, determined by NMR spectroscopy ((Hardman et al., 1995); (Weir et al., 1993); (Jones et al., 1994)). The structures are oriented to show the differences in the relative disposition of helices I and II between the different domains.

The main feature of the HMG-boxes of HMGB1 is the ability to both recognise and produce distorted structures in DNA. HMGB1 binds rather inefficiently to linear

DNA, with no preference for any nucleotide sequence. It shows, on the contrary, high affinity and selectivity to structured DNA, such as 4-way junctions, cisplatin-DNA adducts, and kinked DNA ((Bianchi et al., 1989), (Bianchi et al., 1992)). The binding of HMGB1 to DNA is therefore sequence-independent, but structure-specific. Beyond the ability to recognise distorted structures, HMGB1 can also introduce them in the double helix, thanks to a DNA bending activity. In spite of its low affinity for linear DNA, when present at high concentrations, HMGB1 is capable of inducing DNA bends upon binding, as revealed by ring closure of short DNA fragments (Pil et al., 1993).

As yet, no structure of a complex between HMGB1 and linear DNA has been solved. However, the similarity with other HMG-boxes, for which structural data are available (Werner et al., 1995); (Murphy and Churchill, 2000)), is revealing with respect to the mechanism underlying HMGB1-mediated DNA bending. The DNA-binding surface of the HMG box domain has a hydrophobic surface that conforms to a wide, shallow minor groove. In the centre of this surface, a hydrophobic wedge, usually consisting of four spatially close residues, inserts deep into the minor groove. This partial intercalation introduces a kink into the bound DNA, enhancing the more uniform bend associated with the widening of the minor groove (**Fig. 3A**). The only resolved complex between HMGB1 and a DNA substrate is the X-ray structure of the rat HMG-box A domain complexed to cisplatin-modified DNA (**Fig. 3B**) (Ohndorf et al., 1999). Cisplatin is a drug that damages DNA, creating intrastrand crosslinks between adjacent purines (Pil and Lippard, 1992). These crosslinks bend and unwind the duplex, generating a structure that HMGB1 binds with high affinity, without inducing any further distortion. The analysis of the complex revealed which amino acids are mainly involved in contacts with DNA: particularly important appeared to be a phenylalanine residue in helix II (see **Fig. 2A,B**), which intercalates into the kinked platinated GpG site, and whose substitution dramatically reduces the binding of the domain. Nevertheless, the cisplatin-induced intrastrand crosslinks alter the contact surface for the HMG box on DNA, compared to a linear molecule, and indeed significant differences are observed with respect to LEF1- and SRY-DNA complexes. These proteins induce in DNA a bend similar to the one formed at the kinked platinated site, but the bend locus in this case is near the centre of the protein-binding site. In contrast, the binding surface of HMGB1 box A extends exclusively to the 3' side of the bend, when the platinated strand is used as a reference, and it covers five base-pair steps (**Fig. 3B**). Thus, the relationship between the DNA bend and the protein-binding site is

slightly altered by the cisplatin-induced intrastrand crosslinks. Taking this into account, and considering that the general fold of all HMG boxes is well conserved, a complex between the HMG box of HMGB1 and a linear DNA is likely very similar to those formed by the sequence-specific HMG boxes (**Fig. 3C**).

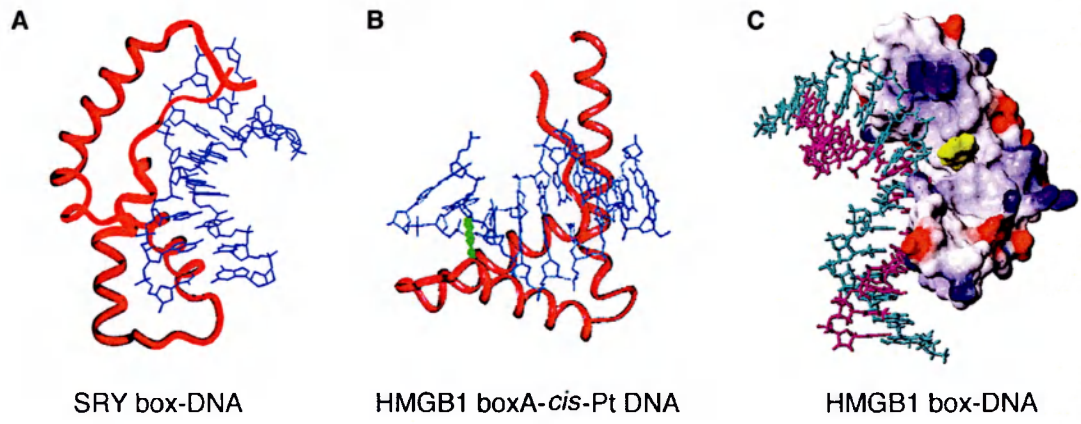


Fig. 3 Structures of HMG box-DNA complexes

A) Structure of the complex between the HMG box (red) of human SRY and the octanucleotide GCACAAAC (blue). The DNA is significantly distorted: the minor groove is widened considerably to accommodate the extended stretch of the HMG box; the planes of the bases are tilted, and the double helix is unwound and bent. The contact between the HMG box and the DNA occurs over a surprisingly large area and with a very tight fit; thus, the energetic cost of distorting the DNA is more than compensated by the chemical energy freed by the protein-DNA interaction.

B) The complex of the HMGB1 box A with cis-platinated DNA (X-ray crystal structure) (Ohndorf et al., 1999). Remarkably, in this structure the orientation of the intercalating Phe group differs from that in the solution structure of the free domain shown in Fig. 2B.

C) Model of HMGB1 box B positioned on 15 bp of DNA. The model is derived from the high similarity between sequence-specific HMG boxes and the HMGB boxes of HMGB1. The electrostatic surface (blue is positive charge; red is negative charge) is shown with the intercalating phenylalanine residue in yellow. DNA strands are shown in magenta and cyan (Wolffe, 1999).

1.1.3 HMGB proteins as architectural regulators

Many studies have been performed over the last few years in the attempt to correlate the characteristic DNA-binding properties of the HMG-box domain to the biological roles of the abundant HMGB proteins. One proposed general function of these proteins is to overcome the barrier imposed by the axial rigidity of DNA and thereby promote the formation of complex nucleoprotein assemblies containing tightly bent DNA (Kahn and Crothers, 1993). Such a role probably requires the bend be precisely placed; a displacement of the binding site by even 1 bp would alter the trajectory of the bent DNA by 30–36°. Because DNA-sequence recognition by the abundant HMGB proteins lacks selectivity, the precise targeting of these proteins must depend on the presence of other sequence-specific DNA-binding proteins in the complex. This indeed appears to be the case.

HMGB1 and HMGB2 have been implicated, from several *in vitro* assays, in both the activation and repression of transcription. A recurring theme is the enhancement of the binding of various transcription factors [e.g. Oct-1 and 2 (Zwilling et al., 1995)), HoxD9 (Zappavigna et al., 1996), p53 (Jayaraman et al., 1998), Rel proteins (Jayaraman et al., 1998), and steroid hormone receptors (Boonyaaratanakornkit et al., 1998) to their cognate DNA binding sites. In most of these cases, the interaction of the HMGB protein with a transcription factor has been detected *in vitro* even in the absence of DNA, and could presumably serve as the mechanism for recruitment of HMGB1, 2 to particular DNA sites (**Fig. 4**). In turn, HMGB proteins bound to their partners increase the protein surface contacting DNA, from both the major and the minor groove, thereby achieving high affinity interactions. In some cases, transfection experiments have also proved functional interactions *in vivo*. The demonstrated interactions *in vitro* are between HMGB1 and/or 2 and a single transcription factor. However, it is probable that *in vivo*, in a natural regulatory context, the DNA bending by HMGB1 or 2 may allow the recruitment of a second transcription factor to the complex, generating a complex network of interactions (**Fig. 4**).

In vitro interactions between HMGB proteins and the basal transcription machinery have also been reported. Human HMGB1 binds to the TATA-box binding protein (TBP) and interferes with the normal binding of TFIIB in the pre-initiation complex, thereby inhibiting TBP function ((Ge and Roeder, 1994); (Sutrias-Grau et al., 1999); both HMGB1 and TFIIB independently enhance binding of TBP to TATA-box

DNA. By contrast, HMGB2 has been reported to stabilize and activate the TBP-containing TFIID–TFIIA complex bound to promoter DNA (Shykind et al., 1995)).

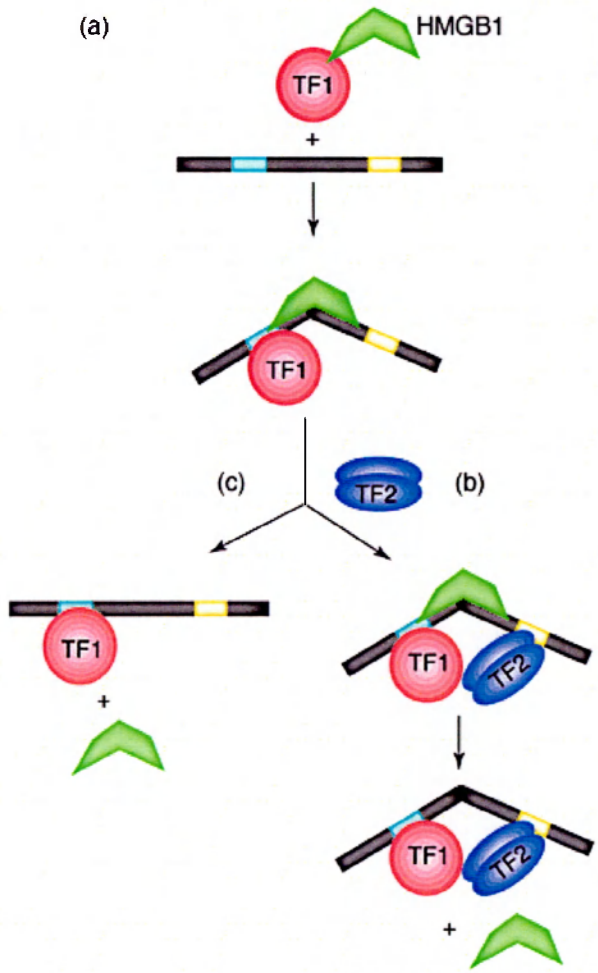


Fig. 4 A possible chaperone role for HMGB1 in facilitation of transcription-factor binding

(**a**) Recruitment of HMGB1 (green) to specific DNA sites through interaction with a sequence-specific transcription factor (TF1; red). Formation of a ternary complex that is probably unstable, but has been detected and/or inferred in some cases. In this ternary complex, HMGB1 might bend the DNA, thus providing the potential for the recruitment of additional DNA-binding proteins (TF2; purple) to the complex (**b**). If no additional protein is recruited, HMGB1 could dissociate from the complex, leaving the transcription factor stably bound (**c**) [in this diagram, the final DNA is shown straight (as in the case of Oct-1 binding), but this is not necessarily the case; for example, the progesterone receptor bends its DNA in the binary complex]. Only a single HMG box is shown because the separate A and B HMG boxes of HMGB1 have been shown to facilitate binding of various transcription factors *in vitro*.

HMGB proteins exert their architectural function also in other nuclear processes involving manipulation of DNA structure. The best example is observed in lymphocytes, during V(D)J recombination, a complex process which allows the generation of a nearly infinite number of antibodies and T Cell Receptors. In developing T and B lymphocytes, antigen receptor genes are somatically assembled from several V (variable), D (diversity), and J (joining) DNA segments, in a process mediated by two lymphoid-specific enzymes, RAG1 and RAG2 (products of Recombination Activating Genes 1-2) (Schatz et al., 1989). These proteins bind specifically to the Recombination Signal Sequences (RSS), regions flanking each antigen receptor segment, which direct the site of rearrangement. Upon binding, the RAG1/2 complex cleaves the DNA, producing a hairpin intermediate with nucleotide overhangs. In the second step of the process, while RAG1/2 remain stably bound to the RSS, the free ends generated by the cleavage are linked to each other by DNA repair activities. During my work for the "Laurea", I could show that HMGB1 and HMGB2 are recruited by RAG1/2 to the RSS, and functionally interact with the complex, facilitating V(D)J recombination (Aidinis et al., 1999). Also in this case, a physical interaction between HMGB1,2 and RAG1/2 enhances the binding of the enzymes to the RSS. The RAG1/2 complex possesses an intrinsic DNA bending activity, and upon binding, it alters the structure of the RSS site even in the absence of HMGB1,2. However, the DNA bending induced by RAG1/2 is very inefficient unless assisted by HMGB1,2, suggesting a crucial contribution of HMGB proteins in stabilising the complex between RAG1,2 and the bent RSS (Aidinis et al., 1999).

The picture emerging from all these data point to HMGB proteins as important and versatile architectural chromatin components, required for the formation and the stabilisation of functional nucleoprotein complexes. This function is sustained by their peculiar DNA binding properties and their ability to modulate the structure of DNA, which guarantee a correct three-dimensional assembly of DNA binding proteins on bent DNA

The molecular analysis of the HMGB1- deficient mice further supports the model of HMGB1 as a co-activator of the steroid hormone receptor in controlling their target genes and – more in general – provides further insight in the model of HMGB1 involvement of regulation of gene expression at various level and in different ways.

1.1.4 Phenotype of HMGB1- and HMGB2- deficient mice

Mice lacking either HMGB1 or HMGB2 were produced in our lab, by conventional knock-out, in order to study the function of the proteins *in vivo* (Calogero et al., 1999, Ronfani et al., 2001). The most dramatic phenotype is displayed by *Hmgb1*$^{-/-}$ mice, which are born, but die within the first day of life as a result of severe hypoglycaemia (**Fig. 5A**) (Calogero et al., 1999). While the level of glucose is decreased in the blood of *Hmgb1*$^{-/-}$ animals, abundant glycogen is present in their liver (**Fig. 5B**), but is not metabolised. Indeed, intraperitoneal injection of glucose in *Hmgb1*$^{-/-}$ newborns permits the survival of some animals, even though the rescued mice remain very sick, with several anatomical abnormalities (**Fig. 5C**).

At the cellular level, *Hmgb1*$^{-/-}$ mice have defects in steroid hormone-dependent gene expression. Fibroblasts from knock-out mice transiently transfected with a reporter plasmid containing a GR responsive element respond weakly to the glucocorticoid analogue dexamethasone, compared to wt fibroblasts (**Fig. 5D**). Moreover, $CD4^+$ $CD8^+$ T cells from *Hmgb1*$^{-/-}$ mice are partially resistant to dexamethasone exposure, while wt $CD4^+$ $CD8^+$ cells undergo massive apoptosis upon treatment (Calogero et al., 1999). All these findings are consistent with the proposal that HMGB1 helps steroid hormone receptors in controlling their target genes.

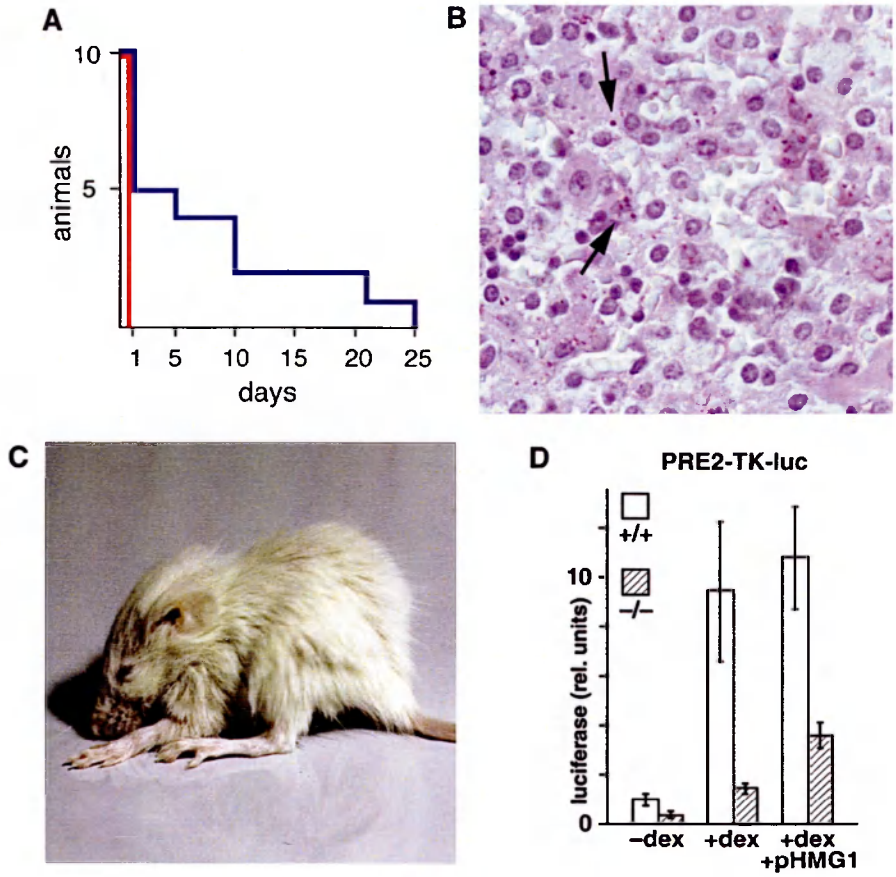


Fig. 5 *Hmgb1*$^{-/-}$ mice develop neonatal hypoglycaemia

A) *Hmgb1*$^{-/-}$ mice die within the first day of life but can survive if given glucose parenterally. Intraperitoneal glucose injections were administered to 10 *Hmgb1*$^{-/-}$ newborns during the first days after birth. Survival is indicated by a blue line. Ten *Hmgb1*$^{-/-}$ control mice, injected with saline solution, all died within day 1 (red line), similar to untreated *Hmgb1*$^{-/-}$ mice.

B) Periodic acid-Schiff (PAS) staining for glycogen (magenta) in livers from *Hmgb1*$^{-/-}$ mice are shown. Despite very low blood glucose concentrations, hepatocytes of *Hmgb1*$^{-/-}$ mice do not completely mobilize glycogen (arrows, glycogen granules).

C) Phenotype of an *Hmgb1*$^{-/-}$ spontaneous survivor at day 25. Spontaneous survivors of the mixed 129Sv/CD1 genetic background are very similar to the most successfully glucose-rescued *Hmgb1*$^{-/-}$ mice of 129Sv/BALB-c background: they have a very reduced size, but respond positively to basic neurological tests. All spontaneous or glucose-treated surviving animals have sealed eyelids, arched backs, long hind paws and abnormal gait.

D) The absence of Hmgb1 reduces the activity of GR in transfection assays. The expression of a GR-controlled reporter is reduced in *Hmgb1*$^{-/-}$ fibroblast cell lines exposed to dexamethasone.

Unexpectedly, however, no defects in V(D)J recombination were found: even though the *in vitro* data implicate HMGB1 in the process, the newborn mice have a normal serum abundance of immunoglobulins, and normal numbers of mature single-positive $CD4^+$, and $CD8^+$ T cells expressing a complete Vß repertoire in the thymus (Calogero et al., 1999). Moreover, in spite of the cooperation between HMGB1 and HOX proteins and the basal transcription machinery, the gene regulation necessary to generate a neonatal animal with fully differentiated tissues is unimpaired in mutant mice.

The most plausible explanation for the lack of defects in the V(D)J products and in the body plan of the *Hmgb1*$^{-/-}$ mice is that HMGB2 could compensate for the absence of HMGB1 in the interaction with RAG1/2 and HOX proteins. HMGB2 is widely expressed during embryonic development, when HOX proteins exert their function, and even after birth it is particularly concentrated in the thymus, which is the site of V(D)J recombination. Therefore, even in the absence of HMGB1, HOX proteins and RAG1/2 could find a suitable partner. By contrast HMGB2 is completely absent from liver in the adult, and this might explain the defects in the GR-mediated functions and the hypoglycaemic phenotype of the *Hmgb1*$^{-/-}$ mice.

In order to test the possibility that HMGB2 might provide partial redundancy to HMGB1, *Hmgb2*$^{-/-}$ mice were also generated, with the perspective to generate double *Hmgb1*$^{-/-}$ *Hmgb2*$^{-/-}$ mutants. Mice lacking HMGB2 are apparently healthy, and only show a reduced fertility in males. Increased apoptosis of germ cells in seminiferous tubules and production of defective spermatozoa appeared to underlie this defect (**Fig. 6**) (Ronfani et al., 2001). This phenotype correlates with the observation that HMGB2 is highly abundant in the testis, even more than HMGB1.

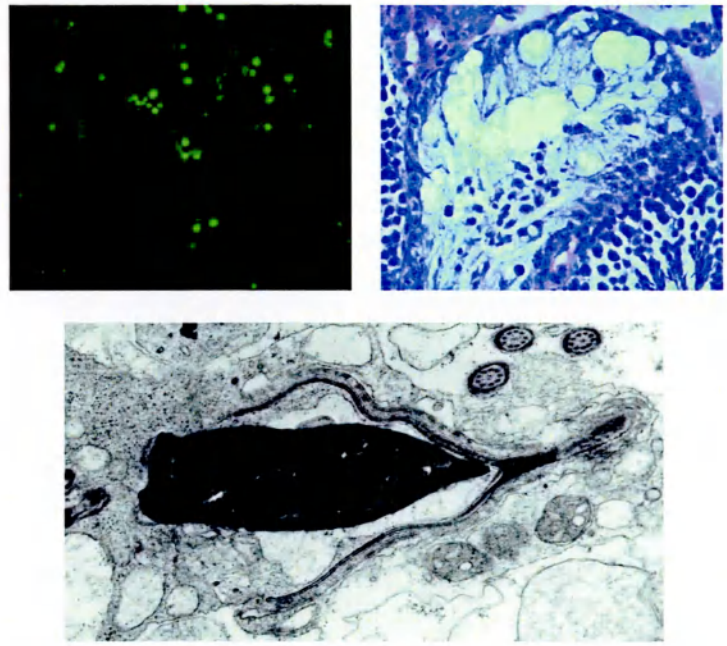


Fig. 6 Abnormalities in the testis of *Hmgb2*$^{-/-}$ mice

A) TUNEL staining for apoptotic cells in testis seminiferous tubules of *Hmgb2*$^{-/-}$ mice, showing the high content of dead cells.

B) Haematoxylin-eosin stained sections of testis from a 163-day *Hmgb2*$^{-/-}$ mouse. The regular periphery-to-lumen succession of spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa is lost, cells are separated by gaps, and degenerated Sertoli cells with large vacuoles are present.

C) Electron micrographs of an elongated spermatid where the acrosome (arrows) is detached from the nucleus, impairing its function.

Taken together, the data obtained from HMGB1- and HMGB2- deficient mice suggest that HMGB1 and HMGB2 are indeed functionally equivalent, and a minimum total amount of HMGB1 plus HMGB2 is required at all times. The lack of HMGB1 is phenotypically more noticeable because HMGB1 is ubiquitous and usually much more abundant than HMGB2. In contrast, as HMGB2 has a more restricted pattern of expression, and in most cases HMGB1 can compensate for the loss of HMGB2, the defects in *Hmgb2*$^{-/-}$ mice are less severe and more localised. In order to definitively prove the redundancy of HMGB proteins, we are currently breeding HMGB1 and 2 knockouts, to generate double mutants. So far, no double knock-out has been found at

embryonic day 14 ($p < 0.05$). This result implies that HMGB1 and HMGB2 have indeed similar functions and a sufficient amount of HMGBs is required for embryonic development.

The functional interaction of HMGB1 and HMGB2 protein during the very first stages of embryonic development is still under investigation. Since embryos do not survive to do day 14 post coitum, we are searching for double mutant embryos at earlier stage: so far, living blastocysts at 3.5 days post- coitum have been found. This result means that the time in which absence of both proteins becomes incompatible with life is between these two dates. The next step, still *in fieri*, consists in searching and analysing embryos at 7.5 days p.c.

Another line of research in our lab is the development of a conditional knock out for HMGB1, in order to be able to excise the Hmgb1 gene in a tissue-specific manner and, therefore, to study in a more detailed way the specific roles of the protein.

The collection of evidence presented above allows depiction of a scenario in which HMGB1 acts as a chaperone in a wide set of events involving chromatin rearrangements, when chromatin structure has to undergo both rapid variations (condensation/ decondensation cycles) and temporary modifications (site-specific recombination, transcriptional activation). To find a "fil rouge" in the mechanism of involvement of HMGB1 in all these phenomena, a better description of its relationship with chromatin becomes necessary.

In spite of the amount of investigation devoted to the issue, the relative interaction between HMGB1 and chromatin is still ill- defined and highly controversial.

Part of my PhD work has been dedicated to add some pieces to this puzzle: we set up a collaboration with Peter Becker and his team, in Munich, who are leaders in the field of gene expression through chromatin remodelling. The results presented in **Chapter 4** are related to the results that I obtained during this collaboration.

1.2 The debate about the functional relationship between HMGB1 and chromatin

1.2.1 HMGB1 does not have a mere structural role in chromatin organisation

The most surprising finding derived from *Hmgb1*$^{-/-}$ mice is that HMGB1 is not essential for the life of cells, in spite of its abundance and evolutionary conservation. This observation impacts on the proposed role of HMGB1 as structural protein involved in packaging DNA into chromatin.

For many years, HMGB proteins have been considered to cooperate with histones in determining chromosome architecture. Both protein families appeared to serve the function of wrapping DNA into arrays of repetitive modules, thereby ensuring the formation of organised high-order structures ((Wisniewski and Grossbach, 1996); (Zlatanova et al., 1999)). On average, a typical cell nucleus contains one molecule of HMGB1 every 10-20 nucleosomal particles, and a role for the protein in promoting the formation of nucleosomes has been suggested (Travers et al., 1994). In addition, HMGB1 has been proposed to stabilize nucleosomes by binding at the DNA crossover site (Lilley, 1992), with a function similar to that of histone H1. Indeed, HMGB1 and histone H1 share several properties. *Xenopus* HMGB1 (xHMG1) binds to nucleosomes *in vitro* in much the same way as histone H1 ((Nightingale et al., 1996); (Ura et al., 1996)); the *Drosophila* HMGB1-like protein HMG-D is a structural component of condensed metaphase chromosomes (Ner and Travers, 1994); histone H1 also binds to four-way junctions (Varga-Weisz et al., 1993). These observations suggested that HMGB1 and H1 could possess similar overall properties but specifically adapted to perform subtly different structural functions.

The first data in conflict with this model were the finding that the association of HMGB1 with chromatin is markedly different from that of histone H1 (Falciola et al., 1997). If cells are treated with NP-40, a non-ionic detergent which permeabilises plasma and nuclear membranes, nuclear proteins not tightly bound to DNA leak into the extracellular medium, whereas stable components of chromatin remain anchored to the nuclear remnants (**Fig. 7A**). Permeabilised cells, both in mitosis and in interphase,

release the vast majority of HMGB1, indicating that the protein is not a structural component of either condensed or decondensed chromatin (**Fig. 7B**). On the contrary, histone H1 remains firmly associated with DNA, as well as the core histones and other chromatin binding proteins. Moreover, immunofluorescence analysis showed that while in interphase HMGB1 is uniformly distributed in the nucleoplasm, like histone H1, in mitosis, contrary to H1, it is displaced from chromosomes (Falciola et al., 1997).

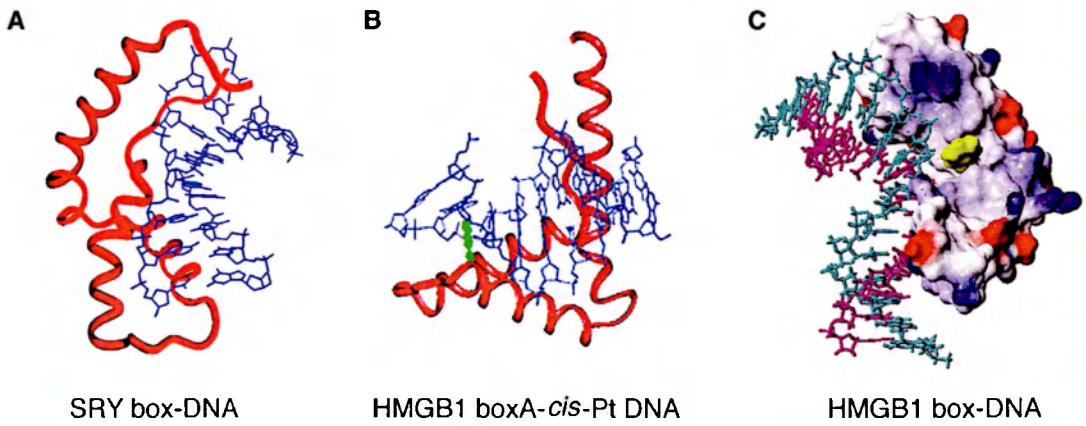


Fig. 7 HMGB1 is not stably associated to chromatin

A) The cartoon shows what happens after NP-40 addition: both cellular and nuclear membranes are permeabilised and soluble proteins (blue squares and red circles) leak out from nucleus and cytoplasm. Thus, nuclear proteins not stably bound to DNA (red circles) are released in the extracellular milieu, whereas histones and other stable component of chromatin (green triangles) are retained in the nucleus.

B) After permeabilisation, the buffer bathing the cells (lanes 1 and 3; S= supernatant) and the cellular remnants, containing chromatin associated proteins (lanes 2 and 4; P= pellet) can be analysed by Western blot with different antibodies, to distinguish between stable components of chromatin and proteins that interact with DNA only transiently. While histone H1 and HMGA1 are found in the pellet, bound to DNA, HMGB1 is released outside the cells both in interphase and in mitosis.

The results described above indicated that HMGB1 and histone H1 do not have equivalent functions in differentiated mammalian cells, but did not rule out the possibility that HMGB1 might substitute for H1 when the linker histone is absent. This is what happens in *Xenopus* and *Drosophila*, where respectively xHMG1 and HMG-D appear to substitute for histone H1 during embryogenesis ((Dimitrov et al., 1993); (Dimitrov et al., 1994); (Ner and Travers, 1994)). Later studies addressed this problem, investigating the association of HMGB1 with chromatin in very early mouse development, a period when histone H1 is present at very low abundance (Clarke et al., 1992). Nonetheless, even in preimplantation embryos, the affinity of HMGB1 for chromatin appeared similar to that observed in somatic cells, and the protein did not remain associated with chromosomes during metaphase (Spada et al., 1998). This result indicated that HMGB1 has non-overlapping roles with the linker histone and does not compensate for its absence during mouse early embryogenesis.

In partial contrast with these observations, however, *in vitro* HMGB1 appeared to bind to reconstituted nucleosomes with high affinity, like H1, forming complexes sufficiently stable to survive band-shift assays (Falciola et al., 1997). I also confirmed and extended these preliminary results during my collaboration with P.Becker. (See **4.2.1**).

Furthermore, our group recently showed by means of photobleaching experiments that the interaction of HMGB1 with chromatin is highly dynamic: the protein has a very high rate of diffusion in the nucleus, which indicates that the binding to specific sites is very transient (Scaffidi et al., 2002).

HMGB1 can bind to nucleosomes with high affinity and structure-specificity *in vitro*; by contrast, *in vivo* its association with chromatin is quite loose. Taken together, these findings disproved a structural role of HMGB1 in packaging bulk DNA, and rather suggested that the protein may establish only transient interactions with chromatin.

Thus, HMGB1 must posses a specific role in chromatin organisation and remodelling that may reconcile the apparently opposite data of the strong affinity for nucleosomes *in vitro* with the high dynamism detected *in vivo*.

1.3 A new field of investigation: HMGB1 is also an extracellular signal

In accordance with its role as an architectural component of chromatin, the localisation of HMGB1 is nuclear in most cell types. Surprisingly, recent data have established that beyond its intranuclear function, HMGB1 also has a pivotal function outside of the cell.

1.3.1 HMGB1 as a potent mediator of inflammation

Wang *et al.* (Wang et al., 1999) have recently identified HMGB1 as a late mediator of endotoxin lethality in mice. HMGB1 was found to be secreted by monocytes/macrophages stimulated by bacterial lipopolysaccaride (LPS, endotoxin), and to accumulate in the serum of the mice, mediating septic shock.

LPS is a component of all Gram-negative bacteria. It is toxic to mammals because it activates the innate immune system (macrophages and neutrophils) to secrete proinflammatory cytokines, such asTNF-α, interleukin 1 (IL-1), and macrophage migration inhibitory factor (MIF) (Tracey and Cerami, 1993); (Bernhagen et al., 1993). These factors drive the acute phase response and mediate the development of shock and tissue injury. In recent years, therapeutic strategies for sepsis have attempted to modulate the excessive inflammatory response by inhibiting cytokines released by macrophages with specific antibodies. However, a major difficulty in targeting cytokine activities derives from the fact that most pro-inflammatory mediators are released early in septic shock, and inhibitors should therefore be administered immediately. In order to broaden the therapeutic window for sepsis, Wang *et al.* initiated a search for a putative macrophage-derived mediator of lethality that appears relatively late after the onset of endotoxemia. This study culminated in the identification of HMGB1 as the proposed late mediator of LPS lethality. The serum concentration of HMGB1 significantly increases 16-32 hours after LPS administration in mice. Administration of anti-HMGB1 antibodies attenuated LPS-induced endotoxemia, and conversely, injection of HMGB1 caused toxic shock. Moreover, septic patients showed increased serum levels of HMGB1, which correlated with the severity of the infection (Wang et al., 1999).

The source of extracellular HMGB1 is provided by activated monocytes/macrophages. Macrophage-like RAW 264.7 cells release the protein 18 hours after stimulation with LPS; confirmation of the inducible nature of HMGB1 release was obtained also in murine primary peritoneal macrophages, and in human primary peripheral blood mononuclear cells (Wang et al., 1999). Pituicytes, which provide an important link between the immune and the neuroendocrine systems, also release HMGB1 in response to specific stimuli like TNF-α and IL-1, suggesting that HMGB1 participates in the regulation of neuroendocrine and immune responses to inflammatory processes (Wang et al., 1999).

Subsequently, HMGB1 was also shown to cause acute lung inflammation when administered intratracheally (Abraham et al., 2000). Antibodies against HMGB1 decreased lung edema and neutrophil migration, whereas they did not reduce the levels of the other proinflammatory cytokines, such as TNF-α, IL-1β or macrophage-inflammatory-protein-2 (MIP2).

One major issue that remains to be investigated so far is how HMGB1 is secreted by monocytes and the other competent cell types: it possesses no signal peptide that would direct it to the endoplasmic reticulum (ER) through the canonical secretion pathway. This lack of a secretion leader peptide is a feature shared with a small number of other secreted proteins, like the cytokine IL-1β (Andrei et al., 1999). Studies in murine erythroleukemia (MEL) cells have shown that HMGB1 export does not involve the ER and the Golgi complex, but is promoted by intracellular Ca^{2+} increase and possibly by activation of a Ca-dependent PKC isoform (Passalacqua et al., 1997).

Anna Rubartelli and co-workers have just published (Gardella et al., 2002) a description of the last steps of HMGB1 secretion, showing that activation of monocytes results in HMGB1 accumulation from nucleus to cytoplasmic organelles that display ultrastructural features of endolysosomes; secretion of the protein is induced by stimuli triggering lysosome exocytosis (LPC: lysophosphatidyl- choline).

In any event, the first step for HMGB1 secretion is re-distribution from nucleus to cytoplasm; the question of how a nuclear protein can be directed to cytoplasm and then to the extracellular space upon specific stimuli was the starting point of my investigation.

Once released, HMGB1 is able to activate several other cells involved in the immune response or inflammatory reactions, and can act as a cytokine itself (Andersson et al., 2000). HMGB1 activates monocytes to secrete a specific subset of proinflammatory cytokines, including TNF-α and IL-1. Stimulation occurs at the level of gene transcription, since cytokine mRNA levels increase after HMGB1 stimulation (Andersson et al., 2000). In comparison with the well-known inflammatory stimulus LPS, HMGB1 causes a delayed and biphasic release of TNF-α, with a first peak at 3 hours, followed by a second peak at 8-10 hours after HMGB1 exposure.

In light of all these data, it is now clear that HMGB1 has a crucial role during phlogystic processes: it is secreted by macrophages in response to proinflammatory stimuli, and also itself provokes a delayed response, thereby prolonging and sustaining inflammation.

1.3.2 HMGB1 in differentiation processes

Before the discovery of the role of HMGB1 in inflammation, it had already been observed that several cells release the protein into their surroundings as part of their differentiation process. Murine erythroleukemia (MEL) cells, exposed to a chemical inducer (hexamethylenebisacetamide, HMBA), release HMGB1 (also called DEF for differentiation enhancing factor) into the medium. In turn, extracellular HMGB1 significantly increases the rate of MEL cell differentiation via specific binding to the external surface of the cell membrane (Passalacqua et al., 1997); (Sparatore et al., 1996).

Likewise, HMGB1 is also secreted by neurons and participates in their differentiation process ((Fages et al., 2000), and references therein). Some years ago, an extracellular, membrane-bound form of HMGB1 was purified from rat brain for its capability of mediating neurite outgrowth. The membrane bound form was named amphoterin because of its dipolar structure, but at the sequence level it is identical to HMGB1. Membrane HMGB1 has been implicated in enhancing neurite outgrowth in cerebral neurons during development, and in regenerating peripheral neurons by binding to a cell surface proteoglycan, syndecan. Membrane HMGB1 is abundant in both neuronal cell bodies and in neurites in neuroblastoma cells, whereas in neurons and

peripheral glial cells (Schwann cells) it is localised in non-nuclear compartments. HMGB1 mRNA is developmentally regulated, and reduced significantly after the rapid perinatal growth phase of the rat brain. The molecular basis for cellular targeting of HMGB1 to membranes remains unclear.

HMGB1 can also be exported to the extracellular space by groups of cells that support the differentiation of other spatially associated cell types. Stimulated astrocytes release HMGB1, which then induces LAN5 neuroblastoma cells to differentiate (Passalacqua et al., 1998). Human promyelocytic HL60 cells, although not secreting HMGB1 themselves, show an accelerated differentiation when exposed to extracellular HMGB1 (Sparatore et al., 1996).

In all these cases, the release of HMGB1 into the extracellular space is well controlled and local: HMGB1 is either secreted in an autocrine manner, or in a paracrine manner by closely associated cells. Remarkably, this spatially restricted secretion of HMGB1 does not seem to trigger an inflammatory response.

1.3.3 HMGB1 mediates cell migration and metastasis

HMGB1 can also promote cell migration. Cells migrate in response to extracellular stimuli, channelled through specific signal transduction pathways that ultimately elicit cytoskeletal remodelling. We showed in our lab that vascular smooth muscle cells respond to extracellular HMGB1 (Degryse et al., 2001). Cells exposed to HMGB1 undergo rapid and transient changes of cell shape, and actin cytoskeleton reorganization, leading to an elongated polarized morphology typical of motile cells.

Even more strikingly, Taguchi *et al.* (Taguchi et al., 2000) showed that HMGB1 is also involved in tumour growth and metastasis: administration of anti-HMGB1 antibodies suppressed metastasis formation by Lewis lung tumour cells implanted under the skin of recipient mice. In this context, HMGB1 promotes both migration and proliferation of tumor cells.

In vivo, cell migration depends to a large extent on the capability of a cell to invade the surrounding tissue, for which the activation of extracellular proteases is required. HMGB1 appears to play a role here as well: it binds to several components of the plasminogen activation system and enhances the activation of t-PA (Parkkinen and

Rauvala, 1991). Moreover, Taguchi *et al.* (Taguchi et al., 2000) showed HMGB1-elicited activation of metalloproteases MMP-2 and MMP-9, which are downstream targets of the plasmin activation cascade. While it enables the degradation of the extracellular matrix, the activation of proteases also leads to degradation of HMGB1, and this might serve as a feedback mechanism.

1.3.4 Signalling mechanisms

The signalling mechanisms by which HMGB1 activates cells to respond are incompletely understood. HMGB1 is rather "sticky", and binds to many different molecules on the cell surface: heparin, proteoglycans, but also sulfoglycolipids and phospholipids (Bianchi, 1988); (Degryse et al., 2001); (Rouhiainen et al., 2000). This could be a mechanism to restrict the diffusion of extracellular HMGB1, and thereby keep the effect of this potentially dangerous molecule local. HMGB1 can also be taken up by the cell in an as yet unidentified manner, and promote the co-uptake of DNA. This property was therefore used to transfect cells (Mistry et al., 1997). It is however unclear whether binding to cell surface glycans and cellular uptake are causally related.

However, at least one high-affinity receptor for HMGB1 exists: RAGE (receptor for advanced glycation endproducts) (Hori et al., 1995). RAGE belongs to the immunoglobulin superfamily and binds a variety of ligands: the glycated proteins that are present in the serum of diabetic patients (advanced glycation endproducts, AGEs), but also calgranulin, a proinflammatory peptide that derives from the proteolytic processing of the cytoplasmic protein S100, and amyloid beta-peptide in Alzheimer patients (reviewed by (Schmidt et al., 2000)). It is expressed on a wide set of cells, including endothelial cells, smooth muscle cells, macrophages/monocytes and neurons, and has been implicated in several pathological processes, such as diabetes, amyloidoses and atherosclerosis. HMGB1 binding on the cell surface itself induces the transcriptional upregulation of RAGE (Li et al., 1998).

The understanding of the signal transduction pathways activated by HMGB1 binding to RAGE is still fragmentary. Extension of neurites requires the small GTPases Cdc42 and Rac, but not the ras-MAP kinase pathway (Huttunen et al., 1999). However, in neural cells, HMGB1 binding to RAGE also activates the Ras-MAP kinase pathway

and leads ultimately to the activation of NF-κB, the transcription factor classically linked to inflammatory processes (Huttunen et al., 1999). During tumour invasion the MAP kinases $p38^{MAPK}$, JNK and p42/p44 MAPK have been shown to be activated by RAGE-HMGB1 (Taguchi et al., 2000). HMGB1-mediated migration of smooth muscle cells also results in activation of the MAP kinase pathway and translocation of phosphorylated ERK 1 and 2 into the nucleus, but also involves a $G_{i/o}$ protein (Degryse et al., 2001). How and if these pathways are linked remains an open question; moreover, different pathways may be activated in different cell types. One potentially interesting observation is that cells that respond to extracellular HMGB1 appear to contain very little HMGB1 themselves, and almost none in the nucleus. In support of this observation, it has been shown that *Hmgb1*$^{-/-}$ embryonic fibroblasts respond better to extracellular HMGB1 in cell migration assays than their wild type counterparts (B. Degryse and M.E. Bianchi, unpublished data).

The second role of HMGB1, as an extracellular signal, has been now extensively documented, and is well established. Together, all the data indicate that, as in the nucleus, also outside the cell HMGB1 is a very versatile molecule, involved in several processes: many cellular types can read out the HMGB1 signal, and depending on the target cells very different biological responses are induced (**Fig. 8**).

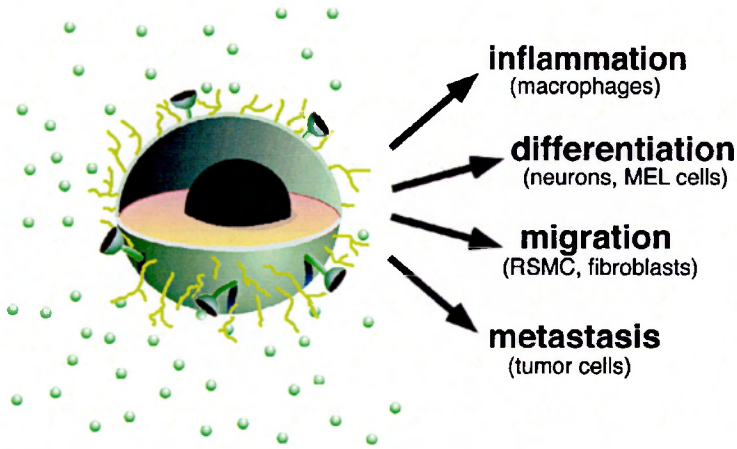


Fig. 8 Different biological responses induced by extracellular HMGB1

Many cells are equipped to recognise HMGB1 in the extracellular milieu. The protein binds with moderate affinity to the proteoglycans covering the surface of every cell (yellow lines). In addition, high affinity receptors like RAGE (green structures) mediate cell-specific responses to HMGB1, triggering different responses, involved in physiological and pathological processes.

1.4 HMGB1: a single molecule for many functions or different isoforms of the same molecule for diverse jobs (and places)?

The discovery of the presence of HMGB1 in the extracellular milieu and of its functions as a signalling molecule has opened an entirely new field of investigation and raised several important questions concerning the signal transduction pathway that regulates its release from the cells, the identity of all its cell surface receptors, and the molecular characterisation of the processes induced by HMGB1 stimulation.

Another major question is how the intracellular and the extracellular roles of HMGB1 are linked to each other. The question of "why" the cell uses a chromatin protein for signalling purposes has recently been partially solved by Paola Scaffidi in her PhD thesis and in the corresponding publication (Scaffidi et al., 2002).

A large part of my thesis work also tried to address these issues: I concentrated primarily on the characterisation of the molecular mechanism employed by the cell to re-distribute a nuclear protein to the cytoplasm and, subsequently, to direct it to secretion.

The problem could be approached from different points of view; since my background was predominantly biochemical, I chose - as starting goal - to identify the biochemical "label" that marks HMGB1's address to the nucleus, the cytoplasm and eventually the extracellular milieu.

The hypothesis that my supervisor and I shaped at the beginning of my work was that HMGB1, a small protein lacking either known regulatory domains, or traditional sequences directing it to specific compartments, does not consists of a single molecule but it is instead composed by a collection of differentially modified isoforms, bearing diverse biochemical/ biophysical properties and thus different functions and subcellular distributions.

CHAPTER 2. MATERIALS and METHODS

2. 1 Plasmids and Nucleic Acids

HMGB1/M1-176 is a truncated form of HMGB1 lacking the COOH-terminal acidic domain of intact HMGB1 molecule; in this thesis had been indicated with different names: HMGB1-boxA+B, -tailles, or -ΔC; the plasmid pRNHMG1/M1-V176 coding for this derivative has been previously described, as well as the plasmid pT7-HMG1bA coding for HMG1boxA a truncated form of HMGB1 containing, aminoacids M1/F89 (Bianchi et al., 1992).

The plasmid pT7-7-rHMG1cm used for expression of full-length HMGB1 in bacteria was a kind gift of prof. J.O. Thomas (Cambridge University. Cambridge, UK).

Plasmids pGEX 2TK-P-CBP (1098-1858) and pGEX2TK-P-PCAF (352-695) expressing the HAT domains of CBP and pCAF respectively, fused with GST, were a kind gift of Dr. J. Bannister (Wellcome/ CRC Institute, Cambrige).

The plasmid pEGFP-N1-HMGB1 contains the open reading frame of HMGB1 fused at the 3' end with the coding region of the Enhanced Green Fluorescent Protein (Enhanced GFP). It was generated inserting a 680 bp fragment of rat HMGB1 cDNA (35 bp upstream the ATG until the stop codon) into the pEGFP-N1 vector (Clontech), using EcoR1 and SacII restriction sites. Both restriction sites were inserted at the end of the cDNA by PCR using appropriate oligonucleotides designed by Paola Scaffidi (Scaffidi et al., 2002).

All the mutant GFP-HMGB1 fusions were generated by PCR according the protocol described in paragraph

2. 2 Mononucleosomes assembly and purification

Nucleosomes were assembled using purified histones and polyglutamic acid (PGA, Sigma P4886) according to (Stein et al., 1979). Histones and PGA were mixed in a 2:1 (w:w) ratio in 0.15M NaCl and incubated for 1hr at RT. Precipitates were removed by

centrifugation at RT for 4 min at 11 krpm, the supernatant (named HP-mix) was stored at –20°C. Different ratios of body labelled DNA and HP-mix were incubated for 3 hr at 37°C and analysed by electromobility shift to reveal optimal conditions for nucleosome assembly. Translational positions of the nucleosomes were separated by electrophoresis on 5% polyacrylamide gels in 0.5x TBE. The gel slice containing the separated nucleosomes was crushed and the nucleosomes were eluted by overnight incubation in EX110 buffer (110 mM KCl, 20 mM Tris pH 7.6, 3 mM MgCl2, 0.5 mM EGTA, 10% Glycerol additionated with 600 ng/µl Chicken Egg Albumin (CEA, Sigma) The final quantity of radiolabelled mononucleosomes of different species were estimated by autoradiography after loading the purified isoforms on a 4.5% polyacrylamide gel in 0.4x TBE, drying it and exposing to X-ray films (Pharmacia Biotech.)

2.3 *E. coli* strains

DH5α: Strain with genotype *supE44 DlacU169 (F80 lacZDM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1.* A recombinant-deficient suppressing strain used for plating and growth of plasmids.

XL1-Blue: Strain with genotype *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'(proAB⁺lacI^q lacZDM15*Tn*10(tet^r)).* It is a recombination- deficient strain that support the growth of vectors carrying some amber mutations. Transfected DNA is modified but not restricted. The F' in this strain allows blue/white screening on X-GAL and permits bacteriophage M13 superinfection.

HB101: *supE44 hsdS20(r_B⁻m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1.* A suppressing strain commonly used for large-scale production of plasmids. It is an *E. coli* K12 X *E. coli* B hybrid that is highly transformable.

BL21(-): Strain with genotype *hsdS gal (□ clts857ind 1 Sam7 nin5 lacUV5-T7 gene1).* A strain used to express at high levels genes under the control of T7 promoter. It lacks the plasmid pLysE, coding for T7 phage lysozyme, which inhibits the RNA polymerase basal activity.

E. coli bacteria were generally grown in LB (Luria-Bertani) Medium, supplemented with ampicillin at a concentration of 100 mg/ml or kanamycin at a concentration of 25 mg/ml as selective agents.

LB (per liter)

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

To express recombinant full length HMGB1, bacteria were grown in M9 minimal medium supplemented with Chloramphenicol at 100 µg/ ml as selective agent:

M9 (per liter):

5 X M9 salts	200 ml
1 M $MgSO_4$	2 ml
1 M $CaCl_2$	0.1%

5 X M9 salts (per liter):

$Na_2HPO_4 \cdot 7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g

2. 4 Preparation of NLS1 and NLS1 mutants and NLSs –GFP construct

Plasmid pEGFP-HMGB1, generated by Paola Scaffidi in our lab was used as template to generate the single mutants in NLS1 and NLS2 in two-step PCR mutagenesis. Oligonucleotides 5'HMG-GFP (5'-ATCCTCgAgACATgggCAAAggAg-3') and 3'HMG-GFP (5'- ACCCCgCggTTCATCATCATCATC-3') were used as external primers and six pairs of internal mutagenic primers (substitutions in bold type):
NLS1KQdir: 5'- gAggAgCAC**CAgCAgCAg**CACCCggATg–3' and
NLS1KQrev: 5'- CATCCgggT**gCTgCTgCT**ggTggTCCTC-3';
NLS1KRdir: 5'- gAggAgCAC**AggAggAgg**CACCCggAT–3' and
NLS1KRrev 5'- CATCCgggTg**CCTCCTCCT**ggTggTCCTC-3';
NLS1KAdir: 5'- gAggAgCAC**gCggCggCg**CACCCggATgC–3' and

NLS1KArev: 5'- gCATCCgggTg**CgCCgCCgC**gTgCTCCTC-3';
NLS2KQdir: 5'- Ag**CCAgCAACAg**AAggAAgAggAAgACgAC-3' and
NLS2KQrev: 5'- **CTgTTgCTg**gCTCTTCTCAgCCTTgAC-3';
NLS2KRdir: 5'- Ag**CAggAgAAgg**AAggAAgAggAAgACgAC-3' and
NLS2KRrev: 5'- **CCTTCTCCT**gCTCTTCTCAgCCTTgAC-3';
NLS2KAdir: 5'- Ag**CgCggCAgCg**AAggAAgAggAAgACgAC-3' and
NLS2KArev: 5'- **CgCTgCCgC**gCTCTTCTCAgCCTTgAC-3'.

The final PCR products were then cloned into the Xho I/Sac II sites of the pEGFP-HMGB1 to obtain the mutants plasmids. Sequences were confirmed by double strand sequencing.

Double mutants were generated using the NLS2 internal mutating primers on the pEGFP-HMG1 mutants in the NLS1 as template. The cloning approach was the same as for the single mutations.

We generated the constructs containing each single NLS1 and NLS2 fused in frame to the GFP by cloning into the XhoI/SacII sites of the vector pEGFP- N1 two cassettes, produced by annealing the following pairs of long-primers, coding for NLS1 and NLS2 sequences, respectively:

NLS1dir:
5'TCTACTCgAgACATGAAgAAgAAgCACCCggATgCTTCTgTCAACTTCTCAgAg TTCTCCAAgAAgCCgCggCTAA-3'

NLS1rev:
5'TTAgCCgCggCTTCTTggAgAACTCTgAgAAgTTgACAgAAgCATCCgggTgCTTC TTCTTCATgTCTCgAgTAgA-3';

NLS2dir:
5'- TCTACTCgAgACATGAAgAgCAAgAAAAAgAAggAACCgCggCTCA-3'

NLS2rev:
5'-TgAgCCgCggTTCCTTCTTTTTCTTgCTCTTCTAgTCTCgAgTAgA-3'.

The primers were temperature-annealed; the annealed cassette was digested with XhoI/SacII restriction enzymes to generate cloning sites, then purified on a 12%AAgel and cloned in the pGFP-N1 vector. Constructs were checked by double strand sequencing.

2.5 Protocol of plasmid DNA extraction

Plasmid DNA was prepared using two classical techniques of plasmid extraction. The first was used to prepare plasmid DNA from 3 ml of many different cultures (1 to 24) of plasmid-containing bacteria (Sambrook et al., 1989). The second one was used to isolate large quantities of plasmid DNA, using anionic exchange cartridges produced by QIAGEN. Bacterial cells were lysed under alkaline conditions and the crude lysates were cleared by centrifugation. The cleared lysate was then loaded onto the anion-exchange tip where plasmid DNA selectively binds under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were removed by a medium-salt wash, and plasmid DNA was then eluted in high-salt buffer. The DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation.

2. 6 Proteins: expression and purification

To produce recombinant HMGB1ΔC (boxA+boxB) and HMGB1boxA we followed the protocol described in (Bianchi et al., 1992); briefly, we inoculated BL21(-) E.coli strain, freshly transformed with appropriate plasmids, in LB medium, supplemented with 0.4% Glucose and 100ug/ml ampicillin. The overnight inoculus was then diluted 1:200 in the same medium and grown at 37°C shaked at 200 rpm. When OD at λ = 600nm, the culture is induced with 1mMIPTG for 90-120 min.

We harvested bacteria by centrifugation and we resuspended them in **Buffer L2** (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), using sonication to clarify them. NaCl was added to a final concentration 0.5M and the extract was centrifuged to pellet the debris, which is discarded. We then perfomed a fractionated precipitation of the supernatant by adding $(NH_4)_2SO_4$ to 60% solubility (corresponding to 3.9g of salt per 10 ml of sample), taking advantage of the capability of HMGB1 to remain in solution at 60% saturation of $(NH_4)_2SO_4$. After 30 min in ice, we centrifuged the extract at 10 kpm for 30 min; The supernatant was collected, filtered and loaded on a Phenyl-Sepharose Column (Amersham Pahrmacia), connected to a FPLC System.

Proteins are eluted in a decreasing gradient of $(NH_4)_2SO_4$ (60-0% saturation) generated mixing the following buffers:

buffer A	20 mM HEPES pH 7.9	**buffer B**	20 mM HEPES pH 7.9
	0.5 mM DTT		0.5 mM DTT
	0.2 mM PMSF		0.2 mM PMSF
	0.2 mM EDTA pH 8.0		0.2 mM EDTA pH 8.0
	0% $(NH_4)_2SO_4$		60% $(NH_4)_2SO_4$

Positive fractions were evaluated on Acrylamyde Gels stained with Coomassie and pooled. At this step, the protocol for purification of the single boxes (boxA and boxB) can be considered completed, while for HMGB1ΔC (boxA+boxB) an additional step of chromatography was required so the positive fractions were dialysed overnight in **buffer A'**(see later) and loaded on a Hi-Trap – SP column (Amersham Pharmacia) connected to a FPLC System (Amerhsam Pharmacia).

HMGB1ΔC was eluted in an increasing gradient of NaCl, mixing the two buffers:

buffer A'	50 mM Hepes pH 7.9	**buffer B'**	50 mM HEPES pH 7.9
	0.5 mM DTT		0.5 mM DTT
	0.2 mM PMSF		0.2 mM PMSF
	20 mM NaCl		1 M NaCl

Positive fractions – evaluated by Coomassie stain in 10-12% SDS PAGE - were collected, pooled, concentrated using a Centricon Cartridge (Millipore); the protein was then desalted using PD-10 columns (Amersham Parmacia) and equilibrated in

Buffer G:

10 mM NaP pH 7.5

100 mM NaCl

0.5 mM DTT

0.1 mM PMSF.

Full-length HMGB1 was produced in BL21(-) *E. coli* strain, following the protocol of Studier and Moffatt (Studier and Moffatt, 1986), but with some modifications: briefly, instead of LB medium used for HMGB1 derivatives, we used M9 medium (see before), completed with:

- Cas-aminoacids 20 g/L
- Glycerol 0.5%
- Yeast Extract 5g/L
- Glucose 0.4%
- Chloramphenicol (instead of Ampicillin) 100 μg/ ml

After an overnight growth, 300 ml of BL21 (-) *E. Coli* strain were inoculated in 3 liters of medium and let grow at 37°C, with a shaking of about 400rpm. When the O.D. was 0.7, IPTG was added to the culture to a final concentration of 0.5mM/1mM; shaking was slowed down to 150/200 rpm and temperature shifted down to 23°C. Bacteria were let grow for other 16 hours, then they were collected by centrifugation.

In principle the protocol is very similar to the one for HMG1 derivatives, until the step of loading on the Phenyl-Sepharose Columns. At this point we didn't elute the protein with a continuous decreasing gradient as described before, but with a stepwise decreasing gradient of 60%- 50%- 40%- 30% of saturation of $(NH_4)_2SO_4$. For each salt-step we waited until the elution-peak was completed and then we went down to the step of lower concentration. Positive fractions were eluted approximatively at the 50% $(NH_4)_2SO_4$ step. Positive fractions were then passed through a Hi-trap MonoQ (5ml column volume) with an increasing gradient from 20 mM to 1M NaCl. Positive fractions were collected, and concentrated in Centricon Cartridges (Millipore). The protein was desalted in final Buffer G and the final concentration was evaluated both by Bradford Method and by Coomassie Staining on 12% SDS-PAGE.

Mammalian HMGB1 was purified from calf thymus according to the protocol kindly provided by Jordi Bernués (CSIC, Barcelona); briefly: the organ is minced in **Buffer1** (0.14 M NaCl, 0.5 mM EDTA, 0.1 mM PMSF) after removal of the fat and the connective tissue.

The homogenate was subjected to three subsequent 5% PCA extractions; supernatants collected after centrifugation were pooled and clarified with TCA (18% final concentration) Fractionated precipitations with Acetone-HCl (400:1 v/v) and acetone

alone were performed to get rid of histone H1 from the sample. The sediment was then dissolved in buffer borate pH 9.0 and passed once or twice (according to the purity of the sample) on a CM-Sephadex C25 column (Amersham Pharmacia), positive fractions were eluted with a NaCl gradient from 0.11M to 0.2M. Typically, different pools were generated according to the purity of the protein, going from very high to mid to low purity of HMGB1. The purified protein was then equilibrated in maintenance Buffer (**Buffer G** or an analogous neutral Phosphate Buffer) with or without addition of 15% glycerol according to the following applications. Aliquots were frozen at Liquid N_2 and kept at –80°C. Alternatively the protein was lyophilised.

GST-fused HATs (GST- CBP and GST- pCAF) were expressed in DH5α *E.coli* bacterial strain and purified using Glutathione Sepahrose 4B (Amersham -Pharmacia), according to manifacturer's protocol: briefly, bacteria freshly transformed with pGEX2TK-PCAF and pGEX2TK-CBP plasmids, were inoculated in LB- ampicillin 100 μg/ml. After an overnight growth, bacteria were diluted 1:100 in 500 ml of fresh medium and grown until the $O.D._{600nm}$ was about 0.7-0.8, then the expression of the protein was induced with IPTG 1mM. Induction was carried on for 2 hours at 37°C. The cells were harvested and resuspended in about about 12 ml of PBS 1X, DTT 1mM, and protease inhibitors cocktail tablets (Boehringer Mannheim). Cells were disrupted by cycles of sonication; Triton1% was added to the extract, that was then centrifuged after 10 min of ice-incubation. We then added the supernatant to Glutathione Sepharose 4B, previously equilibrated in PBS with an approximate ratio of 2.5 mg of GST-protein per ml of slurry. We incubated the protein with the slurry for about 60 – 90 min at 4°C, then we sedimented by centrifugation the beads and we washed them 3- 4 times with 10 bed volumes PBS . The quantity and the purity of the GST-fusions bound to the beads were estimated by loading aliquots of the slurries in 10-12% SDS-PAGE.
Since we needed the GST- fused acetyltransferases for *in vitro* acetylation assays, we did not need to elute the proteins form the slurry, provided that binding to the slurry was not compromising their enzymatic activity (this was appropriately tested).

Histones used to assembly mononucleosomes *in vitro* were extracted from Drosophila embryos and purified by chromatography on a hydroxylapatite column by Ralph

Stroner (Becker's lab, Butenandt Institute, Munich) as described (Simon and Felsenfeld, 1979).

The baculovirus stocks for the expression of Acf1 and C-terminally Flag-tagged Acf1 were kindly provided by Dr. J. Kadonaga. Flag-tagged Acf1 was expressed and purified as previously described (Brehm et al., 2000): SF9 cells were tranfected and recombinant baculovirus was produced using the MaxBac system according to manufacturer's instructions. Exponentially growing SF9 cells were infected with recombinant virus and harvested 48 hours post-infection by centrifugation.
We then resuspended cells in about 20 ml of **HEMG500 Buffer**:

25 mM Hepes pH 7.6
500 mM KCl
12.5 mM $MgCl_2$
0.5 mM EDTA
0.5 mM EGTA
1mM DTT
0.2 mM PMSF
10% glycerol
0.1% NP-40

and submitted to two cycles of freeze-thaw in liquid nitrogen followed by to three cycles of sonication (amplitude 50%, 15 sec/ burst). Total cell lysates were clarified by centrifugation and filtration. The supernatant was collected and incubated with Anti-Flag M2 affinity Gel (Sigma). After incubation on a rotating wheel for 4-6 hours at 4°C, the gel was recovered and washed three times with HEMG1000 (1M KCl) and then 2 times in HMEG100 (100 mM KCl). Bound flagged-Acf1 was eluted with 0.25 mg/ml flag peptide (Sigma) in HEMG100 /0.5% NP-40 incubated 3hours up to O/N at 4°C on a rotating wheel. A second elution is possible (with lower yield) using the same condition.

The ISWI expressing baculovirus was a gift from Dr. C. Wu: recISWI was expressed and purified by Gernot Längst according to the protocol described in (Corona et al., 1999).

2. 7 *In vitro* traslation of recombinant proteins and pull down assays

CRM1 protein was *in vitro* transcribed-translated with the TnT ® Coupled Reticulocyte Lysate System (Promega) following the manifacturer's protocol, using pSGCRM1 a templatge plasmid kindly gifted by Charlotte Kirlstrup in our institute, as the protocol to test the protein – protein interaction.

Seven µl of Met –[^{35}S] labelled CRM1 (freshly made) were incubated with approximatively 1- 4 µM of recombinant HMGB1ΔC protein and derivatives (boxA and boxB), previously cross-linked to Activated Sepharose – CH (Amersham).

Protein GST-NS2 coupled to Gluthatione Sepharose (Amersham) beads was used as positive control of interaction with CRM1 (Askjaer et al., 1998) and BSA-cross linked to Sepharose -CH was used as negative control to test unspecific binding.

The reaction mix for each sample was composed as follows: about 10-20 µl of beads, 7 µl of CRM1, 15 µl **of RAN Buffer** (50 mM Tris/HCl pH 7.5, 200 mM NaCl, 2 mM MgCl2, 10% glycerol), 5µl of 6x **CRM1 buffer** (20mM Hepes/KOH pH 7.5, 80 mM CH_3COOK, 4mM $(CH_3COO)_2Mg$, 250 mM sucrose, 2.5 mM DTT), 1 mg/ml BSA, +/- Leptomycin B 400 nM.

The reaction is performed at 4°C for one hour mixing the slurry on a rotating wheel.

The beads are pelleted by centrifugation and supernatant are collected and dried in the Savant. Beads are then washed 5 times with 50 volumes of PBS additionated with 9% Glycerol, 5 mM $MgCl_2$ and 1% NP-40. Washes are performed at 4°C, incubating the beads 10 minutes /wash on the rotating wheel and pelletting the beads by centrifugation. Washed beads are loaded on a 8% SDS PAGE together with dried output, the fourth wash and one aliquot of the input as a reference. The gel is then blotted onto nylon filter and exposed to X-ray film to detect labelled CRM1.

2. 8 Generation of Sepharose CH-coupled proteins for pull down assays

Sepharose-CH beads (Amersham) conjugated to purified proteins were prepared according to manufacturer's protocol: briefly purified protein are dialysed against **Coupling Buffer** (0.1 M NaHCO3 pH 8.0, 0.5 N NaCl), while the freeze-dried slurry is reswelled in HCl 1mM ice-cold and washed in this chloride buffer 5 times.

Coupling reaction is carried on in the bicarbonate buffer for 2 hours at room temperature or 4 hours at 4°C. After coupling reaction excess ligand is washed away and any remaining active groups are blocked by treatment with ethanolamine 1M, pH 9 for one hour.

The slurry thus generated is washed alternatively with **high pH** (Tris-HCl 0.1M pH 8, 0.5 N NaCl) and **low pH** (acetate 0.1M pH 4, 0.5N NaCl) **buffers** solution four of five times. The slurry is then conserved in different storage buffer depending on the properties of the ligand: we used PBS 1x (with a suitable bateriostaticd agent like NaAzide or Thimerosal 0.01% added) at 4°C.

2. 9 Heterokaryon assay and cooling assay (by Paola Scaffidi)

HeLa cells were plated on glass coverslips in a 6-well dish (100 000 cells/dish). After 16 hours, 200 000 *Hmgb1* -/- mouse fibroblasts were plated on the same coverslips. Following a 3-hour incubation in the presence of 100 µg/ml cycloheximide, the cells were washed with PBS and treated with 100 µl of prewarmed 50% PEG-6000 in PBS for 1 min. After 3 washes with PBS, the cells were incubated with DMEM containing 100 µg/ml cycloheximide for 4 hours, and then fixed with 4% paraformaldehyde. Immunofluorescence was performed using anti-human cytokeratin and anti-HMGB1 antibodies, and chromatin was visualized by DAPI staining. When indicated, 150 nM leptomycin B (Sigma) was added to the medium together with cycloheximide.

For testing passive diffusion of HMGB1, HeLa cells were pretreated with 100 µg/ml cycloheximide at 37°C for 30 min, incubated at 4°C for 4 hours, fixed with 4% paraformaldehyde, and stained with anti-HMGB1 antibodies.

2. 10 Cell cultures and transfections

HeLa cells, 3T3 and 3139 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS from

GIBCO), 100 IU/ml of penicillin and 100 µg/ml streptomycin, in 5% CO_2 humidified atmosphere.

HeLa cells were transfected with pEGFP-N1- HMGB1 vector and mutant derivatives by calcium phosphate co-precipitation (Sambrook et al., 1989): To express HMGB1-GFP, $2x10^5$ cells were plated in 6 cm dishes and were transiently transfected with 8 µg of pEGFP-HMGB1 and derivatives. Cells were observed around 36 hours after transfection. The average amount of HMGB1-GFP in the cell population was between 1 and 3% of HMGB1 (as measured by immunoblotting with anti-HMGB1 antibodies).

Three clones of U937 pro-myelocytic cells (#10, #12, and #34, kindly provided by M. Alfano and G. Poli (HSR, Milano), were grown in RPMI medium (Gibco) supplemented with 10% FCS, L-glutamine, 100 U/ml penicillin 100 µg/ml streptomycin.

To generate a sensitisation to LPS treatment, the cells were first treated for about 3 days with 10^{-7} M Vitamin D3 that induces expression of CD14 receptor and its exposure on the cell membrane; subsequently they were stimulated with 200 ng/ml LPS (Sigma catalogue #L4391) for about 20 hours.

The treatment with 10 ng/ml TSA was instead performed for a time –lag of 3- 6 hours.

Primary human monocytes, purified from peripheral blood (a kind gift from M. Iannacone, Dibit-HSR), were grown in RPMI medium supplemented as described above, and were activated by overnight incubation in RPMI medium supplemented with 200 ng/ml LPS.

SF9 cells, used for baculovirus infection, were cultured in SF900 II Complete Medium with added 1% Pluronic ® F-68 (Life Technologies), 1% L-Glutamine, 1% Penicillin-Streptomycin, 10% Foetal Bovine Serum.

2. 11 Preparation of extracts from cultured cells and organs

Total extracts from cultured cells (Hela, 3T3, U937 and primary monocytes) were prepared using the Freeze-and-Thawn protocol: cells were trypsined if required and pelleted by centrifugation. Pellets were resuspended in **F/T buffer**:

50 mM PIPES pH 7.0

50 mM KCl

5mM EGTA

2mMMgCl$_2$

1mM DTT

proteases inhibitors (one pastille of complete™ Proteinase Inhibitors Cocktail from Boehringer Manheim GmbH was dissolved in 50 ml of buffer)

Four cycles of fast freezing in liquid N_2 followed by thawing at 37°C were performed before centrifuging the lysates at maximum speed for 15 min at 4°C. Pellets containing membrane debries were discarded and supernatant quantified and analysed by SDS-PAGE.

To prepare a lysate from a mouse embryonic thymus, the N_2 frozen organ was powdered by mechanical pressure and then was lysed in an adequate volume of lysis buffer containing 10 mM Hepes, pH 7.3, 0.1 mM DTT, 1 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, 0.2 mM PMSF and a mix of protease inhibitors. The total extract was then mixed with Equilibration Buffer for 2D –PAGE and loaded on strips for the 2D analysis.

2. 12 Detection of proteins by SDS-PAGE: Western blots and Coomassie staining

Samples in SDS-PAGE loading buffer were heated for 5 minutes at 100°C, and applied to SDS polyacrylamide protein gels.

The **separating gel** consisted of:

9%- 12% of Acrylamide-bis stock sol (30% acrylamide, 1% bis)

375 mM Tris pH 8.8

0.1% SDS.

The concentration of the acrylamide-bisacrylamide was variated from 9% to 12%, depending on the size of the proteins to visualise.

The **stacking gel** consisted of:

4% acrylamide-bisacrylamide

125 mM Tris pH 6.8

0.1% SDS.

The **running buffer** contained:

14.4 g/l glycine

3.0 g/l Tris

0.5% SDS.

Gels were run at 5-15 Volt/cm.

In some situations we made use of **Tricine – SDS PAGE** system, that is useful for separation of proteins in the 5 kD to 20 kD range, where normal PAGE-SDS gels have low resolution. The vertical mini-apparatus was used. In this system not only the stacking and running gels are characterised by different AA percentages and different pH values, but also the buffers at the two electrodes have a different composition and chemical properties. This allows gaining a higher resolution in the range of low molecular weighs. The composition of the buffers and of the gel – follows:

Anode bf:	**Cathode bf:**	**Gel bf:**
0.2 M Tris pH 8.9	0.1 M Tris	3M Tris pH 8.45
	0.1 M Tricine (**Sigma #T0377**)	0.3 % SDS
	0.1 % SDS	
	pH is automatically 8.25	

Acrylamide-bis stock sol: 30% acrylamide, 1% bis

Stacking gel	**Separating gel**
6 ml stock acryl-bis	10 ml stock acryl-bis
12.4 ml gel bf	10 ml gel bf
water to 50 ml	3.4 ml glycerol 80 %
	water to 30 ml

Loading buffer 3X (10 ml)

3 ml Tris 0.5 M pH 6.8

0.6 ml mercaptoethanol pure

1.2 g SDS solid

4.5 ml glycerol 80 %

bromophenol blue, some

water to 10 ml

The procedure to mount the apparatus and to pour the gel was the usual one. The gels were run at about 25 V/cm.
For Western blots, the proteins separated by SDS-PAGE (with either methods) were transferred onto nylon Immobilon P membranes (Millipore) using a tankblot system (Hoefer) in 25 mM Tris pH 7.5, 0.192 M glycine, 20 % methanol. Blots were blocked for 1 hour at room temperature in 5% skim milk in **TBST**:

20 mM Tris pH 7.5
137 mM NaCl
0.1% Tween 20.

Washing was performed in TBST and filters were then incubated for 1 hour at room temperature with anti-HMGB1 antibody (working dilution: 1:4000 in TBST/0.01% BSA). After three additional washes in TBST, filters were further incubated for 1 hour with an anti-rabbit antibody conjugated to HRP (Amersham) (1:5000 in TBST/0.01% BSA). The polyclonal antibody R10, a kind gift from B.M. Turner (University of Birmingham Medical School), was used to detect specifically the acetylated form of histone H4. After incubation with secondary antibodies, blots were washed with TBST and developed using the ECL system (Amersham).

Coomassie staining was used to detect proteins and to evaluate their purity and concentration: after electrophoresis, gels were incubated for 45 minutes in staining solution, containing 45% EtOH, 8% acetic acid, 5 g/l Coomassie blue, and then in destain solution, containing 23% EtOH, 8% acetic acid, until proteins were clearly visible. Gels were finally dried on 3MM paper with a gel drier.
Silver Staining, used to detect proteins in 2D gels, is described in the next session.

2.13 2D - gel Electrophoresis separation and detection of protein by Silver Staining

The system used for IEF run is provided by Amersham Pharmacia Biotech. About 50 µg of purified HMGB1 or about 250- 400 µg of total protein of total extracts (generated by about 10 million cultured cells with freezing-thawing method) were added to 350 µl

of rehydration buffer (RB), containing 8 M urea, 2% CHAPS, 20 mM dithioerythritol (DTE), 0.8% IPG buffer (carrier ampholytes, pH 3-10 Non Linear or pH 4-7 Linear).Samples were applied onto ceramic strip holders (Pharmacia Biotech), connecting two electrodes, in contact with 18 cm polyacrylamide gel strips (pH range: 3-10 NL, or pH 4-7 L). Isoelectrofocusing (IEF) was performed on IPGphor (Pharmacia Biotech) with 2 different protocols according to the length of the strips used:

18 cm strips protocol:

-rehydration: 30 min at 20°C

-IEF: 18°C

-S1: step-n-hold 30 V, 10.0 hours

-S2: step-n-hold 200 V, 1.5 hours

-S3: gradient 3500 V, 2.5 hours

-S4: step-n-hold 3500 V, 2.0 hours

-S5: gradient 8000 V, 1.5 hours

-S6: step-n-hold 8000 V, 8.0 hours

7cm strips protocol:

-rehydration: 30 min at 20°C

-IEF 18°C

-S1:step-n-hold 30 V, 10.0 hours

-S2: step-n-hold 200 V, 1.5 hours

-S3: gradient 3500 V, 2.5 hours

-S4: step-n-hold 3500 V, 2.0 hours

-S5: gradient 5000 V, 1.5 hours

-S6: step-n-hold 5000 V, 8.0 hours

The IEF was stopped after 75000- 90000 Vx hrs.

The second dimension electrophoresis was performed using a Protean II apparatus (Bio-Rad). Strips were then soaked first in Equilibration buffer (EB: 6 M urea, 3% SDS, 375 mM Tris pH 8.6, 30% glycerol, 2% DTE), then in EB containing 3% iodoacetamide (IAA) and traces of bromophenol blue (BBP). Subsequently, strips were applied onto 10%-12% polyacrylamide gels. Gels were run at 90 V for about 16 hours and either Silver or Coomassie stained or transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech.) as described before.

Silver Stain of the 2D gels was performed following a protocol developed in our lab that gives a high sensitivity but avoids the long times of other methods.

Briefly, after the second dimension the gel was equilibrated (>/=)3 hours with gentle shaking in 5 gel volumes of 30% Ethanol, 10% Acetic Acid. Two washes of 30 min in 5 gel volumes of 30% Ethanol were then performed, followed by three washes of 10 min in deionised water.

We incubated the gel in 5 gel volumes of 0.1% $AgNO_3$ for 30 min; then we removed the $AgNO_3$ and washed for 20 seconds. The developing step consisted of incubating the gels in 5-10 gel volumes of 2.5% Na_2CO_3, 0.02% Formaldheyde at room temperature

with shaking. Incubation was carried on until spots appeared: this can have a variable duration according to the quality of the extract and of the gel. The staining development was stopped by washing in 1% Acetic acid, then washing several times in deionised water.

After scanning the gels, they were dried at the gel-dryer; to avoid breaking of the gels, we equilibrated them in Ethanol 30%, Glycerol 2% for 30 min with shaking before drying on pre-wetted 3MM paper.

2. 14 Mass Spectrometry Analysis

I performed Mass Spectrometry analysis of calf-thymus purified HMGB1 with the help of Fabio Talamo and under the supervision of Angela Bachi in the Mass- Spectrometry facility of our Institute.

Isolated protein spots were excised from 2D gels stained with colloidal Coomassie, and reduced and alkylated as described (Shevchenko et al., 1996; Wilm et al., 1996). We then performed sequential digestions using different sequence specific proteases and/or chemical cleavage. In particular, Trypsin, Asp-N, Glu-C digestions were performed in 50 mM NH_4HCO_3 buffer pH 8.0 at 37°C, with shaking. Time of digestion varied from 3 hours to overnight according to the efficiency of cleavage. Chemical cleavage with formic acid (Asp-C) was achieved by incubating spots overnight at 56°C in 2% formic acid. Spots blotted on PVDF membranes were incubated in the presence of cyanogen bromide (CNBr) in 70% trifluoroacetic acid for 1 hour at RT in the dark. One µl of digestion products was loaded onto the MALDI target using the dried droplet technique and α-cyano-4-hydroxycinnamic acid (HCCA) or sinapinic acid used as matrix.

MALDI-TOF mass measurements were performed on a Voyager-DE STR time of flight (TOF) mass spectrometer (Applied Biosystems, Framingham, MA, USA) operated in the delayed extraction and reflector mode. Spectra, internally calibrated, were processed via the Data Explorer software.

2. 15 *In vitro* and *in vivo* Acetylation Assays

Assays were performed in **HAT buffer**:
50mM Tris-HCl pH 8.0
10% glycerol (v/v)
1mM DTT
0.1 mM EDTA
0.1 mM PMSF
10 mM NaButyrate

The protein substrate concentration was approximatively of 0.1- 0.2 μg/μl. The concentration of [^{14}C] acetyl-CoA (55 mCi/mmol, Amersham Pharmacia Biotech) was 18 μM, while the concentration of the enzymatic activity (both GST-pCAF and GST-pCBP) conjugated with Glutatione Sepharose was approximatively of 10/ 20 ng/μl. The reaction was performed at 37°C for 20 min and at 4°C for 10 min, stopped by centrifugation 5 min at 1000g that allowed separation of the GST-fused HAT activites from substrates. Supernatants were directly mixed with SDS-gel sample Buffer, boiled and loaded on 10% Tricine-PAGE.
Gels were stained with Coomassie Stain then dried and exposed first to Densitometers Screens (Molecular Dynamics) then to autoradiography films from Amerhsam Phamrmacia Biotech. Films were scanned with a Personal Densitometer IS (Molecular Dynamics) and bands were quantified with ImageQuant software.
In vivo acetylation of HMGB1 in cultured cells (Hela, 3T3, Phonenix,) was performed incubating 70% confluent cells in DMEM medium with different HDAC inhibitors added under the following conditions: TSA 5ng/ml, HC toxin 10 ng/ml, 5mM Butyrate; cells were harvested at different time points by trypsinisation and total cells extracts prepared subjecting the cells to 3 cycles of Freezing-Thawing in Lysis buffer (50 mM Pipes pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1mM DTT, Protease Inhibitor Cocktail from Boehringer Manheim GmbH, 60 μg/ml)
We evaluated the level of acetylation of the extracts by monitoring the acetylation of Histone H4 by Western Blot using anti-H4 acetyl-Lys kindly gifted by Prof. Bryan Turner (Birmingham), produced as previously described (White et al., 1999).

2. 16 Immunoprecipitation of pCAF and p300/ CBP from Hela nuclear extracts and *in vitro* acetylation assay

Rabbit anti-pCAF and rabbit anti-CBP (cross-reacting also with p300) (Santa Cruz Biotechnology) were used to specifically immunoprecipitate the diverse HATs from HeLa total extracts.

About 2 mg of total protein from 3T3 extracts were first precleared by 3 hours of incubation with Protein A-Sepharose, then were incubated overnight at 4°C with antibodies (1:10 dilution, corresponding to approximately 15 µg Ig) in 300 µl **RIPA buffer:**

10 mM Tris-HCl pH 8.0
1% Triton
0.1% SDS,
0.1% Dehoxicolate
140 mM NaCl
1mM DTT
1mM PMSF
1mM EDTA
Protease Inhibitors Cocktail (as before).

Fifty µl of 50% Protein A-Sepharose were washed twice in RIPA Buffer and added to every immunoprecipitation reaction. The mixtures were incubated at 4°C for 3 hours on a rotating wheel, then the beads were pelleted by centrifugation and washed 5 times in RIPA Buffer, while the supernatants were acetone precipitated. Aliquots of the Beads (IP- samples) were loaded on a 10% SDS-PAGE, together with the Supernatants (non IP-samples) and the Inputs, to check the efficiency of Immunoprecipitation.

To test the enzymatic activity of the immuno -precipitated proteins, the HATs linked to the Protein A Sepharose were incubated with about 0.5 µg of protein substrate and [^{14}C] acetyl-CoA to a final concentration of 80 µl in a total volume of 40 µl of buffer HAT. Reaction was carried on at 37°C for 30 min, plus 10 min at 4°C, then the reaction was stopped by adding SDS-gel sample buffer and boiling for 5 min. The level of acetylation was quantified by running the products on 10% Tricine SDS-PAGE and exposing the dried gel to Autoradiography films. The intensities of radioactive bands were quantified as described in the previous paragraph.

2. 17 Immunofluorescence and Imaging on living cells

Cell cultured in LabTek II chambers (Nalgene) or grown in 6-wells plates with a microscopy-glass were directly fixed in 3.7% paraformaldehyde (PFA) in **PHEM buffer:**

36.8 g/l PIPES

13 g/l HEPES

7.6 g/l EGTA

1.99 g/l $MgSO_4$

titrated to pH 7.0 with KOH

Fixation lasted 10 minutes, at room temperature. After fixation, cells were washed with PBS and incubated for 3 minutes at 4°C with HEPES-based permeabilization buffer containing 300 mM sucrose and 0.2% Triton X-100. One hour of incubation in blocking solution (3% Bovine Serum Albumin, 0.05% Tween-20 in PBS) followed. Primary antibodies were then diluted in PBS+BSA 2% to the suitable final concentration and incubation was prolonged for 1 hour at room temperature. After three rinses with PBS containing 0.05% Tween-20 (PB-Tween), cells were incubated with secondary antibodies in PBS+BSA 0.2% for one hour, washed three times with PB-Tween, and then incubated with PBS containing 1.5 µg/ml Hoechst 33342. The polyclonal rabbit anti-HMGB1 was purchased from BD PharMingen (Torrey Pines, CA), and used at 1:1600 dilution in blocking solution. Goat polyclonal antibodies against rabbit IgG (H+L) conjugated to Alexa Fluor 594 (working dilution 1:1000) were purchased by Molecular Probes (Eugene, Oregon, USA).

Living cells expressing HMGB1-GFP, its derivatives and the NLSs-GFP fusions were PFA-fixed as described above, then incubated in PBS containing Hoechst 33342 to stain the nuclei and finally imaged.

Fixed and living cells were imaged using an Olympus 60x/1.4NA Plan Apo oil immersion objective lens on a DeltaVision Restoration Microscopy System (Applied Precision, Issaquah, WA, USA) built around an Olympus IX70 microscope equipped with mercury–arc illumination. Filters were from Chroma Technology Corp. (Brattleboro, VT, USA): Hoechst 33342 excitation 360/40, emission 457/50; GFP excitation 490/20, emission 528/38. Twenty optical sections spaced by 0.5 µm were collected with a Coolsnap_Hq/ICX285 CCD camera (Photometrix, Tucson, AZ, USA)

and deconvolved by the constrained iterative algorithm available in the SoftWoRx 2.50 package (Applied Precision) using 10 iterations and standard parameters. Each image measured 512x512 pixels, and effective pixel size was 0.106 μm.

Due to the spherical morphology of monocytic cells, a quantitative analysis of HMGB1-fluorescence in different subcellular compartments was not feasable, since it would require the normalisation of the fluorescence against the volume of the cell compartment. We tried to bypass this problem with a strict statistical analysis of the redistribution of HMGB1 upon different stimuli: we therefore counted positive cells and normalised this value againsts the total cell number.

In case of both fibroblasts and monocytic cells, when we describe a specific localisation of HMGB1 inside or outside the nucleus, it means that at least 75-80% of the cels observed showed the expected distribution of HMGB1.

2. 18 Bioinformatics

HMGB1 was inspected for potential NLSs using the PredictNLS database, maintained at CUBIC (http://cubic.bioc.columbia.edu/predictNLS) (Cokol et al., 2001). PredictNLS is an automated tool for the analysis and determination of Nuclear Localization Signals (NLS). By submitting a protein sequence or a potential NLS, PredictNLS predicts if the protein is nuclear, or finds out whether the potential NLS corresponds to a known one in the database. The program also compiles statistics on the number of nuclear/non-nuclear proteins in which the potential NLS is found or has a match. Finally, proteins with similar NLS motifs are reported, and the references to the experimental papers describing the particular NLS are given.

2. 19 Nucleosome mobility assay

60 fmoles of nucleosomes with defined translational positions were incubated with ISWI, ACF, HMGB1 and HMGB1ΔC for 0 to 90 min at 16°C. Reaction mixes contained **EX70 buffer**:

70 mM KCl

10 mM Tris

1 mM EDTA

1.4 mM $MgCl_2$

10% glycerol

supplemented with 1 mM ATP, 200 ng /µl CEA and 1 mM DTT.

Reactions were stopped by the addition of 300 ng of competitor cold DNA and differentially positioned mononucleosomes were separated exploiting their different electrophoretic mobility on 4.5% polyacrylamide gels in 0.4x TBE.

Gels were extensively pre-run at 100mV and after loading samples were run for about 2.5 hours at 100mV. Shifts were detected by exposure of dried gels to Hyperfilm autoradiography films or to the phosporimager. Gels were scanned with a PhosphoImager (Fuji) and the individual bands were quantitated with Aida software.

2. 20 Electrophoretic mobility shift assays

Sixty fmoles of purified nucleosomes were incubated with increasing amounts of ISWI, ACF, HMGB1 and HMGB1ΔC in EX80 buffer containing 200 ng/µl CEA and 1 mM DTT. Reactions were incubated for 10 min at RT and then separated on 5% polyacrylamide gels containing 0.4x TBE. The composition of the shifting nucleosome/protein complexes was investigated by using a super-shift approach: after 10 min of binding reaction, the specific antibodies, rabbit anti-HMGB1 (BD Pharmingen) and monoclonal mouse anti-Flag M2 (Sigma) were added to the mix (1:50 and 1:25 working dilution, respectively) and incubated 5-10 min prior loading the sample on an AA gel.

2. 21 Nucleosome footprinting

Footprinting of HMGB1 bound to the nucleosomes was performed by Gernot Längst; briefly, nucleosomes localized between –185 to – 40 relative to the centre of the DNA

at position +16, relative to transcription start site (+1), were purified as described before and used for footprinting assays. Briefly, DNA was 5' end labelled with PNKinase and end-digested with the specific restriction enzime. Optimal HMGB1-nucleosome ratios were estimated in electromobility shift assays. The electromobility shift reactions were scaled up 50-fold and treated with increasing amounts of DNase I. Reactions were stopped by the addition of EDTA to a final concentration of 5 mM and directly loaded on preparative electromobility shift gels. DNase I-treated nucleosomes and HMGB1-nucleosome complexes were isolated from the gel, treated with proteinase K, and analyzed on 6% sequencing gels.

CHAPTER 3: Acetylation of HMGB1 regulates its sub-cellular localisation, switching its function from a chromatin protein to a cytokine.

3.1 INTRODUCTION

3.1.1 First studies on HMGB1 secretion in monocytes

It has recently been shown that activation of monocytes results in the redistribution of HMGB1 from the nucleus to cytoplasmic organelles, which display ultrastructural features of endolysosomes (Andrei et al., 1999). HMGB1 does not traverse the endoplasmic reticulum and the Golgi apparatus, consistent with the absence of a leader peptide in the protein. The early mediator of inflammation interleukin (IL)-1β is also secreted by monocytes through a non-classical pathway involving exocytosis of secretory lysosomes (Andrei et al., 1999). However, in keeping with the roles of IL-1β and HMGB1 as early and late inflammatory factors, the discharge of the secretory vesicles that contain these 2 proteins responds at different times to different stimuli: IL-1β secretion is induced earlier by ATP, autocrinally released by monocytes soon after activation; HMGB1 secretion is triggered by lysophosphatidylcholine (LPC), a lipid generated later in the inflammation site (Andrei et al., 1999).

3.1.2 HMGB1 is variably modified

In activated monocyte secretion of HMGB1 does not involve protein newly made in the cytoplasm, but proceeds through the depletion of nuclear stores. Therefore we reasoned that a change on unknown nature had to occur to the protein already existing; this modification would be able to alter its biochemical properties and causes its redistribution into the cell. The nature of this "alteration " was the first object of my investigation.

Very little is known about functional modulation of HMGB1: structural investigations suggest that regions flanking the HMGbox and in particular the C terminal

acidic tail could modulate the DNA-binding affinity. The indentification in the nucleus of a specific protease that cleaves the C-terminal portion of the protein strengthen this hypothesis and suggests a means for *in vivo* regulation of HMGB1 function (Dyson and Walker, 1984).

Another possibility to regulate HMGB1 resides in post-synthetic modifications of the protein: alternative functions or alternative localizations of proteins often involve post-translational modifications; we therefore imagined that post-translational modifications could be the way to generate different functional isoforms from a single protein and/or to distribute them in different cellular compartments.

HMGB1 is subjected to acetylation and ADP-ribosylation and phosphorylation (Sterner et al., 1979); (Wisniewski et al., 1999).

ADP-ribosylation of HMGB proteins and – among them- of HMGB1 was described long ago, but its significance has still to be clarified, even if several old works relate this modification to cell aging and suggest a role for cell recovery from DNA damage (Prasad and Thakur, 1990; Thakur and Prasad, 1990) and (Tanuma et al., 1985).

Using metabolic labelling with [^{3}H]- acetate and sequential Edman degradation of purified HMGB1 Allfrey and collaborators identified a moderate level of acetylation of lysines 2 and 11 (Sterner et al., 1981).

At the beginning of my PhD, we extended his evidence and hypothesized that PT - modifications could be the means employed by the cell to distribute HMGB1 from nucleus to the cytoplasmic compartments.

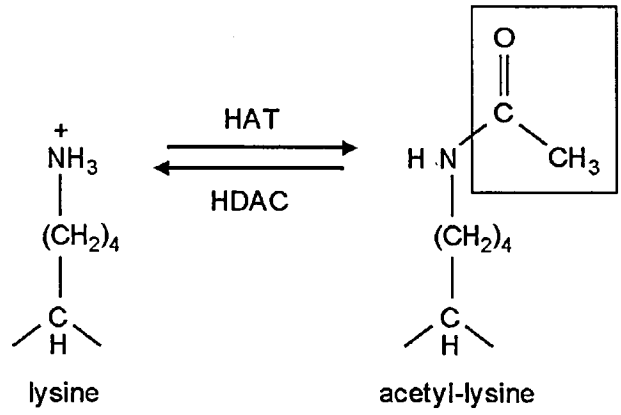
Among the panel of modifications described, reversible acetylation was the most plausible, mainly because acetylation is emerging as a very widespread modification that occurs on an increasing number of nuclear factors in addition to the histone-tails: viral proteins, transcription factors, chromatin components (Chan and La Thangue, 2001) (Kouzarides, 2000) are known to be functionally modulated by acetylation.

3.1.3 HATs and HDAC regulate gene expression and modulate the function of several nuclear factors

Acetylation has been discovered, first, to occur on the histone- octamer tails that protrude from the nucleosome moiety, with the primary effect of loosening the histone–DNA interaction, rendering the chromatin more accessible to transcription factors and therefore more transcriptional active.

Together with the other PT-modifications, acetylation of histone tails is now considered a key control level to the accessibility of chromatin, and therefore of its activation state. As a matter of fact, the transcriptional activity of specific loci in the genome can be strongly correlated to their level of acetylation, which is the result of the balanced and regulated activity of two families of enzymes: acetyltransferases (HAT) and deacetylatses (HDAC) on the tail of the histones.

Acetyltransferase activity consists in the transfer of an acetyl-group to the ε -amino group of a lysine residue and, with the neutralization of its positive charge that results in the decrease of the pI of the protein substrate. HDACs work in the opposite direction, cleaving the acetyl-group from the modified protein.



Both HATs and HDAC consists typically of large, multisubunit enzyme complexes, whose identification was challenging and elusive for many years.

HATs activities were grouped in two general classes, based on their presumptive cellular origin and function: cytoplasmic B-type HATs and A-type HATs, the latter group being involved in transcription– related events and comprising the best studied enzymes GCN5, pCAF, CREB-binding protein (CBP), p300, and TAF250.

The HATs were also organized into four families, differing in their biochemcal properties, summarized in **Table 1**:

Table 1 Characteristics of HAT families

HAT group	HAT (and complexes associated with it)	Interactions with other HATs
GNAT	Gcn5 (SAGA, ADA, A2)	p300; CBP
	PCAF (PCAF)	p300; CBP
	Hat1 (HatB)	
	Elp3 (elongator)	
	Hpa2	
MYST	Esa1 (NuA4)	
	MOF (MSL)	
	Sas2	
	Sas3 (NuA3)	
	MORF	
	Tip60	
	Hbo1 (ORC)	
p300/CBP	p300	PCAF; GCN5
	CBP	PCAF; GCN5
Basal transcription factors	TAFII250 (TFIID)	
	TFIIIC[b]	
	Nut1 (mediator)	
Nuclear receptor cofactors	ACTR[c]	p300; CBP; PCAF
	SRC1	p300; CBP; PCAF

[b]TFIIIC may contain up to three polypeptides with HAT activity (70, 71).

[c]Also known as RAC3, AIB1, PCIP, and TRAM (162).

For an extensive and detailed review on HATs, see (Roth et al., 2001).

HDACs were first described almost 30 years ago and since then have been reported in almost all biological systems, from mammals to yeast (Carmen et al., 1996). In all organisms, the HDACs form a highly conserved protein family, which encode isoforms of the enzyme that may differ in substrate specificity, intracellular localization and post - translational modification. In mammals at least three proteins HDAC1-3 have been identified, that have correspondents in other organism (see reviews: (Turner, 1993); (Grunstein, 1997)).

Both HATs and HDACs are placed at the end of a growing number of signal-transduction pathways exerting a tight regulation; the amount of information in this field is constantly growing but still limited. For reviews see (Urnov and Wolffe, 2001); (Chan and La Thangue, 2001).

An important element of novelty was provided by the discovery that a number of nuclear and cytoplasmic proteins are subject to reversible acetylation, other then histones: transcription factors, transcriptional co-regulators, retroviral gene products and other chromatin components (Kouzarides, 2000) and (Chan and La Thangue, 2001).
Typically, acetylation appears to modulate diverse functions, like DNA recognition, protein–protein interaction, stability and transcriptional potency, working as a rapid and reversible regulatory mechanism, much like phosphorylation.
Remarkably, in 2002 two papers were published that referred to the regulation of nuclear versus cytoplasmic localization by acetylation for transcription factors HNF4 and CTIIA (Soutoglou et al., 2000) and (Spilianakis et al., 2000).
These papers suggest that acetylation posses also a higher- order regulatory effect and add other functional meaning to this post-translational modification.

3.2 RESULTS

In the past, a detailed characterization of post-translational modifications was quite challenging because of lack of appropriate technology; when we started to investigate this field of research, we decided to take advantage of new tools in protein analysis recently developed.

3.2.1 2D electrophoresis as a tool to study protein post-translational modifications

We chose the bi-dimensional electrophoresis as a first qualitative approach because it allows separating proteins according to two independent parameters: the molecular weight, that represents the size of the protein (number of amino acids) and the isoelectric point (pI) that is an indicator of the total charge of the protein. As a matter of fact, a protein consists of a chain of zwitterionic molecules – the amino acids - and therefore can be considered a big zwitterionic molecule *per se*. That means there is a specific pH value at which all the positive charges of the basic residues are balanced by the negative charges of the acid ones, resulting in a neutral total charge. This value depends on the amino acid composition of the protein, therefore it is a highly specific label to mark a polypeptide.

The first step of 2D- electrophoresis consists of an Isolelectrofocusing (IEF) step, where proteins are moved, by an electric field, along a gradient of pH, polymerized on a strip of acrylamide. Each protein is moved by the electric field in either direction of the gradient according to its internal charge until it reaches the position where the external pH equals the pI of the protein. There, the protein is neutral and it is not sensitive anymore to the power of the electric field, therefore it stops in that specific position. At the end of the IEF we assume that every protein has focused in that particular position of the pH gradient corresponding to its pI. The proteins are separated – at this step - only on the base of their charge, so at every pH value we can find peptides of different size but equal total charge.

In the second step, the strip with the focused proteins is loaded "horizontally" on a classical vertical polyacrylamide gel; the gel is run following the classical 1D -

protocol, so that the proteins focused at the same pH value are separated according to their size.

This generates a 2D -matrix where every spot, corresponding to a single protein, is defined - like in a Cartesian Plane - by two coordinates: the X axis corresponds to the pI and the Y axis gives the molecular weight values (**see Fig. 9**)

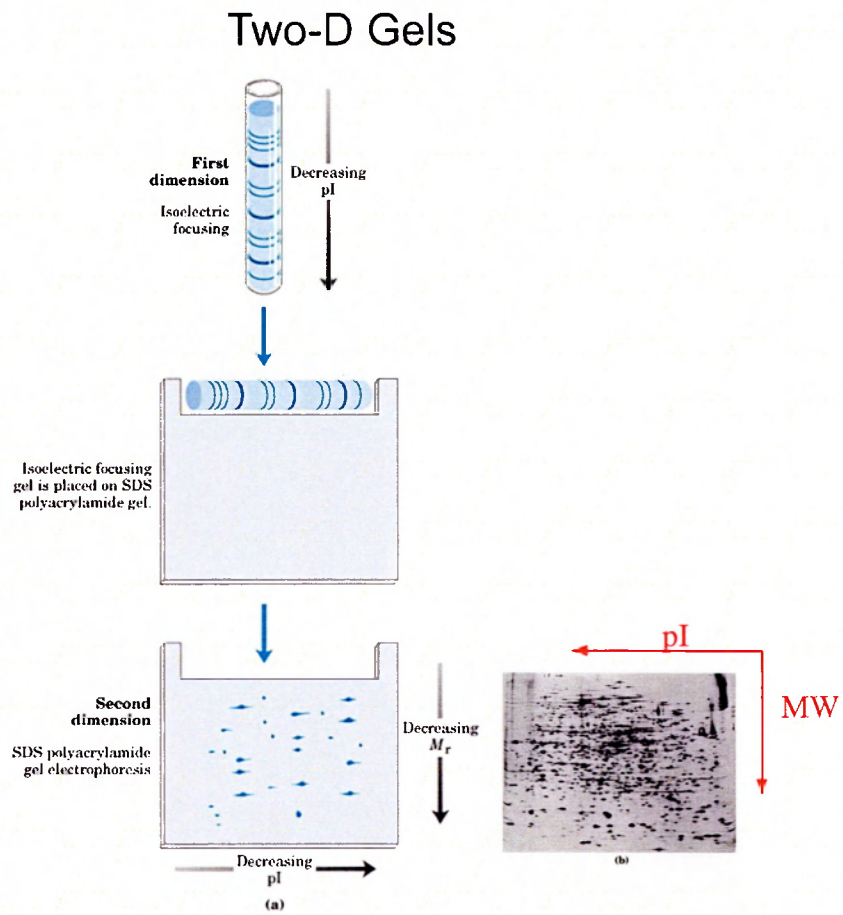


Fig. 9 Explicative scheme of the method of 2D-electrophoresis, showing the two steps of Isoelectrofocusing and PAGE electrophoresis. The result is a matrix, where proteins are represented by spots, characterized by two coordinates (red axis)l likewise points of a Cartesian Plane.

Basing its separating potential onto 2 independent parameters, 2D –PAGE is much more powerful then the classical 1D- approach, allowing the discrimination of a higher number of protein in a total extract.

Modern 2D IEF–PAGE can discriminate a single -charge difference between proteins of average dimension (between 20 and 60 KDa). Therefore it provides a valuable tool to

separate not only different proteins but also differentially modified isoforms of the same one. The addition of small groups, like phosphates or acetyls, to a protein is not sufficient to generate detectable changes in the total mass of the protein, but is enough to generate a pI variation that can be visualized in the IEF step. A protein that is multiply phosphorylated or acetylated can be visualized like a train of spots running at the same position along the MW (= Y axis), but regularly distributed along the pH (= X axis) gradient. The association of this technique with MALDI-Mass Spectrometry analysis, used for high resolution-analysis of polypeptides (see below) provides a powerful technique to study complex patterns of post-translational modifications.

3.2.2 2D electrophoresis separates mammalian purified HMGB1 into several spots, corresponding to differentially modified isoforms

We purified HMGB1 from calf thymus following the protocol kindly provided by Dr Jordy Bernués. Brielfy, a homogenate is prepared from about 1Kg of thymus and subjected to several extractions with PCA, TCA and acetone- HCl; the sample is then passed through a CM-Sephadex C25 column (Amersham Pharmacia); the number of chromatographic cycles depends on the purity of the sample; positive fractions are collected and pooled; the final concentration is assayed by the Bradford method and the purity evaluated by monodimensional SDS-PAGE.

Purified HMGB1 separated into two major bands in monodimensional SDS-PAGE (**Fig. 10, left**); a major one with an apparent molecular weight of 29 KDa, and a minor band that contains HMGB1 modified with ADP-ribosyl moieties (Bernuès, personal communications).

Fifty micrograms of this preparation were separated by isoelectrofocusing (IEF) in a linear pH gradient from 3 to 10, subjected to electrophoresis in a 15% AA gel, and then silver-stained. Eight to ten different spots appeared (**Fig. 10, right**) at similar molecular weight but regularly spaced along the pH gradient.

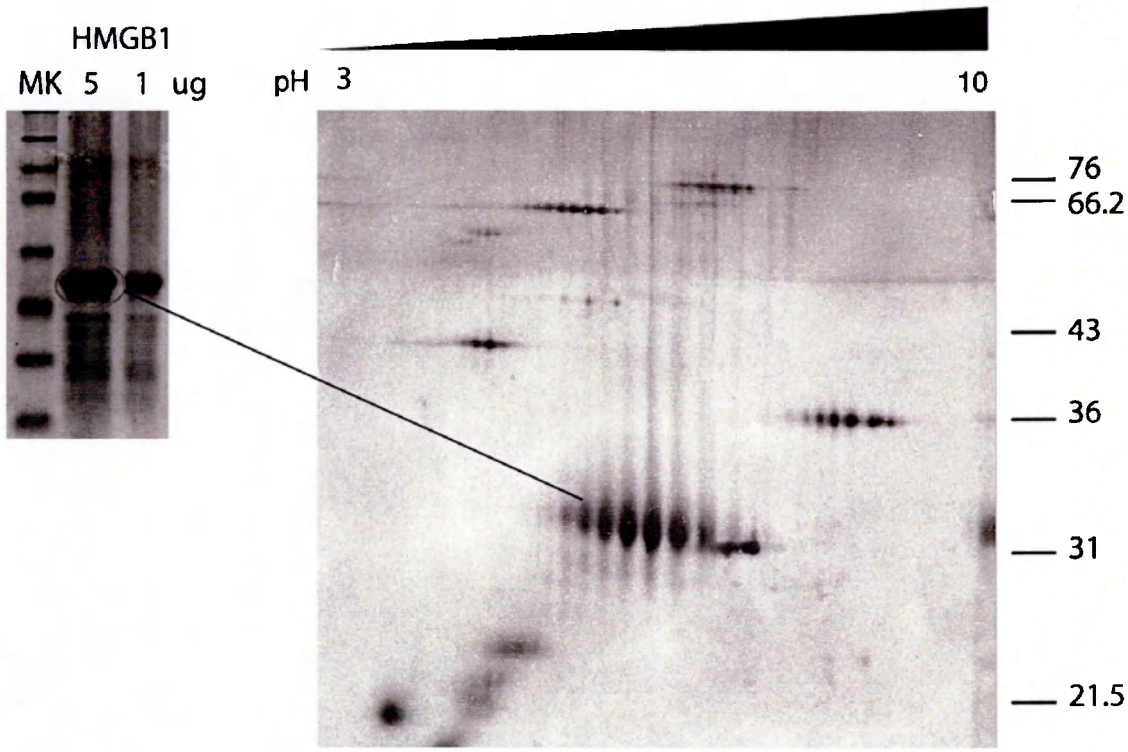


Fig. 10 2D electrophoresis of purified HMGB1

HMGB1 purified from calf thymus gives rise on a monodimensional gel (SDS-PAGE, Coomassie stain, right panel) to two bands (arrows): a major one at about 29 KDa apparent molecular weight, plus a minor one that contains ADP-ribosylated protein. Fifty μg of the same sample of HMGB1 were subjected to 2D gel electrophoresis (silver stain, left panel); 5 μl of 2D Protein Marker from BioRad were loaded together with HMGB1, generating a matrix of spots at the molecular weights listed on the right.

A rabbit anti-HMGB1 polyclonal antibody purchased from Pharmingen (BD) recognized the spots with similar affinity when the 2D gels were blotted on nitrocellulose filter and probed by Western Blot; this confirmed that they corresponded to different isoforms of the protein of interest (not shown).

To exclude the possibility that the spots were purification artefacts we prepared a total extract of thymus from a mouse embryo of 17 days, powdering the N_2-frozen organ by mechanical pressure and then dissolved in a lysis buffer Hepes-based, containing 0.1% Triton. The extract thus generated was analysed using the same approach.

We chose this organ because the thymus is extremely rich in HMGB1 and extremely active as endocrine gland. Thymus reaches its complete development and dimensions

during post-natal life, then it starts to reduce in size and functionality after weaning and is almost totally atrophied at the adult stage.

Twin gels were loaded with approximatively 400 μg of total extract; one gel was silver stained while the other transferred on a filter and probed with anti-HMGB1. A pattern very similar to that seen with purified HMGB1 was seen in the Western Blot, confirming not only that the modifications are physiological but also that they are conserved among different mammalian species (**Fig. 11**).

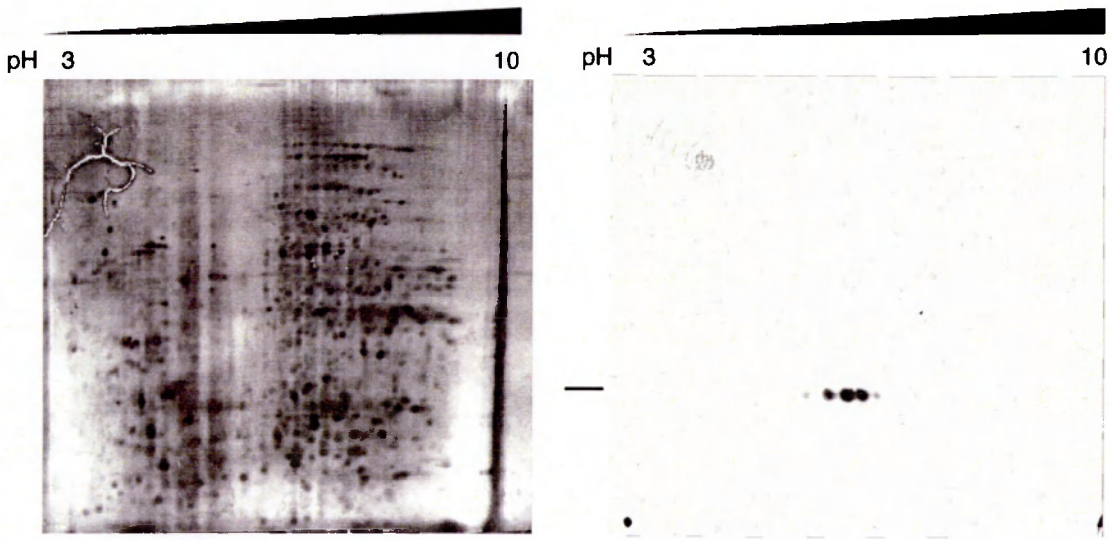


Fig. 11 2D electrophoresis of total extract from mouse thymus

Total mouse thymus contains many isoforms of HMGB1. About 300 μg of protein from a thymus total extract (from a mouse embryo of 17 days) were loaded on two twin 2-D gels; the gel shown on the left was silver stained, while the other one was electroblotted onto a nitrocellulose filter and assayed with a specific anti-HMGB1 rabbit antibody. Note the similarity of the pattern to that obtained from purified HMGB1 (Fig. 10).

The smaller number of spots detected was probably due to the lower quantity of HMGB1 loaded.

At this point, we could conclude that:

1) HMGB1 is composed by a pool of isoforms differing in their position along the pI gradient of the 2D gel;

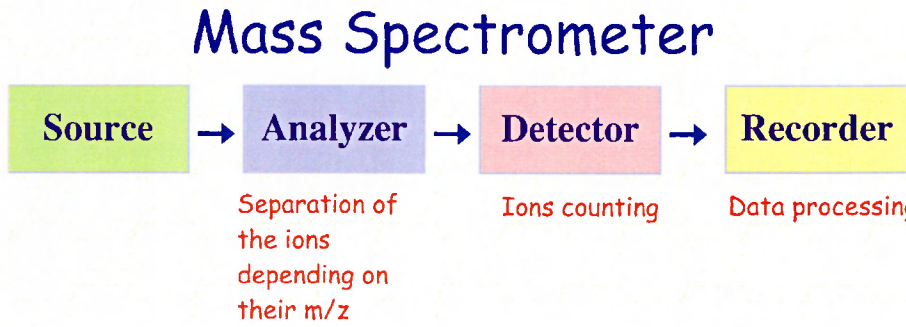
2) the modifications do not change significantly its molecular mass: between the first spot and the last one we detected a very small shift on the vertical axis (MW). PT modifications that induce such effects are, for example, phosphorylation and acetylation; they consist of the attachment of small groups (phosphate- and acetyl-groups) to specific residues, which leads to a change in the total charge of a protein; the phosphate brings a negative charge to the protein, while acetylation neutralizes the positive charge of Lysines and Arginines. The overall effect is a change in the pI of the protein while the effect on the total mass of the protein is limited since all these groups are quite small;
3) the regular positioning of the spots is suggestive of a single type of modification generating the same ΔpI and the same slight increment in molecular weight;
4) the modification is present to a high extent, since the spots detected are at least 8-10. Every spot corresponds to isoforms of HMGB1 that are modified once, twice, three times, etc;
5) the 2D-PAGE can discriminate only between increasing numbers of modification but not among the sites of modification along HMGB1 sequence; thus, every spot corresponds to a collection of all molecules of HMGB1 containing a specific number of modifications, no matter what their position.

In light of these observations, we reasoned that HMGB1 can be modified by the addition of up to 10 modifying groups and that the sites of the modifications, varying along the sequence, are not discriminated by the 2D gel. This means that, for 10 spots there are at least 2^{10} isoforms containing a different number of modifications in different positions along the protein. The huge amount of variability thus generated represented a challenge for our investigation, since our goal was the characterisation of the type and pattern of HMGB1 modification. We had to develop a specific strategy to solve this point.

3.2.3 Principles of Mass Spectrometry to analyse proteins

We chose the Mass Spectrometry approach to investigate the nature of the modification in HMGB1.

MS analysis allows the measurement of the exact mass of a molecule, in this case a protein fragment. A Mass Spectrometer is composed of four elements, represented by the following scheme:



As schematised, the separation principle of mass spectrometry is based on the capability of separating ionised molecules depending on their m/z (mass / charge) ratio.

In a spectrometer, each molecule of a mixture is charged by ionisation, accelerated by a strong Electric Field and then allowed to cover a specific distance represented by the analsyer, by drifting.

In such condition, a specific parameter is associated with every molecule in a sample, the Time Of Flight (TOF), defined as follows: "if ions are accelerated with the same potential at fixed point and a fixed initial time and are allowed to drift, the ions will separate according to their mass-to-charge ratios". In a mathematical form, this is expressed by the following formula:

$$\frac{m}{z} = \frac{2t^2K}{L^2}$$

t	=	**Drift time**
L	=	**Drift length**
m	=	**Mass**
K	=	**Kinetic energy of ion**
z	=	**Number of charges on ion**

The mass/charge ratio of a molecule is proportional to the square of its drift.

In the past, a problem in the application of this technique to big molecules resided in the fact that either they were not ionised enough to be accelerated or that they were broken into pieces by the laser beam used in the ionisation step.

These problem were partially solved by the development of MALDI (Matrix- Assisted Laser Desorption- Ionisation) Mass Spec, where a protein of interest or a mixture of proteins is crystallized on a matrix of variable composition that fulfils different tasks. A typical MALDI matrix compound displays a number of desirable properties:

1) an ability to absorb energy at the laser wavelength whereas the analyte generally does not;
2) an ability to isolate the analyte molecules within some form of solid solution;
3) sufficient volatility to be rapidily vaporized by the laser in the forms of a jet in which intact analyte molecules (and ions) can be entrained;
4) the appropriate chemistry so that matrix molecules excited by the laser can ionise analyte molecules, usually by proton transfer.

The development of more adapted matrixes allowed the analysis of larger molecules, like peptides and even entire proteins. Commonly used matrixes are alpha-cyano-4-hydroxycinnamic acid and sinapinic acid; they are better suited for the analysis of small and large peptides, respectively.

Using this technique, ionised proteins with molecular masses in excess of 200KDa are readily observed –this is considerably more then anything previously achieved. MALDI, in fact, is applicable to a wide range of biopolymer types: not only proteins, but also glycoproteins, oligonucleotides and oligosaccharides, of course with the correct choice of the appropriate matix.

Typically, an average-size protein is cut into pieces by use of an enzymatic or chemical digestion and the pool of peptides are subjected to MS analysis. Therefore, every protein is characterised by a specific "fragmentation- fingerprint" that is visualised by the computer connected to the machine as a pattern of spectra, correlating the mass of each peptide with its relative intensity/ abundace.

The peptide fingerprint is launched in a specific Database (MASCOT or ProteinSearch or others) of experimental and theoretical spectra and the output provided is the match with known protein. In this way it is possible to identify a specific protein.

A different application of Mass Spectrometry, the Electron Spray Ionization (ESI), is based on a more mild principle of ionisation. This technique is less easy-to-handle but prone to a wider range of applications; for example in the very last years it it has been implemented for the micro-sequencing of analyte proteins through their ordered and regulated fragmentation: in this way it is also possible to identify new protein sequences and enlarge the existing databases. ESI technique is also under development in order to gain a straightforward analysis of protein post-translational modifications.

For a general introduction to the concepts and applications of Mass Spectrometry see *Methods in Molecular Biology*, Vol 61: "Protein and peptide analysis by MassSpectometry", Edited by J.R. Chapman, Humana Press Inc.

For a review about the application of Mass Spec approach to the identification of gel-separated proteins see (Patterson, 1995).

At present, the sensitivity reached by these instruments is high enough to perform the analysis of a protein eluted from a band in a 1D gel or even from a spot in a 2D gel; the combination of the separating potential of 2D - PAGE approach with the analytical power of Mass Spec Analysis led to the rise of the Proteomics, a wide-range analysis of the protein-world.

Moreover, MALDI analysis can also be applied to characterize the modifications of a protein by evaluating, with high precision, the difference-value between the experimental masses and the theoretical ones of its fragments: given the sequence of a protein, a specifically designed software predicts the theoretical fragmentation patterns of different proteases; by comparing this prediction with the real mass of the peptides obtained by digestion and ionisation of the protein of interest it is possible to derive difference in masses that correspond exactly to the molecular weight of the modifying groups (acetyls, methyls, phosphate, etc..) or multiple of these values if the protein is multiply modified.

3.2.4 Mass Spectrometry shows that HMGB1 is acetylated

We made use of this second application to study the modifications in HMGB1.

The four most abundant spots from a 4 –7NL pI 2D gel stained in Coomassie were cut from the gel and in-gel digested O/N with Trypsin, a mammalian protease that cleaves the peptidic bond at the C-terminus of Lysines and Arginines. Peptides derived were eluted from the gel, desalted and analysed by MALDI: the mass corresponding to each digestion product was compared with the theoretical mass, obtained by digesting the protein *in silico*. **Fig. 12** shows the steps of the analytical protocol employed, which associates 2D-PAGE technique for the separation of proteins to MALDI analysis to identify and characterise each of them.

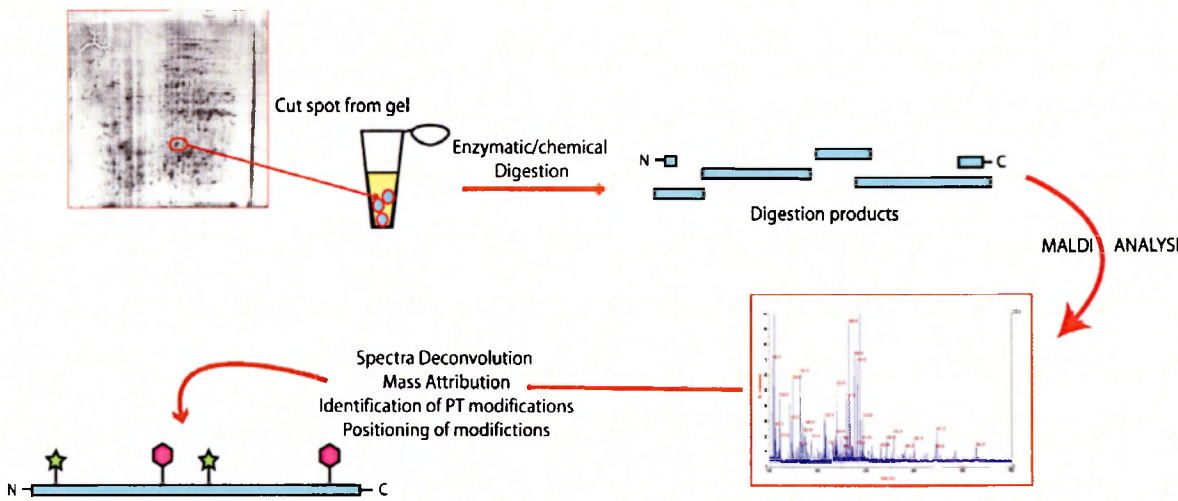


Fig. 12 Strategy for 2D /MALDI-MS analysis of multiply modified HMGB1

Spots were excised from 2D gels and proteolyzed; the complex mixture of oligopeptides was analyzed by MALDI-TOF. A mass was attributed to each peptide in the mixture; the masses corresponding to peptides predicted *in silico* were selected as "anchors", and we searched the complex spectra for further mass peaks corresponding to anchor masses plus multiples of 42 (the mw of an acetyl group); the procedure was iterated for all peptides. We also mined the spectra for evidence of phosphorylation, methylation and glycosylation, without finding any. This information was used to infer the modification pattern on HMGB1.

We found that the mass difference among the peptides detected by MALDI and the corresponding predicted ones corresponded to 42 Da, or multiples of 42 Da: the modification that generates this type of difference is acetylation.

Acetylation consists of the cross-linking of an acetyl group ($-COCH_3$) to the ε-amino group of Lysine ($R-(CH_2)_4-NH3^+$); this leads to the neutralization of the basic charge of Lysine, and therefore decreases the pI of the entire protein moving it towards more acidic values (lower pI).

Acetylation was first discovered for histones; in nucleosomes, acetylation has the immediate effect of loosening the interactions between the octamer particle and the DNA wrapped around it. Acetylation has been found recently in other nuclear proteins, especially transcription factor like p53, GATA-1, E2F, where it seems to fulfil a regulatory function (Chan and La Thangue, 2001).

HMGB1, as nuclear, DNA-binding protein, is likely to be acetylated, and, in fact, Allfrey and his collaborators, more than 20 years ago, published that HMGB1 is acetylated at Lys2 and Lys 11(Sterner et al., 1979) and (Sterner et al., 1981).

Our Mass Spec results were in accordance the pattern of spots in the 2D gel, suggestive of a modification altering the pI of the protein but not its MW; the number of spots were indicated that the number of modification sites was higher than two, as found by Sterner et al.

Given all this, the hypothesis that acetylation could play a role in generating isoforms of HMGB1 with different biological functions looked sound.

3.2.5 A multi-step digestion approach assigns acetylation to specific sites

To further characterise HMGB1 acetylation, we started searching for the sites of modification.

Even today, the analysis of protein mixtures with 10 or more acetylation sites is challenging. Furthermore, HMGB1 contains 43 lysines over a length of just 214 amino acids, and many are clustered within a single proteolytic fragment.

We devised a multiple-digestion strategy: we used different site-specific proteolytic agents (Asp-N, CNBr, trypsin, Glu-C, Asp-C), both singly and in

combination, and analyzed the peptides by MALDI. Thus, one or more acetylations were assigned to large fragments of the protein (but not to specific lysines), and their positions were then progressively restricted inside smaller fragments. This approach is similar to mapping DNA sequences with Restriction Endonucleases.

In the example provided in **Fig.13**, HMGB1 was first digested with Asp-N, and the non-acetylated fragments expected from the digestion were identified in the complex pattern of MS data. Peaks corresponding to the masses of the non-acetylated peptides plus 42 or n-multiples of 42 were presumptively assigned as 1-to-n acetylations of that specific peptide. Then, aliquots of the same peptides were further cleaved with CNBr, and smaller fragments were obtained. The assignment procedure was iterated, until the acetylations could be attributed to specific lysines.

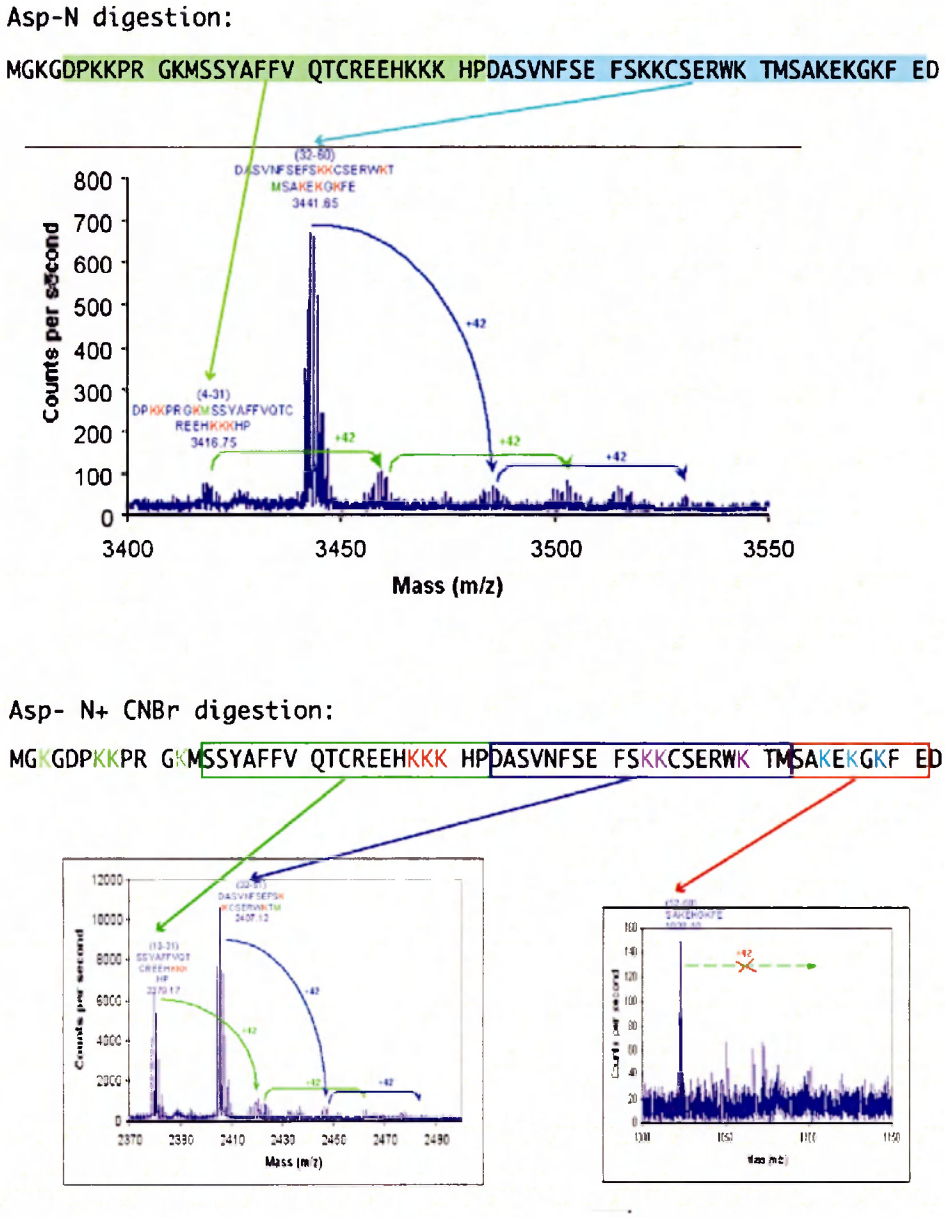


Fig. 13 Example of the multi-digestion strategy adopted to assign specifically acetylation sites along HMGB1 sequence.

HMGB1 was first digested with Asp-N, and acetylations were approximately positioned inside big fragments; then, peptides were chemically cleaved by CNBr, and the smaller fragments produced were analysed further. In this way, the modifications were attributed with higher confidence to residues inside smaller fragments. In the optimal condition of digestion, all the Lysines inside a specific fragment can be characterized unequivocally.

By analysis of a large set of digestion products (not shown), we were able to assign 17 acetylated lysines and to exclude modification of 20 lysines; only 6 lysines out of 43 remained uncharacterized. We could not find evidence of other types of modification; in

particular, we sought for methylation, phosphorylation and glycosylation. MS is not a quantitative technique, but it was very clear from the data that no lysine was acetylated in all HMGB1 molecules in the sample. In principle, if each of the lysines we assigned can be acetylated independently, there can exist 2^{17} molecular species of HMGB1; however, it appeared that several lysines tend to be acetylated as a cluster

Digestion I	Site specificity I	Digestion II	Site specificity II
Trypsin	Lys- C Arg-C;	–	–
Asp -N	Asp -N	–	–
CNBr	Met -C	–	–
V8	Glu-C	–	–
CNBr	Met- C	**Asp -N**	Asp-N
Asp -N	Asp -N	**CNBr**	Met- C
CNBr	Met- C	**FA**	Asp- C
CNBr	Met- C	**V8**	Glu-C

Table 2 Summary of all the combinations of proteases used for our analysis.

Collecting and comparing all products obtained form the digestion performed, summarized in **Table 2**, we were able to assign 17 modifications to specific positions and to exclude 20 Lysines from modification; only 6 Lysines out of 43 were uncharacterised due to no sequence coverage.

Among the acetylated Lysines, we were able to distinguish between those that were always found modified and the others, that are modified less frequently: the sites acetylated with a frequency of 100% were only 7 and accumulated in two main clusters, one inside the boxA and the second in the stretch of aminoacids linking the boxB with the acidic tail at the C-terminus of the protein. Eight Lysines were also found acetylated but with a lower frequency; they also show clustering.

Remarkably, 20 Lysines were excluded from modification; this provides an important result *per se*, suggesting that acetylation is not really spread homogeneously all over

HMGB1 sequence. Six Lysines remained uncharacterised because the corresponding fragments were not found in the MALDI spectra, probably because they are not properly ionised and thus they do not fly well. As a confirmation, the unidentified residues are grouped in the same areas of the protein.

The number of characterised residues (37/43) was sufficient to gain a good confidence in the results of our analysis. They are summarised in **Fig. 14**.

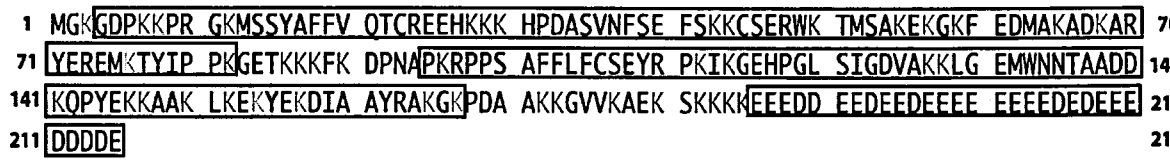


Fig. 14 Distribution of acetylation sites along the sequence of HMGB1

Lysines (K) are marked with different colours: **blue Lys** are never acetylated; **violet Lys** are the uncharacterised residues because because of lack of sequence coverage; **red Lys** and **green Lys** are the acetylated ones, with the difference that red ones were found acetylated in all the analysis performed, while green were found to be acetylated less frequently.

The sites attribution is correlated to the domain organisation of the protein (blue box = HMGB1boxA and boxB, respectively; red box = acidic tail).

The immediate observation was that HMGB1 seems to be acetylated to a higher degree than other proteins studied so far.

Typically, proteins are modified only two or three times along the entire sequence; in the case of HMGB1, the most abundant spots correspond to the isoforms modified in 4/5 sites, while both the isoform that is acetylated 9-10 times and the unmodified one are quite rare.

Remarkably, acetyl-groups are not uniformly spread along the protein, but they tend to generate clusters and to leave some regions completely untouched; for example the internal regions of the two HMG-boxes are rarely modified.

We cannot exclude that the clustering is a mere consequence of the fact that some regions of the protein are more exposed to the acetyltransferases while others are

hidden in the protein core. Alternatively, the peculiar concentration of acetylation in two main clusters could have a functional meaning.

3.2.6 *In vitro* acetylation of HMGB1 by recombinant and cellular acetyl-transferases

After discovering acetylation as a major PT modification of HMGB1, we tried to modify experimentally the acetylation level of the protein both *in vitro* and *in vivo*.
In eukaryotes, a class of enzymes called acetyl-transferases catalyses transfer of an acetyl group from Acetyl- CoA to the side chains of Lysines. The first members of this family were discovered by a genetic approach in yeast as general transcription co-activators capable of stimulating the expression of some genes and to compensate mutations in other factors involved in transcription, like GCN5 (Georgakopoulos et al., 1995).

Further investigation revealed that their function is based on their acetyltransferases activity on histone tails, which causes the decondensation of regions of chromatin with concomitant increase in the expression of the genes. Therefore, these histone-tails modifying enzymes were called Histone-Acetyl-Transferases, or HATs.
In the following years, this class of enzymes increased in numerosity, with the addition of several other members with high homology between different organisms. In parallel with this, other substrates were found, as described in the introdcuction.

In search of the enzymes that acetylate HMGB1, we set up *in vitro* acetylation experiments using recombinant GST-fusions of known HATs: pCAF and p300 /CBP (CREB-Binding Protein). The constructs were kindly provided by A. J. Bannister (CRC, Cambridge).
We expressed in bacteria the recombinant GST-fusions and purified them on Glutathione-Sepharose beads. We decided to keep the proteins bound to the beads and – without eluting them – we incubated the slurry with bacterially purified HMGB1 in presence of ^{14}[C]- Acetyl-CoA. In this way, we could easily separate by centrifugation the enzymatic activity from the reaction product that was loaded AA gels. Gels were Coomassie Stained, dried and exposed to X-ray films.

The yield of the reaction was measured by autoradiography and quantified by densitometry: the intensity of radioactivity was normalised over the total quantity of protein estimated by Coomassie staining.

We found that HMGB1 was efficiently acetylated *in vitro* by recombinant pCAF; the reaction efficiency was evaluated as 50% of the efficiency of the same enzyme when it modifies recombinant histones H3 and H4, its natural substrates and that were used as positive controls, **Fig. 15**.

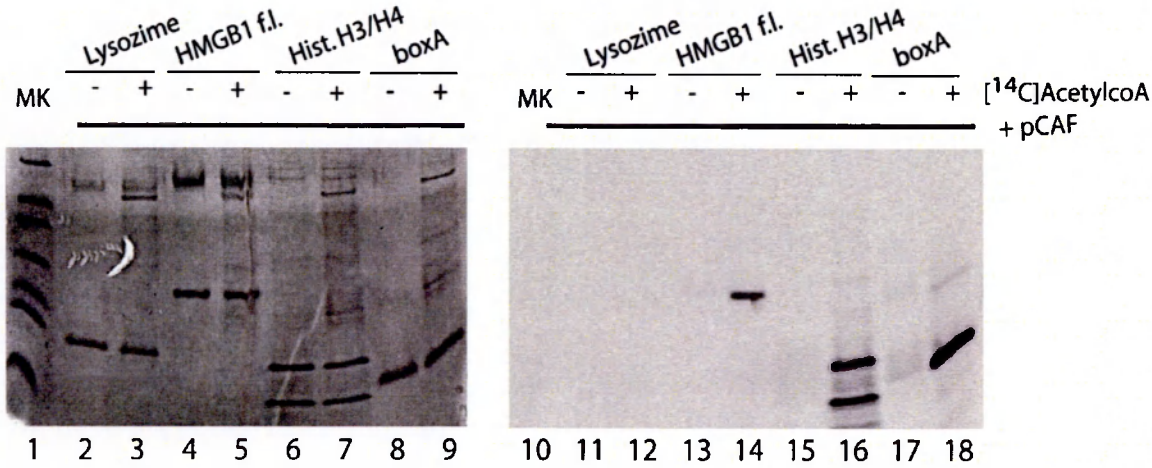


Fig. 15 ***In vitro*** **acetylation of HMGB1**

A set of soluble purified proteins were incubated with gluthatione-Sepharose-bound GST-pCAF (+) or control gluthatione-Sepharose (-) and [^{14}C]-acetyl-CoA. After incubation, the reaction products in the supernatant were separated by centrifugation from the beads and electrophoresed. The gel was Coomassie stained (left side), dried and autoradiographed (right). Both bacterially produced full-length HMGB1 and the isolated boxA were efficiently acetylated (lanes 14 and 18, respectively), like histones H3 and H4 (lane 16); the negative control, lysozyme, was not acetylated (lane 12).

Also the single boxes, boxA and boxB were tested and showed similar modification efficiency (see **Fig. 15**, lanes 8, 9 and 17, 18 for boxA, while the acetylation yield for boxB is not shown). We used lysozyme as negative control to detect unspecific reaction, but no radioactive bands were found on the autoradiographic film corresponding to its position.

Analogous results were obtained when recombinant CBP/p300 were tested as modifying activity even if with lower efficiency (data not shown).

Since the pCAF and CBP/p300 are the most common and the best-known acetyl-transferases, we did not extended our investigation to other HATs that were less characterised.

We then tried to confirm this result by using native acetylatransferases immuno-purified from total cell Lysates with specific antibodies. pCAF (about 90 KDa) could be efficiently immuno-precipitated from HeLa or 3T3 cell extracts (**Fig. 16, left**), and its enzymatic activity was tested using recombinant histones H3 and H4 as substrates. Protein p300/CBP was immunoprecipitated as well, even if with less efficiency, due to its high molecular weight (about 240 KDa) that renders the purification and characterisation procedures much more challenging.

Once we were sure that the enzyme was bound to the beads, HMGB1 was incubated with it in presence of radioactive Acetyl-CoA, (see Methods, section 2): this reaction produced a less efficient, but specific, labelling of HMGB1 (**Fig.16, right**).

This result confirms that native HATs, purified by total extracts, can recognise our protein as specific substrate. In this type of experiments the amount of purified enzymes used in every assay is much lower then in the previous assay performed with recombinant HATs, therefore the idea that HMGB1 acetylation was due to a specific recognition of a "real " substrate (and not only due to the proximity of two unrelated reagents at high concentration) gained further support.

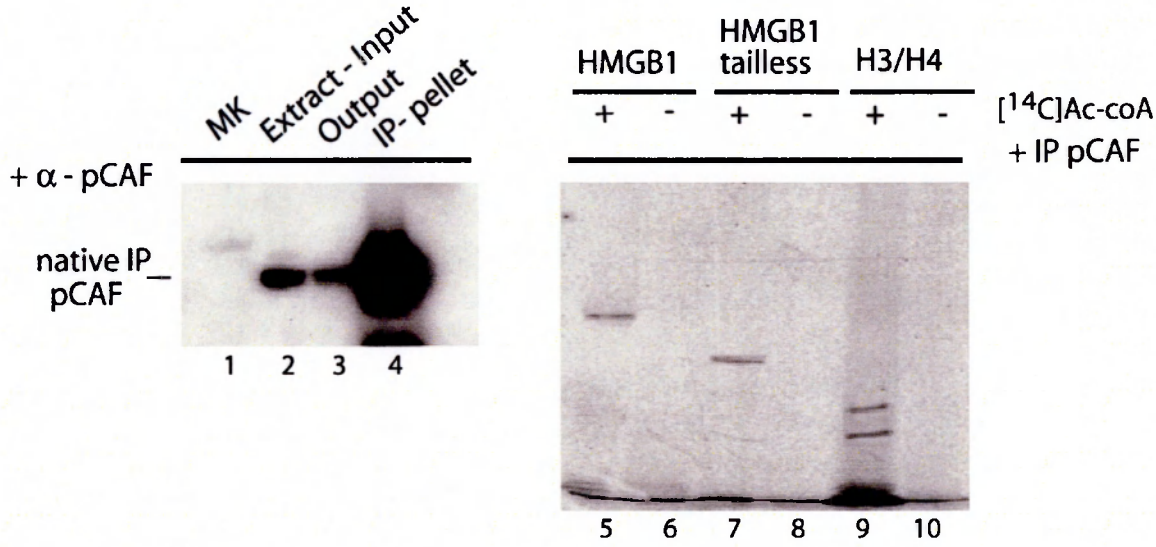


Fig. 16 Acetylation of HMGB1 by immunopurified pCAF

Native pCAF was immunoprecipitated from mouse 3T3 fibroblasts with specific anti-pCAF polyclonal antibodies and Protein-A Sepharose. The efficiency of immunoprecipitation was tested by western blotting: the immunoprecipitated protein (IP-pellet, lane 4) was compared to aliquots of pre-incubation extract (Input) and of flow-through (Output) (lanes 2 and 3, respectively). Immunoprecipitated pCAF was incubated with recombinant histones H3 and H4, recombinant full length HMGB1 and its ΔC derivative (lanes 5, 7, 9, respectively). As negative control, no [^{14}C]-acetyl-coA was added to the reaction mix in lanes 6, 8 and 10.

3.2.7 *In vivo* Acetylation of HMGB1

Based on the results of the *in vitro* acetylation assays, we speculated that HMGB1, like histones, undergoes in the cell cycles of acetylation/ deacetylation due to the activity of acetyltransferases and deacetylates.

Enzymatic activities classified as "HDAC (Histone- DeACetylases)" cleave acetyl-groups from Lysines residues. Specific drugs are known that inhibit this reaction; the effect of these drugs is, therefore, the hyperacetylation of histone tails and of other nuclear factors (Johnson and Turner, 1999).

Commonly used inhibitors are Sodium Butyrate, Trichostatin A and, more recently, also HCtoxin. They were first studied as anti-cancer drugs and then employed also in basic research; they differ for their specificity and toxicity: Sodium Butyrate, for example, has a general cell toxicity and therefore is the less used in studies *in vivo*, while the last

discovered – HCtoxin – is much less dangerous for the cells. Usage of these inhibitors allowed to define the existence of a dynamic cycle of acetylation /deacetylation, whose steady state correspond, for every single protein, to a specific level of modification.
The kinetics of both acetylation and deacetylation are quite high: every protein in basal conditions constantly undergoes cycles of acetylation /deacetylation with a specific steady state where the two reaction are balanced. Upon specific stimuli, proteins get hyper- or under- acetylated. By using deacetylase inhibitors, we can unbalance the steady stated towards the hyper acetylation of a protein that is a natural substrate of native HATs: we treated 3 different cell – lines (3T3 mouse fibroblast, Hela human cells, Phoenix cells) with the three known HDAC inhibitors.

We first performed titration experiments to define the minimal concentration of drugs that is effective in inducing hyperacetylation of histone tails without eliciting toxic effects. Total extracts from treated cells were prepared, loaded on SDS-PAGE and then transferred on PVDF filters; the abundance in acetylated Lysines in histone H4 was determined by western blot using a specific anti- acetyl-H4 kindly provided by my external supervisor, Prof. Bryan Turner (Birmingham).
Instead, to determine the level of acetylation of HMGB1 we had to use the 2D PAGE, since we could not take advantage of a specific anti-acetyl-HMGB1 antibody: therefore, the extracts were loaded on 2D gels and blotted on nitrocellulose filters. Each extract from different treatments (TSA, NaButyrate, HC toxin) was compared with the extract obtained from untreated cells: **Fig. 17** shows that, while untreated cells showed only two spots corresponding to two different isoforms of HMGB1, all HDAC treatments generated a train of spots, similar to the pattern detected with the purified protein.
The new spots are shifted to the more acidic part of the pH gradient, that is highly suggestive of acetylation.

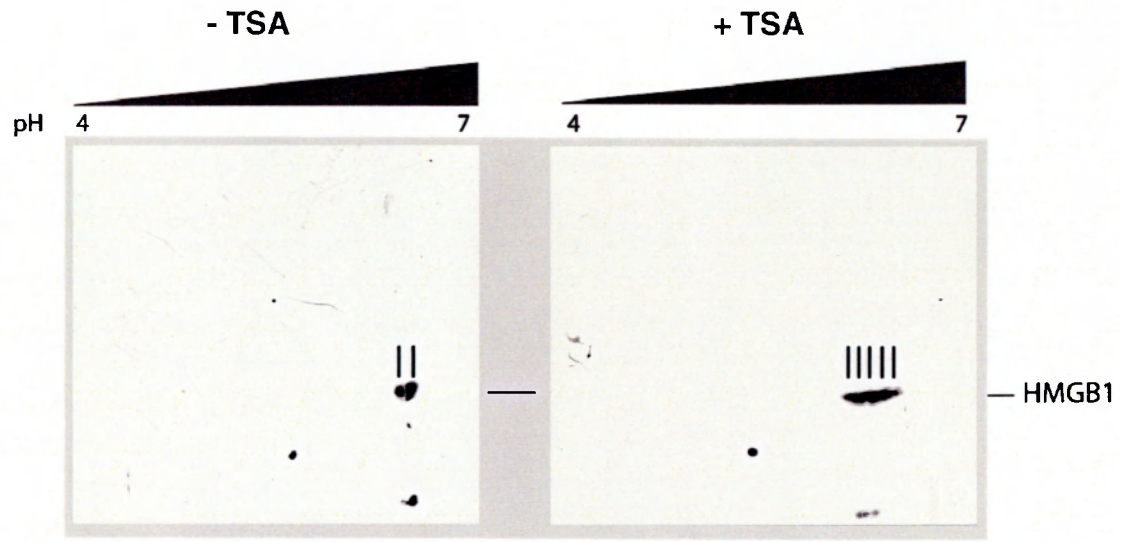
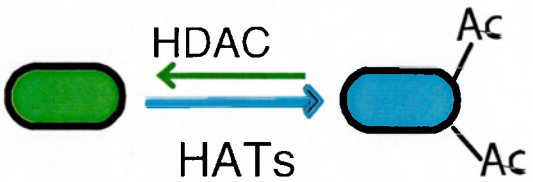


Fig. 17 HDAC inhibitors trigger hyperacetylation of HMGB1

3T3 cells were incubated for 6 hours in DMEM, or DMEM supplemented with 10 ng/ml TSA, then collected and lysed. About 400 µg of whole cell extracts was loaded in duplicate on two 2D gels and then blotted on nitrocellulose; patterns of HMGB1 were revealed with polyclonal anti-HMGB1 antibody. Upon inhibition of HDACs (right), several HMGB1 spots appeared, compared to only 2 in control fibroblasts (left).

Our conclusion from this experiment is that, in basal conditions, the steady state of HMGB1 acetylation/ deacetylation maintains the protein to a level represented in 2D-PAGE by two spot:



Whenever this balance is perturbed by inhibition of deacetylation, HMGB1 gets hyperacetylated

The time course of inhibition indicated that the maximal level of acetylation is reached between 3- 4 hours of treatment, but that already 90 minutes are sufficient to detect a train of spots: this confirms the evidence found in literature that referred to a quite dynamic process and of a high acetylation/deacetylation rate (**Fig. 18**).

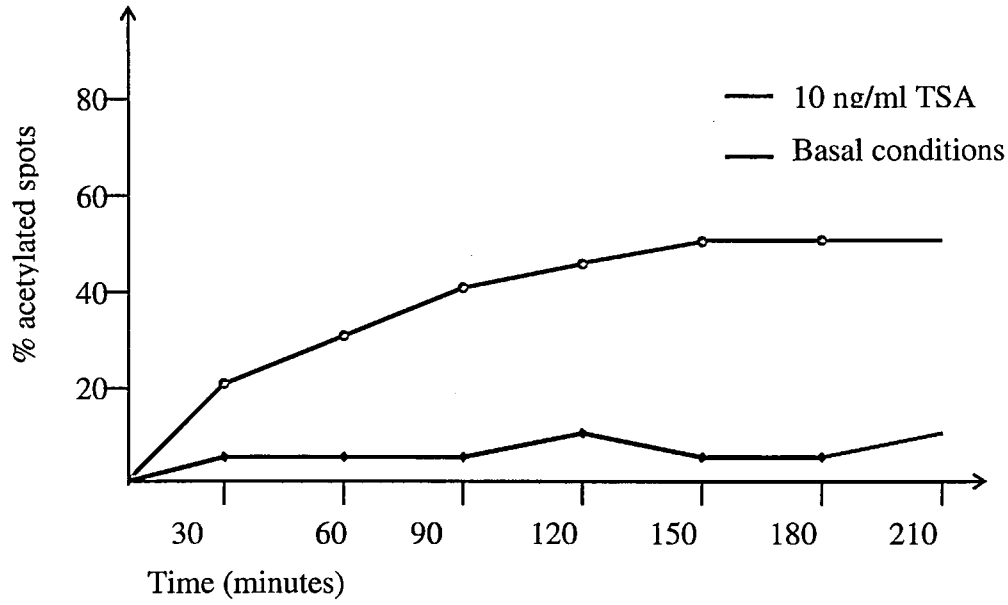


Fig. 18 Time course of HMGB1 acetylation upon TSA treatment

3T3 cells were treated with 10 ng/ml TSA and samples were stopped at different time points; total cell lysates were prepared by freeze-thawing and acetylation of HMGB1 was detected by 2D electrophoresis and Western Blotting using anti-HMGB1 antibody. The efficiency of the reaction is determined by evaluationg the intensity of novel spots produced upon drug treatment normalised against the intensity of basal spots.

3.2.8 Lysines 27-43 define the boundaries of the Nuclear Localisation Signal of HMGB1

Mass Spec analysis, performed on a sample of protein purified from calf thymus, provided intriguing hints about a preferential clustering of the acetylation sites in HMGB1. We reasoned that this clustering could have specific meaning, and decided to investigate the functional effect of these modifications on the activity of HMGB1 in the cell.

Like most nuclear proteins, also HMGB1 should contain a sequence, named Nuclear Localisation Signal that selectively directs proteins to the nucleus, mediating their interaction with the protein of the nuclear pore complex. As a matter of fact, HMGB1 is small enough to pass by simple diffusion through the nuclear pore; however, NLSs mediated nuclear import does not simply regulate nuclear import by size, but more

generally appears as an answer of the cell to the demand of selectivity and specificity for nuclear localisation: only the correct cargoes are imported (and exported) at the proper time. NLS signals contribute crucially to the fidelity of this system, but they are not the only players in the classical nuclear import mechanism: importins α and β, and Ran are also involved (for a review see (Gorlich, 1998)).

The NLSs have been precisely characterised in several nuclear proteins, both by introducing mutations that destroy their function and by fusing the regions of interest to cytoplasmic proteins with the aim to test their capability to redirect them to the nucleus. This approach showed that these signals can be located almost anywhere in the amino acid sequence and that they can have different lengths, but that they require at least one main feature: abundance of positively charged residues Lysines and Arginines, with the presence also of one or two prolines.

Most NLSs can be grouped in two classes: monopartite NLSs that consists in a short stretch rich in basic amino acids (5 – 8 residues long) and bipartite NLSs, in which the positively charged residues are split into two blocks of two to four aminoacids each, separated from each other by about 10 - 12 amino acids. Bipartite NLSs were found in well known nuclear proteins like p53, hPR, etc.

Luca Falciola in our lab had done preliminary experiments that pointed to region 27 - 43 in HMGB1 as its Nuclear Localization Signal, with the characteristic consensus of classical bipartite NLSs: two stretches of 2-3 basic residues (Lys or Arg), separated by 12 amino acids (**Fig 19**)

Fig. 19 Identification of NLS1 in HMGB1

Amino acids 27-43 of HMGB1 were compared with previously characterized bipartite NLSs: this sequence matches with the NLSs of human p53, progesterone receptor and *Xenopus* nucleoplasmin.

To prove the functional significance of the presumptive NLS, we chose the classical fusion approach: we assembled a DNA- cassette coding for sequence 27 - 43 and we cloned it at the 5 end of the GFP gene inside the vector pEGFP-N1 (see paragraph 2.2).

We compared the NLS-GFP construct to the GFP expressed in transiently transfected HeLa Cells. Unfused GFP was distributed all over the cell, whereas about 90% of fluorescence from the NLS-GFP fusion was concentrated in the nucleus (**Fig. 20 A**). Thus, amino acids 27-43 can provide a functional NLS for HMGB1.

Since lysines 27, 28, 29 are among the most frequently acetylated, we examined the possibility that acetylation of the NLS was related to the sub-cellular localization of HMGB1. We therefore mutated lysines 27-29 of the HMGB1–GFP fusion into glutamines to mimic acetylation; we also fully destroyed the NLS by changing the 3 lysines into alanines.

Surprisingly, neither mutation affected the nuclear localization of HMGB1–GFP, suggesting that an element other then aa 27-43 might contribute to locate HMGB1 into the nucleus (**Fig. 20B**).

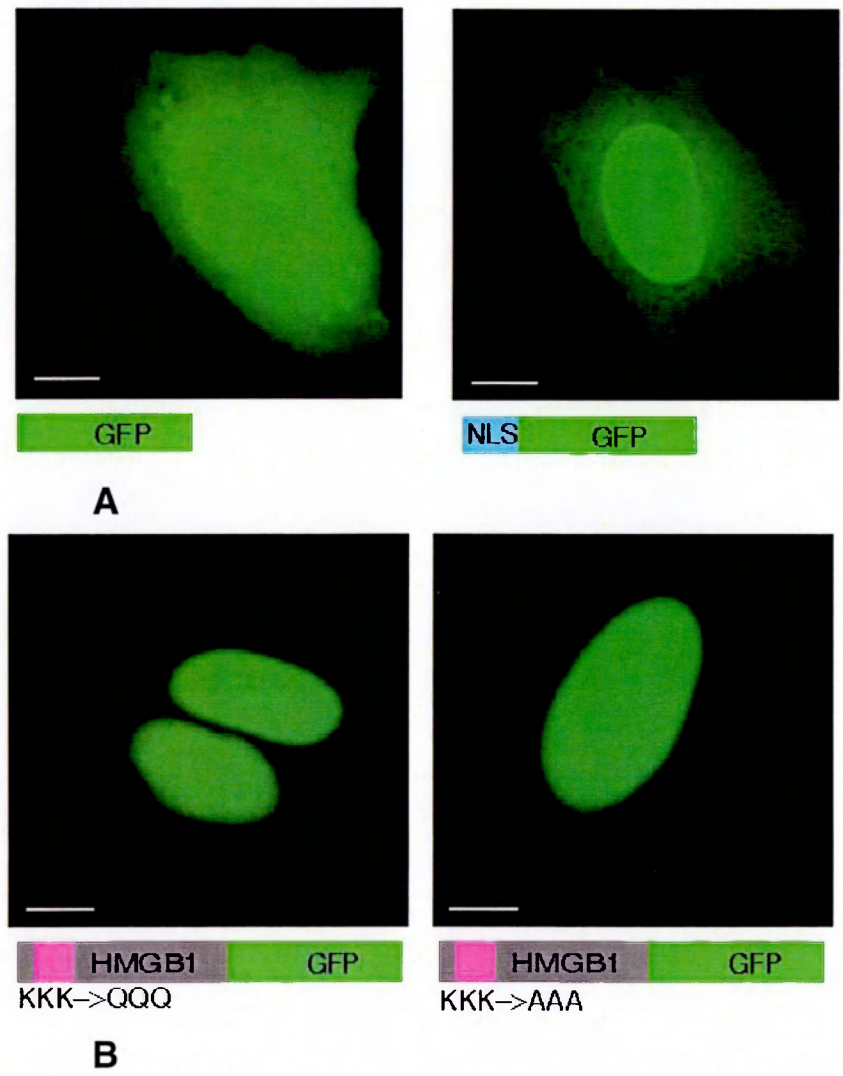


Fig. 20 Experimental identification of NLS1 and point mutation approach.
(**A**) The presumptive bipartite NLS of HMGB1 was fused to GFP and expressed in HeLa cells: while the NLS–GFP fusion is predominantly nuclear, GFP alone is broadly diffuse. (**B**) Lysines (K) 27, 28, and 29 of HMGB1–GFP were changed into glutamines (Q) or alanines (A): neither mutation altered the nuclear localization of the fluorescent protein.

This hypothesis was further strengthened by the observation that other proteins of the HMG-box family make use of two independent NLSs, (Sudbeck and Scherer, 1997). We then started hunting for other NLSs in HMGB1.

3.2.9 HMGB1 has a second monopartite NLS (NLS2)

By visual inspection of the HMGB1 sequence we could not find any other canonical NLS, so we took advantage of the database available at *http://cubic.bioc.columbia.edu/predictNLS*, which offers a collection of experimentally verified plus *in silico*-generated NLSs (Cokol et al., 2001), to help us predict additional NLSs in HMGB1.

The region between aa 178-184 appeared as a good candidate for a monopartite NLS motif: typically, these are characterized by a cluster of basic residues preceded by a helix-breaking residue, but some variations are possible (Boulikas, 1993). When this sequence was launched in the database, it was not identified as an already known NLS, but it matched at highest similarity with several proteins, 93% of which were nuclear (**Fig. 21A**).

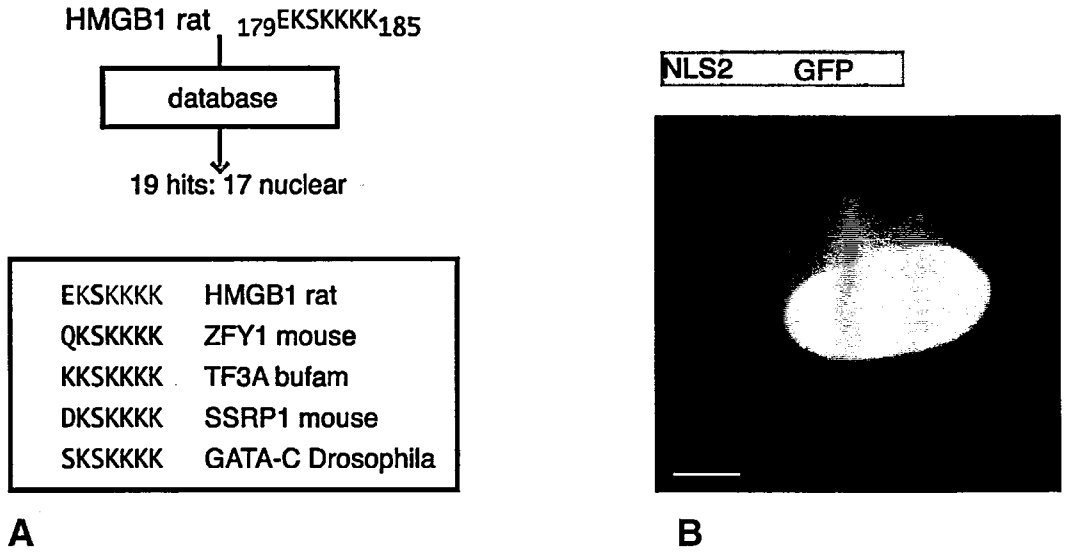


Fig. 21 Bioinformatic and experimental identification of a second NLS in HMGB1

Sequence segments of HMGB1 were launched into the PredictNLS database: the 178-184 segment matched with 19 proteins in the database, 17 of them nuclear proteins. This constitutes a good indication for a potential NLS, that we named NLS2. The presumptive NLS2 was fused to GFP and expressed in HeLa cells, showing a predominantly nuclear distribution.

Based on this, we tested the nuclear-translocation activity of aminoacids 178-184 of HMGB1. The DNA sequence coding for it was fused in frame at the 5' of the GFP in

the pEGFP-N1 vector: the pNLS2-GFP construct was thus generated. We transfected HeLa cells with pNLS2-GFP and inspected its localisation: more then 90% of the fluorescence was concentrated into the nucleus, proving that also this region works as a Nuclear Localisation Signal (**Fig. 21B**).

Thus, HMGB1 is endowed with 2 motifs with NLS activity: we called the bipartite motif NLS1 and the monopartite motif NLS2. Remarkably, also NLS2 corresponds to a cluster of hyperactylated lysines.

Point mutation analysis was operated on Lysines 181-183; as before, we switched Lysines into Glutamines and Alanines, to test the effect of mutations mimicking acetylation and inactivation respectively: the mutants showed no relocalization in the cytoplasm but behaved exactly like the wild type fusion (**Fig. 22**).

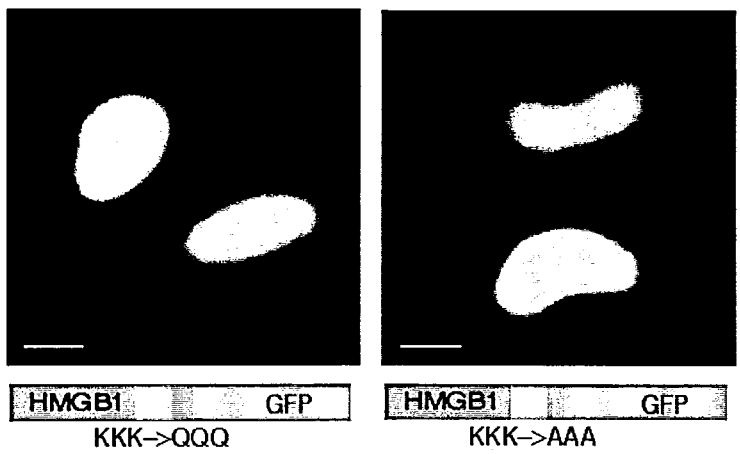


Fig. 22 Point mutation approach to investigate the role of acetylation on NLS2
Lysines 181, 182 and 183 of HMGB1-GFP were mutated into glutamines (Q) or alanines (A) and mutants transiently transfected in HeLa cells: neither mutation caused the cytoplasmic localization of HMGB1–GFP.

Thus, we concluded that each motif is *per se* sufficient to drive HMGB1 to the nucleus, but not necessary: destructive mutation of a single NLS at a time is not enough to induce HMGB1 accumulation in the cytoplasmic compartment.

3.2.10 Double mutation of NLS1 and NLS2 induces redistribution of HMGB1 in the cytoplasm

Double mutants of HMGB1–GFP, where both lysine clusters in NLS1 and NLS2 are changed to glutamines or alanines, were then constructed.

With both mutants, fluorescence was redistributed to the cytoplasm, with comparable efficiency (**Figure 23 A, B**). The double NLS1/NLS2 mutant where lysines are changed to arginines (K->R), showing a nuclear localisation, confirmed that the mutagenesis *per se* is not perturbing the natural destiny of the protein (**Fig. 23 C**).

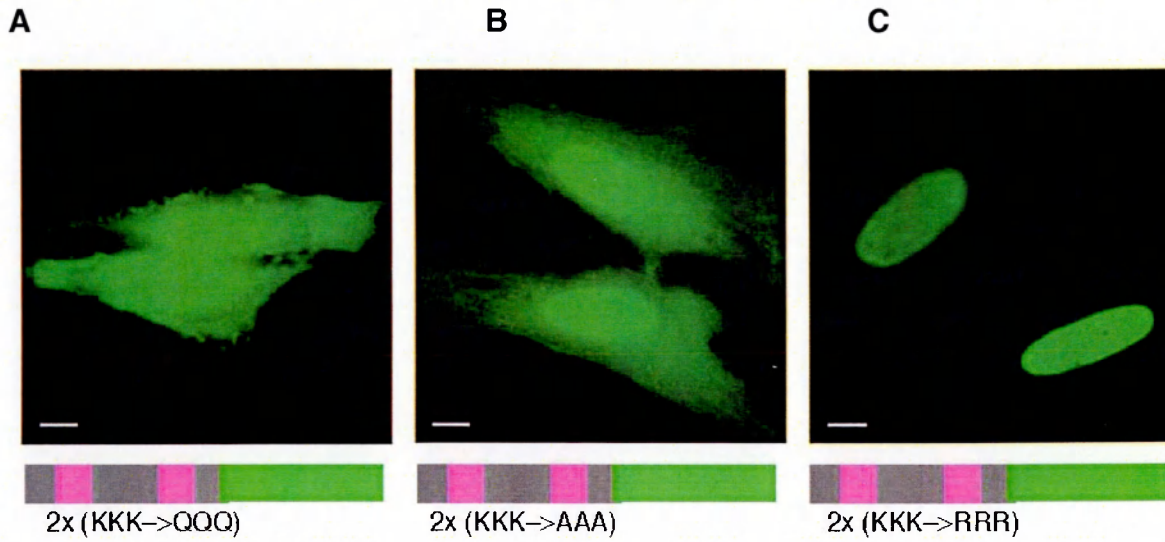


Fig. 23 Inactivation of both NLSs redistributes HMGB1–GFP to the cytoplasm
In HeLa cells, mutations of the 6 lysines in the 2 NLSs into glutamines (as a mimic of acetyl-lysine, **A**) or alanines (**B**) produce a clear cytoplasmic fluorescence of comparable intensity. As negative control, a mutant in which the 6 lysines are mutated to arginines did not alter the nuclear localization of HMGB1–GFP (**C**). Bar represents 10 μm in all panels.

On the other side, the cytoplasmic location of double K->Q and K->A suggested to us that acetylation on the two NLSs modulates their function and therefore influence the nuclear accumulation of HMGB1.

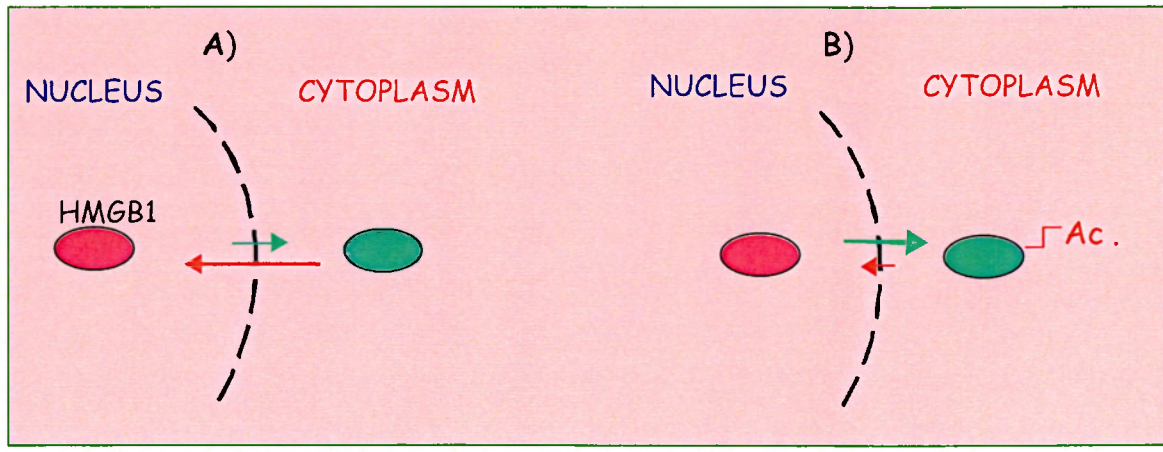
One important point is that the cytoplsmic accumulation of K->A and K-> Q mutants is still not complete and a fraction of the protein is still detectable in the nucleus. The size

of the protein does not exclude that some molecules manage to cross the nuclear pore by simple passive diffusion.

Then, the NLSs are not the sole activities that may direct HMGB1 to the nucleus; presumably, the interaction of HMGB1 with its partner proteins and with DNA tilts somewhat its diffusion equilibrium towards the nucleus.

As a third possibility, HMGB1 may have other NLSs: at least other two regions rich in Lys and Arg could fulfil the consensus requirements of a monopartite NLS. Anyway, we did not investigate further this aspect that remains to be clarified.

From a biochemical point of view, acetylation neutralises the positive charges of Lysines, which are the "label" of all known NLSs. Since NLSs are the target of acetylation, acetylation may prevent the nuclear accumulation of HMGB1. This assumes that HMGB1 also migrates from nucleus to cytoplasm.



As represented in the scheme above, under basal conditions the steady state of HMGB1 shuttling in and out of the nucleus in unbalanced towards import and the protein accumulates into the nucleus (**A**). When HMGB1 gets hyperacetylated in its NLSs, the import is strongly reduced; this results in accumulation of HMGB1 in the cytoplasmic compartment (**B**).

In this case acetylation regulates a protein function by altering its natural distribution in cellular compartments. There are very few cases known where a post-translational modification exerts a regulatory role via such a mechanism: so far, a similar regulation has been reported for HNF4 and CTIIA (Soutoglou et al., 2000); (Spilianakis et al., 2000) but with the opposite effect (acetylation promotes nuclear accumulation). Very

recently, acetylation was shown to restrict E1A to the cytoplasm (Madison et al., 2002). We will discuss this point further in the next section.

3.2.11 HMGB1 has non-classical Nuclear Export Signals

Our model that acetylation reduces the efficiency of HMGB1 nuclear import causing its accumulation in the cytoplasm is based the hypothesis that HMGB1 recycles between nucleus and cytoplasm and that nuclear import is somehow balanced by a corresponding export. The investigation was carried on in collaboration with Paola Scaffidi in our group, who provided preliminary *in vivo* experiments described in this paragraph; I completed her work with the *in vitro* interaction assays.

To test whether HMGB1 (mw 25 kDa) can pass to and from the nucleus by simple diffusion across nuclear pores, Paola incubated HeLa cells at 4°C for 4 hours, a condition that impedes active, GTP-driven nuclear import/export. After cold incubation, a part of HMGB1 diffused back to the cytoplasm; the same was true for HMGB1-GFP (not shown).

However, many small proteins that are able to cross the nuclear membranes by passive diffusion are also endowed with a Nuclear Export Signal (NES) that promotes active nuclear extrusion of the protein. To test for active export, *Hmgb1-/-* mouse embryonic fibroblasts (Falciola et al., 1997) were fused with HMGB1-positive human HeLa cells. Human cytoplasm was identified by staining with anti-human cytokeratin antibodies (red, **Fig. 24**), mouse nuclei by their bright DAPI-positive heterochromatic whitish spots over a blue background. Cells with human cytokeratin and a mouse nucleus were heterokaryons. HMGB1 is 99.5% identical in mouse and human, and its nuclear presence was scored with green fluorescent antibodies. Shortly after fusion, mouse nuclei in heterokaryons had very little HMGB1 (not shown), but 4 hours at 37°C (in the presence of cycloheximide to inhibit protein synthesis) were generally sufficient to equilibrate evenly HMGB1 between the human and the mouse nucleus (**Fig. 24 A and B**).

By itself, this result just indicates that HMGB1 can traverse the nuclear membranes

from the nuclear side to the cytoplasmic side, and does not show that the export is active. However, active export that involves the CRM1 exportin can be inhibited by leptomycin B. We then treated heterokaryons with leptomycin, and noticed that re-equilibration of HMGB1 between human and mouse nuclei was strongly reduced.

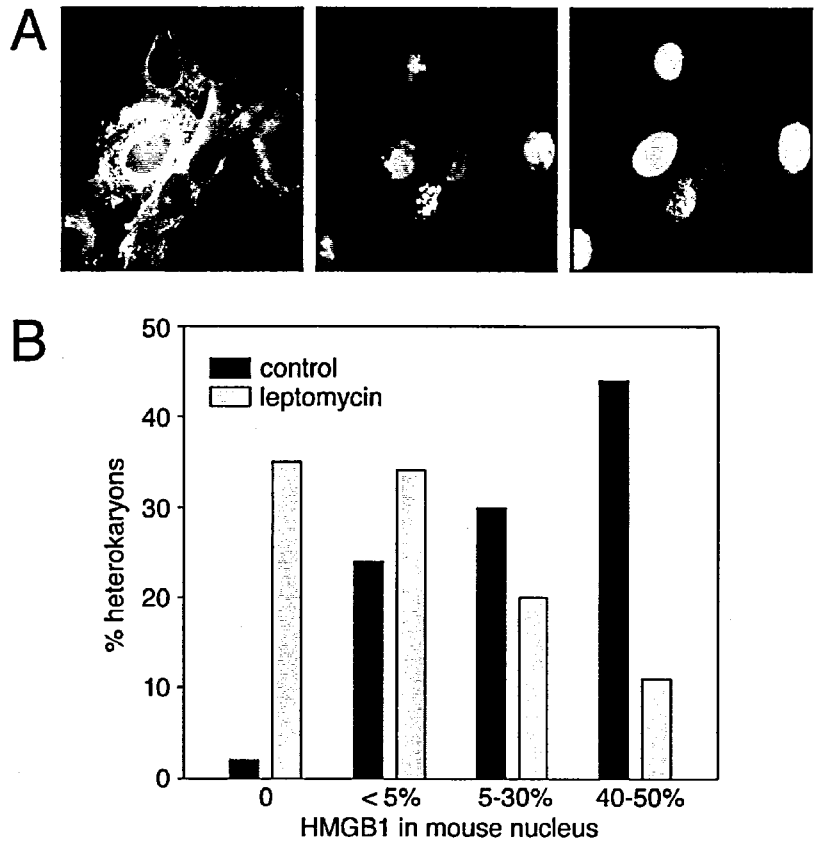


Fig. 24 HMGB1 migrates from nucleus to cytoplasm by both passive and active transport (courtesy of P. Scaffidi)

(A) Heterokaryons were formed by fusing HMGB1-expressing HeLa cells (human) and *Hmgb1-/-* mouse embryonic fibroblasts. Human cytokeratin and HMGB1 were stained red and green, respectively, with specific antibodies; nuclei were stained blue with DAPI. Cells with human cytokeratin staining and two nuclei, one of which with bright DAPI-positive heterochromatic spots characteristic of mouse cells, were considered heterokaryons. After incubation at 37°C for 4 hours, HMGB1 re-equilibrated from human to mouse nuclei, indicating that it could pass from the nuclear to the cytoplasmic side of the nuclear membranes, and be taken up by the other nucleus.

(B) Treatment of (number) heterokaryons (produced and scored as in panel A) at 37°C for 4 hours with leptomycin B substantially reduces, but does not abolish, HMGB1 transfer between human and mouse nuclei. The 50% level of HMGB1 in recipient mouse nuclei is equivalent to complete equilibration.

By visual inspection, an approximative quantitation of the intensity of green fluorescence suggested that in the vast majority of leptomycin-treated heterokaryons approximatively less than 5% of HMGB1 had migrated to the mouse nucleus after 4 hours (in the controls, almost 50% of nuclei had equilibrated).

These results indicate that HMGB1 has a NES that interacts with the CRM1 exportin (the target of leptomycin). I then synthesized labeled CRM1 in a reticulocyte extract, and passed it over columns that contained GST-NS2 (NS2 is strong nuclear export signal used as positive control), BSA (a negative control), HMGB1ΔC (BoxA+B), BoxA or BoxB. CRM1 bound to both HMG boxes, indicating that each contains an NES. When 400 nM leptomycin was added to the reticulocyte extract containing CRM1, the labeled protein did not bind to the either NS2 or HMGB1ΔC, that contains both NESs (**Fig. 25**).

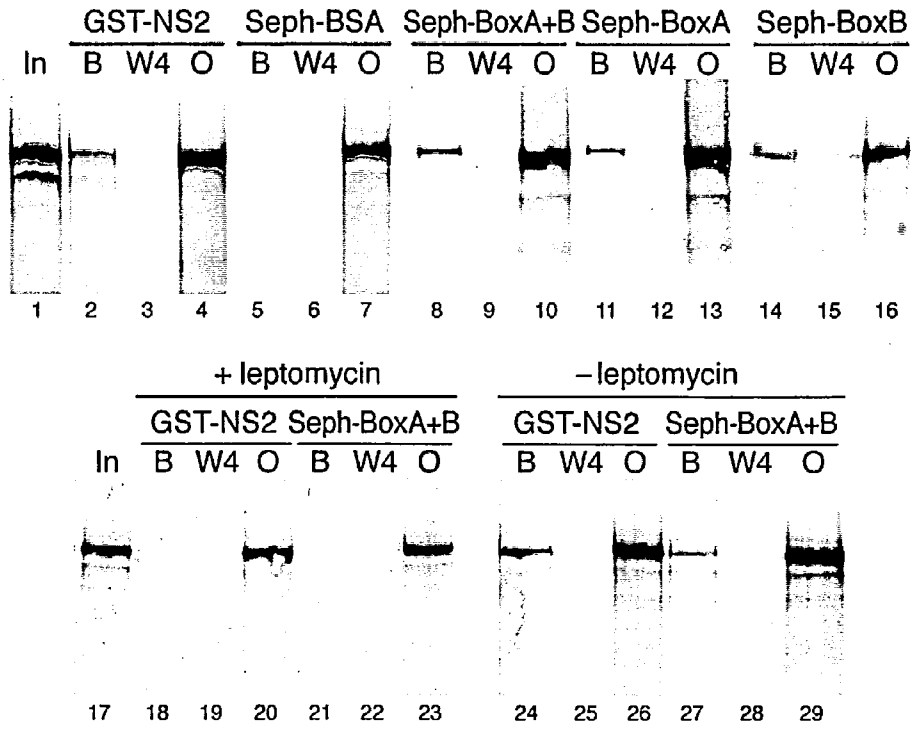


Fig. 25 Pull down assays to test the interaction of HMGB1 and derivatives with CRM1

In vitro translated CRM1, labelled with [^{35}S]-Methionine, was incubated with Sepharose beads conjugated with recombinant HMGB1 (boxA+boxB), the single boxes (boxA and boxB) and BSA and GST-NS2, respectively. After incubation slurries (**B**) were washed 5 times (**W4**= fourth wash) and then loaded on a Laemmli Gel, together with an aliquot of the input (**In**) and output (**O**). The gels were then dried and exposed to X-ray film to detect the presence of labelled CRM1 in different fractions.

3.2.12 HMGB1 secreted by LPS-activated macrophages is hyper-acetylated

Despite its usual nuclear localisation, several cell types under specific circumstances also secrete HMGB1. The HMGB1 secretion pathway is obviously interesting, and is likely to involve the import-export signals that we had identified.

We focused on the secretion of HMGB1 in monocytic cells described recently by Gardella et al. (Gardella et al., 2002). Resting monocyes from human peripheral blood contain nuclear HMGB1; upon challenge with LPS, monocytes accumulate a major portion of HMGB1 into cytoplasmic vesicles. Translocation takes about 16 hours and is not inhibited by cycloheximide. Secretion of HMGB1 does not follow the classical pathway via ER and Golgi apparatus since the protein does not possess the leader sequence driving it to the ER. Like other leaderless proteins, HMGB1 follows an alternative pathway, still not thoroughly characterised. Secretion of HMG1, involves a specific type of vesicles, called secretory lysosomes (Gardella et al., 2002).

The coincidence between NLSs and acetylation clusters in the HMGB1 purified from calf thymus suggested that vescicular accumulation might follow from cytoplasmic localisation and would depend on the acetylation of HMGB1.

To confirm our model, we estabilished a collaboration with the group in Genoa to study the biochemical properties of secreted HMGB1. Rubartelli's group has the expertise to prepare cultures of monocytes from Buffy Coats, to activate them via pro-inflammatory molecules, and to also to purify the endolysosomal vesicles from a total cell extract by ultracentrifugation.

We treated with LPS monocytes purified from Buffy Coats and checked HMGB1 accumulation in cytoplasmic vesicles by imaging (**Fig. 26**).

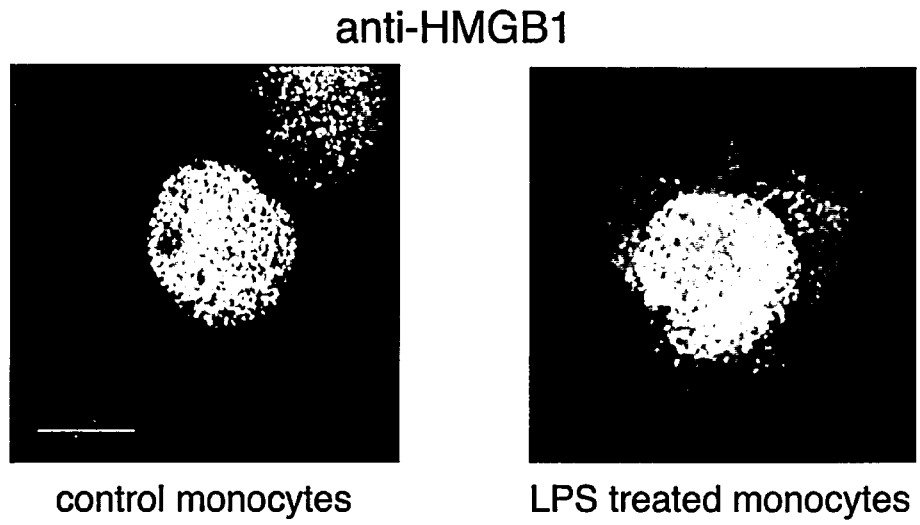


Fig. 26 LPS-activated human monocytes accumulate HMGB1 in cytoplasmic vesicles

Primary monocytes purified from peripheral blood cells were cultured overnight, in the presence or absence of 100 ng/ml LPS. Aliquots of activated and control monocytes were then fixed and immunostained with anti-HMGB1 rabbit antibody (red). Note the exclusively nuclear localization of HMGB1 in unstimulated monocytes, as opposed to nuclear plus vesicular HMGB1 localization in LPS-activated monocytes.

We generated total extracts from resting and activated monocytes and analysed them by 2D electrophoresis and western blotting.

Remarkably, while resting monocytes show only two isoforms of HMGB1, likewise all the other cell lines tested so far; in LPS –activated monocytes the redistribution of HMGB1 in to cytoplsmic vesicles parallels the appearance of a major additional spot (**Fig. 27**).

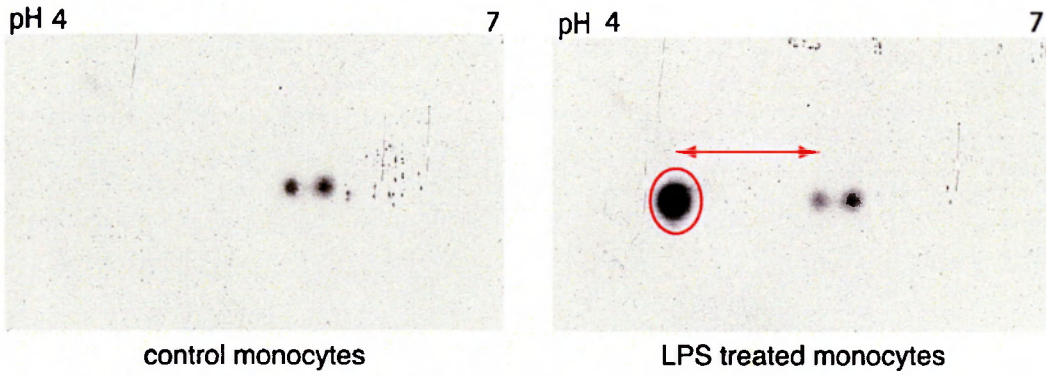


Fig. 27 2D electrophoresis of control and activated monocytes reveals HMGB1 hyperacetylation
Aliquots of untreated and LPS-activated monocytes were lysed by freeze-thawing, and about 400 μg of total protein extract were loaded on 2D gels, blotted onto nitrocellulose filters, and immunodetected with anti-HMGB1. Note the major additional HMGB1 spot in activated monocytes (right).

The spot is approximatively at the same molecular weight of the two "baseline" ones, but at a lower pI. The software ImageMaster 2D, specifically designed to calculate the pI of a spot form its position in a gel and to estimate the number of specific modifications corresponding to a defined ΔpI, predicts that the novel spot corresponds to HMGB1 acetylated 4 or 5 times more than the rightmost baseline spot. The absence of intermediate spots between "activated" and baseline spots suggests that in monocytes the acetylation of HMGB1 is not gradual but highly concomitant.

All this evidence establishes for the first time a correlation between HMGB1 distribution in the two major compartments of the cell and its level of acetylation: macrophages, and in a similar way others cells, may make use of acetylation to exclude HMGB1 from the nucleus and accumulate it in the cytoplasm, to subsequently direct it to the secretory pathway.

3.2.13 Acetylation of HMGB1 determines its relocation to the cytoplasm in fibroblast and to vesicles in moncytic cells

The results showed above provided a first correlation between acetylation of HMGB1 and its sub-cellular localization in primary monocytes.
To further confirm the role of acetylation, we performed experiments with fibroblasts, that do not contain secretory lysosomes, and with U937 monocytic cells, that do contain them.

TSA treatment for 3 hours of 3134 mouse fibroblasts transiently expressing GFP-HMGB1 was sufficient to relocate a significant amount of HMGB1 to the cytoplasm (**Fig. 28**), suggesting that hyperacetylation can force HMGB1 to relocate to the cytoplasm in most cells.

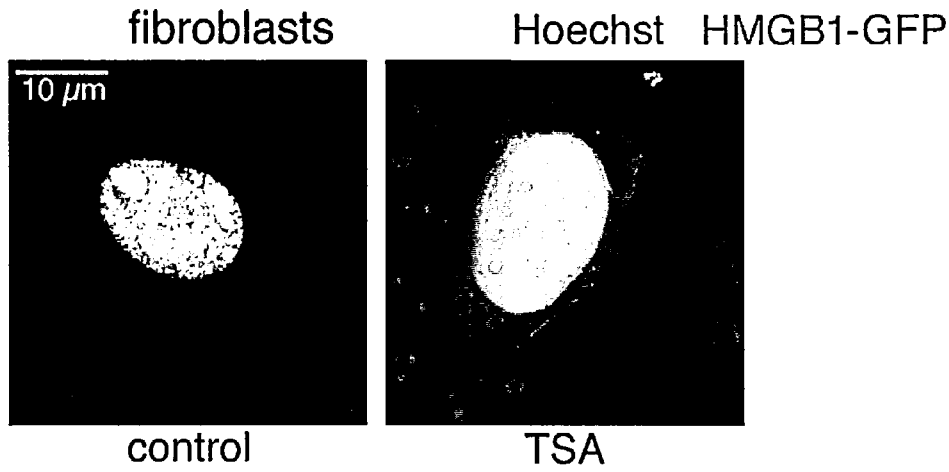


Fig. 28 GFP-HMGB1 relocation upon TSA treatment in 3134 mouse fibroblasts
3134 fibroblasts were left untreated (left) or exposed to 10 ng/ml TSA for 3 hours (right); cells then were fixed with PFA and directly imaged by fluorescence microscopy (green): a significant relocation of HMGB1-GFP to the cytoplasm could be detected; no vesicles are recognizable, due either to lack of these structures in fibroblast cells or to inability of the GFP fusion to be loaded inside vescicles.

The same experiment was then performed with U937 cells; we chose U937 as they are a promyelocytic cell line that can be activated to a monocytic-like stage upon treatment with differentiating factors. Therefore, they provide a good model to study response of monocytes to stimuli, with the advantage that, being a cell line, they have a more consistent and more homogenious behaviour than primary monocytes, whose high

variability in different donors had complicated our work.

Different subclones of U937 cells perform differently in cytokine secretion; for this reason, we chose a defined subclone, 12[-], with a well-established profile of plasma membrane molecular markers and functional responses (Biswas et al., 2001); (Bovolenta et al., 1999).

U937.12 cells paralleled closely the behavior of primary monocytes: resting cells contained HMGB1 in the nucleus, whereas LPS-activated ones redistributed a significant fraction of the protein into cytoplasmic vesicles (**Fig. 29**, lower panels). However, the relocation was much faster than in monocytes, and was clearly visible starting 1 hour after stimulation.

To test whether HMGB1 localization was directly determined by its acetylation status, we treated U937.12 cells with 10 ng/ml TSA for 3 hours: a fraction of HMGB1 started to shift to cytoplasmic vesicles (**Fig. 29**, arrows). Remarkably, this occurred in the absence of the cytoplasmic expansion, nuclear reshaping (form circular to horseshoe) and substrate adherence that accompanies activation by LPS.

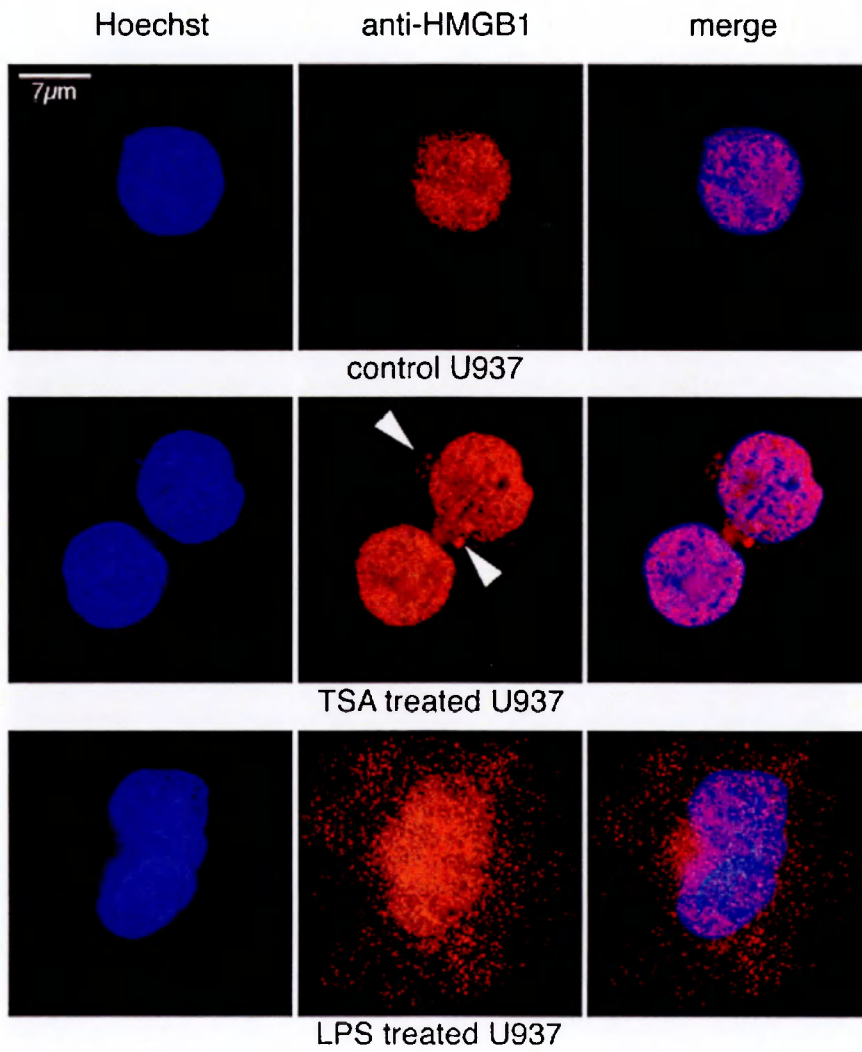


Fig. 29 HMGB1 relocalisation after LPS and TSA treatment in U937 promonocytic cells
U937.12 cells were cultured without stimulation or in the presence of 10 ng/ml TSA for 3 hours or 100 ng/ml LPS for 20 hours. Stimulated and control cells were fixed and immunostained with anti-HMGB1 antibody (red). Note the nuclear localisation of HMGB1 in resting monocytes, and the nuclear plus vesicular localisation of HMGB1 in LPS-activated monocytes, that display a much larger cytoplasm and horseshoe-shaped nuclei. TSA treated monocytes do not show cytoplasmic expansion or nuclear reshaping, but a fraction of HMGB1 is relocalized to cytoplasmic vesicles.

In parallel, A. Agresti, in our group, also tested whether hypoacetylated HMGB1 could be taken up by secretory lysosomes: she incubated U937.12 cells for several hours at 4°C, causing the passive diffusion of a significant fraction of HMGB1 to the cytoplasm, and then raised back the temperature to 37°C. Within 5 minutes, HMGB1 was accumulated in secretory lysosomes. Likewise, HMGB1 liberated into the cytoplasm by

the breakdown of the nuclear membrane during mitosis (Falciola et al., 1997) was also accumulated into secretory lysosomes (not shown).

The results of these two experiments provided a further confirmation of what we had already derived from the experiments performed by P.Scaffidi: nuclear import is an active process that requires energy and therefore the right temperature to take place, while nuclear export consists of two processes, one active and the other passive.

The cytoplasmic accumulation of HMGB1 in vesicles provides an important piece of information: acetylation is not absolutely required for a specific translocation of HMGB1 into the endolysosomes, contrary to our expectations.

The model emerging would rather suggest that acetylation only plays a role in switching HMGB1 from nucleus to cytoplasm; once HMGB1 is accumulated into the cytoplasm, by default - or at least without any other known signals- it is loaded into the vesicles. So, the two events are temporarily connected, but only the former process so far seems to be regulatable.

This aspect and its implications will be debated in the discussion (3.3)

We also tried to express – either transiently or stably – the construct HMGB1-GFP, and the NLSs mutants in the U937.12 clone.

GFP-HMGB1 expressed in U937.12 was shuttled from nucleus to cytoplasm in response to LPS and TSA, but was unable to proceed further into secretory lysosomes, and much less to be secreted (not shown). Also the constructs coding for the double NLS1/NLS2 mutants (double K->A and double K->Q) fused with GFP were both transiently and stably expressed in U937: their localisation is mainly cytoplasmic in resting U937 cells, confirming their phenotype in fibroblast cells (not shown). The double substitution from lysines to arginines was lethal to U938.12 cells, and could not be tested.

We did not go further in this analysis: the inability of the GFP-fusion to be translocated into secretory lysosomes could be due to the size of the protein or to a specific problem of folding. We judged that it did not provide a good model to study vesicular accumulation.

3.2.14 Acetylation of HMGB1 is controlled via the ERK MAP Kinase pathway (in collaboration with A. Agresti)

The experiments reported above indicate that U937.12 cells acetylate and accumulate HMGB1 in vesicles in response to activation. Therefore, acetylation or deacetylation activities (at least on HMGB1) must be controlled by signal transduction pathways that start with the engagement of receptors for TNF-α, IL-1β, LPS, HMGB1 itself and/or other pro-inflammatory ligands. The secretion of TNF-α, IL-1, IL-8, and PGE_2 by LPS-stimulated monocytes can be blocked by inhibiting ERK phosphorylation (Scherle et al., 1998). A. Agresti therefore tested whether the same was true for HMGB1 acetylation and vesicular accumulation. U937.12 cells were exposed to 200 ng/ml LPS in the presence of 10 μM U0126 (a specific inhibitor of ERK phosphorylation) or 1 μM SB203580 (a specific inhibitor of the p38 kinases). Control cultures were not exposed to LPS, or exposed to LPS but no inhibitors. HMGB1 translocation was substantial in cells exposed to LPS alone, or LPS plus SB203580, but was almost completely blocked in cells exposed to LPS plus U0126 (**Fig. 30**; more than 95% of the cells had the morphology shown). Both kinase inhibitors had no effect on U937.12 cell viability, nor on cytoplasm expansion and nuclear reshaping that follow activation.

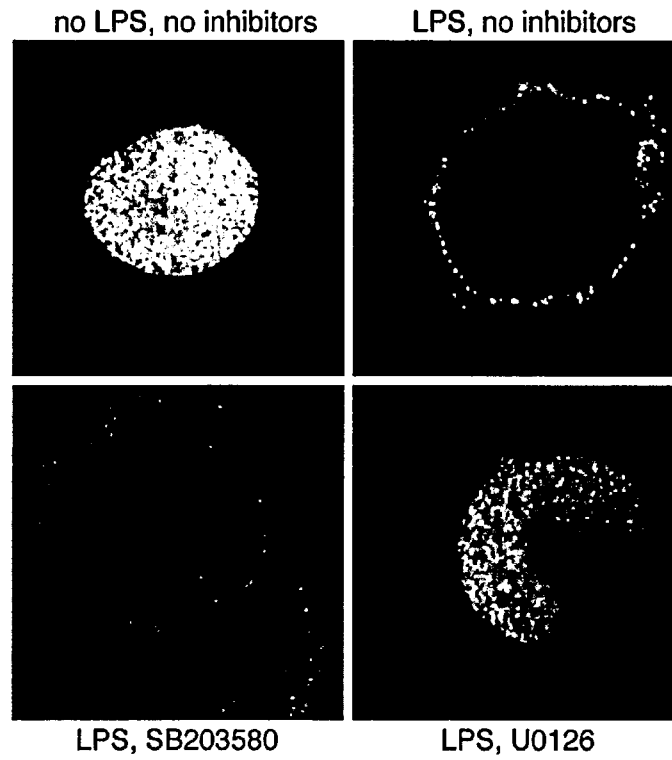


Fig. 30 LPS-induced relocalisation of HMGB1 is regulated through the MAPkinase pathway (courtesy of A. Agresti)

U937.12 clone cells were stimulated with 200 ng/ml LPS in the presence of 1 μM SB203580 (lower left panel) or 10 μM U0126 (lower right panel). Control cultures were not exposed to LPS, or exposed to LPS but no inhibitors (upper left and right, respectively). HMGB1 translocation was substantial in cells exposed to LPS alone, or LPS plus SB203580, but was almost completely blocked in cells exposed to LPS plus U0126 (1cm = 8 μm). Blue= DAPI, red= α-HMGB1.

These results indicate that ERK kinases (but not p38 kinases) are involved in the control of HMGB1 acetylation and translocation. ERK1 and 2 migrate to the nucleus when phosphorylated by the kinase MEK1 (the step that is inihibited by U0126), and, in turn, phosphorylate several transcription factors that promote the expression of a number of genes. For example, secretion of TNF-α involves transcriptional upregulation of the *TNF* gene, which depends on the expression of the EGR1 transcription factor, which in turn depends on the ERK-mediated phosphorylation of the transcription factor ELK1 (Guha and Mackman, 2002).

In contrast, HMGB1 relocation does not depend on LPS-activated expression of specific genes: U937.12 cells stimulated with 200 ng/ml LPS for 3 hours in the presence

of 10 μg/ml cycloheximide still translocate HMGB1 to cytoplasmic vesicles (**Fig. 31, left**). The same was true for human monocytes (although on a time scale of 16-20 hours).

These results indicate that ERK kinases have a direct control over HMGB1 acetylases or deacetylases.

Preliminary results indicate that LPS treatment does not affect the amounts of HATS, either pCAF or CBP: we analysed by western Blot the amounts of the two proteins in total extracts taken from resting and activated U937: no difference was detected in the quantity prior to and after treatment, suggesting that the kinase affect the activities of these enzymes rather then their quantity (not shown).

HMGB1 destiny was also followed when U937.12 cells were LPS-activated in presence of Leptomicin B, that blocks HMGB1 export form the nucleus, as previously shown by P. Scaffidi: no cytoplsmic vesiculation was detected (**Fig. 31, right**). This completes our investigation and strongly indicates that acetylation is necessary for HMGB1 to accumulate in the cytoplasm and then to be loaded into the vescicles.

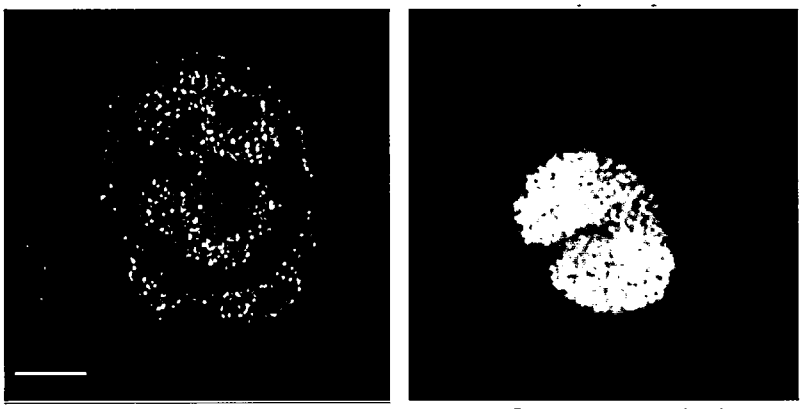


Fig. 31 Regulation of HMGB1 translocation by cyclohexymide and LMB (courtesy of A. Agresti)
U937.12 cells were incubated with 200 ng/ml LPS in presence of 10 μg/ml cycloheximide: the protein still translocates in the vescicles also when translation is blocked (left). LPS stimulation was also carried out in the presence of 150 nM leptomycin B that blocks active export (right): HMGB1 is not translocated into vesicles but remains into the nucleus. Treatments were carried on for 3 hours then cells were PFA-fixed and immunostained with rabbit anti-HMGB1 as primary antibody and Alexa Fluor 594 anti-rabbit as secondary antibody. Blue= DAPI, red= α-HMGB1.

Previous work indicated that CBP phosphorylated by ERK kinase increases its HAT activity on histones (Ait-Si-Ali et al., 1999): it is very likely that this activity can be extended also to other protein substrates like HMGB1 even if we are still unable to verify this hypothesis.

The general model derived form this work will be presented and thoroughly described in the discussion.

3.3 DISCUSSION

HMGB1 is unique among nuclear proteins in that it can be secreted, both by cells of myeloid origin (which use it as a proinflammatory cytokine) and by developing neural cells (where its role is much less understood) [reviewed by (Muller et al., 2001)].
During my PhD, most of my time was dedicated to investigate how HMGB1 is distributed in alternative subcellular locations: in the nucleus, where it serves its primary function as an architectural factor, and in the cytoplasm or in cytoplasmic vesicles, which serve as intermediate stations towards its eventual secretion.
I collected evidence that the final location of HMGB1 depends from the acetylation status of its nuclear localization sequences (NLSs). Details about this model will be widely discussed in the next paragraphs.

3.3.1 HMGB1 can be acetylated at multiple sites

We first established that HMGB1 is multiply acetylated. In fact, the pioneering work of Allfrey and coworkers indicated that lysines 2 and 11 of HMGB1 are subject to acetylation (Sterner et al., 1979). This certainly holds true for most tissues and cell lines, but in thymus, in monocytes and probably in all cells of myeloid origin at least 17 different lysines within HMGB1 can be acetylated, and a single HMGB1 molecule can be acetylated up to 10 times. Although acetylated species are the vast majority in HMGB1 from thymus, and a significant fraction in HMGB1 from cultured cells, no single lysine appears to be acetylated in all HMGB1 molecules We did not detect any other post-translational modification (other than poly-ADP-ribosylation), but in principle this level of acetylation allows for more than 100 000 different HMGB1 molecular species if all lysines were acetylated independently. In practice, several lysines appear to be acetylated concomitantly and, remarkably, the two areas of concordant lysine acetylation correspond to sequences with NLS function.
The fact that acetylation is not spread all along HMGB1's sequence but, on the contrary, shows some clustering is suggestive that the actetyltransferases involved are processive once they have recognised HMGB1 as a substrate.
An attempt to characterise presumptive consensus sequences for acetylation has been

tried, like for tyrosine-phosphorylation, but with little success; the more the number of acetylated proteins increases, the clearer it is that a strong consensus for acetyl transferases does really not exist. And, in the case of HMGB1, we did not find any match with presumptive consensus sequences.

3.3.2 Acetyltransferase and Deacetylase activities in the cells

The discovery of a high extent of acetylation in HMGB1purifed from an organ (**3.2.1-3.2.3**) and the presence of up to eleven different isoforms of the protein in a 2D gel suggests that there is a wide spectrum of of acetylation levels for HMGB1 that probably can be correlated to different functions in the cell; so far, we managed to understand the role of one pattern of acetylation in a restricted cell lineage.

In contrast to the variability detected in organs and tisyuuu32ewwwwwwwsues, anyway, all the cell types analysed by 2D electrophoresis (Hela, 3T3, Phoenix, 3134, U937) had a basal level of acetylation represented always by two spots. This suggests that hyperacetylation of HMGB1 as response to external stimuli, can be only partially reproduced in a cell colture.

Using both HDAC inhibitors and LPS (in case of U937 and monocytes) we perturbed the basal state of the cells. Acetylation of HMGB1, like any other protein, is the result of the balanced activity of HATs and HDACs, which are regulated in the cells in order to respond to the stimuli from the environment.

In U937 cells we saw that this response is quick (one hour is enough to trigger cytoplasmic accumulation of HMGB1), suggesting that the acetylation-deacetylation rates are quite high. This was confirmed when we treated the cells with HDAC-inhibitors (paragraph 3.2.6, Fig. 20 and 21): 30 minutes are enough to raise significantly the acetylation levels of HMGB1 and after 60 minutes this level is already saturated.

In light of this, acetylation appears to correlate to rapid responses of the cells to sudden variations or stimuli, in a very similar way to how phosphorylation works.

The issue of the identity of the HATs responsible for HMGB1 acetylation is still debated: the *in vitro* experiments showed that the most common nuclear HATs – CBP, p300 and pCAF – can all recognise HMGB1 as substrate and acetylate it with a good efficiency. However, these experiments may lack specificity and provide no hints about

the preferential enzyme acting on HMGB1 *in vivo*.

The experiments performed inhibiting LPS-mediated relocalisation of HMGB1 with different drugs suggest the involvement of the MAP-kinase pathway for the activation of the HATs that modify HMGB1. Moreover, works are published that correlate LPS – mediated activation of the same cells with an increased activity of CBP (Ait-Si-Ali et al., 1999).

This strongly supports the involvement of at least this enzyme in HMGB1 acetylation *in vivo*, but a definitive confirmation is still missing.

However, taking into account the lack of any real consensus site for acetylation and the high number of acetylation sites in our protein, it is very likely that more the one acetyl-transferase activity may be responsible of HMGB1 acetylation, similarly to what happens for histones and other nuclear substrates.

In this perspective, the heterogeneus panel of acetylation sites detected by MassSpec might be the result of the activity of different enzymes, with different efficiency and site-preference.

According with the clustering of acetylation sites, we imagined that whenever CBP or pCAF or p300 or any other still uncharacterized HAT have estabilished a contact with HMGB1, they would transfer acetyl- groups to more then one Lysine in more then one cycle of acetylation, working in a processive way.

The processivity extensively modifies HMGB1 and therefore alters significantly its total charge, as we detected by 2D electrophoresis of untreated and LPS-activated monocytes. This in the end causes a quick but massive change in the properties (here the localization) of the protein.

3.3.3 Functional effect of acetylation of HMGB1 and future fields of investigation

Most frequently, acetylation regulates the function of proteins likewise all the other PT-modifications: by affecting their affinity for the DNA-target or for other protein partners. This kind of regulation has been proposed also for HMGB1: Pashev and collaborators have recently shown that acetylation at Lys 2 and Lys11 enhances HMGB1 binding activity for distorted DNA (Ugrinova et al., 2001).

In our work we did not investigate this aspect; however, given the high number of

acetylation sites found in HMGB1 sequence and considering that some of them are outside the two NLSs, it is very likely that acetylation can influences the interaction of HMGB1 with several of its natural partners in the nucleus, with profound impact on several processes, like transcription, V(D)J recombination, etc. Moreover, the availability of several functional *in vitro* assays together with the production of recombinant mutants in the acetylated residues, open the possibility to investigate this issue quite easily.

For example, acetylated HMGB1 might show a different affinity for the nuclesome particle, impacting the efficiency of nuclesome sliding itself (see above).

In this case acetylation of HMGB1, together with the modification of the histone tails, would provide a supplementary epigenetic mechanism of regulation of gene expression.

Another possibility is that acetylation of NLS2 could modulate the documented interaction between the C-terminal tail of HMGB1 with the two basic boxes.

Several investigators observed that the C-terminal acidic tail of HMGB1 controls the DNA binding properties of the HMG boxes (Lee and Thomas, 2000; Muller et al., 2001; Stros et al., 1994). In past years I also collected several pieces of unpublished data suggesting that deletion of the C-terminal domain increases the activity of the HMG boxes in protein-protein interaction and transactivation assays.

The peculiar position of the second hyperacetylated cluster of lysines, adjacent to the tail, suggests that acetylation in this region might affect the interaction of the tail with the boxes; in such a case acetylation would provide a supplementary way to regulate HMGB1 activity.

With the present work we propose a higher level of regulation accomplished by acetylation on the subcellular distribution of a protein.

This type of effect is less commonly documented but not completely novel since there are other examples in literature of a similar effect of acetylation of nuclear factors as HNF4, CTIIA and E1A (Soutoglou et al., 2000); (Spilianakis et al., 2000); (Madison et al., 2002).

The peculiarity of HMGB1 acetylation resides in the high extent to which the modification is carried, and the effectivness of the events thus triggered. The mechanism described is most interesting because it provides an explanation for the

question about the identity of a molecular switch that redirects a nuclear protein from one subcellular location to another.

3.3.4 HMGB1 Shuttles Continually between Nucleus and Cytoplasm

HMGB1 (mw 25 000) is small enough to diffuse passively through nuclear pores, and in fact a significant portion of the protein diffuses to the cytoplasm if cells are incubated for a few hours at 4°C, a condition that blocks energy-driven transports. However, HMGB1 also contains two independent NLSs and two NESs, defined as CRM1-interacting surfaces in the protein. One NLS matches perfectly to classical bipartite NLSs, the other one is rather loosely related to a monopartite NLS. The 2 NESs may be related to each other as they occur in the two HMG boxes, but have no sequence similarity to other ones known to date, so it was not possible to identify them using bioinformatics tools. The experimental characterisation of these regions should require a deletion approach and it is in future plans.
Two NLSs and (at least) one NES are also present in group E Sox proteins (Sox8, 9 and 10), and are essential for their transcriptional activity (Sudbeck and Scherer, 1997); (Rehberg et al., 2002).

From an evolutionary point of view the presence of two NLSs in a protein can be considered as a way to ensure the proper location of a nuclear protein also in case of random mutations inactivating one of them. The evidence from the double mutants experiments that, even with the inactivation of both NLSs, the presence of HMGB1 into the nucleus is not completely abolished, raises the possibility of other regions accomplishing this functions: the CUBIC database suggested at least other two sequences with presumptive NLS-function, and we already started to generate the GFP-fusions constructs to esperimentally test their function. It is also possible that a fraction of HMGB1 lacking its own NLS function may be transported into the nuclear compartment by estabilishing contacts in the cytoplasm with other nuclear partners.

Functionally, the presence of both import and export signals ensures that HMGB1 shuttles actively between the nuclear and cytoplasmic compartments in all cell

types, at the basal level the nuclear import exceeds the export, defining a nuclear accumulation of the protein.

General deacetylase inhibitors cause the hyperacetylation of HMGB1 and the relocation of part of the protein to the cytoplasm. This presumably happens in all cell types, and is due to the acetylation of lysines within both NLSs. The mutation of the lysine clusters within the NLSs to glutamines, which most resemble acetylated lysines, is sufficient to abrogate NLS function.

We managed to uncover one of the signals that lead to the perturbation of the import/export equilibrium in U937 and monocytes; in other cell types not responsive to LPS, acetylation of HMGB1 is probably modulated in response to other starting stimuli, still to identify.

We propose a scenario (**Fig. 32**) where HATs and HDACs that are common and conserved in mammals have been recruited to control the flow of HMGB1 across the nuclear envelope: deacetylation or low-level acetylation allows for the default nuclear accumulation of HMGB1, whereas hyperacetylation causes the accumulation of HMGB1 in the cytoplasmic compartment.

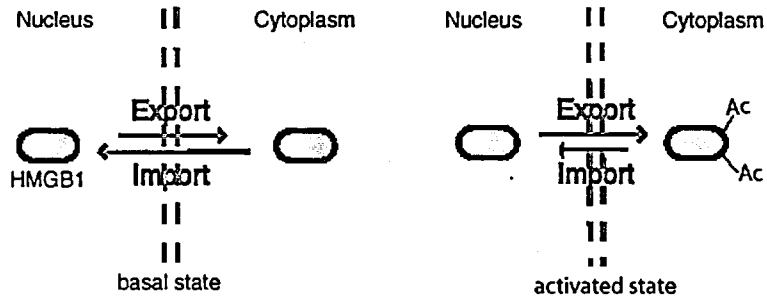


Fig. 32 Model I: acetylation regulates HMGB1 distribution between nucleus and cytoplasm
At basal state HMGB1 is hypoacetylated and nuclear import exceeds export: HMGB1 accumulates in the nucleus; upon stimulation, HMGB1 is hyperacetylated by HATs in the NLSs and import is inhibited: export exceeds import and HMGB1 accumulates in the cytoplasm, that serves as temporary location for subsequent events (see model in **Fig. 33**). This model can be applied to all the cell types possessing HATs and HDACs that act on HMGB1.

The regulation of nuclear vs cytoplasmic localization by acetylation has been described before for transcription factors HNF4 and CTIIA; however, in these cases lysine acetylation promotes nuclear accumulation, in a process that appears to be related to

NES function and protein export mechanisms. Very recently, acetylation of the NLS of viral protein E1A has been shown to cause its cytoplasmic accumulation (Madison et al., 2002). The process we describe is similar, save for the scale: around a million HMGB1 molecules per cell must be acetylated in a limited time-span.

3.3.5 HMGB1 as a signal for tissue damage

We have recently shown that HMGB1 is a very mobile component of chromatin, both in interphase and mitotic chromosomes (Scaffidi et al., 2002). The protein constantly shuttles between nucleoplasm and chromatin, with a residence time of less than 2 seconds. This is consistent with the proposed role of HMGB1 as an architectural factor, or even a "chromatin chaperone" (Travers et al., 1994). As a consequence of its dynamic and reversible interaction with chromatin *in vivo*, HMGB1 is passively leaked out from cells (together with all other soluble proteins) when the integrity of membranes is lost, either because of the addition of detergents to the medium bathing the cells (Falciola et al., 1997), or because of cell necrosis (Scaffidi et al., 2002). The release of HMGB1 by necrotic cells differs from active secretion, as it is a totally passive process and a direct consequence of the transient nature of the association HMGB1 with chromatin: HMGB1 dilutes in the extracellular milieu following the concentration gradient. Thus, we expect that all forms of HMGB1, either acetylated or not, will be leaked out in a similar fashion. Remarkably, however, HMGB1 is irreversibly bound to chromatin in cells undergoing apoptosis, a condition that is concomitant with a generalized histone deacetylation (Scaffidi et al., 2002). HMGB1 remains chromatin-bound, and thus indiffusible, even when apoptotic cells lose the integrity of their membranes and leak out other soluble cellular components (secondary necrosis). This is not a common fate for chromatin proteins: most are either firmly bound to chromatin all of the time (like histones), or weakly bound to chromatin all of the time (like HMGN proteins or transcription factors) (Scaffidi et al., 2002). A "window of opportunity" therefore exists to use HMGB1 as a signal for necrosis, as opposed to apoptosis or secondary necrosis. The advantage for the organism to distinguish between primary and secondary necrosis is obvious: primary necrosis is caused by trauma, hypoxia or poisoning, and is associated with damage to the tissues

that need repair; apoptosis is programmed and is not associated with traumatic tissue damage. In this scenario, the evolution of receptors to detect extracellular HMGB1 endows cells that are not directly hurt by tissue damage with the ability to recognize, at a distance, that damage has occurred. Some cells may simply divide to replace dead cells, some will migrate to replace them, and some will amplify and relay the tissue damage signal(s) to distant districts in the body. At least one receptor for extracellular HMGB1 has been identified: RAGE (the receptor for advanced glycation endproducts) binds several ligands, including HMGB1, with nanomolar affinity (Hori et al., 1995). Upon RAGE engagement, smooth muscle cells can both migrate up the HMGB1 gradient (Degryse et al., 2001), and divide (R. Palumbo and M.E.B., unpublished). Neutrophils and other inflammatory cells are recruited to the site of necrosis (Degryse et al., 2001), and are activated to secrete TNF-α and other proinflammatory cytokines (Andersson et al., 2000).

3.3.6 Myeloid Cells Control HMGB1 Acetylation

Monocytes and promyelocytic cells must have developed a specific ability to acetylate massively a chromatin component in order to reroute it to secretion and use it as a cytokine. Nuclear export, vesicular accumulation and secretion are separate steps, whose occurrence depends on the completion of the previous step. Thus, it is only natural that HMGB1 accumulated in secretory lysosomes should be acetylated, as this is necessary for cytoplasmic relocation of the nuclear protein. However, HMGB1 acetylation is not necessary for vesicular accumulation: secretory lysosomes do capture some of the hypoacetylated HMGB1 protein that diffuses to the cytoplasm during incubation at 4°C of resting U937.12 cells. Most of all, secretory lysosomes also take up hypoacetylated HMGB1 released into the cytoplasm during mitosis. Thus, it appears that vesicular accumulation of cytoplasmic HMGB1 is a default process that simply requires the presence of secretory lysosomes. This further highlights the physiological importance of lysophosphatidylcholine (LPC) as a second signal for HMGB1 secretion (Gardella et al.,

2002); if a second signal were not required, some secretion of HMGB1 from newly divided cells would be unavoidable. A model for the regulation of HMGB1 secretion by monocytes is summarised in **Fig. 33**. This model concentrated on the specific cell type that we investigated and at the same time extends the one presented in Fig. 32: the scheme is based on the evidence collected in paragraphs 3.2.10 – 3.2.12

Monocytes and promyelocytic cells only reroute HMGB1 when activated. Activation is triggered by binding of inflammatory molecules (IL-1β, TNF-α, LPS, HMGB1 itself) to their own receptors, and is concomitant with cell differentiation. However, also non-activated U937.12 cells can transport HMGB1 to vesicles when TSA causes hyperacetylation. TSA could potentially also cause the same sort of differentiation that is triggered by inflammatory molecules, but this appears unlikely, as several morphological markers of activation are absent (cell and nucleus reshaping plus other evidence not shown).

Binding of proinflammatory-signals to surface receptors in monocytes activates a number of signaling pathways, including calcium signaling through calmodulin and NFAT/calcineurin, NF-κB and all MAP kinases (ERK, Jnk and p38 routes). We have shown that inhibition of ERK phosphorylation blocks the LPS-induced translocation of HMGB1 to secretory lysosomes. ERK kinases must control directly the enzymes responsible for HMGB1 acetylation, rather than indirectly through the phosphorylation of transcription factors that control the expression of specific genes, since cycloheximide treatment of either LPS-activated U937.12 cells or monocytes does not prevent HMGB1 relocation from the nucleus to cytoplasmic vesicles.

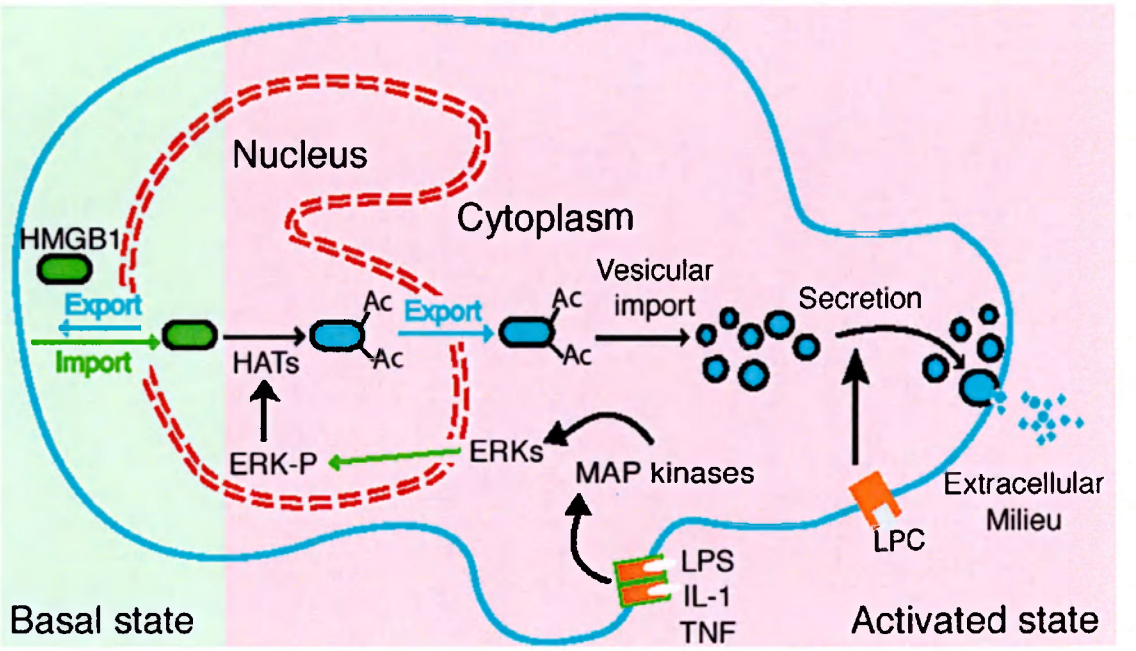


Fig. 33 Model II: the control of HMGB1 secretion in inflammatory cells

In all cells, including resting inflammatory cells (light blue panel), HMGB1 partitions between nucleus and cytoplasm: nuclear import is mediated by the NLSs, and the protein re-diffuses back to the cytoplasm through the nuclear pores via passive diffusion and CRM1-mediated active export. When the NLSs are not acetylated, the rate of nuclear import exceeds that of rediffusion or export, and the protein appears predominantly or solely nuclear. Upon stimulation of inflammatory cells (pink panel) through binding of IL-1β, TNF-α, LPS or HMGB1 itself to their own receptors, the NF-κB (not shown) and MAP kinase pathways are activated. Phosphorylated ERKs migrate to the nucleus, where directly or indirectly they activate histone acetylases, or inhibit histone deacetylases. This in turn promotes the acetylation of the 2 NLSs of HMGB1. Exported acetyl-HMGB1 cannot return to the nucleus. Myeloid cells are equipped with a special variety of lysosomes that can be secreted upon appropriate stimulation (Jaiswal et al., 2002), and that can accumulate IL-1β (not shown) or HMGB1, presumably through the action of specific transporters embedded in the lysosomal membrane (Andrei et al., 1999); (Gardella et al., 2002). Upon the engagement of LPC (lysophosphatidylcholine, an inflammatory lipid) to its own receptor, the secretory lysosomes carrying HMGB1 fuse with the plasma membrane and secrete their cargo.

3.3.7 Monocytes relay and amplify the tissue damage signal

Remarkably, starting about 16 hours after activation, monocytes and macrophages can secrete HMGB1 (Wang et al., 1999). This creates a closed feedback loop with inbuilt delay: as inflammatory cells secrete the same protein that can serve as a signal for tissue damage, the signal is sustained in time, and HMGB1 serves both as an early and a late inflammatory signal. This circuit is conceptually simple, economical and elegant, but can have complicated outputs in terms of intensity of the inflammatory signal. For example, if after 16 hours the sum of passively and actively released HMGB1 is larger than the amount of HMGB1 released as the initial trigger of inflammation, the inflammatory response can be self-amplifying and can lead to dramatic outcomes like toxic shock. Significantly, one might observe toxic shock-like symptoms even in the absence of LPS or bacterial pathogens, provided that the initial tissue damage is extensive enough.

In our interpretation, the ability of monocytes and macrophages to provide as output the same protein that initially served as input for inflammatory signaling is an example of molecular mimicry, and must have evolved after the evolution of HMGB1 as a tissue damage signal. A clear advantage for using the same protein as early and late inflammatory signal is that the organism can re-use the same components already available, both as ligands and receptors.

3.3.8 Myeloid cells use pre-existing components towards HMGB1 secretion

HMGB1 is a small protein that could pass easily through nuclear pores. In all cells, NLSs shift the equilibrium of HMGB1 distribution strongly towards a nuclear localization.

NLS acetylation might have evolved *ad hoc*, or simply be a by-product of bystander acetylation of a chromatin protein. Within the nucleus, HMGB1 interacts directly with nucleosomes ((Falciola et al., 1997); (Nightingale et al., 1996), and will come in close contact with the HAT and HDAC activities that reversibly (de)acetylate histone tails. The exact specificity of these activities towards their substrates is not known, but are likely to be somewhat relaxed, as the same enzymes can acetylate and

deacetylate transcription factors and coactivators in addition to histones (Kouzarides, 2000). In all likelihood, most HATs and HDACs can accept HMGB1 as a substrate, and align its acetylation status with that of the histones.

In any event, acetylation of both NLSs would free HMGB1 from the Ran-driven cycle of nuclear accumulation. This event must be rare spontaneously, as HMGB1 is tightly confined to the nucleus in cultured cells, even promyelocytic ones like U937. We also note that the irreversible deposition of HMGB1 onto chromatin during apoptosis is concomitant with histone deacetylation (Scaffidi et al., 2002) thus, HMGB1 deacetylation (whether controlled or bystander) would clear the cytoplasmic pool of HMGB1, and prevent leakage from that pool. HMGB1 deposition onto underacetylated chromatin and depletion of its cytoplasmic pool are thus aligned, whether by chance or design.

The existence of an HMGB1 acetylation cycle offers the "window of opportunity" to use such a cycle, and the cytoplasmic pool of HMGB1, for secretion. Most cells cannot secrete HMGB1, whereas cells of the myeloid lineage can. We postulate that the vesicles for HMGB1 accumulation must pre-exist the evolutionary invention of HMGB1 secretion. To some extent, this prediction is supported by the fact that myeloid cells appear to secrete IL-1β, another proinflammatory cytokine, precisely via the leaderless accumulation of IL-1β into vesicles, and their regulated exocytosis (Andrei et al., 1999). However, myeloid cells must have invented or deployed a mechanism to acetylate HMGB1 after receiving inflammatory signals. Thus, in myeloid cells acetylation or deacetylation activities (at least of HMGB1) must be controlled by signal transduction pathways that start with the engagement of receptors for TNF-α, IL-1β, LPS and/or other "innate immunity" ligands.

The molecular mimicry of HMGB1 passive release by activated monocytes/ macrophages is not perfect, however, since inflammatory cells secrete only appropriately hyperacetylated HMGB1, whereas necrotic cells would release all species of HMGB1, acetylated or otherwise. Appropriate antibodies towards the acetylated NLSs of HMGB1 might distinguish between passively and actively released HMGB1, and would potentially afford the ability to block the late HMGB1 response without affecting the early response. Just as possibly, the difference between passively and actively secreted HMGB1 could be perceived by alternative receptors in mammals.

In conclusion, we have shown that inflammatory cells can redeploy a nuclear

component to a cytokine function by controlling its movement from nucleus to cytoplasm. In turn, this depends from the acetylation of the 2 NLSs of HMGB1.

CHAPTER 4. Investigating HMGB1 activity within the nucleus: a chaperoning role in chromatin remodelling?

(In collaboration with P. Becker and G. Längst – Adolf Butenandt Institut, Munich)

4.1 INTRODUCTION

4.1.1 Mechanisms of chromatin remodelling

Chromatin is intrinsically involved in the regulation of all nuclear processes, most prominently transcription, replication, repair and recombination (de La Serna et al., 2000); (Flanagan and Peterson, 1999) (Fyodorov and Kadonaga, 2001) (Muchardt and Yaniv, 1999) (Kwon et al., 2000). DNA and histones within nucleosomes are held together by a large number of weak interactions, which confer a significant stability to the nucleoprotein structure, and movement of a nucleosome has to overcome a significant activation energy (Luger et al., 1997) (**Fig. 34**).

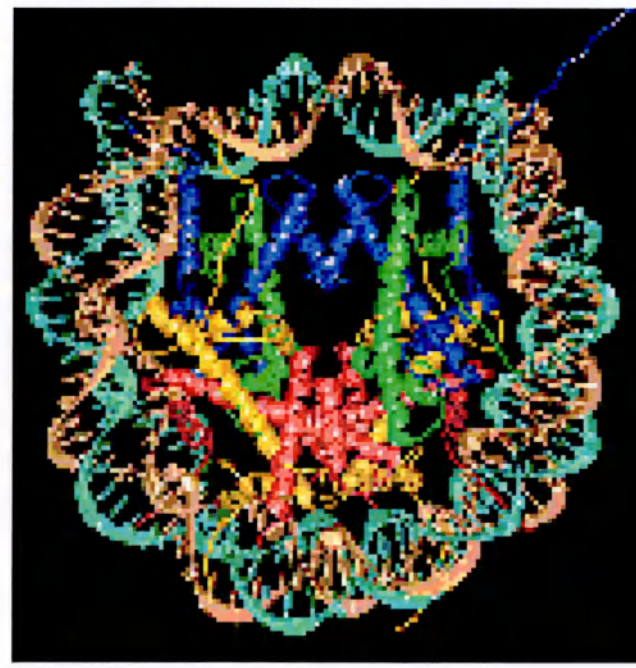


Fig. 34 Crystal structure of the nucleosome core particle

The fundamental packing unit of chromatin is the nucleosome, which consists of a short length DNA wrapped around a core of eight histone protein molecules (octamer). The X-ray crystal structure allowed analysing in atomic detail how the histone protein octamer is assembled and

how the 146 base pairs of DNA are organised into a superhelix around it. Both histone/histone and histone/DNA interactions depend on the histone fold domains and additional, well -ordered structure elements extending from this motif. Histone N-terminal tails pass over and between the gyres of the DNA superhelix to contact neighbouring particles (from (Luger et al., 1997))

In vitro, specific conditions (temperature, ionic strength) can weaken these interactions, rendering the nucleosome a more dynamic structure (Beard, 1978) (Guschin and Wolffe, 1999) (Pennings et al., 1991) (Widom, 1999), but the efficiency of these spontaneous rearrangements is usually low.

Yet, nucleosomes have to be moved around all the time, for example to allow the passage of elongating RNA polymerase. At least two different mechanisms affect nucleosome sliding: covalent modification of histones themselves (reviewed extensively in (Grunstein, 1997); (Urnov and Wolffe, 2001); (Strahl and Allis, 2000); (Jenuwein and Allis, 2001); (Turner, 2002), and mobilization by ATP-dependent machines, called nucleosome remodelling factors.

The multi-protein complexes that covalently modify nuclesomes can add or remove many chemical moieties; acetylation, phosphorylation and methylation of histone N-termini have received the most attention. The chemical and enzymatic activities of many of these complexes are well understood, but the major questions still to be investigated concern how these covalent modifications impact on the structure of the template and the ability of other complexes (e.g. those in the transcription machinery) to function coordinately.

The so-called nucleosome remodelling factors are multi-subunit complexes containing dedicated ATPases, able to convert the energy stored in ATP into displacement of nucleosomal DNA relative to the histone octamer (Kingston and Narlikar, 1999); (Narlikar et al., 2002); (Peterson, 2000; Peterson and Logie, 2000) (Aalfs and Kingston, 2000); (Workman and Kingston, 1998). They can be grouped in three main families -SWI2/SNF2 type, ISWI type and CHD type (reviewed in (Varga-Weisz and Becker, 1998); (Vignali et al., 2000) and summarized in **Table 3.**

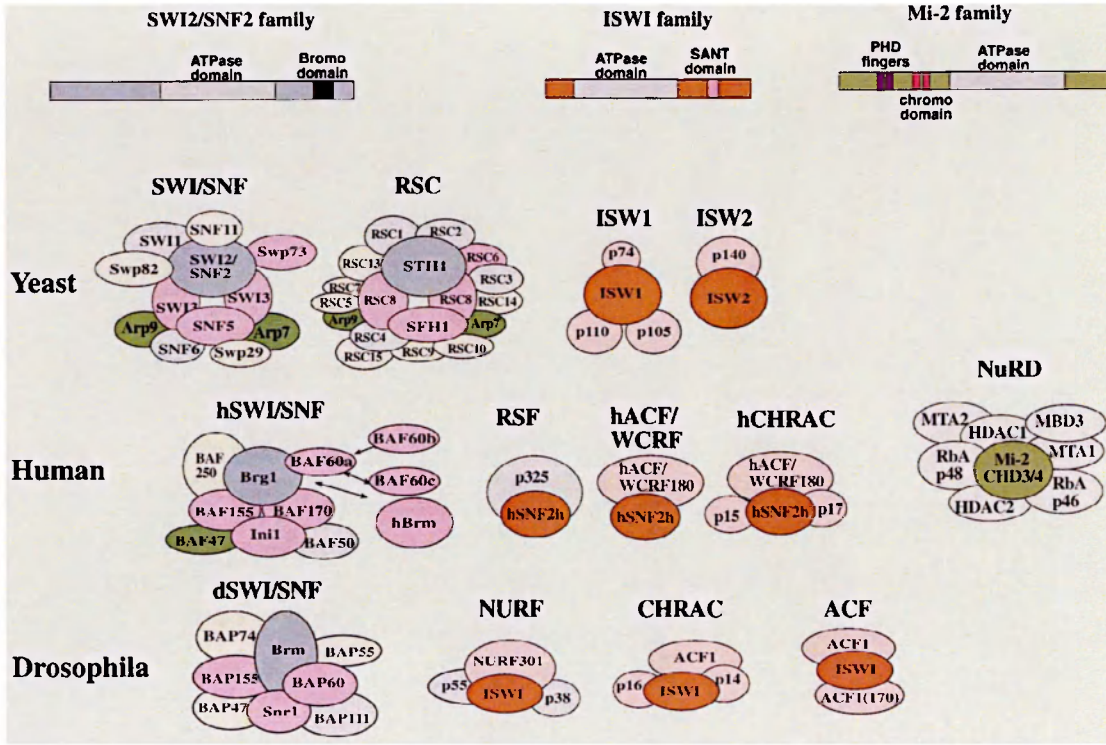


Table 3. ATP-dependent chromatin remodelling complexes

ATP-dependent remodelling complexes use ATP hydrolysis to increase the accessibility of nucleosomal DNA. These complexes can be divided into three main classes based on the identity of their catalytic ATPase subunit. These ATPase subunits display homology only within the ATPase domain and contain different additional domains. Further, each ATPase subunit forms complexes with different additional proteins, whose identity is still not completely defined. Up to now, most of investigation has been directed to the central ATPase activity, which can alter chromatin structure in the absence of the remaining subunits (Kingston and Narlikar, 1999); (Langst and Becker, 2001b); (Wang and Zhang, 2001). The biochemical characterization of the activity or the remaining subunits has begun to provide evidence for mechanistic differences among the three classes of complexes.

All these multiprotein complexes contain an ATP-ase subunit, working as the "engine" of the entire machine, but they differ for the composition of other subunits and for the specific effect on nucleosome organisation. Anyway they are all able to alter histone-DNA interaction such that nucleosomes can move (reviewed in Becker and Hörz, 2002; (Langst and Becker, 2001b); (Vignali et al., 2000)).

4.2.2 Mechanisms of nucleosome sliding: the bulge model

In spite of the several assays developed to study the activity of ATP-dependent chromatin remodelling complexes (reviewed in (Narlikar et al., 2002)), the mechanism by which remodelling machines relocate DNA over the histone octamer still remains elusive. Although rather extensive nucleosome disruption is effected by the family of SWI/SNF-related remodelling factors (Lorch et al., 1999), the two most popular scenarios assume that not all the weak histone-DNA interactions are broken simultaneously, but only a small number at any given time.

Nucleosome sliding presumably involves the detachment of a DNA segment from the histone octamer surface, possibly as a local loop or bulge, or as otherwise distorted DNA, at sites where the DNA enters its path around the particle (Becker and Horz, 2002). Propagation of this detached segment over the surface of the histone octamer would lead to displacement of nucleosomal DNA with respect to landmarks of the histone octamer surface. (**Fig. 35**)

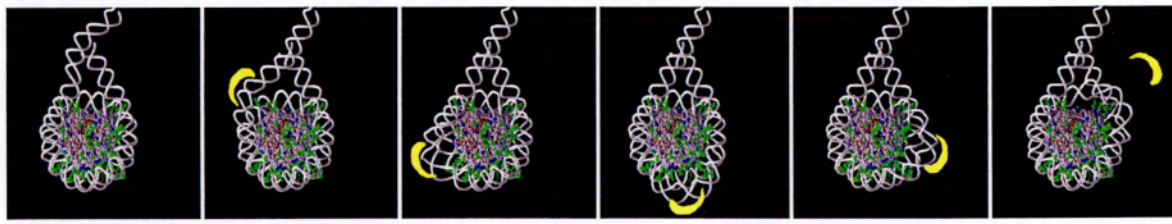


Fig. 35 Mechanism of nucleosme sliding by diffusion of a DNA bulge around the histone particle
A small region of DNA detaches form the histone octamer surface with the breakage of a small number of bonds. Some of the bulges abort by a general relaxation of the entire nuclesome unit; in some cases the bulge is maintained and starts to diffuse all around the histone core particle, for the 1.6 turn that correspond to the wrapping of the DNA around the histone core; this diffusion results in a relative circular movement of the DNA respect to the histone core that corresponds, in a linear way, to the sliding of the two entities one respect to the other.

The "twist diffusion model" argues that a small set of histone-DNA interactions is disrupted when the topology of DNA is altered, for example by varying the DNA twist. The bond-breakage would be propagated over the surface of the nucleososme, creating a translational shift of one base pair of DNA at the time relative to the histone

core. Support for this model is provided by recent work in Owen-Hughes' group (Havas et al., 2000), showing that ISWI and SWI/SNF complexes introduce superhelicity into linear DNA fragments.

In the "bulge model" local loops of DNA are detached from the histone octamer surface, starting at the sites where the DNA enters its path around the particle, and are displaced progressively along the length of nucleosomal DNA. Such looping could be initiated by thermal energy or by specific conditions of high ionic strength, and remodelling machines are postulated to catalyse events that proceed quite inefficiently when spontaneous.

Models of this kind are attractive because they explain how a nucleosome can be mobilized even though at any given time during the process only a limited number of histone-DNA contacts are disrupted. Nucleosome remodelling enzymes may catalyse the initial detachment of a DNA segment, possibly by some kind of distortion, and may endow its 'propagation' through the nucleosome surface with directionality.

Recently, Längst and co-workers (Langst and Becker, 2001a) tested the validity of the "twist diffusion" model by introducing randomly positioned nicks in the nucleosomal DNA substrate: the nicks, although leading to relaxation of any topological stress, did not prevent sliding. This argues against involvement of DNA twist in nucleosome mobilization. Remarkably, a single nick in the spacer DNA at the entry of the nucleosome facilitates sliding, which suggests that the nick in the spacer DNA between nucleosomes might increase the flexibility of DNA and might therefore facilitate the kind of DNA distortion necessary for initiating the nucleosome sliding process.

Our collaboration with Längst and Becker elaborated on this idea: if the rate-limiting step in nucleosome sliding is the distortion of linker DNA, e.g. the formation of a small local loop or bulge, proteins that can generate and/or stabilise such distortions might facilitate nucleosome sliding. HMGB1 is the archetype of proteins that bend and distort DNA, and, because it has been shown to interact with linker DNA at sites overlapping ISWI binding sites, is a good candidate to help "lubricate" nucleosome sliding.

4.2 Results

4.2.1 Interaction of HMGB1 with mononucleosomes

Several proteins, among them histone H1 and HMGB1, that are able to interact with bent or otherwise distorted DNA (Lilley, 1992; Varga-Weisz et al., 1994), are also known to bind DNA at the entry points into the nucleosome. Several investigators already documented the interaction of HMGB1 with nucleosomes (An et al., 1998); (Nightingale et al., 1996); (Schröter and Bode, 1982). Recombinant HMGB1 also interacts efficiently with the mononucleosomes that are used as substrates in nucleosome sliding assays (**Fig.36**). Electrophoretic mobility shift assays (EMSA) document the binding of HMGB1 to a nucleosome assembled centrally on a 248 bp DNA fragment derived from the ribosomal RNA promoter. The titration of HMGB1 yields a single retarded band, confirming the existence of one preferred binding site (**Fig 36 A**; lanes 4-6). HMGB1 also supershifts a nucleosome positioned at the end of the same DNA fragment (lanes 10-12). This interaction does not lead to disruption of the nucleosome: excess unlabelled DNA added to an EMSA reaction before gel loading, effectively competes HMGB1 off the labelled nucleosome, which is released intact (**Fig. 36 B**; lanes 6-9).

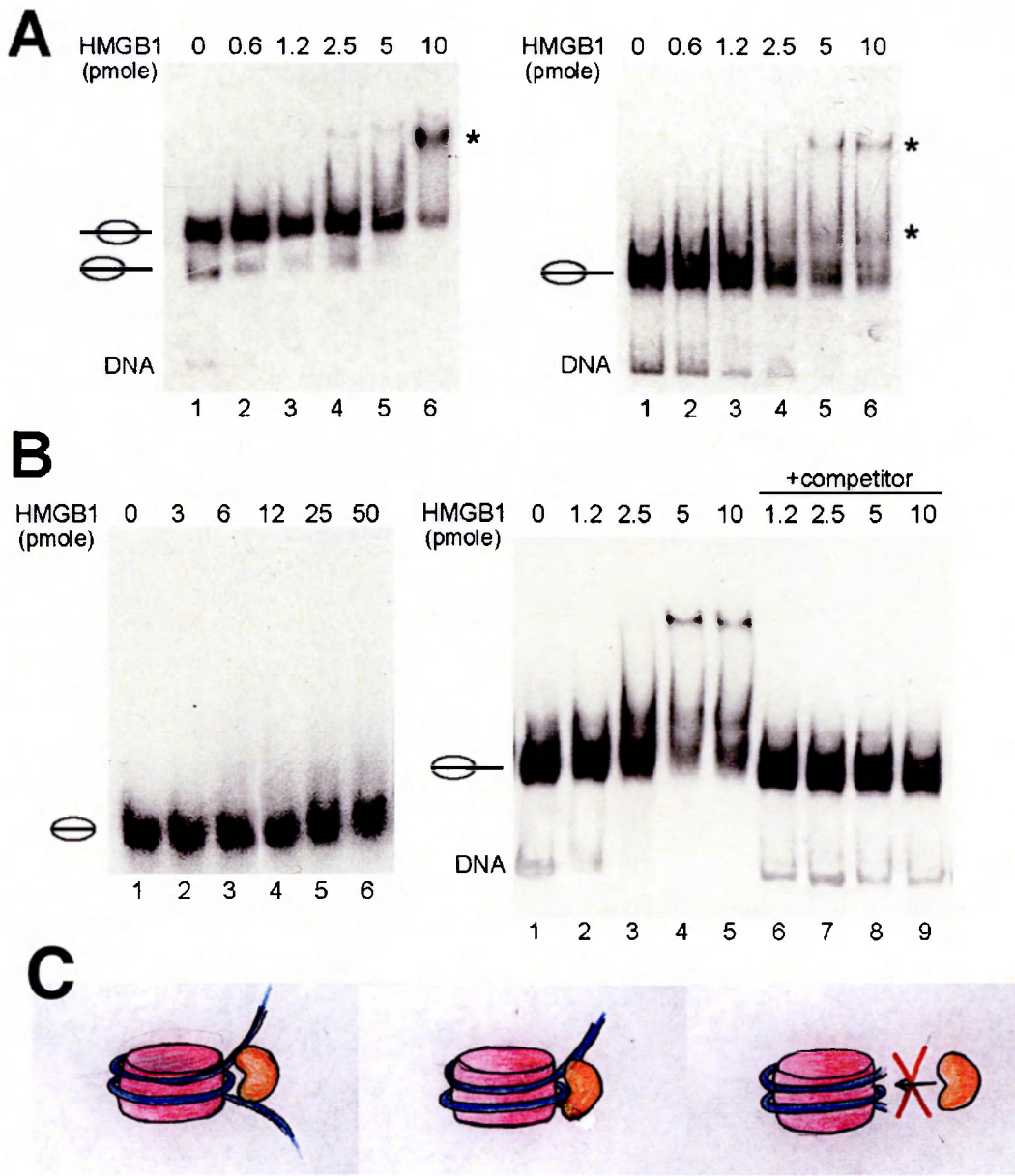


Fig. 36 Specific interaction of HMGB1 with nucleosomes exhibiting protruding DNA

(**A**) Increasing amounts of HMGB1 full length (as indicated) were incubated with nucleosomes (60 fmol) positioned at the centre (left panel) or at the end (right panel) of the 248bp rDNA Fragment. The complexes were resolved by electrophoresis on a native polyacrylamide gel. An autoradiograph of the gel is shown. The major nucleosome-HMGB1 complex is marked by an asterisk. Nucleosomes are schematized by ellipses with protruding DNA. (**B**) Nucleosomes (60 fmol) assembled on a 146bp DNA fragment were incubated with increasing amounts of HMGB1 as indicated and analysed by electromobility shift assay as above (left panel): no specific binding was detected with increasing amounts of HMGB1. HMGB1 binding to the nucleosome does not disrupt the histone octamer (left). HMGB1 binding was analysed as described above without (lanes 2-5) and with the addition of competitor DNA (lanes 6-9) prior to gel loading. (**C**) Picture schematising HMGB1 binding specificity for different mononucleosome isoforms.

In agreement with earlier results (An et al., 1998); (Nightingale et al., 1996) and (Schröter and Bode, 1982), stable interaction of HMGB1 requires the presence of some linker DNA protruding from the nucleosomal core. It is unable to associate with a nucleosome core particle consisting of 146 bp DNA wrapped tightly around a histone octamer, even at very high concentrations (**Fig.36 B**, lanes 4-6). The interaction of HMGB1 with nucleosomes thus is reminiscent of the properties of the remodelling ATPase ISWI: ISWI cannot bind to a nucleosome core, but requires linker DNA at least on one side of the nucleosome particle (Brehm et al., 2000); (Langst and Becker, 2001b).

We attempted to document the preferred interaction of HMGB1 by footprinting (**Fig.37**). Centrally positioned nucleosomes were isolated, incubated with HMGB1 and partially digested with DNase I. HMGB1-nucleosome complexes were identified on native polyacrylamide gels, excised and the purified DNA was analysed on sequencing gels. While HMGB1 protects free DNA non-specifically from DNase I digestion (panel 3) the protection of nucleosomal DNA was modulated (panel 5). Since the 'central nucleosome' actually corresponds to a series of nucleosomes with different translation positions between position –20 and –190 on the DNA the footprint naturally lacks some precision. However, obvious protection of DNase I digestion on the nucleosomal fragment is seen in the area corresponding to one end of the nucleosomes (the –190 position), but not at the other end (the –40 position).

This interaction is reminiscent of earlier studies involving model nucleosomes, which described asymmetrical interactions. Enhancements of DNase I cleavage are also seen close to the nucleosomal pseudodyad, which suggests a close interaction of HMGB1 with the nucleosome, rather than a detached interaction with linker DNA.

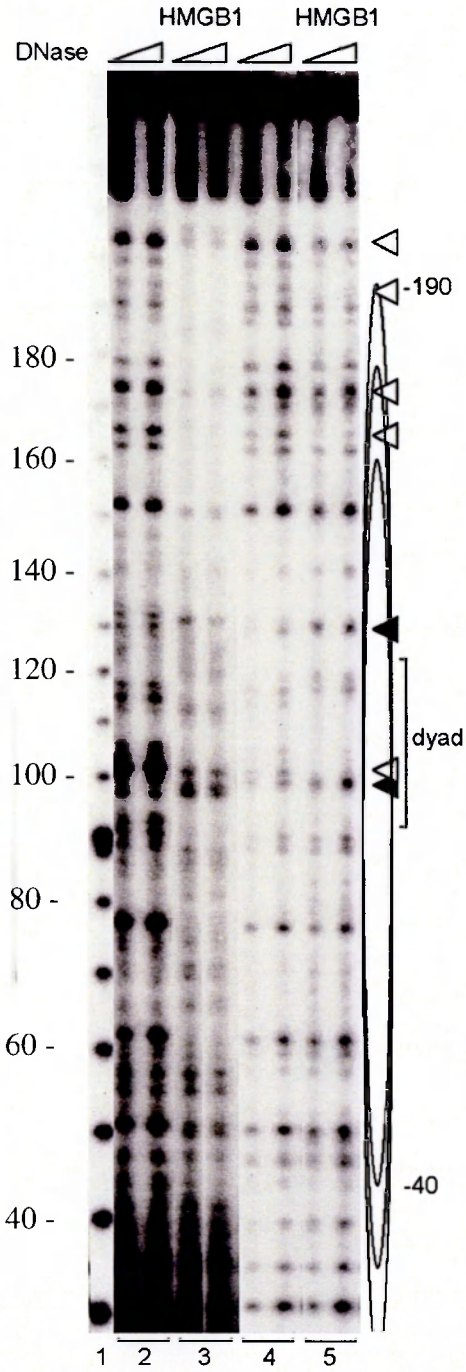


Fig. 37 Footprinting HMGB1 on the mononucleosome

DNA (panels 2 and 3) or purified nucleosomes (panels 4 and 5) were incubated with HMGB1. DNase I-treated free DNA, nucleosomes and nucleosome-HMGB1 complexes were resolved by PAGE, the DNA was isolated from the corresponding bands and analyzed on sequencing gels. The cluster of central nucleosome positions is indicated by white ellipses and the area of perturbation of histone-DNA interaction upon HMGB1 binding is marked by white (DNase protection) or black triangles (DNase hypersensitivity).

The fact that HMGB1 and ISWI approach nucleosomes with overlapping binding specificity raises questions about the consequences of the presence of HMGB1 in ISWI-induced nucleosome sliding.

4.2.2 An *in vitro* tool to study chromatin remodelling at the molecular level

In order to test for an effect of HMGB1 on ISWI- and ACF-induced nucleosome mobility, we employed an established assay that allows visualization of single nucleosome movement catalysed by ATP-dependent nucleosome remodelling factors. The system was developed in Becker's group and is already well documented in several works (Brehm et al., 2000; Clapier et al., 2001; Eberharter et al., 2001; Langst et al., 1999). The starting point to develop the mononucleosome remodelling experiment was the observation that wrapping of short DNA fragments around histone octamers frequently led to multiple translational positions and that those isoforms could be separated electrophoretically on a 5% AA gel. In our case, nucleosomes occupy prominent positions on a 248 bp fragment derived from the rDNA promoter: a dominant internal position is dictated by the anisotropic flexibility of the DNA sequence, and in addition nucleosomes can abut the fragment ends, as depicted in **Fig. 38 A**:

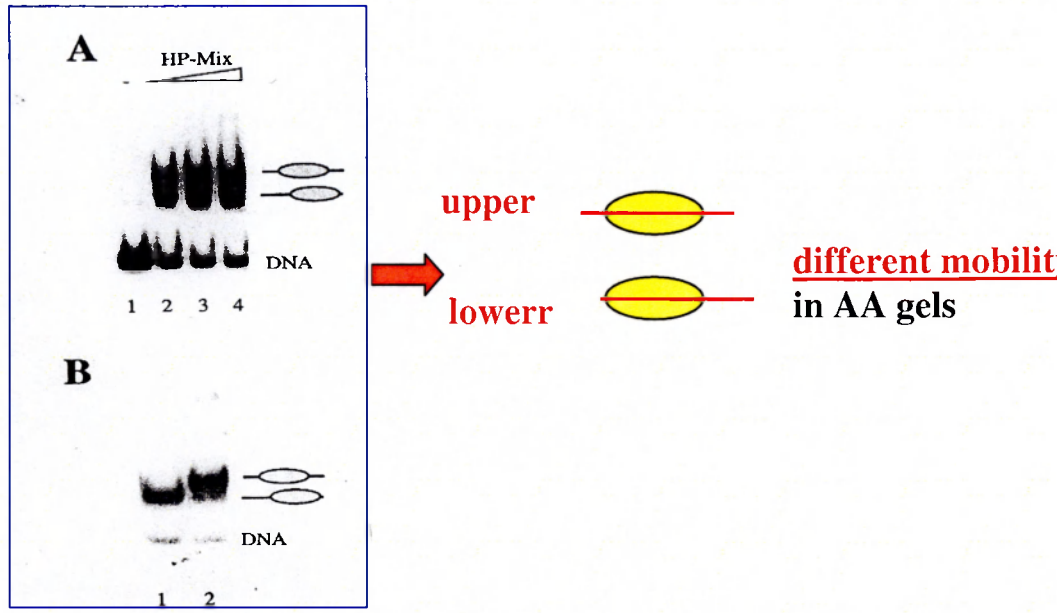


Fig. 38A. Assembly of mononucleosomes *in vitro*

Mononucleosomes are assembled *in vitro* by incubating a stretch of DNA, 248 bp long, with increasing amounts of histone-core proteins (**A**, see Methods); the incubation products are loaded on a 5% acrylamide gel and exposed to X-ray film. (**B**): two major bands can be detected, representing different translational positions of the histone core with respect to the DNA stretch. Different isoforms run diffently and can be separated in an acrylamide gel: the centrally positioned nucleosome is slower then the pheripherally positioned one.

Isolated nucleosomes positioned either at the centre or at the periphery of the DNA fragment serve as substrate for remodelling machines (**Fig. 38 B**).

Becker and collaborators previously showed that the remodelling ATPase ISWI is able to move nucleosomes from internal positions to fragment ends (Langst and Becker, 2001b); (Langst et al., 1999). Association of ACF1 with ISWI reconstitutes the heteromeric ACF (Ito et al., 1997). ACF1 not only increases the efficiency of ISWI-depending remodelling by an order of magnitude, it also leads to a qualitative change in nucleosome sliding: in contrast to isolated ISWI, ACF can catalyse the movement of nucleosomes from fragment ends to more central positions (Eberharter et al., 2001). While these simple model reactions on short linear DNA fragments are unlikely to reflect all physiological constraints of nucleosome movements in nuclei, they provide an excellent opportunity to study fundamental parameters that affect nucleosome mobility.

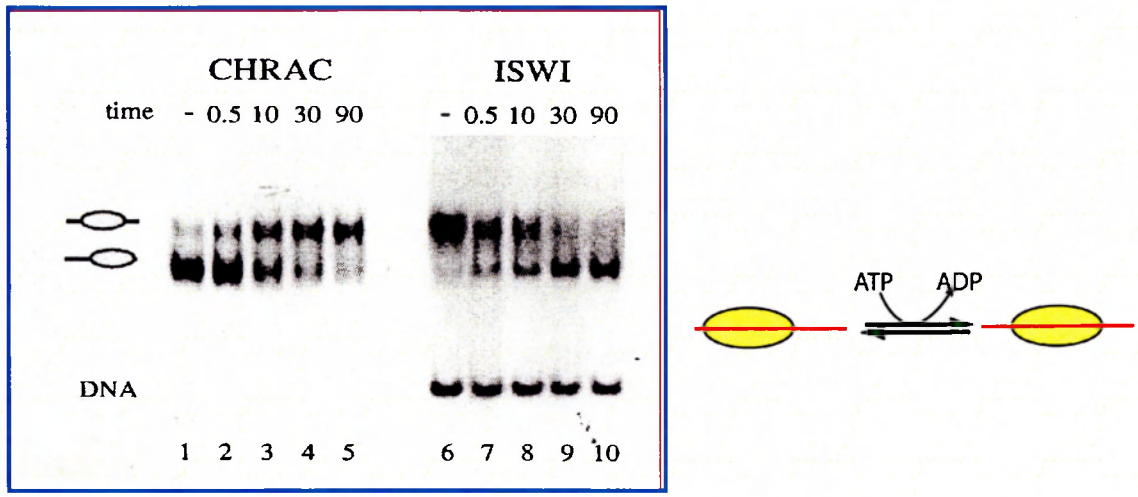


Fig. 38 (From (Langst et al., 1999)) *In vitro* sliding assay of mononucleosomes with chromatin remodelling machines

Differentially positioned mononuclesomes are used as substrates in *in vitro* remodelling assays by incubating them with remodelling machines (ISWI, CHRAC and ACF) in the presence of ATP. The energy derived from hydrolysis of ATP is used to slide the histone core with respect to the DNA stretch, with a different directionality depending on the type of enzyme used: CHRAC and ISWI, for example, recognise and remodel different mononucleosomes. The reaction is stopped by loading the mix on an AA gel; the labelled mononucleosomes are detected by autoradiography.

4.2.3 HMGB1 affects the sliding activity of ACF and CHRAC

The first evidence for an effect of HMGB1 on nucleosome sliding was obtained in reactions catalyzed by native CHRAC (**Fig. 39A**). We measured the kinetics of nucleosome sliding as a function of fixed amounts of HMGB1 by stopping the reactions at different time points with competitor DNA followed by native gel electrophoresis. We found that nucleosomes moved significantly faster in the presence of HMGB1 (compare lanes 2, 3 to lanes 6, 7), but the overall level of nucleosome mobilization at the end of an extended incubation was not changed (Figure 2A, compare lanes 5 and 9). We next explored whether HMGB1 would also affect the kinetics of nucleosome sliding by recombinant two-subunit ACF (Eberharter et al., 2001). In the presence of 2 pmoles of HMGB1 a clear increase of nucleosome sliding was detectable, particularly at the early time points (**Fig.39B**, lanes 2-4 and 8-10). A similar stimulation was observed if

HMGB1 concentrations were increased 5-fold, but raising the concentrations of remodelling factor abolished the effect (data not shown).

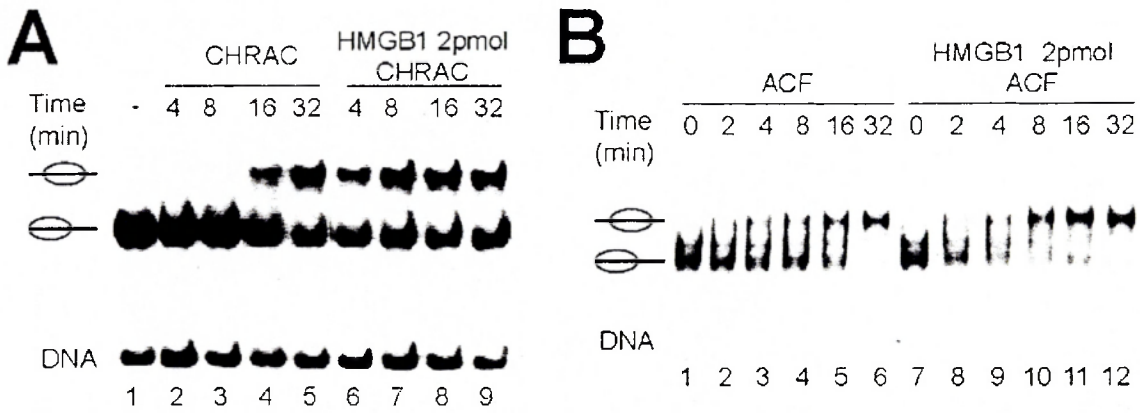


Fig. 39 HMGB1 stimulates CHRAC and ACF-mediated nucleosome remodelling

(**A**) 60 fmol of nucleosomes positioned at the end of the DNA fragment (lane 1) were incubated with 5 fmol of native CHRAC and ATP, in the absence (lanes 2-5) or presence of 2 pmol of HMGB1 (lanes 6-9). The reactions were incubated at 16°C and stopped at the indicated time points by the addition of competitor DNA. Nucleosome positions were subsequently analysed by electrophoresis. Nucleosomes are schematized by ellipses with protruding DNA.

(**B**) Nucleosome remodelling assay, as described in (**A**), using 5 fmol of recombinant ACF in the absence (lanes 1-6) or presence of 2 pmol of HMGB1 (lanes 7-12).

In order to document better the kinetic effect of HMGB1 on nucleosome sliding we quantified the degree of nucleosome sliding throughout the sliding reaction, and determined the degree of stimulation by HMGB1 for each time point. The graph in **Fig. 40** displays the average data derived from 4 independent experiments. It documents a kinetic effect of HMGB1 on nucleosome sliding which is mainly observable at the early times.

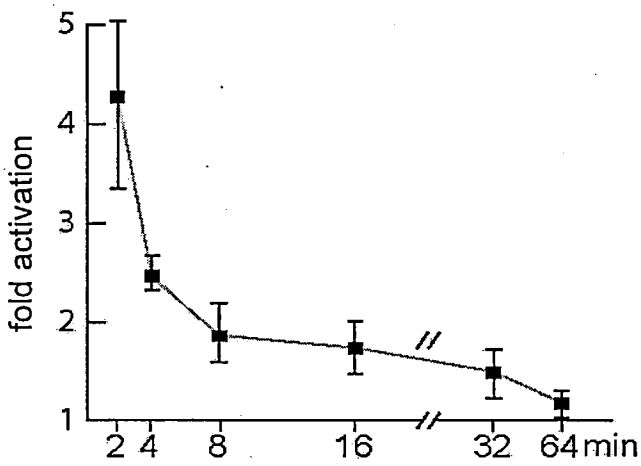


Fig. 40 Quantification of the HMGB1 effect on nucleosome remodelling

The ratio of nucleosomes moved in the presence of HMGB1 versus nucleosomes moved in the absence of HMGB1 was calculated for 4 independent experiments and displayed in a graph.

In order to establish whether ISWI by itself would also profit from the presence of HMGB1 we added the factor to reactions in which ISWI mobilised a centrally positioned histone octamer to slide to a fragment end (**Fig. 41**). Surprisingly, two pmol and higher amounts (data not shown) of HMGB1 did not affect the kinetics of ISWI-dependent nucleosome sliding, suggesting a critical contribution of the ACF1 moiety for the functional synergism with HMGB1.

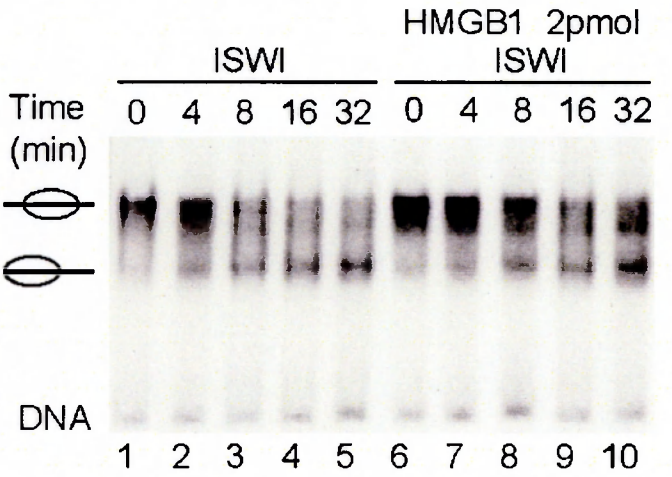


Fig. 41 HMGB1 does not affect the kinetics of ISWI – mediated nucleosome sliding
Nucleosomes positioned at the centre of the DNA fragment were incubated with 30 fmol of ISWI and ATP, in the absence (lanes 1-5) or presence of HMGB1 (lanes 6-10, 2 pmol) and assayed as described in (Fig. 39).

4.2.4 HMGB1 promotes ACF binding to the nucleosome

The stimulatory effect could be due to interactions of HMGB1 with ACF, with the nucleosomal linker DNA, or both. We have used mouse HMGB1 in the context of a nucleosome remodelling machinery from Drosophila to minimize the probability of direct interactions and accordingly we were so far unable to detect any significant interactions between HMGB1 and ACF in biochemical pull-down experiments (data not shown). Therefore we next analysed the effect of HMGB1 on the interaction of the remodelling factors with the nucleosomes. Binding of ACF to the nucleosome can be visualised by EMSA as a single, prominent band if no competitor DNA is added prior to gel loading (**Fig. 42**, lanes 4-6). As before, HMGB1 shifts the nucleosome into a position close to, but distinct from the ACF-nucleosome complex (**Fig. 42**, lanes 13, 14). Importantly, the presence of 0.6 pmol of HMGB1 does not shift the nucleosomal band significantly (lane 10). However, if this amount of HMGB1 was included in a nucleosome binding reaction with ACF, four fold lower concentrations of ACF were required to shift the nucleosome (compare lanes 1-6 to 15-20).

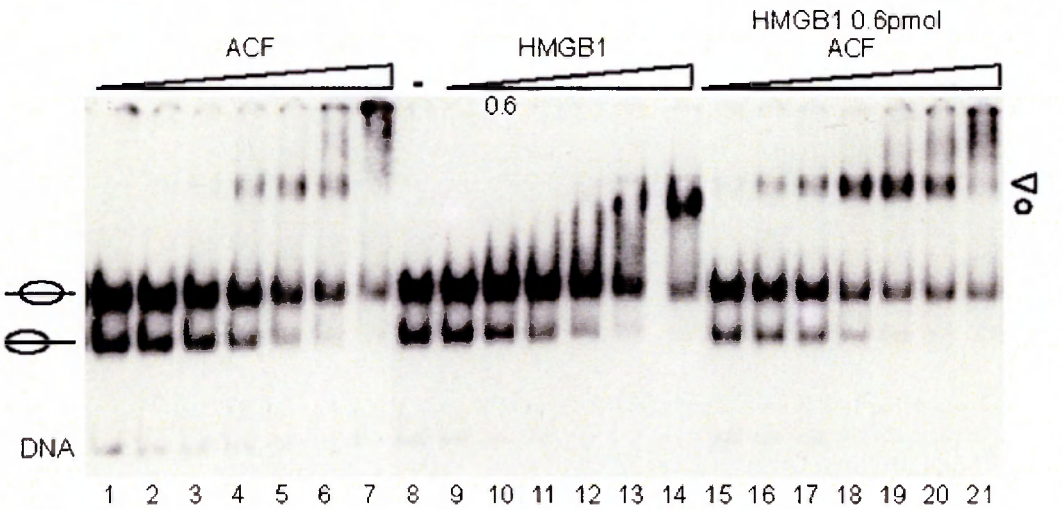


Fig. 42 HMGB1 promotes the formation of a ACF-nucleosome complex

Nucleosomes preferentially positioned at the centre of the DNA fragment were incubated with increasing amounts of ACF (lanes 1-7; 15, 30, 60, 120, 240, 480, 960 fmol ACF), HMGB1 (lanes 9-14; 0.3, 0.6, 1.2, 2.4, 4.8, 9.6 pmol) and increasing amounts of ACF in the presence of 0.6 pmol HMGB1 (lanes 15-21). Complexes were analysed in electromobility shift assays, as before. Nucleosome-ACF complexes are marked by a triangle and nucleosome-HMGB1 complexes are indicated by a circle.

At higher, saturating concentrations of ACF the degree of nucleosome binding was unaffected by HMGB1, in close correspondence with the nucleosome sliding profiles. Under these conditions no evidence for the existence of a ternary complex of ACF, HMGB1 and the nucleosome was found using specific antibodies in 'supershift' strategies (data not shown). Thus, HMGB1 may function as a DNA chaperone that facilitates ACF binding to the nucleosome at suboptimal conditions, without being a stable component of the complex.

A similar experiment, in which we assayed for an effect of HMGB1 on the ISWI-nucleosome interaction revealed no stimulation (**Fig.43**).

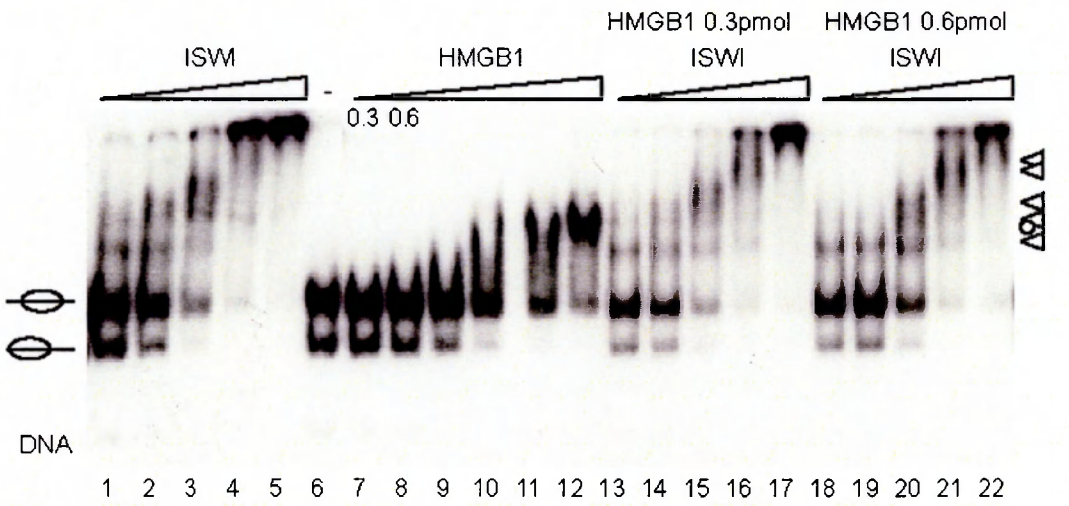


Fig. 43 HMGB1 does not affect the binding affinity of ISWI for the nucleosomes

Positioned nucleosomes were incubated with increasing amounts of ISWI (lanes 1-5; 30, 60, 120, 240, 480, 960, 1920 fmol), HMGB1 (lanes 7-12, 0.3, 0.6, 1.2, 2.4, 4.8, 9.6 pmol) and ISWI with fixed amounts of HMGB1 (lanes 13-17, 0.3 pmol HMGB1; lanes 18-22, 0.6 pmol HMGB1). Nucleosome-ISWI complexes are marked by triangles and the nucleosome-HMGB1 complex is indicated by a circle.

We concluded that the potential of HMGB1 to facilitate nucleosome sliding correlates with its ability to lead to enhanced interaction of the remodelling machine with the nucleosomal substrate. The data identify the ACF1 moiety of ACF as crucial for this effect.

4.2.5 A HMGB1 mutant that binds nucleosomes with high affinity inhibits nucleosome sliding

Previously, several investigators observed that the C-terminal acidic tail of HMGB1 controls the DNA binding properties of the HMG boxes (Lee and Thomas, 2000); (Stros et al., 1994) (Muller et al., 2001). In agreement with these studies I observed that deletion of the C-terminal domain increased the activity of the HMG boxes in protein-protein interaction and transactivation assays (unpublished observations). We, therefore, wished to test a C-terminally deleted HMGB1 (HMGB1ΔC= boxA+boxB) in nucleosome sliding assays.

Considering the absolute amounts of input protein, HMGB1ΔC interacted about 100 fold better with free DNA than full length HMGB1; similarly, HMGB1ΔC interacted two orders of magnitude better than HMGB1 with either peripheral or central nucleosomes (**Figure 44B**, compare to **Fig. 36A**). Higher concentrations of HMGB1ΔC yielded several bands, which, in light of the pattern obtained by interaction of HMGB1ΔC with free DNA (**Fig. 44A**), presumably are due to interaction of several molecules with the free DNA next to the nucleosome.

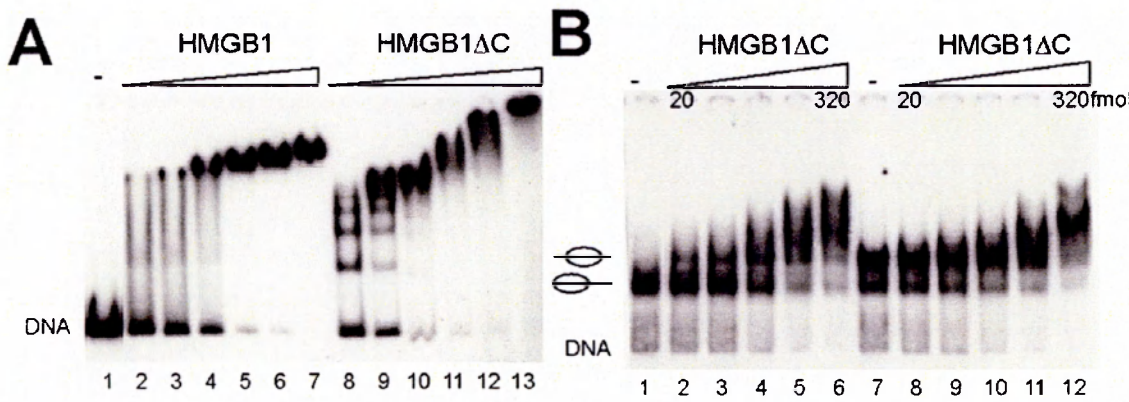


Fig. 44 HMGB1ΔC binding to free DNA and to mononucleosomes

(**A**) Comparison of HMGB1 and HMGB1ΔC binding to the 248bp rDNA fragment. The DNA fragment was incubated with increasing amounts of HMGB1 (lanes 2-7; 1.5, 2.3, 3.4, 5, 7.6, 11 pmol) and HMGB1ΔC (lanes 8-13; 50, 75, 112, 169, 253, 380 fmol). HMG-DNA complexes were separated on native polyacrylamide gels.

(**B**) HMGB1ΔC interacts with nucleosomes positioned at the border or the centre of the rDNA fragment. Positioned nucleosomes were incubated with increasing amounts of HMGB1ΔC (20, 40, 80, 160, 320 fmol). Compare the binding efficiency with that of HMGB1 in Fig.36

Surprisingly, when HMGB1ΔC was titrated into the nucleosome mobility assay, it inhibited the nucleosome sliding catalysed by a fixed amount of ACF (**Fig. 45**), rather than facilitating it. Nucleosome movement decreased as a function of HMGB1ΔC and complete inhibition was achieved upon addition of 80-160 fmoles. This amount of HMGB1ΔC is similar to the amount required to yield the first complex with the nucleosome in the EMSA, which presumably reflects the interaction of a single HMGB1ΔC molecule with its preferred binding site (lane 4).

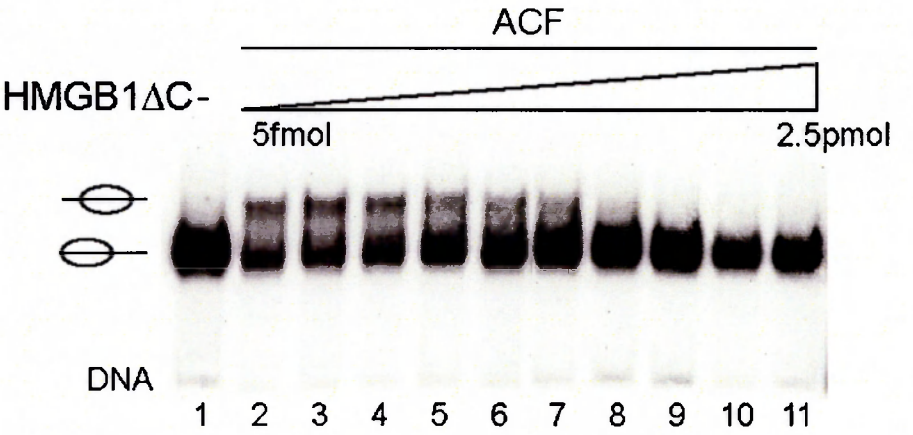


Fig. 45 HMGB1ΔC inhibits ACF-dependent nucleosomes remodelling

ACF-mediated nucleosome remodelling is inhibited by the addition of HMGB1ΔC. Nucleosomes positioned at the border of the DNA fragment were incubated with ACF, ATP and increasing amounts of HMGB1ΔC (lanes 2-11; 5, 10, 20, 40, 80, 160, 320, 640, 1240, 2560 fmol). Remodelling reactions were stopped by the addition of competitor DNA and nucleosome positions were analysed on a 4.5% polyacrylamide gel.

This result suggests that the tight binding of HMGB1ΔC to nucleosomes prevents efficient nucleosome mobilisation by ACF. Our interpretation of these results will be summarised in the model presented in the next section.

4.3 Discussion

4.3.1 HMGB1, a hallmark of dynamic chromatin

Nucleosome remodelling complexes containing the ATPase ISWI are likely to contribute significantly to the flexibility of chromatin enabling dynamic structural transitions throughout the lifetime of a cell. Among the Drosophila ISWI complexes only the Nucleosome Remodelling Factor (NURF) seems to be involved in targeted transcriptional regulation (Mizuguchi et al., 1997; Mizuguchi et al., 2001); (Xiao et al., 2001). By contrast, the features of the related ACF and CHRAC complexes suggest more global roles in the assembly and maintenance of dynamic chromatin (Langst and Becker, 2001b). Whether or not the activity of these abundant factors is restricted to particular subnuclear domains, or otherwise regulated is unknown. The experiments described above indicate that HMGB1, one of the most abundant non-histone proteins in nuclei, is able to increase the efficiency of ACF-dependent nucleosome sliding in a defined model system. This observation may point to an important, novel function of HMGB1, but at the same time reveals unexpected insight into the mechanism underlying catalysed nucleosome sliding.

HMGB1 is a hallmark of dynamic chromatin: it diffuses throughout the nucleus with highest rates, indicating a rather dynamic association with chromatin (Scaffidi et al., 2002). Since HMGB1 binds nucleosomes at sites overlapping those recognised by the linker histone H1, HMGB1 and related proteins in Drosophila may compete with histone H1 for nucleosomal binding sites (Ner et al., 1993); (Nightingale et al., 1996); (Varga-Weisz et al., 1994). Association of histone H1 prevents spontaneous nucleosome sliding at elevated salt and temperature (Pennings et al., 1994) and moderately inhibits the remodelling action of the SWI/SNF complex on mononucleosome substrates (Hill and Imbalzano, 2000). Whether histone H1 affects nucleosome sliding or other ATP-dependent functions of ISWI has not been determined in a defined system yet (but see (Varga-Weisz et al., 1995; Varga-Weisz and Becker, 1995)). Given, that the interaction of H1 with linker DNA (Widom, 1998) is likely to interfere with ISWI binding, some kind of inhibition is expected. Therefore, HMGB1 may facilitate nucleosome remodelling in two ways: (1) by 'unlocking' the nucleosome

through displacing linker histones, and (2) by stimulating the activity of ACF/CHRAC as shown here and discussed below.

4.3.2 Catalysed nucleosome sliding: mechanistic considerations

ISWI-induced nucleosome sliding does not involve the disassembly of the histone octamer (Hamiche et al., 1999); (Langst et al., 1999). Given the large number of histone-DNA contacts in the nucleosome, it is likely that nucleosome sliding involves disruption of a limited number of histone-DNA contacts at any time. Prevalent models assume that the critical step in nucleosome mobilisation is the tight bending (or bulging, looping) of a DNA segment at the edge of the nucleosome by the remodelling ATPase, effectively replacing the histone-DNA contacts within this segment by analogous ones involving an equivalent linker DNA segment. This would lead to the formation of a local loop, or bulge, on the surface of the histone octamer (Langst and Becker, 2001a); (Havas et al., 2000); (Kingston and Narlikar, 1999). We presume that this initial DNA distortion is rate-limiting the overall reaction: once the bulge is introduced into nucleosomal DNA its propagation over the nucleosomal surface is presumed to require relatively little further energy input (Langst and Becker, 2001b); (Becker and Horz, 2002). These types of models are supported by recent observations of Owen-Hughes and colleagues, who documented the ability of ISWI to deform DNA in a reaction depending on the presence of nucleosomes and ATP (Havas et al., 2000).

We recently observed that single stranded nicks in DNA close to the entry into the nucleosome - the site of ISWI interaction - facilitated ATP-dependent nucleosome sliding (Langst and Becker, 2001a). We hypothesised that the presence of these nicks might enhance the flexibility of DNA at this site, such that it could be easier bent or otherwise distorted. The current finding that HMGB1 - the prototype of proteins that preferentially bind distorted DNA and which bend DNA upon binding - facilitates ACF-dependent nucleosome sliding, leads us to the model depicted in **Fig. 46**. We presume that HMGB1 acts as a DNA chaperone, kinking or bending DNA (or stabilising an intrinsic bend) at the nucleosomal edge. The presence of this bend might either enhance the ability of ACF to interact with this site, or alternatively, enhance its ability to

introduce the rate-limiting DNA distortion. Although a direct, allosteric effect on ACF cannot be excluded in light of the fact that HMGB1 is able to interact with several regulators of chromatin structure (Dintilhac and Bernues, 2002), this appears not to be the basis of our current findings since we have been unable to detect a direct interaction of HMGB1 with ISWI and ACF in the absence of nucleosomal DNA. HMG domains may be more generally involved in the recognition and modification of nucleosomal substrate of remodelling factors, since the BAF57 subunit of the SWI/SNF-like complexes contains an HMG domain (Wang et al., 1998). Similarly, the BAP111 subunit of the Drosophila BRM complex, which is important for BRM function, contains an HMG box (Papoulas et al., 1998).

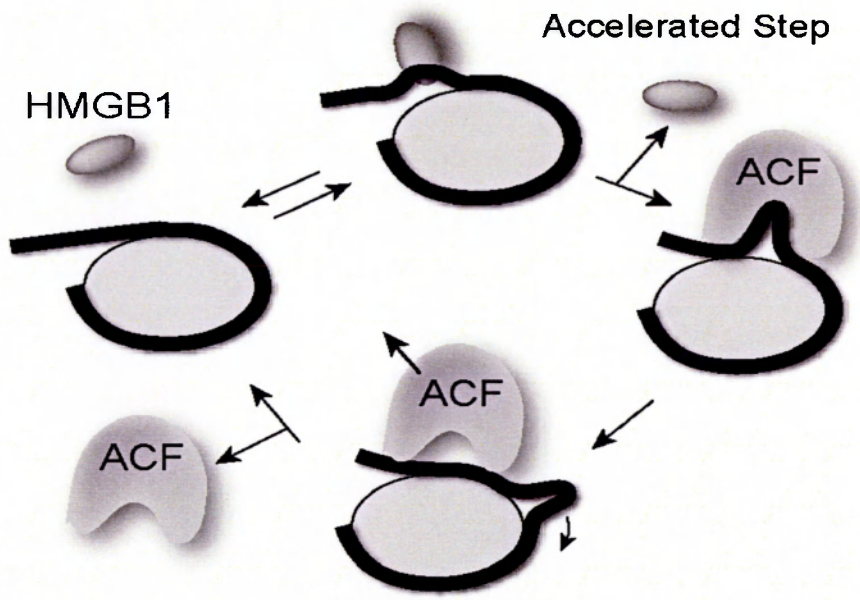


Fig. 46 Model showing the stimulatory effect of HMGB1 on ACF-mediated nucleosome remodelling

HMGB1 may introduce a kink ar an initial bulge at the nucleosome side this intrinsic bend is recongised by ACF, whose binding affinity for the nuclesomes is thus increased. The wrapping of DNA (black line) around a histone octamer (grey sphere) is shown sideways.

Interestingly, HMGB1 potentiated the sliding competence of ACF, but did not enhance the ability of the ATPase, ISWI alone. Previous work highlighted several important distinctions between the remodelling reactions catalysed by these two factors. The association of ACF1 with ISWI to form ACF affects the activity of the ATPase both quantitatively and qualitatively (Eberharter et al., 2001). First, ACF mobilises nucleosomes by an order of magnitude better than ISWI alone at similar levels of ATP hydrolysis. At the same time, ACF and ISWI appear to approach the nucleosomal substrate somehow differently since ISWI only triggers the sliding of nucleosomes to fragment ends whereas ACF is able to move nucleosomes from ends to more central positions on DNA. This phenomenology is likely to reflect profound differences in the initiation of nucleosome mobilisation. The current data identify yet another aspect in which ISWI and ACF function differs: the ability to profit from the action of HMGB1. In the presence of low amounts of HMGB1, ACF, but not ISWI, interacts better with the nucleosomal substrate. Since these amounts of HMGB1 do not form ternary complexes with ACF and the nucleosome, we speculate that ACF1 might enable the remodelling factor to recognise DNA structure within the nucleosomal linker and hence sensitise it for pre-bent DNA. Remarkably, ACF1-like molecules appear to be obligatory subunits of ISWI-containing remodelling ATPases and therefore seem to be tightly involved in the remodelling process itself.

4.3.3 Facilitated nucleosome sliding involving the DNA chaperone function of HMGB1

The observation that catalytic concentrations of HMGB1 affect the rate with which ACF interacts productively with the nucleosomal substrate points to highly dynamic association of HMGB1 with nucleosomes. We envision that the transient association of HMGB1 with its target will introduce a DNA distortion, which will subsequently be bound by ACF while HMGB1 dissociates (**Fig. 46**). Such a DNA chaperone function of HMGB1 is not without precedent. HMGB1/2 molecules are known to facilitate the DNA binding of various regulatory factors, such as HOX proteins, steroid hormone receptors, p53 and the site specific recombinase RAG1 (Aidinis et al., 1999); for review of the older literature, see (Bianchi and Beltrame, 1998)).

The general idea is strongly supported by the observation that an HMGB1 derivative lacking the acidic C-terminus and hence able to interact with the substrate 100-fold better than full length HMGB1 does not stimulate sliding, but rather represses it. We consider that, due to its higher affinity for the substrate, HMGB1ΔC might resist displacement by ACF, remain bound to the nucleosome and hence continue to 'lock' the nucleosome, perhaps in analogy to histone H1.

Obviously, and in agreement with previous findings (Lee and Thomas, 2000); (Muller et al., 2001), its acidic C-terminus is responsible for the dynamic interaction of HMGB1 with nucleosomal DNA. Differential calorimetry experiments, both in the presence (Muller et al., 2001) or the absence of DNA (Ramstein et al., 1999), indicated that the acidic tail binds to one of the HMG boxes of HMGB1 (most likely Box A). By dynamic competition with DNA this intramolecular binding might render the HMG box-DNA interaction reversible and transient, consistent with a chaperone function of HMGB1. The DNA bulge released from box A could then be recognised by ACF.

Our model experiments highlight the importance of bulges for nucleosome sliding. HMGB1 merely served as a paradigm for a class of factors which, due to their distinct DNA binding features may function as DNA chaperones (Travers et al., 1994). Other HMG-box proteins, including the single-HMG-box protein HMG-D in Drosophila (Ner et al., 2001), might share the ability to present bulges or kinks to nucleosome remodelling machines. In fact, TBP binding to the TATA box of the IFN-β gene promotes the relocation nucleosome that obstructs the transcription start site (Lomvardas and Thanos, 2001), thus suggesting that any protein capable of bending DNA might promote nucleosome sliding.

CHAPTER 5. CONCLUSIONS

During these years I had the opportunity to develop two independent but related fields of investigation that allowed deepening my knowledge on HMGB1 as a "doubleface" protein: a nuclear chaperone and a signalling molecule.

Searching for a definition of the peculiar duplicity of HMGB1, we coined the term "molecular moonlighting" that expresses well the concept that this molecule has two jobs in the cell, independent one from the other as far as we know

P. Scaffidi discovered that a passive release of HMGB1 by necrotic cells can work as a "danger signal".

My PhD work provides a step further in the understanding the molecular mechanism used by healthy cells to actively release HMGB1 using a danger signal as a specific and rapid response to an inflammatory condition; the results in Section 3 suggest also that the inflammatory cells may have employed a machinery common to all the cell types (the HATs and HDACs activities) to develop this quick response via a cascade of PT modifications.

On the other side, my collaboration with Becker's group in Munich provided some hints for the understand of the role of HMGB1 in modulating the mechanism of chromatin remodelling. As thoroughly discussed in **Sections 1.2** and **4**, the debate around the structural and functional relationship between HMGB1 and chromatin is still controversial.

Showing that HMGB1 is indeed able to enhance the activity of native CHRAC and ACF, apparently by increasing the affinity of ACF for its nucleosomal substrate, we propose a model in which HMGB1 distorts DNA at its entry into the nucleosome and interacts with it in a highly dynamic manner thus effectively 'presenting' the DNA bend to ACF. These results are particularly relevant in light of the recent discussion about the mechanisms governing nucleosome sliding and they allowed identifying HMGB1 as a potential regulator of ATP-dependent nucleosome remodelling processes.

Both lines of investigation require further development, since the results I collected in Sections 3 and 4 are preliminary, even if they represent an important progress in the two distinct fields.

Still, an organic perspective, capable of declinating the two aspects of my study in a coherent model, is difficult to define; likely, this goal will be reachable only when more information will be collected about the issue of HMGB1 as a signalling molecule.

As a general consideration, one common message I could derive from the two lines of research is the concepts of dynamism and mobility, as main features of the biological world at all levels, in my case from the cellular level to the molecular one.

Dynamism that I could detect in the interactions between molecules: the role of HMGB1 on sliding is mainly based on its capability to estabilish very dynamic contacts with the other partners. A proof is given by the fact that, whenever this dynamism is lost due to excessive affinity between partners (like with HMGB1ΔC), the mechanism collapses.

Regarding HMGB1 acetylation, dynamism could be detected at many different levels: HMGB1 moves quickly inside the nucleus, estabilishing specific but loose contacts with nucleoplasm; HMGB1 shuttles continuously in and out of the nucleus, with a final location that is always the result of a very dynamic processes.

The acetylation level of HMGB1 is itself the result of a dynamic balance between two enzymatic activities (HATs and HDACs) that are highly and quickly regulatable.

Finally, in monocytes, the quick kinetics of HMGB1 secretion upon LPS is the result of the sum of all these dynamic processes occurring at all levels and in all subcellular compartments.

In perspective, HMGB1 emerges like a special archetype of highly dynamic molecules, being located in several places, where it plays different roles with different partners: in many more sense then one it is a High Mobility Protein.

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