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Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene

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ABSTRACT

Members of the HMG-I(Y) family of mammalian nonhistone proteins are of importance because they have been demonstrated to bind specifically to the minor groove of A·T-rich sequences both *in vitro* and *in vivo* and to function as gene transcriptional regulatory proteins *in vivo*. Here we report the cloning, sequencing, characterization and chromosomal localization of the human HMG-I(Y) gene. The gene has several potential promoter/enhancer regions, a number of different transcription start sites and numerous alternatively spliced exons making it one of the most complex nonhistone chromatin protein-encoding genes so far reported. The putative promoter/enhancer regions each contain a number of conserved nucleotide sequences for potential binding of inducible regulatory transcription factors. Consistent with the presence of these conserved sequences, we found that transcription of the HMG-I(Y) gene is inducible in human lymphoid cells by factors such as phorbol esters and calcium ionophores. Detailed sequence analysis confirms our earlier suggestion that alternative splicing of precursor mRNAs gives rise to the major HMG-I and HMG-Y isoform proteins found in human cells. Furthermore, the gene's exon-intron arrangement fully accounts for all of the previously cloned human HMG-I(Y) cDNAs (1,2). Also of considerable interest is the fact that each of the three different DNA-binding domain peptides present in an individual HMG-I(Y) protein is coded for by sequences present on separate exons thus potentially allowing for exon 'shuffling' of these functional domains during evolution. And, finally, we localized the gene to the short arm of chromosome 6 (6p) in a region that is known to be involved in rearrangements, translocations and other abnormalities correlated with a number of human cancers.

INTRODUCTION

The mammalian HMG-I(Y) family of 'high mobility group' (HMG) chromosomal proteins is composed primarily of the isoform proteins HMG-I and HMG-Y (1,2) and the closely related HMGI-C (HMG-I′) nonhistone protein (3). Members of the HMG-I(Y) family are distinguished from other groups of HMG proteins (4) by their ability to specifically bind in the narrow minor groove of A·T-rich sequences of DNA *in vitro* (5–10). The peptide domains of the HMG-I(Y) proteins that specifically interact with A·T-sequences, the so-called 'A·T-hook motifs', have been determined (10) and results from recent two-dimensional ^{1}H NMR studies (11) are consistent with the original crescent-shaped model predicted for the backbone of these DNA-binding domain (BD) peptides (10). Furthermore, the A·T-minor groove binding ligands distamycin, netropsin and Hoechst 33258 (which also have planar crescent shapes) have been shown to interact with similar DNA sequences as the HMG-I(Y) proteins and to compete efficiently with these proteins for substrate binding (9), providing additional support for the proposed BD peptide structure.

In vivo the HMG-I(Y) proteins have been immunolocalized to the A·T-rich G/Q and C bands of mammalian metaphase chromosomes (12), suggesting they may play an important role in chromosome structural changes during the cell cycle cycle (9,12,13). Cell cycle-dependent p34^{cdc2}-like kinases have been shown to phosphorylate the DNA-binding domains of HMG-I(Y) proteins both *in vitro* and *in vivo* (14–17). Phosphorylation by these kinases has been demonstrated to greatly reduce the substrate-binding affinity of the HMG-I(Y) proteins *in vitro* (15,16). Such alternations of DNA-binding affinity may well serve as an important regulatory mechanism for modulating the function(s) of these nonhistone proteins during the cell cycle (12).

In addition to their possible role as chromatin structural components, considerable interest in the HMG-I(Y) proteins has been generated by recent publications suggesting that they may also function as general transcription regulatory factors for several

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genes *in vivo* (18–20). Given their potential *in vivo* function as gene regulatory proteins it is perhaps not entirely surprising that numerous earlier reports have documented a striking correlation between elevated levels of HMG-I(Y) gene products and both neoplastic cell transformation (1–3,21–24) and metastatic tumor progression (25,26). In this regard, a number of human genes have recently been identified that contain amino acid sequences with similarity to the A·T-hook domains of the HMG-I(Y) proteins and these conserved protein regions have been found to be rearranged by chromosomal translocations in certain acute leukemias (27).

Here we report the cloning, sequencing, characterization and chromosomal localization of the human HMG-I(Y) gene. The structure, organization and transcriptional expression of the HMG-I(Y) gene is one of the most complex reported for any mammalian nonhistone chromosomal protein. These findings are a first step toward elucidation of the intricate molecular mechanisms regulating expression of the HMG-I(Y) gene in both normal and cancerous cells.

MATERIALS AND METHODS

Cloning of an intron-specific probe using the polymerase chain reaction (PCR)

An intron specific probe was isolated following the strategy outlined by Davies et al. (28). PCR amplifications were carried out in 100 μl volumes containing 2 μg human placental genomic DNA (Clonetech), 50 mM KCl, 10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM (each) dATP, dCTP, dGTP, and dTPP, 50 pmoles of each primer, and 2.5 units of Taq polymerase (Promega). The primers chosen were: (#1) 5′TGAGTCGAGCTCGAAGTCCAGCCA3′ (sense' corresponding to genomic nucleotides (nt) # 3902–3925 of this paper; (nt# 6–29 of HMG-I cDNA in Fig. 3 of Johnson et al., [2]); and, (#2) 5′GGTCTCTTAGGTGTTGGCACTTC3′ (antisense, corresponding to genomic nt # 5844–5866, [nt# 148–170 in Fig. 3 of Johnson et al., [2]). All PCR reactions were carried out in a programmable DNA Thermal Cycler (Perkin Elmer Cetus) and consisted of 30 cycles of 1 min at 94°C, 2 min at 55°C, and 4 min at 72°C followed by one final extension for 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis on 1% gels, excised and purified using the Geneclean glassmilk procedure (BIO 101). The identity of the PCR-generated intron fragment was confirmed both by sequencing and by Southern Blotting to both HMG-I cDNA and to a synthetic oligonucleotide spanning nt # 3999–4031 (nt# 103–135 in Fig. 3 of Johnson et al., [2]) in the cDNA which would be expected to be immediately 5′ adjacent to the amplified intron sequence. The ends of the duplex PCR fragment were filled in using Klenow fragment DNA polymerase (Boehringer Mannheim) and subcloned into the *Sma I* site of pBluescriptSK(–). The DNA sequence of the probe ends was determined from either single- or double-stranded DNA using the Sequenase kit (U.S. Biochemicals). In order to generate a probe containing only intron sequence, primers were synthesized corresponding to sequences at the ends of the intron fragment. These were: (#3) 5′CCTTTGCTTCACTTGGTTTACCC3′ (sense, corresponding to genomic nt # 4045–4067, this paper); and, (#4) 5′GGTAGTCAGGGACAGTCATCAC3′ (antisense, corresponding to genomic nt #5794–5815, this paper) and used in a PCR reaction to amplify an intron-specific probe termed INTRON.

Screening of the human genomic library

A human male Caucasian placenta genomic library, (Stratagene, cat. # 946203) was screened with the nick-translated PCR-generated intron specific-probe INTRON and with the previously isolated HMG-I6A cDNA (2). Four positive clones were isolated from approximately 5×10^5 plaques. Restriction analysis showed three clones to be identical and one of these, clone 2, was subjected to further analysis. DNA fragments were excised with *Xba* I or *Not* I and subcloned into pBluscript SK(–) cloning vector and grown in *Escherichia coli* XL-1/BLUE cells.

Southern blot hybridization

Plasmid DNA was prepared from the genomic subclones and subjected to restriction endonuclease digestion analysis. The resulting fragments were separated on agarose gels and transferred to Nytran nylon membrane (Schleicher & Schuell) according to Ausubel et al. (29). In order to establish a restriction map of the various regions of the gene structure, the hybridizations were carried out using either an HMG-I cDNA probe, the intron-specific probe, or a synthetic oligonucleotide containing a 5′ untranslated region (UTR) sequence common to all the previously cloned human cDNAs (2).

Nucleotide sequencing analysis

The *Xba* I subclones were further fragmented by digestion with either *Xho* I, *Eco* RI, *Pst* I or *Sac* I. These smaller fragments were all subcloned into pBluescriptSK(–) and their nucleotide sequence determined using nested deletions and dideoxy sequencing (29). Each fragment was digested with the appropriate 3′ and 5′ overhang restriction enzyme cutters that did not cut within the fragment and subjected to Exonuclease III digestion for 30 sec intervals. The fragments were then treated with S1 Nuclease for 30 min at 22°C, blunt-ended with Klenow fragment DNA polymerase, religated and grown in *Escherichia coli* XL-1/Blue host. The majority of sequences were determined by a modified dideoxy, alkaline-denatured double stranded DNA method (30). For regions rich in G·C residues or secondary structure, the chemical sequencing method of Maxam and Gilbert (31) was employed.

Statistical analysis of exons containing DNA-binding domains (BDs)

As outlined in Results, we observed that each of the three individual HMG-I(Y) DNA-binding domains (BDs) were contained on separate exons. To examine the possible relationship between exons containing different BDs, the three human HMG-I(Y) gene BD exons were aligned with each other and with the corresponding putative BD exons from the mouse HMG-I(Y) (1) and HMGI-C (3) cDNAs. For the mouse cDNAs (1), likely intron/exon boundaries were predicted by comparison with homologous regions and sequences of the human HMG-I(Y) gene. The sequence alignment was done by anchoring the highly conserved Arg-Gly-Arg-Pro core of the DNA-binding domains, and then using the Pileup program from the University of Wisconsin's Genetic Computer Group (GCG) Sequence Analysis Software Package version 7.2 (32) with gapweight = 1.0 and gaplengthweight = 0.3. The region corresponding to the shortest of the nine exons was then run through the GCG Plotsimilarity program. To determine the significance of the observed degree of similarity, each of the nine sequences was randomized and the Plotsimilarity program repeated; this was done 100 times.

Pairwise comparisons between exons containing DNA-binding domains were also performed using the GCG Gap program.

Chromosomal localization of the human HMG-I(Y) gene

A panel of somatic cell hamster-human hybrids (I-7, 636−46 and 3.1.0.A; ref. 33) and radiation hybrids (R3, R10, R24, R46, R61, R73 and R82 and R86; ref. 34) which retained various fragments of the short arm (6p) of human chromosome 6 was used to regionally locate the position of the authentic HMG-I(Y) gene. DNA from these radiation hybrid cell lines was used as a template for PCR amplifications using primers (2) and (3) above. The PCR amplifications were carried out essentially as described earlier except that in certain cases 35 amplification cycles were performed. Aliquots of the PCR amplifications were separated by electrophoresis on 1% agarose gels and transferred to a nylon membrane (Genescreen, NEN). The blots were hybridized to a 701 bp *Pst* I-*Pst* I fragment (nt #4927−5628) corresponding to intron #5 sequence lying within the region amplified by the PCR reactions, and washed in 0.1×SSC, 0.1% SDS at 67°C. The presence of the human HMG-I(Y) gene was determined by the specific hybridization of a 1.8 kb fragment corresponding to intron #5.

Cell culture, RNA isolation and Northern hybridization

The human K562 erythroleukemic cell line (American Type Culture Collection; ATTC #CCL243) and the human HUT-78 lymphoid cell line (ATCC # TIB161)were maintained in culture at a density of ~1−4×10^6 cells/ml in RPMI medium (Flow Laboratories, Inc., McLean, VA) supplemented with either 5% calf serum (K562) or 5% fetal calf serum (HUT-78) (both obtained from Tissue Culture Biologicals, Tulare, CA) and antibiotics (100 μg/ml each of penicillin-G and streptomycin). Where indicated, cultured cells at a density of 4×10^6/ml were stimulated with either 10 ng/ml 12-myristate 13-acetate (PMA; Sigma Co., St. Louis, MO) or with 10 ng/ml PMA plus 1 μM ionomycin (Sigma Co.). Cell samples (1×10^8 cells) were collected at various times after chemical stimulation and RNA isolated using the guanidinium isothiocyanate/cesium chloride method (29). The RNA pellet was resuspended, quantified and employed for Northern blot hybridization analysis as previously described (35). Equivalent loading of RNA was determined by ethidium bromide staining of ribosomal RNA and by detection of actin or β-tubulin mRNA on Northern blots as described by Wingett et al (35). Quantitation of transcript levels was determined by densitometric analysis of autoradiographs using an LKB XL Laser Densitometer (LKB AB, Sweden).

RESULTS

Isolation of the HMG-I(Y) genomic clone

Owing to the existence of a number of related DNA sequences in the human genome, early attempts to clone the human HMG-I(Y) gene were unsuccessful and resulted in the isolation of a number of processed pseudogenes (2). Consequently, in order to isolate the authentic human HMG-I(Y) gene the strategy used by Davies et al. (28) was followed to isolate an intron-containing gene in the presence of several intronless pseudogenes. Based on the sequence and structure of a number of isolated cDNAs, we previously proposed the existence of an intron at a specific position in the authentic human HMG-I(Y) gene where alternative splicing occurs to produce HMG-I and HMG-Y isoform mRNAs (2). PCR was carried out on human placental DNA using primers flanking this predicted intron and an amplified DNA product of about 2 kb was produced (data not shown). Sequencing of the ends of the 2 kb amplified product showed the expected HMG-I(Y) cDNA sequences flanking the intron. From the deduced intron sequence, new primers were synthesized and used in a PCR reaction to amplify a fragment containing most of the 2 kb intron sequence while lacking any cDNA sequence. This intron-specific fragment, in conjunction with the HMG-I cDNA, was subsequently used to screen a human genomic library and clone the authentic human HMG-I(Y) gene. Four positive clones were isolated (out of 5×10^5 plaques), and three were shown to be identical by restriction endonuclease analysis. The fourth clone appeared to have a similar restriction map to the others but apparently also contained an additional 1 kb in the 3′ direction. One of the three identical clones was chosen for complete sequence analysis.

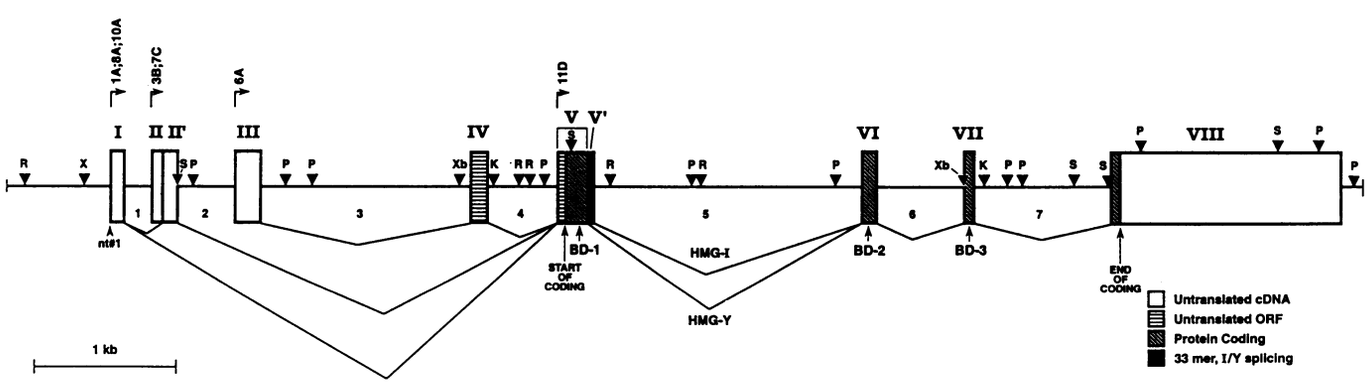

Figure 1. Diagram of the human HMG-I(Y) gene showing patterns of transcript initiation and alternative splicing. The human HMG-I(Y) gene is greater than 10 kb in length and contains eight exons (Roman numerals I-VIII) and seven introns (Arabic numbers #1−7). Nucleotide #1 indicates the start of exon I and arrows show putative transcription start sites that correspond to the beginning of previously cloned cDNAs coding for HMG-I(Y) mRNAs (ref. 2). Solid lines connecting the various exons indicate different alternative splicing patterns that result in the production of all of the different cDNA clones isolated to date (ref. 2). BD-1, -2, -3 indicate the DNA-binding domains of the protein (ref. 10) and the important protein coding and untranslated regions of the gene designated by the shading key shown in the lower right hand corner of the diagram. Restriction enzyme cut sites: K, *Kpn*-I; P, *Pst*-I; R, *Eco*-RI; S, *Sac*-I; Xb, *Xba*-I; X, *Xho*-I.

Table 1. Transcript start sites, alternative exon splicing patterns and regulatory factors

mRNA* (cDNA) Clones	Exon at Transcription Start Site	Potentially Inducible by Transcription Factors+	Spliced Exons in 5′ UTR	Protein Coding Spliced Exons	Form of Mature mRNA
1A & 8A	I	AP2, PEA3, Oct1 KROX-24	I, II′, V	V,VI,VII,VIII	HMG-Y
3B	II	AP2, HMG-I(Y)	II, II′	V,VI,VII,VIII	HMG-Y
7B	II	AP2, HMG-I(Y)	II, II′	V′,VI,VII,VIII	HMG-I
6A	III	AP2, KROX-24 CREB	III, IV	V′,VI,VII,VIII	HMG-I
11D	V	AP2, AP1, PEA3, NFκB,CREB, CP2, HSP70,MT-1,insulin	–	V′,VI,VII,VIII	HMG-I

*From Johnson et al. (1989); reference (2).
+From Faisst and Meyer (1992); reference (38).

Gene structure, transcription initiation sites and alternative splicing

The HMG-I(Y) gene is 10,144 nt long and has seven introns (#s 1–7) and eight exons (#s I-VIII). Figure 1 diagrams the intron/exon arrangement of the sequenced genomic clone (along with 627 nt 5′ upstream of the first exon) with annotations indicating some of its important structural features. Overall, the gene is 58% G+C with exons varying significantly in G+C content (see below). Nucleotide numbering begins at exon I (nt #1) which is arbitrarily designated as the 5′-most gene boundary based on the fact that it contains the transcription start sites for the mRNAs (e.g., cDNA clones 1A & 8A; Table 1 and ref. 2) known to be most 5′-distal from the translation initiation codon common to all HMG-I(Y) messages. However, since there are multiple transcription initiation start sites in the gene and the 5′-UTR is involved in a complex patterns of alternative mRNA splicing (see below) the actual 5′ terminus of the functional HMG-I(Y) gene is somewhat uncertain. The first four exons (I–IV) contain the 5′ untranslated regions (UTRs) of different human cDNAs isolated previously (2). Exon V contains the translation start sites, as deduced from cDNA clones, of all mRNAs coding for both the HMG-I and HMG-Y isoform proteins (2). The 3′ end of the gene was inferred from the position of the potential polyadenylation signal at nt #9327–9332 common to all known human cDNA clones (2) and from the divergence of the genomic sequence from all cDNAs just downstream of this site. Interestingly, each of the three independent DNA-binding domains present in the HMG-I(Y) family of proteins is located on a separate exon (exons V, VI and VII; Fig. 1).

Primer extension analysis performed earlier (2) defined three main RNA species which corresponded to three size classes of 5′-UTRs in HMG-I(Y) mRNAs from human K562 erythroleukemia cells. This finding suggested that the HMG-I(Y) gene most likely contained multiple mRNA transcription initiation start sites and probably several promoter regions (2). This interpretation is confirmed by the structure of the genomic gene (Figs. 1). The common transcription start sites for the most abundant mRNA species, represented by the 2B, 3B and 7C cDNA clones (Table 1; and ref. 2), are located at the 5′ end of exon II (nt #280). The start site for the next most abundant transcripts (clones1A and 8A) are at the 5′ end of exon I (nt # 1). So far, no primer extension products have been observed for mRNA species corresponding to either the 6A or 11D cDNA clones whose transcription start sites would likely be located at the 5′ ends of exons III (nt #742) and V (nt #3852), respectively, probably as a result of scarcity of these transcripts in K562 cells (2). As shown in Fig. 1, a complex pattern of alternative mRNA splicing between the first six exons, combined with the various putative 5′-UTR transcription initiation sites, can fully explain all of the HMG-I(Y) cDNA clones isolated to date (Table 1; ref. 2). The various intron splicing patterns occurring in the 5′ UTRs of the different cDNA clones appear to be independent of alternative splicing that occurs in the protein coding region of the gene which results in mRNAs coding for either HMG-I or HMG-Y proteins. Exon IV, found in the 5′ UTR of cDNA clone 6A, contains a long ORF which could potentially encode a new protein (2). However, no protein has so far been isolated corresponding to this open reading frame (37).

Exon V (nt #3852–4031) contains the 45 bp common 5′ UTR to all cloned cDNAs as well as the translation ATG start codon (beginning at nt #3897) and the sequence coding for the first 34 amino acids. This exon also includes the first DNA binding domain (BD-1) and the 33 bp region coding for another 11 amino acids which is spliced away in the HMG-Y isoform cDNAs (Fig. 1; Table 1). Exon VI, encompassing nts #5832–5915, encodes amino acids 46–73 and contains the second DNA-binding domain (BD-2). Exon VII, at nt positions #6591–6641, encodes amino acids 74–90 and contains the third DNA-binding

Table 2. Intron-exon junctions of the human HMG-I(Y) gene

Exon	Acceptor (intron/exon)	Donor (exon/intron)
I(10A)	* CCCTGCCCCTGGGTC/G	GGC/GTGAGT
I(1A)	* CCCTGCCCCTGGGTC/G	AAG/GCGTGA
II(7C)	* GGGGGCCTGGGCGGC/G	# CAG/ATCCGC
II′(1A,7C)	CTCTCCCGGTTTCAG/A	GCG/GTGAGT
III(6A)	* AGCTGCGGCGAGCGC/G	AGG/GAAGTT
IV(6A′)	TTTCTTTTAAATTAG/A	CAG/GCATGA
V	TTTTCTGTCTCCCAG/C	CCG/GTGAGT
V′	TTTTCTGTCTCCCAG/C	CAG/GTGGGT
VI	CCTCTGTCTTTACAG/A	CGG/GTGAGA
VI′(IID)	CTGTCTTTACAGAAG/G	CGG/GTGAGA
VII	TCTCTAACCCTCTAG/A	CTG/GTAGGT
VIII	CTGCCCACCTCACAG/G	----------
CONSENSUS♦	(T) NCAG/G (C)n T	CAG/GTAAGT A G

♦Consensus sequences from Mount (1982); reference (40).
* Not observed to be a functional splice acceptor.
# Not observed to be a functional splice donor.

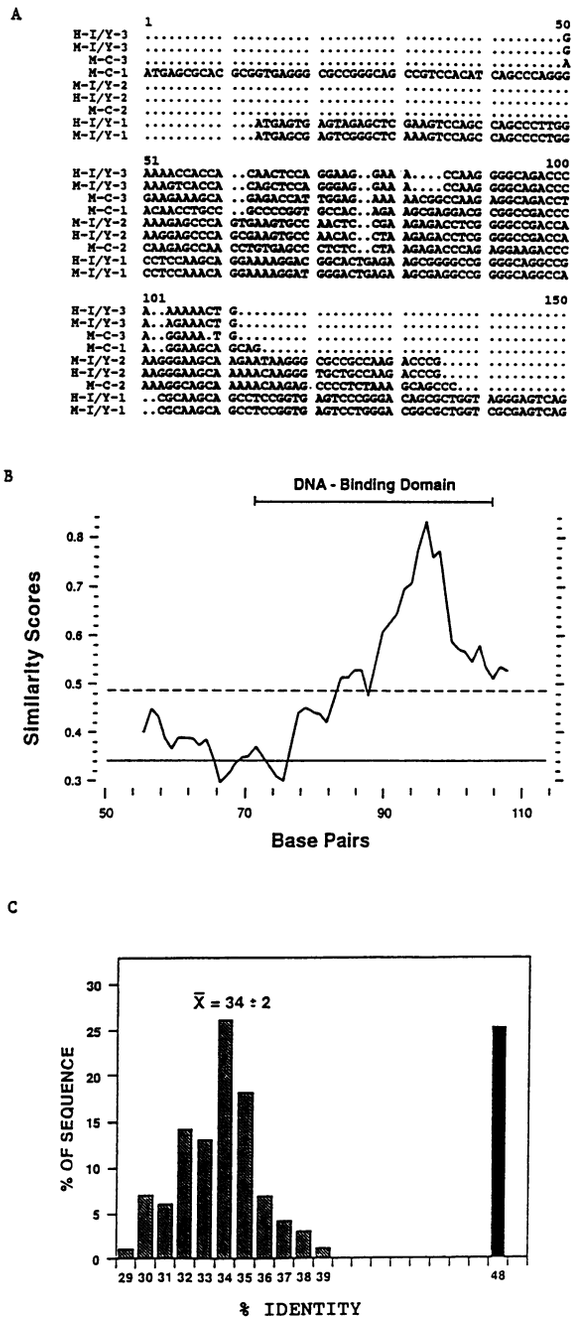

Figure 2. Comparison of HMG-I(Y) and HMGI-C exons containing DNA-binding domains. (**A**) Alignment of human HMG-I(Y) Exons containing DNA binding domains and mouse HMG-I(Y) and HMGI-C putative exons containing DNA binding domains; (**B**) Percentage identity between exons of human HMG-I(Y) and mouse HMG-I(Y) and mouse HMGI-C; (**C**) Histogram of random sequences with the same base pair composition as the exons contained in DNA binding domains. **Key**: H-I/Y-1, -2, -3, human HMG-I(Y) genomic exon sequeces containing peptide DNA-binding domains -1, -2 and -3, respectively; M-I/Y-1, -2, -3, mouse HMG-I(Y) cDNA sequences (2) containing peptide DNA-binding domains -1, -2 and -3, respectively; M-C-1, -2, -3, mouse HMGI-C cDNA sequences (3) containing putative peptide DNA-binding domains -1, -2 and -3, respectively.

domain (BD-3). The last exon (VIII), at nt positions #7953–9349, encodes the final 17 amino acids comprising the 'acidic tail region' of the HMG-I(Y) proteins and contains 1345 bp of 3′ UTR.

Putative promoter/enhancer regions

Primer extension data (2) and the gene structure indicate that there are likely to be at least four different promoter/enhancer regions for control of HMG-I(Y) mRNA transcription initiation (5′ upstream of exons I, II, III and V; Fig. 1; Table 1). For example, full-length HMG-I(Y) cDNA clones have been previously isolated that precisely start at the beginning of either exon I (clones 1A and 8A), exon II (clones 2B, 3B and 7C), exon III (clone 6A) or exon V (clone 11D) (Fig. 1 and ref. 2). Computer sequence analyses indicate that each of these four putative promoter/enhancer regions contain potential binding sites for a number of different inducible transcriptional regulatory proteins (Table 1; see ref. 38 for a recent compilation of vertebrate transcription factors). These include, as noted in Table 1, potential binding sites for AP-1, AP-2, PEA3, and KROX-24 (inducible by phorbol esters, serum, retinoic acid and numerous mitogens), CREB (inducible by Ca^{++} or cAMP), NFκB sites (inducible by phorbol esters, cAMP and various growth factors), CP-2 (inducible by serum and certain growth factors) and insulin (c.f., 38). The presence of such a variety of potential transcription factor binding sites in the putative regulatory regions suggests that the HMG-I(Y) gene may be inducible by a variety of extacellular mitogenic signals, a prediction confirmed by direct experimental analysis (see below). However, whether all of these potential transcription factor binding sites are indeed functional elements in the different putative promoter/enhancer regions remains to be demonstrated.

Overall the gene contains 58% G+C with some areas, such as that before exon II, containing as high as 84% G+C. However, other areas are also exceptionally G+C rich, particularly in the region before exon I and in intron #2 and exon III. All of these areas conform to the criteria of HTF islands(39) in that they have a high incidence of CpG and GpC dinucleotides, multiple Hpa II restriction sites and numerous putative Sp-1 transcription factor binding sites. These features suggest that perhaps some transcripts initiated at exon I (for example, cDNA clones 1A and 8A) or at exon III (e.g., cDNA clone 6A) might be under constitutive control and thus classified as 'housekeeping' transcripts.

Intron/exon junctions

Table 2 shows the sequences surrounding the intron-exon junctions which generally follow the intron donor-acceptor, 'GT-AG' rule (40) although sequences on the donor side of several of the exons deviate somewhat from the canonical consensus motif. In the case of exons I, II and III, the 5′ ends of the exons do not contain a sequence corresponding to a canonical splice acceptor site, while the 3′ ends of the exons do contain sequences corresponding to consensus splice donor sites. This suggests, in agreement with earlier primer extension and other data (2), that the 5′ ends of these exons most likely represent sites of transcription initiation rather than being generated as a result of splicing of longer precursor transcripts. In the case of exon II, splicing events can occur both within the exon (at nt position #333) and at the 3′ end of the exon (at nt #446). At the splice site within the exon there is a splice acceptor consensus sequence (Table 2, exon II′), but no consensus donor site. Thus, alternative splicing and transcription initiation at exon II could result in the formation of transcripts with two different 5′-UTRs, one transcript initiating before exon I and joining directly with exon II′, and the other transcript initiating before exon II and containing all of this exon (II + II′) (Fig. 1; Table 2).

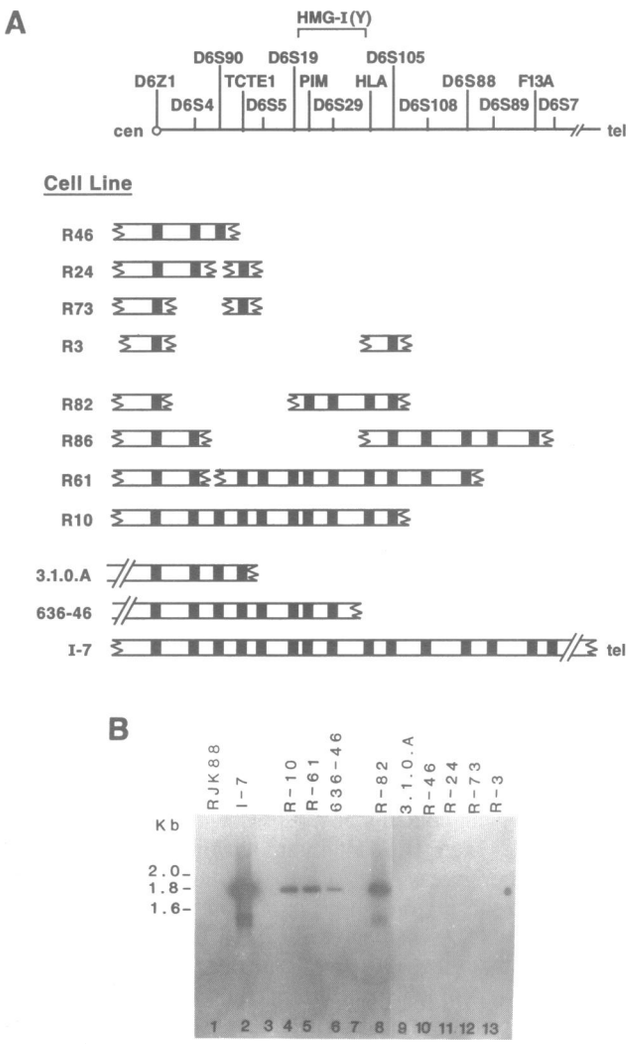

Figure 3. PCR Amplification of HMG-I(Y) intron fragment in hamster-human hybrid and radiation hybrids. **(A)** Schematic diagram illustrating the DNA contents of selected somatic cell hybrids and radiation hybrids (indicated by 'R') retaining various segments of chromosome 6 (redrawn, with modifications, after ref. 34). The diagram of human chromosome 6p on top is not drawn to scale. **(B)** Autoradiographs of PCR amplified DNA from different hybrid cell lines hybridized to an intron-specific HMG-I(Y) gene probe. **Lanes**: 1) RJK88, parental hamster DNA; 2) I-7, full length 6p DNA; 3) blank; 4) R-10; 5) R-61; 6) 636−46; 7) blank; 8) R-82; 9) 3.1.0.A; 10) R-46; 11) R-24; 12) R-73; 13) R-3. DNA molecular weight markers (in kb) indicated on the left. For convenience and reference, the cell line designations are also indicated above the appropriate lanes in the figure.

In mammalian cells the HMG-Y protein is identical in sequence to the HMG-I isoform protein except that it is shorter as a result of an internal deletion of 11 internal amino acid residues (1,2). Exon V is the site of the two alternative splice events which generate either the longer HMG-I mRNA or the 33 nt shorter, internally deleted, HMG-Y transcript. The intron-exon junction at the 5′ end of exon V conforms to a consensus splice acceptor site, while within and at the 3′ end of the exon there are two different splice donor sites positioned 33 bp apart (Table 2, exon V, V′, Figure 2). Consequently, the shorter HMG-Y isoform mRNA is generated by alternative splicing from the donor site inside the exon (at nt #3998; Table 2, donor site V) directly to the acceptor site at the 3′ end of intron #5 (i.e., at nt #5832

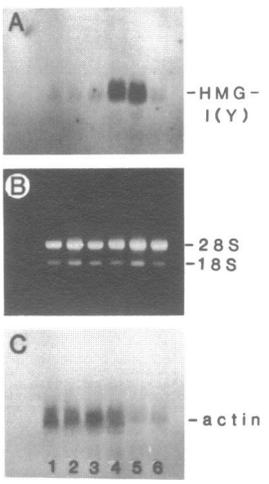

Figure 4. HMG-I(Y) gene expression induced by phorbol esters and calcium ionophore stimulation. Panel **A:** Northern blot showing of HMG-I(Y) mRNA induction in growing human HUT-78 cells treated for various times (hr) with a combination of the phorbol ester PMA plus the calcium ionophore ionomycin. Position of HMG-I(Y) mRNA migration indicated beside panel. Panel **B**: Parallel gel to that shown in panel A stained with ethidium bromide to show equal RNA loading of lanes. Position of 18S and 28S ribosomal RNAs indicated beside panel. Panel **C**: The Northern blot shown in panel A was stripped of all radioactivity and reprobed with a radiolabeled α-actin cDNA. Position of α-actin mRNA migration indicated beside panel. **Lanes,** hours of stimulation with PMA/ionomycin (from left to right in all panels): 1) 0 hr; 2) 1 hr; 3) 2 hr; 4) 4 hr; 5) 7 hr; 6) 21 hr.

at the 5′ end of exon VI; Table 2). On the other hand, the longer HMG-I transcript is formed as a result of alternative splicing from the consensus donor site found at the 3′ end of the exon (nt #4031; Table 2, donor site V′) through to the same acceptor site at the 3′ end of intron #5. Interestingly, one previously isolated variant HMG-Y cDNA clone (11D; ref. 2) has a 36 bp deletion which would arise from splicing at the same internal donor site as the HMG-Y isoform directly to a 'wobble' splice acceptor site 3 nt downstream of the start of exon VI (Table 2, exon VI′).

Comparison of exons containing DNA binding domains

The observation that each of the three DNA-binding domains of the human HMG-I(Y) proteins is contained in a separate exon (exons V, VI and VII) and the fact that both the amino acid and nucleotide sequences of the different BDs are very highly conserved (10), raised the possibility that the three exons might have arisen from one or more ancestral exon duplication events. Furthermore, the cDNA for another member of the HMG-I(Y) family has been isolated from mice (HMGI-C) which is apparently transcribed from a related, but distinct, genomic sequence. The murine HMGI-C protein also contains highly conserved amino acid regions which correspond to the DNA-binding domains and the acidic tail region of the HMG-I(Y) protein family. A statistical comparison of the nucleotide sequences of the exons containing the DNA-binding domains of the human HMG-I(Y) gene, the mouse HMG-I(Y) cDNA and the mouse HMGI-C cDNA (see Methods and Methods for details), revealed 48% identity between all of these nine exon (or putative exon) sequences (Fig. 2A and B), with the greatest degree of identity between the exons being in the region of the DNA-binding domains themselves. To determine the significance of this similarity, the same nine exon sequences were computer

randomized and realigned 100 times. The mean percentage identity for these randomized exon sequences was 34% with a standard deviation of 2% (Fig. 2C). The 48% identity observed for the actual BD-encoding exons is seven standard deviations away from the mean value obtained for the randomized exon sequences, and is therefore considered significant. Based on these analyses, it seems probable that the three BD-containing exons of the HMG- I(Y) gene arose either by triplication of a single ancestral exon or, perhaps more likely, arose as a result of two separate duplication events during evolution.

Pairwise sequence comparisons (see Fig. 2A−C) between the three human HMG-I(Y) BD-containing exons (BD-1, BD-2 and BD-3; Figs. 1) and the three putative BD-containing mouse HMG-I(Y) and HMGI-C exons revealed that the first BD-containing exons (BD-1) of these different genes are more similar to each other than they are to either of the other two BD-containing exons within the genes. The same is true for both the second (BD-2) and third (BD-3) DNA-binding domain containing exons. In other words, the degree of sequence identity and the linear polar arrangement of the different BD-containing exons (BD-1, BD-2, BD-3) is conserved within both the human and mouse genes. This strongly suggests that the human and murine HMG-I(Y) genes, as well as the related murine HMGI-C gene, probably descended from a common ancestral genomic sequence.

Chromosomal localization of the human HMG-I(Y) gene

Employing genetic linkage analysis Johnson et al. (41) localized the functional murine HMG-I(Y) gene (*Hmgi*) near the *Pim*-1 proto-oncogene in the *t*-complex region of mouse chromosome 17. Due to extensive regions of homology between the mouse and human genomes (42), this finding suggests that the human HMG-I(Y) gene is located on chromosome 6 (41). The human PIM locus is located on the proximal short arm of chromosome 6 near position 6p21 (33,43). A panel of somatic cell hamster-human hybrids (33) and radiation hybrids (34) which retained various fragments of the short arm of human chromosome 6 was therefore screened to determine whether the HMG-I(Y) gene was indeed localized to this arm of the chromosome. Figure 3A shows a schematic diagram illustrating the human chromosome contents of the selected 6p somatic cell hybrids and radiation (R) hybrids that were screened. Fig. 3B shows the results of Southern blots of PCR-amplified hybrid cell DNAs hybridized to an intron probe specific for the functional HMG-I(Y) gene. As shown in the autoradiographs of Fig. 3B, the intron-specific probe hybridized with amplified DNA from the hybrid cell line containing all of human 6p (I-7, Lane 2) as well as to DNA from cell lines R-10, R-61, 636−46 and R-82 (Lanes 4−6 and 8, respectively). In contrast, the probe did not hybridize above background to amplified DNA from either the parental hamster cell line RJK88 (Lane 1), nor to DNA from cell lines 3.1.0.A, R-46, R-24, R-73 or R-3 (Lanes 9−13, respectively). Of particular interest is hybridization of the probe to the DNA fragment R-82 (Lane 8) which contains two different regions of 6p, one encompassing D6Z1 and the area around the centromere, and the other spanning the area from approximately D6S19 to about D6S105. This indicates that the HMG-I(Y) gene maps to 6p, in a region spanning the loci D6S19 and D6S105. Moreover, since the R-3 fragment, which contains DNA surrounding the D6S105 locus, did not hybridize to the probe (Lane 13), the HMG-I(Y) gene is most likely located in a 12−14 centimorgan region distal to D6S19 and proximal to HLA. This places the human gene in a region near the PIM locus at 6p21 (33, 43), which is analogous to the localization of the functional *Hmgi* on chromosome 17 of mouse (41).

Inducible expression of the HMG-I(Y) gene

Cellular levels of HMG-I(Y) gene products are known to vary as a function of growth state, being low in differentiated and quiescent cells and increasing substantially in actively dividing and neoplastically transformed cells (1, 7, 22−26). The presence in all four of the potential promoter/enhancer regions of the HMG-I(Y) gene (Figs. 1; Table 1) of numerous consensus nucleotide binding sites for inducible transcription factors, such as those responsive to phorbol esters, calcium and serum, suggest that expression of the gene is under inducible control. To investigate this possibility, Northern blot analyses were performed on the human HUT-78 lymphoid T cell lymphoma line to determine whether treatment with phorbol ester, or a combination of phorbol ester plus ionomycin (a Ca^{++} ionophore), could induce HMG-I(Y) mRNA transcription. The results shown in Fig. 4A indicate that growing HUT-78 cells treated with a combination of the phorbol ester PMA plus ionomycin respond within 2−4 hours following exposure by significantly increasing their steady state levels of HMG-I(Y) mRNA. A maximum accumulation of HMG-I(Y) mRNA occurs by about 4−7 hours following PMA/ionomycin stimulation (Lanes 4 and 5) and has dropped significantly by 21 hours post stimulation (Lane 6). Panel B of Fig. 4, shows a parallel gel that was stained with ethidium bromide and panel C an autoradiograph of the same blot shown in panel A after 'stripping' and rehybridization with a radiolabeled α-actin probe. From these results it is evident the pattern of α-actin mRNA accumulation after PMA/ionomycin treatment of HUT-78 cells does not follow the same pattern of induction as the HMG-I(Y) mRNA in the same cells. A qualitatively similar pattern of HMG-I(Y) mRNA induction is seen following exposure of growing HUT-78 cells to PMA alone (data not shown). Similar induction of HMG-I(Y) mRNA in response to phorbol ester treatment has also been observed with the human K562 erythroleukemia cell line (unpublished observations). Since we have previously shown that HMG-I(Y) mRNA is very stable in all phases of the mammalian cell cycle ($T_{1/2} > 6$ hours; ref. 24), the simplest interpretation of these data is that both PMA alone, and in combination with a Ca^{++} mobilizer, induces increased transcription from the HMG-I(Y) gene as a result of promoter/enhancer elements that respond to these factors.

DISCUSSION

Earlier work from this laboratory suggested that there are 2−10 copies of sequences related to the HMG-I(Y) cDNAs present in the haploid human genome (2). Initial attempts to clone the HMG-I(Y) gene resulted in the isolation of several HMG-Y processed retropseudogenes lacking introns (2, and unpublished data). In order to isolate the authentic human HMG-I(Y) gene from this background of processed pseudogenes, an intron-specific probe was generated using PCR with primers flanking a putative intron site which upon splicing, would result in a 33 bp deletion that characterizes the HMG-Y transcript. The location of this intron was confirmed and using the intron-specific probe, the authentic HMG-I(Y) gene was isolated and sequenced (Fig. 1).

The human HMG-I(Y) gene with 627 nt of 5′ upstream promoter is 10,144 bp long (Fig. 1) and overall is slightly G+C-rich (58%) with certain regions (e.g., exon II through exon III)

containing over 80% G+C residues. It is comprised of eight exons separated by seven introns which give rise to different cDNAs by a complex pattern of different transcription starts and alternative splicing (Fig. 1). A number of conserved sequences for potential binding of inducible transcription factors are found upstream of each of the four different potential transcription start sites (exons I, II, III and V; Fig. 1; Table 1). These include consensus binding sites for transcription factors induced by serum, growth factors, mitogens, phorbol esters and calcium activators. That at least some of these potential sites are indeed involved in inducible gene expression is confirmed by the demonstration that both PMA and PMA plus a calcium ionophore significantly induce HMG-I(Y) mRNA levels in HUT-78 cells within 2–4 hours after treatment (Fig. 4). Which of the different promoter/ehancer regions and potential regulatory sequences are actually involved in these gene inductions are unknown but are currently under investigation. Recently Lanahan et al. (44) also reported that both HMG-I(Y) and HMGI-C mRNAs are induced in quiescent mouse 3T3 fibroblasts within 2–4 hours of treatment with either serum or purified growth factors (PDGF or FGF), several hours prior to induction of DNA synthesis by these same factors. Together these observations, along with our findings, place the HMG-I genes in the family of 'delayed early response' genes (44) whose inducible transcription in the pre-S phase of the cell cycle raises the question of whether these nonhistone proteins might play a role in the G_1-to-S transition.

Computer analysis and comparisons of the BD-containing exons in the human and mouse HMG-I(Y) genes and the mouse HMGI-C gene strongly support the hypothesis that all of these BD-containing exons descended by duplication(s) from a common ancestral precursor sequence and diverged in each case by accumulation of a variety of point mutations (Fig. 2). The fact that each of the three BD regions of the HMG-I(Y) proteins is encoded by a separate exon also potentially allows for 'exon shuffling' of these DNA-binding regions to other genes (perhaps, for example, to the human HRX gene (27)) during evolution.

The structure of the human HMG-I(Y) gene is among the most complex so far reported for any mammalian chromatin protein and, in general, is markedly different from the genes coding for other families of HMG proteins (4). Superficially, however, there are regions of the HMG-I(Y) gene that do share some similarity with portions of the human and chicken HMG-14/−17 genes. For example, both families of HMG genes contain limited G+C-rich areas that conform to 'HTF' islands which might be involved in constitutive production of gene transcripts. On the other hand, in contrast to other HMG genes (4), the HMG-I(Y) gene also potentially contains several different inducible promoter/enhance elements, multiple transcription start sites and an intron/exon pattern that facilitates complex transcript splicing events.

Alternative splicing of pre-mRNAs is widespread in eukaryotes and has become recognized as an important developmental, and/or tissue specific post-transcriptional mechanism for regulating gene expression (45–48). For example, alternative splicing is the primary mechanism involved in the regulatory hierarchy of sex determination in *Drosophila* (48) and in many examples of tissue-specific gene expression (49). When alternative splicing occurs in either the 5′- and/or the 3′-untranslated regions of transcripts, it is often used to quantitatively regulate gene expression by giving rise to prematurely truncated open reading frames, by regulating translational efficiency, or by altering message stability (50–53). Given the extensive alternative splicing patterns found in the 5′-untranslated region observed in different mRNAs (Fig. 1), it seems likely that these various processed transcription products may likewise be involved in differential regulation of HMG-I(Y) gene expression.

We localized the HMG-I(Y) gene to a region between the D6S19 and HLA loci on the short arm of chromosome 6 (Fig. 4) in the vicinity of the PIM locus at position 6p21 (42, 43). This location of the HMG-I(Y) gene is of considerable interest because numerous chromosomal abnormalities (translocations, rearrangements and amplifications) of this region of 6p have been observed in a variety of human cancers including subtypes of acute nonlymphocytic leukemia (ANLL) (54,55), acute and chronic myeloid leukemias (56,57), chronic lymphocytic leukemia (58), malignant T cell lymphoma (59) and neuroblastoma (60). Since HMG-I(Y) mRNA and protein levels are know to be significantly elevated in many types of cancerous cells (1–3, 21–26), the intriguing possibility exists that a certain subgroup of such malignancies might be associate with chromosomal abnormalities in the vicinity of the HMG-I(Y) gene.

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