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Chapter 28

Cell cycle regulation and functions of HMG-I(Y)

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Members of the HMG-I(Y) family of "high mobility group" (HMG) proteins are distinguished from other nonhistone chromatin proteins by their ability to preferentially recognize the structure of the narrow minor groove of A·T-sequences of B-form DNA. *In vivo* the HMG-I(Y) proteins are localized in the A·T-rich G/Q bands and in the "scaffold-associated regions" (SARs) of metaphase chromosomes. These proteins also share with some of the other "HMG box" proteins the ability to recognize non-B-form structures, such as cruciforms (four-way junctions), as well as the possessing the capacity to introduce both bends and supercoils in substrate DNAs. These characteristics, along with their ability to specifically interact with a number of known transcription factors, enable the HMG-I(Y) proteins to function *in vivo* as structural transcription factors for a number mammalian genes. The HMG-I(Y) proteins are also *in vivo* substrates for the cell cycle regulated Cdc2 kinase which phosphorylates the DNA-binding domain(s) of the protein and, as a result, decreases their substrate binding affinity. This reversible *in vivo* pattern of Cdc2 kinase phosphorylations during the cell cycle is likely to play a major role in mediating the biological function(s) of the HMG-I(Y) proteins.

INTRODUCTION

The nonhistone HMG-I(Y) "high mobility group" (HMG) proteins are of interest because they are founding members of a newly described category of nuclear proteins called "architectural transcription factors" (1) that *in vivo* appear to be involved in regulation of both chromatin structure and transcriptional activity of an increasing large number of genes. In mammalian cells the HMG-I(Y) family (not to be confused with the unrelated HMG-1 and -2 family of "HMG box" proteins (2-6, 31,32)) consists of two small isoform proteins, HMG-I (~11.9 kD) and HMG-Y (~10.6 kD), produced from a single gene by alternative mRNA splicing (5-7) plus a third member, HMG-I' (or HMG-IC) coded for by a closely related gene (8,9). *In vivo* the HMG-I(Y) proteins exhibit considerable additional heterogeneity as a result of a variety of secondary biochemical modifications (2,4,10) certain of which, e.g., reversible phosphorylations (11-13), are cell cycle correlated. The HMG-I(Y) proteins were the first fully characterized mammalian proteins demonstrated to preferentially bind, both *in vitro* and *in vivo*, to A·T-rich sequences of DNA. *In vitro* HMG-I(Y) proteins have been shown to bind to the narrow minor groove of short stretches of A·T-DNA (14-18). *In vivo* they have been immunolocalized to the A·T-rich G/Q and C bands of mammalian metaphase chromosomes (19), suggesting that they play an important role in chromosome structural changes during the cell cycle (18,20). More recently, confocal microscopic analyses and immunologicalization procedures have demonstrated HMG-I(Y) to be distributed *in vivo* along the longitudinal length of the backbone scaffolding, or "A·T-queue", of native chromosomes, including co-localization in the G/Q bands which are postulated to represent tightly coiled scaffold-associated regions (SARs) (21,22). These *in vivo* observations are consistent with the earlier demonstration that purified HMG-I(Y) proteins preferentially bind to isolated SAR fragments (23) and, in fact, effectively out-compete histone H1 for binding to such A·T-rich sequences in *in vitro* competition experiments (unpublished data, (24)).

HMG-I(Y) RECOGNIZES DNA STRUCTURE

In vitro binding experiments employing purified proteins (4,16,17,25) indicate that HMG-I(Y) recognizes the structure of certain stretches of A·T-residues rather than their particular nucleotide sequences (25). In linear duplex DNA, the affinity and specificity of such HMG-I(Y)-structural recognition is significantly influenced by both the length and sequence of the particular A·T-stretches (15,17,26) and by the "context" of flanking or adjacent nucleotide sequences (16,17,26,27). The peptide domains of the HMG-I(Y) proteins that preferentially interact with A·T-DNA have been experimentally determined and a synthetic 11 amino acid peptide (T-P-K-R-P-R-G-R-P-K-K) corresponding to a "consensus" binding domain (BD) sequence was found to footprint to the minor groove of a stretch of 5-6 bp (or about one half helical turn) of A·T-DNA in a manner similar to binding of the intact protein (25). Each HMG-I(Y) protein has

three separate BD motifs separated by stretches of flexible peptide backbone. Thus, the tandem binding of all three BDs in an HMG-I(Y) protein should occupy the minor groove of ~ 15-18 bp (or about one and a half helical turns) of contiguous A·T-residues. As illustrated in **Figure 1**, the peptide backbone of each of the three BD regions are predicted (25) to have a planar, crescent-shaped structure (referred to as the A·T- hook motif) that has general similarities to the antiviral and antitumor peptide drugs distamycin A and netropsin and to the fluorescent dye Hoechst 33258, ligands that also bind to the minor groove of A·T-rich DNA. The structural similarity of these ligands and the BD peptides of HMG-I(Y) is further supported by the striking similarity of their footprints on A·T-DNA (18) and by their competition with each other, both *in vitro* (18,25), and *in vivo* (unpublished data), for substrate binding. Recent two-dimensional ^{1}H NMR solution studies have confirmed crucial features of the proposed planar crescent shaped structure of the BD peptide's backbone (28-30) as well as demonstrating its minor groove binding (30).

Interestingly, HMG-I(Y) also has the capacity to recognize and preferentially bind to certain types of structures formed by non-A·T-rich DNA sequences, a characteristic shared with certain of the "HMG box" proteins (1,31,32). For example, it has been found *in vitro* that the HMG-I(Y) protein ((33), unpublished observations), as well as the A·T-hook motif itself (34), binds to synthetic cruciform (four-way-junction) structures in preference to linear duplex DNA molecules of identical sequence. Likewise, HMG-I(Y) recognizes and binds to non-B-

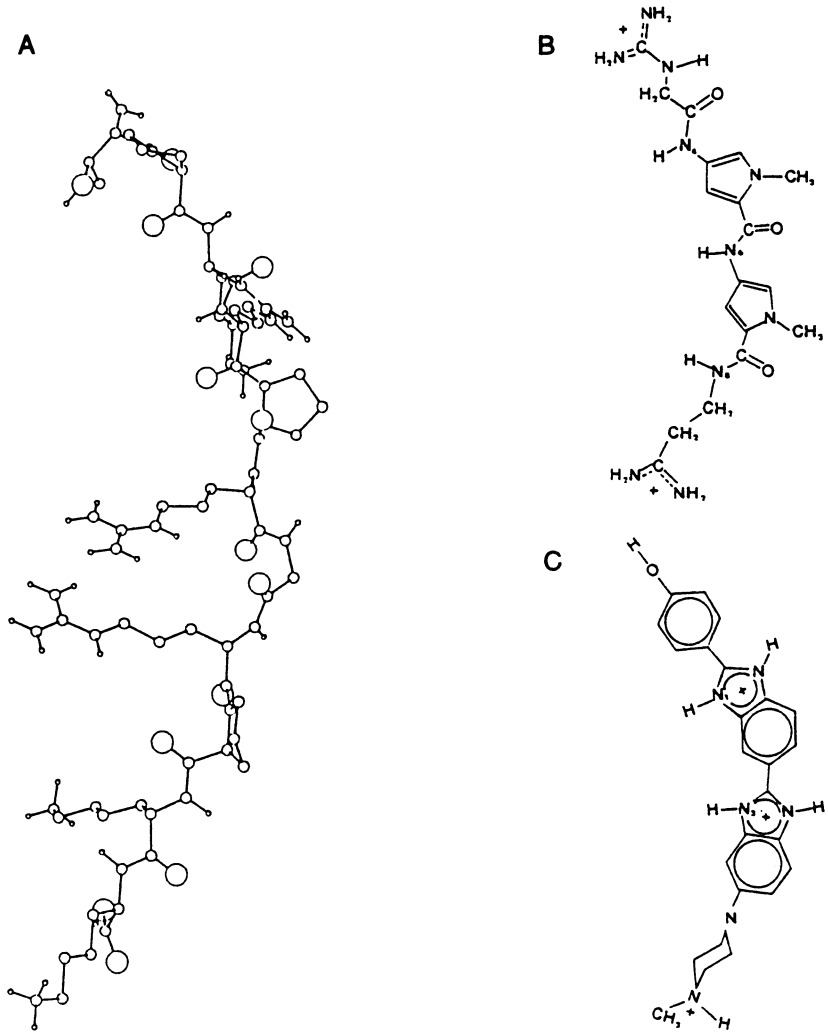

Figure 1. Comparison of the predicted planar crescent-shaped backbone structure of (A) the consensus DNA-binding domain (BD) peptide of the HMG-I(Y) family of proteins with those of minor A·T-DNA binding ligands (B) netropsin and (C) Hoechst 33258.

form DNA structures found in supercoiled plasmids (35) as well as to certain distorted regions of DNA found on isolated nucleosome core particles (36). The mode of interaction of the HMG-I(Y) BD peptides (i.e., the A·T-hook regions) with these non-B-form DNA structures is unknown. However, the inherent rotational flexibility about the glycine residue in the middle of the BD peptide allows for pliancy to adopt certain alternative, thermally-stable, backbone conformations that could potentially accommodate binding of the HMG-I(Y) proteins to such altered DNA structures (29).

HMG-I(Y) INDUCES BENDS AND SUPERCOILS IN DNA

Circular dichroism studies (37), circular permutation/electrophoretic mobility shift analyses (unpublished data) and plasmid relaxation assays (35) all suggest that HMG-I(Y) binding markedly alters DNA conformation by introducing both bends and other distortions in the substrates. Given its predicted mode of interaction with the minor groove of linear or relaxed plasmid DNAs (20,25,35), the most likely physical explanation for such protein-induced bending is by asymmetric phosphate charge neutralization (38). Nevertheless, HMG-I(Y)-mediated strand unwinding also contributes significantly to the proteins ability to introduce distortions in substrate DNAs (35). For example, the electrophoretic results shown in **Figure 2** demonstrate that binding of increasing concentrations of HMG-I(Y) to a relaxed circular plasmid (pBLT) in the presence of Topoisomerase-I, results in the introduction of increasing numbers of supercoils in the DNA. Detailed analyses of this phenomenon revealed that such supercoiling derives from a combination of both protein-induced bending and strand unwinding (35). An additional finding of considerable interest from these studies is that an *in vitro* produced mutant HMG-I protein lacking the negatively charged carboxyl-terminal domain binds A·T-DNA with about the same affinity as the full-length wild type HMG-I(Y) protein and yet is about 8-10 fold more effective at introducing negative supercoils (35). This suggests that the highly acidic C-terminal region of HMG-I(Y) may function as a regulatory domain influencing the amount of topological change induced in DNA substrates by binding of the protein.

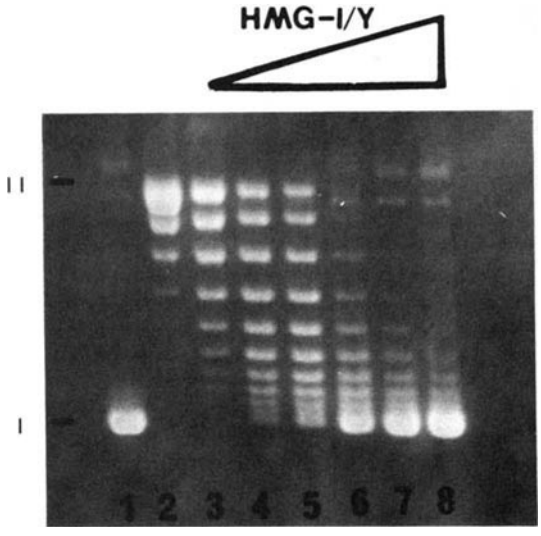

Figure 2. HMG-I(Y) proteins can induce supercoils in topoisomerase I-relaxed circular plasmid DNA. Topoisomerase-I (Topo-I) mediated relaxation assay of the pBLT plasmid in the presence of increasing concentrations of recombinant human HMG-I (35). Lanes: (1) supercoiled pBLT plasmid DNA with no added Topo-I (form I DNA); (2) Topo-I relaxed, closed circular plasmid DNA (form II) without any added HMG-I; Lanes (3)-(8), increasing concentrations of HMG-I protein added to the relaxation reactions at protein to nucleotide molar ratios of 10:1, 12.5:1, 15:1, 20:1, 25:1 and 30:1, respectively.

HMG-I(Y) PROTEINS AS STRUCTURAL TRANSCRIPTION FACTORS

The HMG-I(Y) proteins have all of the characteristics of what have become known as "architectural transcription factors" (1): [i] the ability to recognize DNA structure rather than sequence; [ii] the ability to induce bends/distortions or supercoils in DNA; and, [iii] the ability to specifically interact with other known transcription factors (see below). It is therefore not surprising, as shown in **Table 1**, that numerous reports have appeared directly implicating the HMG-I(Y) proteins in *in vivo* transcriptional regulation (either positive or negative) of mammalian genes lying in close proximity to A·T-rich promoter/enhancer sequences. The first example of *in vivo* transcriptional regulation by the HMG-I(Y) protein was reported by Fashena et al (39) in studies of the promoter region of the murine lymphotoxin (LT; a.k.a., tumor necrosis factor-β) gene that is constitutively expressed in transformed B-cell lines. Mutation and promoter deletion analyses delineated a 5' poly(dA-dT) upstream activating sequence (UAS) that was an important component of LT transcriptional activation *in vivo*. Additional experiments demonstrated that recombinant HMG-I specifically bound this UAS *in vitro* and that nuclear extracts from LT expressing mouse cells contained an HMG-I-like protein with identical UAS binding characteristics. This "reactive" nuclear extract protein was also recognized by an anti-HMG-I(Y) specific antibody. In electrophoretic mobility shift analyses (EMSA) using LT promoter DNA incubated in nuclear extracts, the antibody gave "super-shifts" of bands identical to those observed when the antibody was reacted with promoter

Table 1. Genes Proposed to be Regulated *in vivo* by HMG-I(Y) Proteins

Positive Regulation	Negative Regulation
Murine Tumor Necrosis Factor-β (TNF-β) (39)	Human Interleukin-4 (IL-4) (87)
Human Interferon-β (IFN-β) (40,41)	Murine GP91-PHOX (26)
Human IL-2 Receptor-α (IL-2Rα) (43)	Murine ε-Immunoglobulin (ε-IgG) (88)
Human E-Selectin (45)	

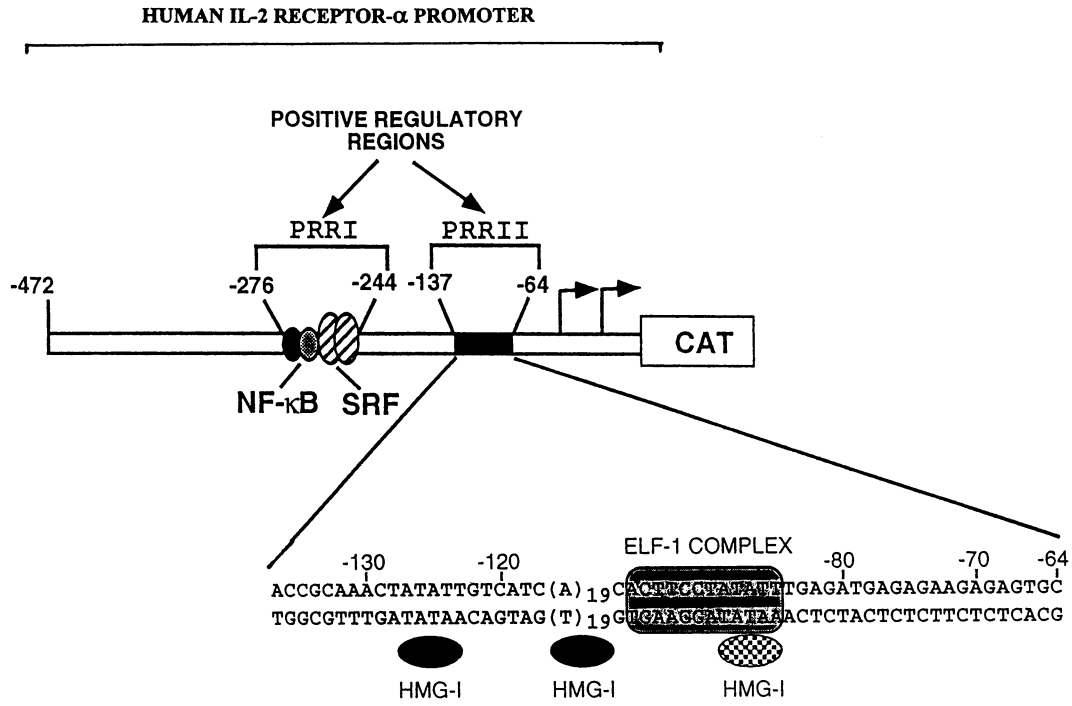

Figure 3A. Schematic diagram of the human IL-2R α gene 5' regulatory region between nucleotides -472 to +109 including the upstream and downstream positive regulatory regions (PRRI and PRRII) attached to the chloramphenicol acetyltransferase (CAT) reporter gene used for *in vivo* expression analyses. The diagram shows the binding sites for transcription factors NF-κB, serum response factor (SRF), Elf-1 and HMG-I(Y) and is redrawn (with modifications) from the article by John et al. (43).

DNA that had only recombinant HMG-I protein bound. And, finally, EMSA combined with antibody reactivity analyses revealed that at least one additional protein was present in the nuclear extracts that specifically bound to both HMG-I and to the UAS, suggesting that HMG-I (probably in combination with this unknown protein) facilitates the formation of an active transcription complex necessary for the LT gene expression (39).

Since this initial report, several additional examples have appeared documenting the *in vivo* involvement of HMG-I(Y) in the positive induction of transcription from promoters/enhancers containing A·T-rich sequences including the human genes coding for β-interferon (40,41), the α-subunit of the interleukin-2 receptor (43) and E-selectin (45). Examples are also known of instances where HMG-I(Y) binding to promoter regions appears to be involved in negative transcriptional regulation (**Table 1**). So far, all of the reports documenting an *in vivo* role for HMG-I(Y) in positive gene regulation have suggested that the protein probably functions by both bending DNA and by directly interacting with other transcription factors to facilitate the formation of a multiprotein complex than brings together upstream promoter/enhancer elements and the basal transcription apparatus during transcription induction. For example, during their investigations

of the mitogen-inducible expression of the gene coding for the α-subunit of the IL-2 receptor (IL-2Rα) in human T cells, John et al (43) identified and characterized a new positive regulatory region (PRRII) in the gene's promoter (between nucleotides -137 to -64) that binds both HMG-I(Y) and the lymphoid cell-specific transcription factor Elf-1. Cell transfection experiments demonstrated that transcription of the IL-2Rα promoter (ligated to the reporter gene chloramphenicol acetyltransferase (CAT)) **(Figure 3A)** was inhibited when either the Elf-1 or the HMG-I(Y) binding sites were mutated. Furthermore, coexpression of both Elf-1 and HMG-I(Y) proteins in transfected COS-7 cells activated transcription from a truncated promoter containing only the PRRII element. Previous experiments had identified an inducible promoter sequence (PRRI) farther upstream of the transcription start site (between nucleotides -276 and -244) that contained binding sites for two additional transcription proteins, serum response factor (SRF) and NF-κB. Coimmunoprecipitation experiments, as well as EMSA super-shift assays, employing specific antibodies against each of the various components of the system, demonstrated that Elf-1 is physically associated with HMG-I(Y) and also with NF-κB p50 and c-Rel *in vitro*. These findings strongly suggest that protein-protein associations functionally coordinate the interactions of the upstream (PRRI) and the downstream (PRRII) positive regulatory elements leading to the formation of a stereospecific protein complex necessary for inducible IL-2Rα gene expression **(Figure 3B)**.

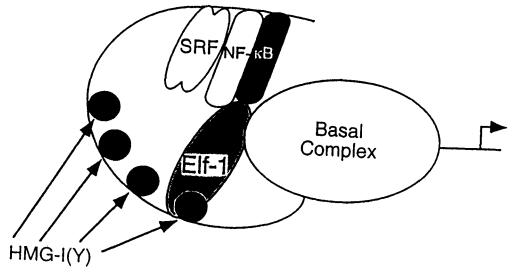

Figure 3B. Model of the human IL-2Rα gene promoter after mitogen stimulation showing the interactions of the lymphoid-specific transcription factor Elf-1 with both HMG-I(Y) and NF-κB to form a hypothetical stereospecific, inducible transcription complex with the basal transcription apparatus. Redrawn with modifications from (43).

Additional examples supporting the *in vivo* involvement of HMG-I(Y)-mediated protein-protein interactions in the formation of active transcription initiation complexes come from studies of the inducible human interferon-β (IFN-β) and E-selectin (45,46) genes. The laboratory of Maniatis (40,41,46) has demonstrated *in vivo* that HMG-I(Y) plays a causal role in the virus-induced expression of the IFN-β gene. Induction is dependent on the simultaneous binding of HMG-I(Y) and the transcription factors NF-κB and ATF-2 to two separate "positive regulatory domains" (PRDII and PRDIV) sites in the gene's 5' enhancer region. Importantly, in free solution HMG-I(Y) was demonstrated to interact directly with both NF-κB and ATF-2, thereby significantly increasing the binding affinity of both of these transcription factors for their cognate DNA recognition sites. As anticipated, HMG-I(Y) bound to the minor groove, whereas NF-κB and ATF-2 bound to the major groove, of substrate DNA and thus did not present any steric impediments to each other's binding. As in those systems described above, the proposed function of HMG-I(Y) in this situation is as a mediator for the assembly of a multiprotein complex (including NF-κB, ATF-2 and c-Jun) on the IFN-β enhancer that is obligatory for *in vivo* virus-induced transcriptional activation. Interestingly, in this experimental system HMG-I(Y) can either stimulate or inhibit the binding of different ATF-2 isoform proteins to the IFN-β promoter depending on whether not these variant transcription factors contain a short stretch of basic amino acid residues near their basic-leucine zipper domains that is required for HMG-I(Y) binding (46). This differential interaction of HMG-I(Y) with alternative ATF-2 isoforms determines whether a functional ATF-2 dimer molecule is formed that is capable of enhancer binding and thus, by inference, regulates whether an inducible transcription factor complex is formed on the IFN-β promoter *in vivo*.

ABERRANT EXPRESSION AND REGULATION OF HMG-I(Y) GENES IN CANCER CELLS

Given the persuasive experimental evidence that HMG-I(Y) functions as a transcription factor *in vivo*, it is not surprising that a number of laboratories have observed a striking correlation between abnormally high levels of expression of the HMG-I(Y) gene and the cancerous transformation and/or increased metastatic potential of tumor cells. HMG-I(Y) proteins and mRNAs are expressed at only very low levels in normal cells (4-6,47,48). In contrast, cancerous cells (3,8,47,49-52) and embryonic cells that have not yet undergone overt differentiation (53) often contain exceptionally high concentrations of HMG-I(Y) gene products. For example, either spontaneously derived tumors or normal cells experimentally transformed by treatment with chemicals, ionizing radiation, UV, or viral oncogenes (*v-src, v-ras, v-mos, v-myc*) have all been reported to contain abnormally high levels of HMG-I(Y) proteins and mRNAs (4-6,8,50-52).

Significantly, the elevated HMG-I(Y) levels in tumors are independent of cellular growth rates since untransformed normal cells proliferating at about the same rate as their transformed counterparts consistently contain much lower levels of HMG-I(Y) gene products than do the malignant cells (8,49,50). Johnson et al. (5,6,47) have estimated that certain cancer cell lines constitutively contain 15 to >>50 times the level of HMG-I(Y) mRNAs found in non-transformed normal cells. Levels of total HMG-I(Y) mRNA vary with the rate of cell proliferation, being relatively low in nonproliferating or quiescent cells and increasing during exponential cellular growth. For example, there is about a four-fold increase in mRNA concentration in partially synchronized mouse NIH3T3 cells during the transition from quiescence to active proliferation (47). Nevertheless, superimposed on this usual increase of mRNAs in dividing *vs* non-dividing cells, the observation has been consistently made that there is a much higher level of HMG-I(Y) mRNA expression in proliferating malignant cells than in normal cells replicating at about the same rate.

Indeed, the correlation between cancerous transformation and high constitutive levels of HMG-I(Y) gene products is so striking that Goodwin et al. (8,51,52) have suggested that these elevated concentrations are a characteristic and diagnostic feature of the transformed cellular phenotype. Schalken's laboratory (49) has also identified increased levels of HMG-I(Y) mRNAs as a progression marker for prostate cancer metastasis in the Dunning rat model system demonstrating that the extent of HMG-I(Y) over-expression directly correlates with the degree of metastatic aggressiveness of the tumors. More recent studies by this same group have extended these findings to human prostate cancers in a retrospective *in situ* RNA hybridization study of HMG-I/Y mRNA levels in paraffin-embedded materials obtained from patients presenting different Gleason grades of metastatic prostate cancer (54). Similar correlations also suggest that increased levels of HMG-I(Y) mRNA are a reliable biochemical marker for tumor progression from preneoplastic to highly aggressive metastatic cancers in a well characterized mouse mammary epithelial cell system (50). The reverse situation also appears to be true, namely that when undifferentiated, highly aggressive tumor cells are induced to undergo overt cellular differentiation they lose both their high constitutive levels of HMG-I(Y) gene products and their *in vivo* tumorigenic potential (53).

Specific chromosome translocations are frequently found in human lymphomas and leukemias (42,55) and recently the human mixed-lineage leukemia (MLL) gene (56) (also called ALL-1 (57) or HRX (58)) involved in number of such rearrangements has been isolated and sequenced. Significantly, the N-terminal region of the MLL (ALL/HRX) gene was found to code for an amino acid sequence almost identical to the "A·T-hook" DNA-binding motif of the HMG-I(Y) proteins and it is this region of the gene that is frequently translocated in a large number human leukemias (55-58). These findings raise the intriguing possibility that in certain human cancers, chromosomal translocation and fusion of an A·T-hook-like motif to a new cellular protein may convert the resulting hybrid into a transforming oncoprotein as a result of DNA mistargeting. Support for such a scenario has recently been provided by the demonstration that the A·T hook peptide motif found in the MLL gene, the translocation of which is involved in several human cancers (reviewed in (55)), is capable of specifically binding to both A·T-rich sequences and to cruciform DNA structures *in vitro* (34).

CELL CYCLE PHOSPHORYLATION OF HMG-I(Y) BY CDC2 KINASE

Along with histone H1, the HMG-I(Y) proteins are among the most highly phosphorylated proteins in the nucleus and the extent of such phosphorylation is cell cycle dependent (4,10-13,59,60). In mammals the extensive phosphorylation of histone H1 that occurs in proliferating cells during mitosis (61-63) is catalyzed by "growth associated histone H1 kinase" (63,64), an enzyme homolog of the yeast cyclin-dependent kinase (cdk) $p34^{cdc2/CDC28}$ kinase (a.k.a., Cdc2 kinase). Cdc2 kinase, together with its positive regulatory subunit cyclin B, forms the heteromeric maturation-promoting factor (MPF), the apparent "universal regulator" of mitosis and cell cycle progression in all eukaryotic cells (65-68). Cyclin-activated Cdc2 kinase phosphorylates serine or threonine residues within the consensus sequence Ser/Thr-Pro-(Xaa)-Lys/Arg, where the presence of Xaa (usually a polar residue) is variable (69,70). Inspection of the "consensus" DNA-binding domain sequence of mammalian HMG-I(Y) proteins (see above) suggests that the threonine at the amino-terminal end of the BD peptide (***T-P-K**-R-P-R-G-R-P-K-K) is a potential Cdc2 kinase phosphorylation site. There is an important difference, however, between the sequence of this "consensus" BD peptide and the actual amino acid sequences found at the N-terminal ends of several of the various BD peptides present in native human and mouse HMG-I(Y) proteins (5-7). For example, of the three BD peptides found in the human HMG-I protein, only two have potential Cdc2 phosphorylation sites (at residues Thr^{53} and Thr^{78}) and of the three BDs found in the murine protein, only one has a potential phosphorylation site (at residue Thr^{53}).

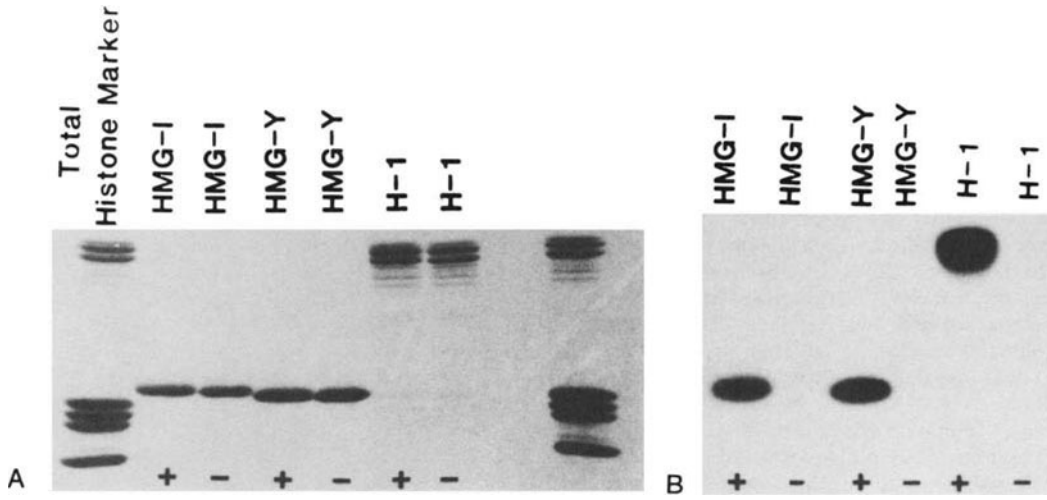

Figure 4. ***In vitro*** **phosphorylation of HMG-I, HMG-Y and histone H1 by Cdc2 kinase.** Purified rat Cdc2 kinase was used to phosphorylate the indicated proteins with (+) or without (-) the addition of kinase. (A) Coomassie-stained SDS/PAGE gel. (B) Autoradiogram of (A). Reproduced from (11) with permission.

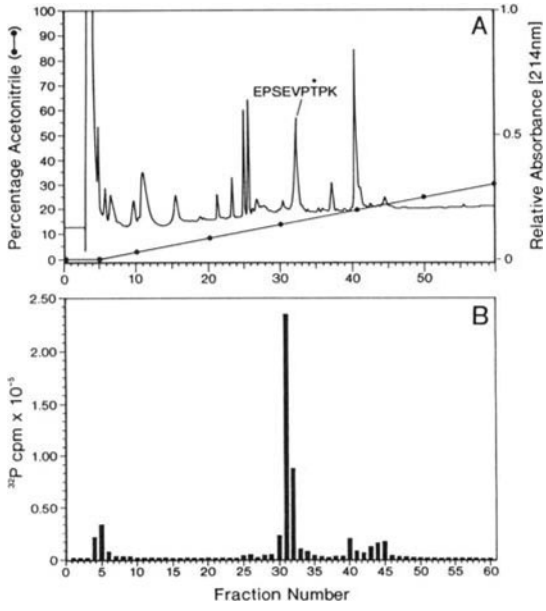

Figure 5. Tryptic peptide analysis of ***in vitro*** **Cdc2 kinase ^{32}P-labeled murine HMG-I protein.** Tryptic peptides were separated by RP-HPLC (A) and counted for radioactivity (B). The indicated ^{32}P-labeled peptide was sequenced, and the site of *in vitro* Cdc2 phosphorylation was determined (*). This phosphorylation site corresponds to threonine residue 53 at the amino-terminal hook end of the principal DNA-binding domain of the murine HMG-I protein. Reproduced from (11) with permission.

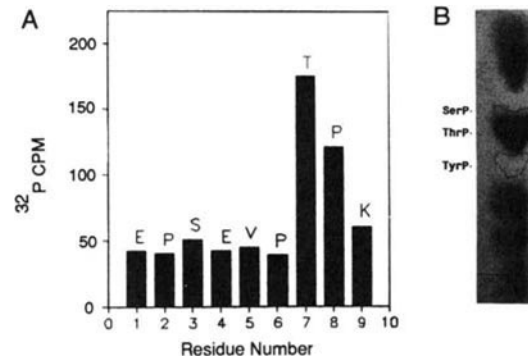

Figure 6. Phosphoamino acid analysis of murine HMG-I phosphorylated by Cdc2 kinase ***in vitro*****.** (A) Histogram of ^{32}P-labeled amino acids (in one letter code) released during the automated sequencing of the *in vitro*-labeled peptide shown in Fig. 5. (B) Acid hydroylosate of Cdc2 kinase ^{32}P-labeled murine HMG-I protein and the resulting amino acids and peptide fragments separated by thin layer chromatography. [^{32}P]-phosphoamino acids (as well as peptide fragments and free inorganic phosphate) were visualized by autoradiography to confirm the Cdc2 kinase phosphorylation of threonine residues in the murine protein. Dashed lines show the position of carrier phosphoaminoacids stained with ninhydrin. Reproduced from (11) with permission.

Nevertheless, as predicted from sequence analysis, activated Cdc2 kinase enzymes isolated from mammalian cells (11), as well as from starfish oocytes and sea urchin eggs (13), have been demonstrated to efficiently phosphorylate both human and murine HMG-I and HMG-Y proteins *in vitro* (**Figure 4**). Separation (**Figure 5**) and microsequencing (**Figure 6**) of the tryptic peptic fragments of *in vitro* enzymatically ^{32}P-phosphorylated murine HMG-I protein confirmed that the predicted single threonine residue (Thr^{53}) at the N-terminal end of one of the A·T-hook BD-peptides is indeed phosphorylated by Cdc2 kinase. Furthermore, *in vivo* [^{32}P]phosphate-labelling studies of partially synchronous cultures of mouse cells demonstrated that this same Thr^{53} residue is radiolabeled in HMG-I isolated from metaphase (but not from G_1, S or nonproliferating) cells (11). Similar *in vivo* studies in human cells have likewise demonstrated that, as expected, both Thr^{53} and Thr^{78} are phosphorylated in mitotic, but not in nondividing, cells (13). Together these results clearly indicate that the mammalian HMG-I(Y) proteins are *in vivo* substrates for Cdc2 kinase and that the extent of phosphorylation of the DNA-binding domains varies in a cell cycle-dependent manner.

The biological role played by Cdc2 kinase phosphorylation of HMG-I(Y) proteins is unknown. However, as shown in **Figure 7**, phosphorylation of two of the three conserved threonine residues in the DNA-binding domains of recombinant human HMG-I by purified Cdc2 kinase greatly reduces the *in vitro* binding affinity (~ 20-fold at physiological ionic strength; Kd= 320 nM *vs* 16 nM) of the intact protein for A·T-rich DNA (12). Somewhat surprisingly, *in vitro* mutagenesis studies revealed that replacement of the conserved Cdc2 kinase-modifiable threonine residues in the recombinant human protein (i.e., Thr^{53} and Thr^{78}) with non-phosphorylatable alanine residues did not markedly change the DNA-binding affinity of the doubly mutant protein from that of the non-phosphorylated wild type HMG-I protein (71). Therefore, the observed weakening of DNA-binding affinity of the Cdc2 kinase-phosphorylated wild type HMG-I(Y) is most easily explained as a consequence of negative charge repulsion between the phosphates of the backbone of the DNA substrate and those of the enzymatically modified protein (20,71). The threonines on the N-terminal ends of the BD peptides are thus postulated to be "regulatory residues" for modulating DNA binding affinity as a result of reversible phosphorylation by Cdc2 kinase (71). Such marked modulations of DNA-binding affinity by Cdc2 kinase phosphorylation might reasonably be expected to have significant physiological effects on the *in vivo* function(s) of the HMG-I (Y) proteins during the cell cycle (20,71).

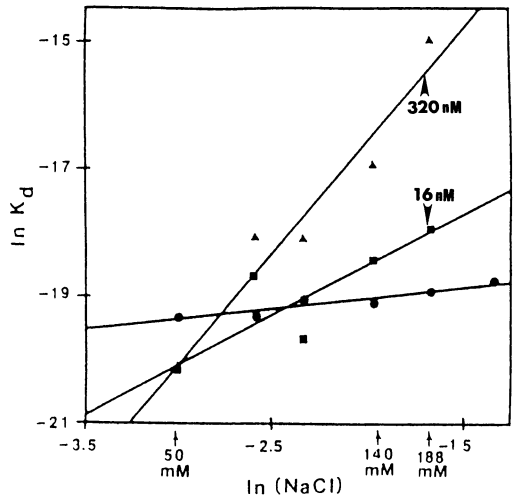

Figure 7. Ionic strength dependence of binding of unphosphorylated and Cdc2 kinase-phosphorylated recombinant human HMG-I to A·T-rich DNA. The ln Kd of HMG-I binding to substrate is plotted as a function of increasing concentrations of salt, ln [NaCl]. The Kd values of Hoechst 33258 dye (●), unphosphorylated HMG-I (■), and Cdc2-phosphorylated HMG-I (▲) were determined at increasing NaCl concentrations from 50 to 188 mM. At 188 mM NaCl there is a difference of ~20-fold in the binding strength of the unphosphorylated protein (Kd=16 nM) compared to the Cdc2-phosphorylated HMG-I protein (Kd= 320 nM). Reproduced from (12) with permission.

HMG-I(Y), HISTONE H1 AND THE OPENING OF CHROMATIN DOMAINS

What are the possible *in vivo* biological functions of Cdc2 kinase-induced, cell cycle-dependent variations in HMG-I(Y) phosphorylation levels and substrate binding affinities? The answers may at least partially lie with the *in vivo* requirements for the chromatin structural changes occurring during the mitotic condensation and decondensation of chromosomes (18,20,72). As previously noted, the HMG-I(Y) proteins have been immunolocalized *in vivo* to the G/Q- and C-bands (19), as well as to the longitudinal, SAR-containing, "A·T queue" backbone structures (21,22) of metaphase chromosomes. As a consequence of Cdc2 kinase activity, it is precisely in such metaphase chromosomes that the HMG-I(Y) proteins are maximally phosphorylated and probably also most loosely bound to substrate DNA. Like HMG-I(Y), the H1 histones are also specific *in vivo* substrates for Cdc2 kinase (62,64,69) and are maximally phosphorylated during mitosis (61,64). These H1 phosphoproteins, likewise, also have a substantially reduced DNA-binding affinity and

yet still remain associated with metaphase chromosomes (73,74). Nevertheless, both histone H1 and HMG-I(Y) appear to be necessary for chromosome condensation since distamycin (which displaces both H1 (75) and HMG-I(Y) (18,76) from SARs and other A·T-rich sequences) induces chromatin fibers to become substantially less compacted *in vitro* (77). *In vivo* distamycin causes a marked decondensation of centromeric heterochromatin and a considerable metaphase chromosome elongation in treated cells (72,78). Therefore, one plausible function of the Cdc2 kinase phosphorylation of both histone H1 and HMG-I(Y) during mitosis is to loosen (but not completely dissociate) the attachment of these normally tightly bound proteins in order to facilitate other processes that directly lead to chromosome condensation, for example by allowing association of the hyperphosphorylated tails of H1 and HMG-I(Y) with other chromatin proteins that are causally involved in compaction (20).

Another likely function of the cyclic phosphorylation of HMG-I(Y) relates to the *in vivo* role of the protein as a transcription factor and its relationship to histone H1. Like the BD peptides of HMG-I(Y), the peptide tails of H1 histones preferentially bind to narrow DNA minor grooves (reviewed in (79)). Furthermore, HMG-I(Y) competes with histone H1 for binding to A·T-rich sequences *in vitro*. If either histone H1 (80,81) and/or nucleosomes (reviewed in (82-84)) bind to gene promoter/enhancer regions, transcription of the associated gene by RNA polymerase is greatly inhibited or completely repressed. It was therefore speculated (20) that a probable *in vivo* function of the HMG-I(Y) proteins would be to act as antirepressor molecules (80,81) that compete with, or displace, inhibitory histone H1 and/or nucleosomes binding to A·T-sequences and thus help to establish an open or accessible chromatin structure in crucial DNA regulatory regions. Once such an "open" chromatin structure has been established by HMG-I(Y) binding, this accessible configuration would then be faithfully propagated from one cellular interphase to the next as both HMG-I(Y) and histone H1 change their Cdc2 kinase-induced phosphorylation levels, and hence their relative DNA-binding strengths, in a coordinated manner during mitosis.

Considerable support for the above scenario has recently come from the demonstration by Laemmli's laboratory (24) that HMG-I(Y) not only acts as an antirepressor molecule by preventing histone H1 binding to SAR sequences, but it also functions as a true derepressor by displacing previously bound H1 proteins thereby relieving histone H1-mediated repression of transcription of reporter genes *in vitro* (24). Based on the ability of HMG-I(Y) to function as a derepressor molecule, these workers proposed a model for the involvement of both SARs and HMG-I(Y) in establishing the overall pattern of both inactive and transcriptionally competent "open" or active chromatin domains during cellular differentiation (24,75). In this model, inactive chromosome loops or domains (85,86) are thought to be compacted and stabilized by "nucleating" histone H1 molecules that initially bind tightly to A·T-rich SAR sequences located at the base of the loops. Following this H1 nucleation event subsequent cooperative H1-H1 protein interactions, "spread" their inhibitory influence throughout a topologically defined chromatin region or domain. These compact, H1-containing domains remain transcriptionally inactive until HMG-I(Y) (or another "D" or distamycin-like protein) binds to the SARs and "mobilizes" histone H1: i.e., HMG-I(Y) binding is proposed to interfere with the ability of SARs to serve as nucleation sites for cooperative histone H1 assembly (75). As a consequence of HMG-I(Y) binding, the equilibrium of histone H1 association is shifted towards a reduction in occupancy of nucleosome linker regions in the chromatin domain thus resulting in its "opening" into a transcriptionally competent or active region (24,75). The biological controls that regulate the patterns and specificity of repressed and open chromatin domains are unknown, but once having been established they are quite stable and are faithfully propagated from one cell generation to the next. If, during cellular differentiation, the histone H1 and HMG-I(Y) proteins are indeed involved in the initial establishment of active and repressed chromatin states, the coordinated and reversible phosphorylation of these proteins during the cell cycle by Cdc2 kinase may well play a dominant role in the maintenance of these structural features as cells transit through mitosis.

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