Existence of a Transcription Factor for the Human HMG2 Gene Positively Related to the Level of HMG2 mRNA in the Cells†

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ABSTRACT: A functional gene encoding high mobility group 2 (HMG2) protein, which is an abundant eukaryotic DNA-binding protein, has been isolated. The expression of the HMG2 gene is enhanced in exponentially growing cells and in cells transformed with various viral genes and oncogenes. We attempted to identify and characterize the HMG2 gene structure and transcription factor(s) participating in the expression of the gene. Chloramphenicol acetyltransferase assays to characterize the 5′-flanking region of the human HMG2 gene revealed that the nucleotide sequences in two regions are necessary for expression of the HMG2 gene: one (−85 to +44 region) as a core promoter, and the other (−621 to −493 region) as a *cis* regulatory element(s). Electrophoresis mobility shift assay with a DNA fragment containing the *cis* regulatory element and a crude nuclear extract from HeLa cells gave several complexes. Chemical footprint and competition assays indicated that the component giving one of the major complexes recognizes a nucleotide sequence of −499 to −486 in the *cis* regulatory element. Chloramphenicol acetyltransferase assay indicated that the component giving the major complex is required for the effective transcription of the HMG2 gene. The binding component named HMG2TF (HMG2 transcription factor) contained a protein in apparent molecular size of 85 000, as determined by a UV cross-linking experiment. The amount of HMG2TF in the growing cells and transformed cells increased in positive relationship to the level of expression of HMG2 mRNA in the cells. These results suggest that HMG2 gene expression may be regulated by the relative amount of this transcription factor.

High mobility group (HMG)[1] proteins are non-histone components in chromatin of relatively low molecular weight. HMG1 and HMG2 proteins show remarkable structural and presumably functional similarities to each other. These proteins show a preferential affinity for single-stranded DNA (Bidney & Reeck, 1978; Isackson et al., 1979; Yoshida & Shimura, 1984; Hamada & Bustin, 1985), unwind double-stranded DNA (Yoshida & Shimura, 1984; Makiguchi et al., 1984; Javaherian et al., 1978), remove the transcriptional blocks caused by left-handed Z-form DNA (Waga et al., 1988) and cruciform DNA (Waga et al., 1990), and stimulate *in vitro* transcription (Waga et al., 1988, 1990; Tremethick & Molloy, 1986, 1988). They also function as general class II transcription factors (Singh & Dixon, 1990; Ge & Roeder, 1994). The novel DNA-binding motif first identified by Reeck et al. (1982) of these HMG proteins is also present in several nuclear and mitochondrial transcriptional factors (see references of citation Laudet et al., 1993). However, the cellular functions and roles of HMG1 and HMG2 have not been clarified.

Recently, we found that the level of HMG2 mRNA is enhanced in exponentially growing cells and in cells transformed with various viral genes and nuclear oncogenes (Yamazaki et al., unpublished experiments). These observations suggest that HMG2 protein is closely related to cell proliferation. In this study, we characterized the structure of the human HMG2 gene, which we had first isolated (Shirakawa & Yoshida, 1992), and identified a necessary *cis* element and a transcription factor which binds to it. The amount of transcription factor containing a protein in apparent molecular size of 85 000 was increased in the growing and transformed cells, showing a positive relationship with the respective mRNA level for HMG2 in the cells. We discuss the possible functional implications of these observations.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized using an Applied Biosystems Model 381A DNA synthesizer. After deprotection with ammonium hydroxide, they were purified using NENSORB PREP cartridge columns (Du Pont) and recovered in accordance with the instruction manual.

Polymerase Chain Reaction. Polymerase chain reaction (PCR) was carried out in a total volume of 100 μL containing 40 pmol of each primer, 50 ng of each template plasmid, 160 μM dNTP, *Pfu* DNA polymerase buffer (Stratagene), and 2 drops of mineral oil (Sigma). After heating at 95 °C for 5 min to denature the template DNA, 2.5 units of *Pfu* DNA polymerase (Stratagene) was added and subjected to 25 cycles at 95 °C for 1.5 min, 55 °C for 1.5 min, and 72 °C for 3 min.

Cell Culture. HeLa cells were grown in Eagle's minimum essential medium supplemented with 10% newborn calf serum. TIG-1 (Ohashi et al., 1980), 3Y1 (Kimura et al.,

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoresis mobility shift assay; HMG, high mobility group; np, nucleotide position(s); PCR, polymerase chain reaction.

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1975), and XhoC (Tsutsui et al., 1990) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. TIG-1 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB), and 3Y1 and XhoC cells from Dr. K. Oda.

Plasmid Construction. Each chloramphenicol acetyltransferase (CAT) plasmid was constructed by ligating the 5′-flanking region of the human HMG2 gene amplified by PCR into the *Hin*dIII site upstream from the CAT gene of pSV0 cat (Laimins et al., 1982). Briefly, the template DNAs for PCR were a series of deletion clones prepared for nucleotide sequence determination of the human HMG2 gene (Shirakawa & Yoshida, 1992). Complementary oligonucleotides of pBluescriptII KS(+) nucleotide position (np) 627−648 and the human HMG2 gene np +31 to +50 and np −100 to −77 were synthesized as the PCR primers. All primers contained a *Hin*dIII site. After amplification, the DNA fragments were digested by *Hin*dIII, followed by ligation into pSV0 cat (Laimins et al., 1982) pretreated with *Hin*dIII and bacterial alkaline phosphatase, and then transformed into *Escherichia coli* JM109. The nucleotide sequence of each construct was confirmed by the dideoxy sequencing method using Sequenase (USB). The plasmids were prepared by the alkaline−SDS method, followed by purification with equilibrium density-gradient centrifugation in cesium chloride (Sambrook et al., 1989).

DNA Transfection and CAT Assay. The respective CAT plasmid (5 μg) was cotransfected with pCH110 (1 μg, Pharmacia) as an internal standard expression plasmid into HeLa cells by a cationic liposome method using Transfection reagent (Boehringer Mannheim). DNA−liposome complex was added to semiconfluent HeLa cells in Hepes-buffered saline and incubated for 4 h. After transfection, the medium was exchanged for Eagle's minimum essential medium supplemented with 10% newborn calf serum, and the cells were incubated for another 48 h. The harvested cells were suspended in 150 μL of 0.25 M Tris-HCl, pH 7.8, followed by 3 cycles of freezing and thawing. The supernatant obtained by centrifugation at 12000*g*, 4 °C, for 10 min was transferred to another tube and used for subsequent analysis. An aliquot of the cell extract was measured for β-galactosidase activity (Rosenthal, 1987) and protein concentration using the protein assay kit (Bio-Rad). The cell extract was then incubated at 60 °C for 10 min and centrifuged at 12000*g* for 5 min. The CAT activity in the supernatant was measured according to Rosenthal (1987). The ratio of acetylated to unacetylated chloramphenicol was determined by a BAS2000 Imaging Analyzer (Fuji Film).

Preparation of Labeled DNA. A DNA fragment containing np −624 to −478 of the human HMG2 gene was prepared for electrophoresis mobility shift assay (EMSA). Briefly, a DNA fragment of np −624 to −478 amplified by PCR using a 5′-primer (np −624 to −612 plus an *Xho*I restriction site) and a 3′-primer (complementary to np −492 to −478 plus a *Hin*dIII restriction site) was ligated into pBluescriptII KS(+). After excision with *Xho*I (for labeling the noncoding strand) or *Hin*dIII (for labeling the coding strand), DNA was labeled in 7 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 20 mM NaCl, 7 mM $MgCl_2$, 0.1 mM dATP, dGTP, TTP, [α-^{32}P]dCTP, and Klenow fragment and then treated with the other restriction enzyme, respectively. The lower molecular weight DNA fragments were recovered by electrophoresis on 4% polyacrylamide gel. The DNA fragment of np −533 to −478 was used for chemical footprint assay. For this, a plasmid similar to that for EMSA was digested with *Hin*dIII (for labeling the noncoding strand) or *Pst*I (for labeling the coding strand), treated with alkaline phosphatase, labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, and finally treated with the other restriction endonuclease, respectively. For the UV cross-linking experiment, a synthetic coding strand (np −502 to −475) and a noncoding strand (np −485 to −475) were annealed and filled with dATP, dGTP, 5-bromo-dUTP, and [α-^{32}P]dCTP using Klenow fragment.

Preparation of Nuclear Extract, and Electrophoresis Mobility Shift Assay. The nuclear extracts were prepared from HeLa, TIG-1, 3Y1, and XhoC cells according to the procedure of Dignam et al. (1983) and dialyzed against 20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, and 17% glycerol. The samples for electrophoresis mobility shift assay were prepared in 10 μL of solution containing 12 mM Hepes-KOH, pH 7.9, 60 mM KCl, 7.5 mM $MgCl_2$, 1 mM EDTA, 1.2 mM DTT, 10% glycerol containing the nuclear extract, ^{32}P-labeled DNA fragment (approximately 10^4 cpm), and 1 μg of poly(dI-dC)·poly(dI-dC). After 30 min of incubation at 0 °C, the mixture was electrophoresed on 4% polyacrylamide gel using 0.5× TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA) buffer, at 10 V/cm at 4 °C, and then processed for autoradiography.

Chemical Footprint Assay. The ^{32}P-labeled DNA fragment was incubated with HeLa nuclear extract at a scale 10-fold that of EMSA. The resulting complex separated by electrophoresis was digested in polyacrylamide gel by chemical nuclease, 1,10-phenanthroline−copper ion complex (Sigman, 1986) according to the method of Kuwabara and Sigman (1987). The DNA fragments, eluted from the gel slices containing shifted and unshifted bands, were denatured at 95 °C for 3 min, analyzed in 10% polyacrylamide/urea gel, and then processed for autoradiography.

UV Cross-Linking Experiment. A ^{32}P-labeled bromodeoxyuridine-substituted DNA fragment was complexed with HeLa cell nuclear extract. After gel electrophoretic separation, the protein−DNA complexes were irradiated in the gel under an UV lamp (Spectrolinker, Japan Genetics) for 5 or 30 min. Bands containing complexes were analyzed according to the method of William et al. (1992). The gel slices, washed with 125 mM Tris-HCl, pH 6.8, 3 mM DTT, and 1% SDS, were heated in the same buffer at boiling temperature for 5 min. The slices were placed on an SDS−Laemmli running gel of 8−20% gradient polyacrylamide and filled with stacking gel. After electrophoresis, the gel was dried and processed for autoradiography.

Northern Blot Analysis. Total RNA was extracted from the cells by the guanidine hydrochloride method (MacDonald et al., 1987), separated on 1.1% agarose gel, and transferred to a cellulose nitrate filter according to Thomas (1983). Pig thymus HMG2 cDNA (Shirakawa et al., 1990) and mouse β-actin cDNA (Tokunaga et al., 1986), which were ^{32}P-labeled using a Takara random primer DNA labeling system (Kyoto), were used as the probes for hybridization.

RESULTS

Identification of the Cis Regulatory Element. Several CAT-plasmid constructs containing serial human HMG2

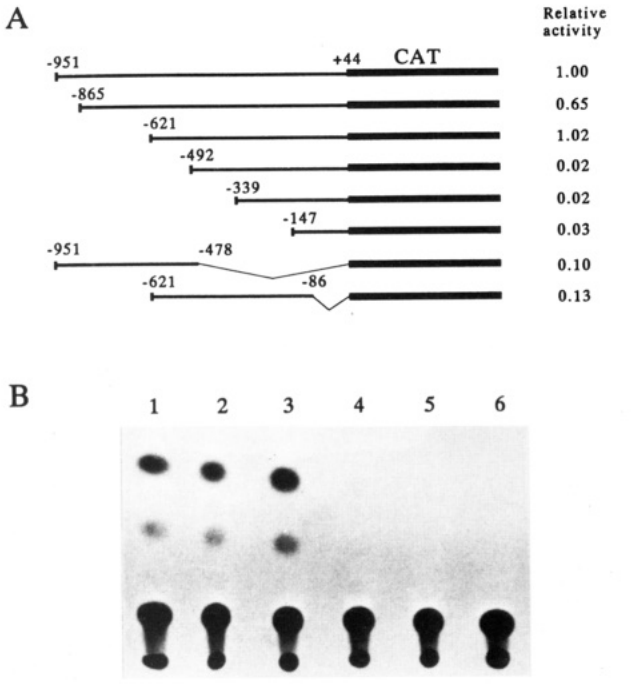

FIGURE 1: Transient expression of CAT plasmids containing various 5′-flanking regions of the HMG2 gene in HeLa cells. *Panel A*: A diagram of CAT constructs containing 5′-flanking regions of the human HMG2 gene. Thin lines represent HMG2 5′-flanking sequences, and thick lines represent the CAT gene. The CAT activities in the right-hand column are expressed as values relative to that of the np −951 to +44 construct. The relative values are means of three determinations. *Panel B*: Determination of CAT activity for the constructs. Each respective CAT plasmid (5 μg) was cotransfected with pCH110 plasmid (1 μg) into HeLa cells as described in Materials and Methods. β-Galactosidase enzyme activity and protein concentration were measured in duplicate for each cell extract. After incubation of [^{14}C]chloramphenicol and acetyl-CoA with the cell extracts containing β-galactosidase at the same activity as an internal standard, acetylated and unacetylated chloramphenicol were separated by thin-layer chromatography, and the radioactivities were determined by a BAS2000 Imaging Analyzer. Lane 1, np −951 to +44 construct; lane 2, np −865 to +44 construct; lane 3, np −621 to +44 construct; lane 4, np −492 to +44 construct; lane 5, np −339 to +44 construct; lane 6, np −147 to +44 construct.

gene 5′-flanking regions upstream from the CAT gene of pSV0 cat were transfected into HeLa cells. At 48 h after transfection, the cells were harvested and their CAT activity was measured. The activities were expressed as the value relative to that of the np −951 to +44 construct. Whereas the constructs containing np −621 to +44 in the 5′-upstream region showed substantial CAT activity, those containing np −492 to +44 and with further deletion showed no activity (Figure 1). The construct lacking the transcription initiation site (np −85 to +44) showed hardly any activity. These results indicate that at least the sequence np −621 to +44 is necessary for expression of the HMG2 gene. Since the construct which contains np −951 to −478 did not show CAT activity, the sequence np −621 to −493 may not function as the promoter. Although the canonical 5′-regulatory motif, the TATA box, is absent from this gene (Shirakawa & Yoshida, 1992), the sequence np −85 to +44 must therefore function as a core promoter. Moreover, the results of the CAT assay suggest that there is (are) *cis* regulatory element(s) for HMG2 gene transcription in the sequence np −621 to −493.

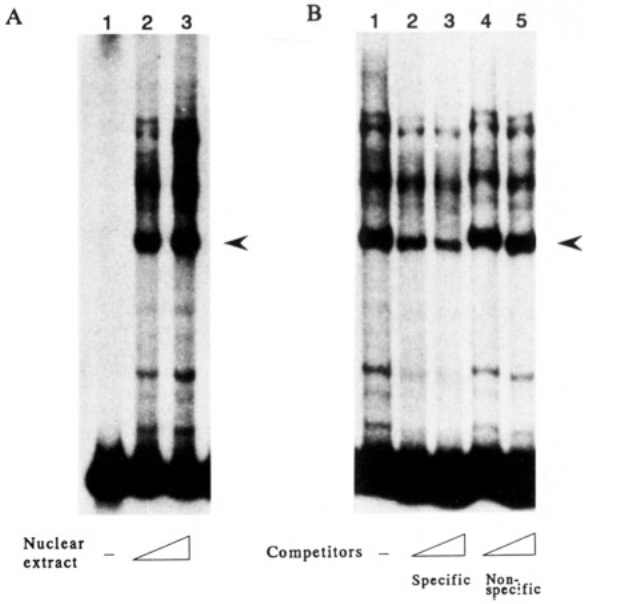

FIGURE 2: Binding of nuclear protein from HeLa cells to the HMG2 *cis* regulatory element. *Panel A*: EMSA was carried out using a DNA fragment containing np −624 to −478 and an unfractionated nuclear extract from HeLa cells. The arrow shows the major complex. Each reaction mixture contained 5 ng DNA fragment (approximately 10^4 cpm). Lane 1, without nuclear extract; lane 2, nuclear extract containing 1 μg of protein; lane 3, nuclear extract containing 2 μg of protein. *Panel B*: EMSA was carried out in the presence of specific or nonspecific competitors in addition to 5 ng DNA fragment containing np −624 to −478 (approximately 10^4 cpm) and unfractionated nuclear extract containing 1 μg of protein. Lane 1, without competitor; lanes 2 and 3, in the presence of a 5- and 10-fold molar excess of specific competitor DNA (np −624 to −478); lanes 4 and 5, in the presence of a 5- and 10-fold molar excess of nonspecific competitor 210-bp DNA fragment containing pBluescriptII KS(+) multicloning sites obtained by PCR amplification.

Identification and Characterization of the Transcription Factor for the Human HMG2 Gene. Electrophoresis mobility shift assay (EMSA) was performed using a DNA fragment containing np −624 to −478 to determine whether any *trans* element(s) exists to bind the *cis* regulatory element-(s). A ^{32}P-labeled DNA fragment was incubated with crude nuclear extract from HeLa cells and electrophoresed on polyacrylamide gel. As shown in Figure 2A, several major and minor complexes were observed. These were rather specific for the DNA fragment and dependent on the amount of nuclear extract used for incubation, as indicated by the competition experiments (Figure 2B). It was expected that some of these complexes were formed from transcription factors(s) bound to the *cis* regulatory element(s). To determine the binding nucleotide sequence in the most prominent complex, a DNase I footprint assay and a methylation interference analysis were performed. However, neither apparent footprint nor interfered bases were obtained (data not shown). Instead, more sensitive analysis with a chemical nuclease, 1,10-phenanthroline−copper ion complex was applied. As shown in Figure 3, the minimal protection region against the chemical nuclease was located at np −499 to −485 (or −486) on both the coding and noncoding strands. The protected sequence ($^{-499}$CCTAAATGGTGGTC^{-486}) was consistent with the minimum sequence determined by EMSA using various DNA fragments and competitive synthetic oligonucleotides of this region (data not shown). A nucleotide sequence AAATGG contained in the protected

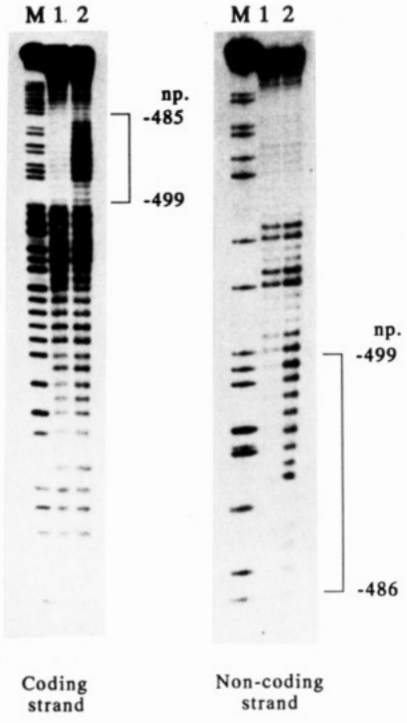

FIGURE 3: Determination of the binding nucleotide sequence of the *trans* element in the nuclear extract. The DNA fragment (np −533 to −478) was ^{32}P-labeled as described in Materials and Methods. Each coding or noncoding strand DNA (6 ng) was incubated with crude nuclear extract from HeLa cells (30 μg). The shifted and unshifted complexes separated by electrophoresis were digested in polyacrylamide gel using 1,10-phenanthroline−copper ion complex, and the DNA fragments recovered from the gel slices were analyzed in 10% polyacrylamide/urea gel. *Left panel* is the autoradiogram for the coding strand and *right panel* that for the noncoding strand. Lane 1, digestion product of shifted complex; lane 2, digestion product of unshifted complex; lane M, G+A specific sequencing ladder.

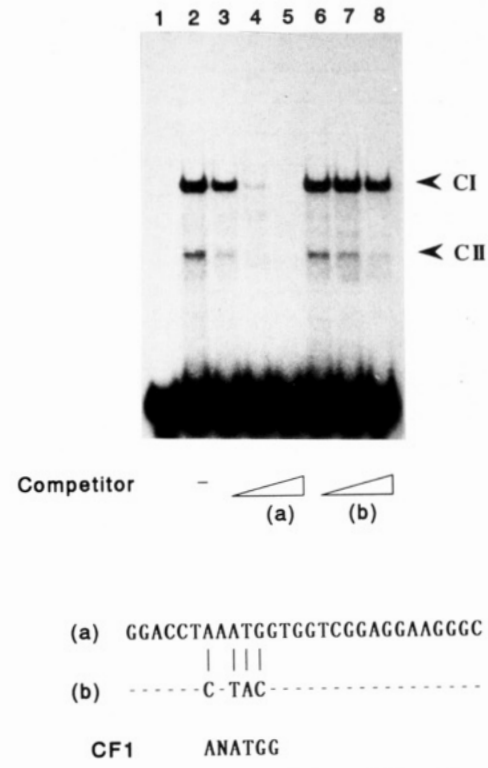

FIGURE 4: Binding of nuclear protein from HeLa cells to the 28-bp fragment. A 0.5-ng portion of the 28-bp fragment (np −502 to −475, approximately 10^4 cpm) and 3 μg of protein of the crude nuclear extract of HeLa cells were incubated in the presence or absence of unlabeled competitor DNAs and then subjected to EMSA. The competitors indicated at the bottom of the panel were (a) the wild-type sequence of 28 bp (np −502 to −475) and (b) a mutant sequence of 28 bp in which the nucleotides in the CF-1 motif were replaced with others. Lane 1, in the absence of nuclear extract or competitor; lane 2, in the absence of competitor; lanes 3−5, in the presence of a 10- , 100- , or 1000-fold molar excess of the wild-type competitor, respectively; lanes 6−8, in the presence of a 10- , 100- , or 1000-fold molar excess of the mutant competitor, respectively. The CI is the major complex, and the CII the minor one.

region is similar to the consensus motif in transcriptional factor CF-1, which regulates the expression of the c-*myc* gene in plasmocytoma (Kakkis et al., 1989). When a 28-bp fragment (np −502 to −475) containing the protected nucleotide sequence was subjected to EMSA, a distinct band (CI) was observed together with a minor one (CII) (Figure 4, lane 2). In the presence of the wild-type competitor at a maximum 10-fold molar excess, the bands disappeared (lanes 3−5). On the other hand, a mutant competitor, in which the consensus nucleotides in the CF-1 motif were replaced with CATACG, hardly inhibited formation of the major complex even at a 1000-fold molar excess (lane 8). These results suggest that the factor in the most prominent complexes in EMSA using a DNA fragment containing np −624 to −478 (the band marked by the arrowhead in Figure 2) corresponds to one giving the major band (CI) in EMSA using a 28-bp DNA fragment. In order to examine whether this factor giving the major complex affects the transcription of the HMG2 gene, a CAT-plasmid containing np −951 to +44 in the 5′-upstream region, in which the consensus nucleotides in the CF-1 motif were replaced with CATACG, was constructed. CAT activity in HeLa cells transfected with the mutant construct was 33% of that in the cells transfected with the wild construct. This result shows that the binding of the factor to the consensus nucleotides in the CF-1 motif is required for effective transcription of the HMG2 gene. Thus, this factor, named HMG2TF after HMG2 transcription factor, may recognize the same nucleotide sequence motif with CF-1 and regulate the level of expression of the HMG2 gene. In order to estimate the apparent molecular weight of HMG2TF, a UV cross-linking experiment was performed. A preliminary attempt showed that this factor was able to bind with the 28-bp fragment (np −502 to −475) in which bromodeoxyuridine was substituted for thymidine (data not shown). The bromodeoxyuridine-substituted DNA fragment−factor complex was cross-linked under UV irradiation in the polyacrylamide gel, heated at boiling temperature, and analyzed by SDS−polyacrylamide gel electrophoresis (Figure 5). The autoradiogram gave a clear band corresponding to an apparent molecular size of 85 000. This suggests that the component recognizes and binds to the nucleotide sequence in the 28-bp DNA fragment, although the molar number of the component involved in the binding is unclear.

In order to examine whether it is possible to determine the relative amount of HMG2TF in nuclear extract, an aliquot of crude nuclear extract from TIG-1 or HeLa cells was incubated with a ^{32}P-labeled 28-bp fragment (np −502 to −475) and analyzed by EMSA. The major complex (CI) increased in parallel with the amount of protein in both the nuclear extracts used for complex formation (Figure 6A). The other complex (CII) which was minor for HeLa cell

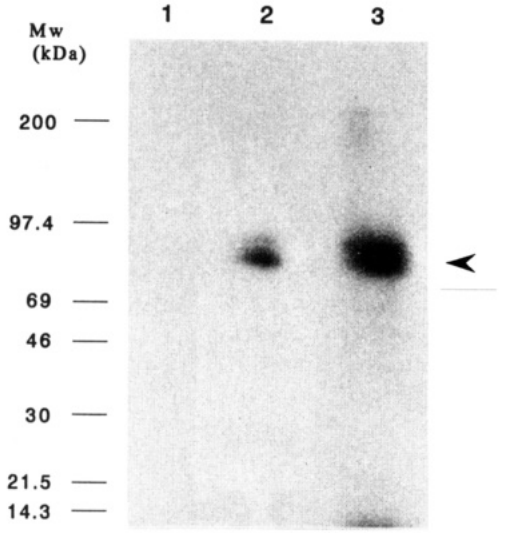

FIGURE 5: Estimation of the apparent molecular size of HMG2TF. The complex between ^{32}P-labeled bromodeoxyuridine-substituted DNA fragment and HMG2TF separated by electrophoresis was cross-linked by UV irradiation in polyacrylamide gel, heated at boiling temperature, and analyzed by SDS−polyacrylamide gel electrophoresis. Lane 1, without UV irradiation; lane 2, UV irradiation for 5 min; lane 3, UV irradiation for 30 min. The molecular weight markers are indicated in the left-hand column in kDa.

nuclear extract (see also Figure 4) was major (CII) for TIG-1 cells (lanes 2−6). The densitometric tracing (Figure 6B) showed that the relative amount of HMG2TF−DNA complex was proportional to the amount of nuclear extract used for the incubation. It may therefore be acceptable to presume the relative amount of HMG2TF in the nuclear extract by applying this method.

Relationship between the Amount of Transcription Factor and the Level of HMG2 mRNA in Cells. The 28-bp fragment was incubated with crude nuclear extracts prepared from TIG-1 cells in the growth and stationary phases. The relative amount of HMG2TF (CI) in the growing cells was about double that in the stationary cells (Figure 7A). For comparison, the total RNAs prepared from the TIG-1 cells at both cell phases were analyzed by Northern blot hybridization using pig thymus HMG2 cDNA (Shirakawa et al., 1990) as a probe. Expressed relative to the amount of β-actin mRNA, the level of HMG2 mRNA was enhanced in the growing cells 2−3-fold in comparison with that in the stationary cells (Figure 7B), consistent with our previous observation (Yamazaki et al., unpublished experiments). The similar results were obtained in 293 (Graham et al., 1977) and 321 (Sawada et al., 1988) cells, which are human embryonic kidney cells transformed with the adenovirus type 5 *E1* gene and type 5/12 *E1* genes, respectively (data not shown). In a previous study, the relative amount of HMG2 mRNA was found to be increased 3- or 4-fold in rat embryonal fibroblast 3Y1 cells after transformation with the adenovirus *E1a* and *E1b* genes (Yamazaki et al., unpublished experiments). Nuclear extracts from 3Y1 cells and the derivative cells transformed with the adenovirus *E1a* and *E1b* genes, XhoC cells, were prepared and subjected to EMSA (Figure 7C). The relative amount of DNA−HMG2TF complex was 2 times higher in the transformed cells than in the 3Y1 cells. The relationship between the amount of HMG2TF (CI) and the level of HMG2 mRNA in the various cells is listed in Table 1. The comparison

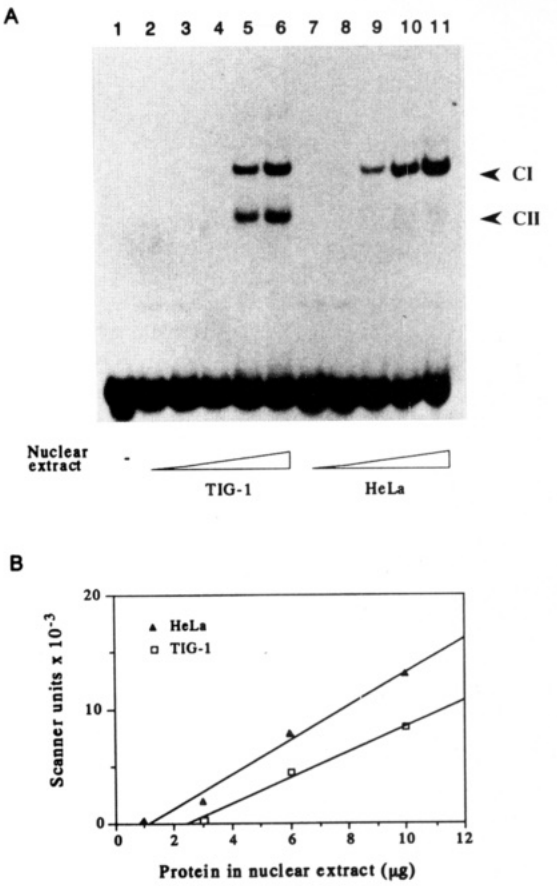

FIGURE 6: Determination of the relative amount of HMG2TF in nuclear extract by EMSA. *Panel A*: EMSA was carried out with 0.5 ng of the 28-bp fragment (np −502 to −475) and crude nuclear extracts from TIG-1 or HeLa cells. Lane 1, no nuclear extract; lanes 2−6, TIG-1 cell nuclear extract; lanes 7−11, HeLa cell nuclear extract. The amounts of protein in the nuclear extract are 0.5 μg (lanes 2 and 7), 1 μg (lanes 3 and 8), 3 μg (lanes 4 and 9), 6 μg (lanes 5 and 10), and 10 μg (lanes 6 and 11). *Panel B*: Determination of the amount of complex by densitometric tracing. The amounts of complex are indicated in scanner units against the amounts of protein in the nuclear extracts.

suggests that the amount of HMG2TF is positively related to the level of expression of HMG2 mRNA in the respective cells. This suggests that HMG2 expression may be regulated by the amount of HMG2TF.

DISCUSSION

We have revealed the primary structures of pig HMG1 and HMG2 proteins deduced from their cDNA sequences (Tsuda et al., 1988; Shirakawa et al., 1990), and the gene structure for human HMG2 (Shirakawa & Yoshida, 1992). The mRNA level of HMG2 protein was enhanced in exponentially growing rat embryonal fibroblasts in comparison with the arrested cells and by transformation with various viral genes and oncogenes. These results are consistent with the previous observations at the protein level that the levels of HMG2 proteins parallel the proliferative activity of various organs (Seyedin & Kistler, 1979; Craddock & Henderson, 1980). The 5′-flanking region of the human HMG2 gene does not contain the canonical TATA box, although several transcriptional initiation sites have been identified (Shirakawa & Yoshida, 1992). The construct containing np −621 to +44 in the 5′-upstream region showed a similar CAT activity to that containing np −951 to +44. The construct containing

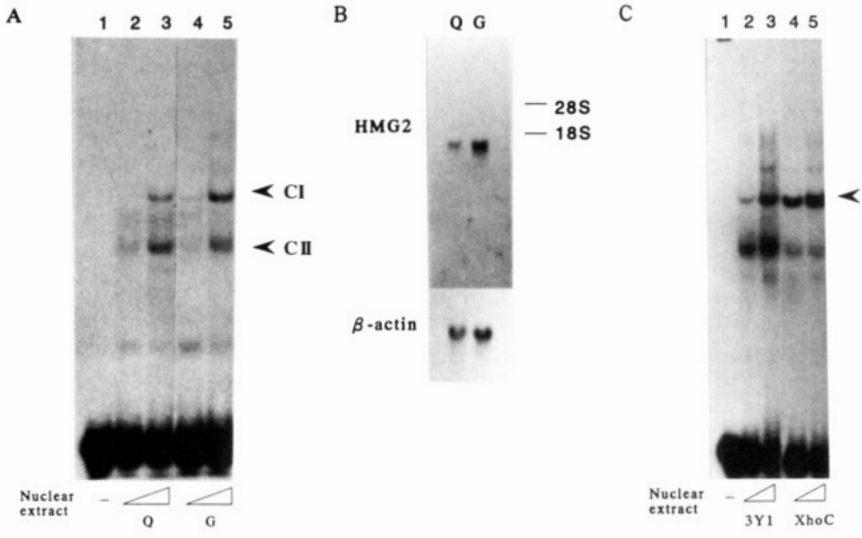

FIGURE 7: Comparative determination of the amount of HMG2TF with the level of expression of HMG2 mRNA. *Panel A*: Determination of the relative amount of HMG2TF in TIG-1 cells by EMSA. The assays were carried out using a 28-bp fragment (np −502 to −475, approximately 10^4 cpm) and the crude nuclear extract from TIG-1 in stationary phase by serum starvation (Q) and in the growth phase (G). Lane 1, without nuclear extract; lanes 2 and 3, 1 and 3 μg of protein of nuclear extract in the stationary cells; lanes 4 and 5, 1 and 3 μg of protein of nuclear extract in the growing cells. CI corresponds to the major complex and CII to the minor one in HeLa cell nuclear extract. *Panel B*: Expression of HMG2 mRNA in human fibroblast TIG-1. Fifteen micrograms of total RNAs prepared from TIG-1 cells in stationary phase (Q) and growth phase (G) were separated on 1.1% agarose gel and transferred to a cellulose nitrate filter. The hybridization was probed with ^{32}P-labeled pig thymus HMG2 cDNA (top) and mouse β-actin cDNA (bottom). The migrations of size marker RNAs are indicated in the right-hand column. *Panel C*: Determination of the relative amounts of HMG2TF in 3Y1 rat fibroblasts and their derivative cell line XhoC. EMSA was carried out using a 28-bp fragment (np −502 to −475, 10^4 cpm) and the crude nuclear extract of 3Y1 or XhoC cells. Lane 1, without nuclear extract; lanes 2 and 3, 3 and 6 μg of protein in 3Y1 nuclear extract; lanes 4 and 5, 3 and 6 μg of protein in XhoC nuclear extract. The arrow shows the HMG2TF−DNA complex.

Table 1: Summary of the Relative Amount of HMG2TF (CI) and the Level of HMG2 mRNA in the Cells[a]

animal	cells	HMG2TF (CI)	mRNA
human	HeLa(G)	1	1
	TIG-1(G)	0.57	0.50
	TIG-1(Q)	0.36	0.21
	293(G)	2.7	4.3
	321(G)	2.7	2.3
rat	3Y1(G)	1	1
	XhoC(G)	2.1	3.6

[a] The relative amount of HMG2TF (CI) per 1 μg of protein in nuclear extract was determined densitometrically and standardized against that of HeLa cells for human cells or that of 3Y1 cells for rat cells. The relative amount of HMG2 mRNA was determined densitometrically and standardized against that of α-tubulin mRNA (Lewis et al., 1985) in the cells. The amounts of HMG2 mRNA relative to that of HeLa cells for human cells or that of 3Y1 cells for rat cells are presented. G and Q in the brackets refer to cells in the growth and stationary phases, respectively. The relative amounts are means of three determinations.

np −865 to +44, however, showed lower activity in comparison with those constructs (Figure 1A). These results suggest that a negative control element and an antirepression element exist between np −865 to −622 and np −951 to −866, respectively. The present analysis also showed that in the majority of cases, gene transcription is initiated at the major promoter sequence deduced from primer extension analysis (Shirakawa & Yoshida, 1992). That is, both the sequences np −85 to +44 as a core promoter and np −621 to −493 as an enhancement *cis* element are necessary for expression of the HMG2 gene. In addition, we recognized the presence of a transcription regulation factor, HMG2TF, which binds specifically to the sequence np −499 to −485 in the *cis* element and stimulates the level of expression of the HMG2 gene. The region of np −499 to −485 contains a sequence very similar to the binding site in CF-1, which is a regulatory factor of c-*myc* gene expression first observed in mouse plasmocytoma, and thereafter in several other cell lines (Kakkis et al., 1989). HMG2TF recognizes the same sequence motif as CF-1. It is unclear whether HMG2TF is the same as CF-1, because the molecular characteristics of CF-1 have not been reported.

The relative amounts of HMG2TF in fibroblasts were enhanced in growing cells or transformed cells in comparison with cells in stationary phase or parental cells (Figure 7). It was noticeable that the amount of HMG2TF in these cells seemed to be positively related to the respective HMG2 mRNA level and also to the amount of HMG2 protein. The similar positive relationship was observed in all the cell lines which we examined (Table 1). The most likely explanation for these positive relationships is that transcription of the HMG2 gene is regulated by the amount of HMG2TF bound to the *cis* regulatory sequence far upstream from the HMG2 coding sequence.

A faster-migrating complex derived from TIG-1 cell nuclear extract (CII) was a minor component in EMSA using HeLa cell nuclear extract (Figures 6A and 7A). Competition assays suggested that this additional factor in TIG-1 cells may recognize a nucleotide sequence similar to HMG2TF. The level of HMG2 mRNA in HeLa cells was elevated in comparison with that in TIG-1 cells (data not shown), and the HMG2TF level in HeLa cells was also elevated (Figure 6B). These results suggest that HMG2 gene transcription in TIG-1 cells may be regulated by these two competitive factors which bind to the same sequence, although the real function of the component in the faster-migrating complex (CII) is unknown.

We have confirmed that HMG protein stimulates *in vitro* transcription (Waga et al., 1988, 1990). The transcriptional

activation is thought to be due to alteration of the chromatin structure by HMG protein. If this is the case, then the increase in the amount of HMG2TF during cell proliferation and upon cell transformation stimulates the transcription of the HMG2 gene. Studies along these lines are now under way.

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REFERENCES

Bidney, D. L., & Reeck, G. R. (1978) *Biochem. Biophys. Res. Commun. 85*, 1211−1218.

Craddock, V. M., & Henderson, A. R. (1980) *Carcinogenesis 1*, 445−450.

Dignam, J. D., Lebovitz, R. M., & Roeder, R. G. (1983) *Nucleic Acids Res. 11*, 1475−1489.

Ge, H., & Roeder, R. G. (1994) *J. Biol. Chem. 269*, 17136−17140.

Graham, F. L., Smiley, J., Russell, W. C., & Nairn, R. (1977) *J. Gen. Virol. 36*, 59−72.

Hamada, H., & Bustin, M. (1985) *Biochemistry 24*, 1428−1433.

Isackson, P. J., Fishback, J. L., Bidney, D. L., & Reeck, G. R. (1979) *J. Biol. Chem. 254*, 5569−5572.

Javaherian, K., Liu, L. F., & Wang, J. C. (1978) *Science 199*, 1345−1346.

Kakkis, E., Riggs, K. J., Gillespie, W., & Calame, K. (1989) *Nature 339*, 718−721.

Kimura, G., Itagaki, A., & Summers, J. (1975) *Int. J. Cancer 15*, 694−706.

Kuwabara, M. D., & Sigman, D. S. (1987) *Biochemistry 26*, 7234−7238.

Laimins, L. A., Khoury, G., Gorman, C., Howard, B., & Gruss, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6453−6457.

Laudet, V., Stehelin, D., & Clevers, H. (1993) *Nucleic Acids Res. 21*, 2493−2501.

Lewis, S. A., Lee, M. G., & Cowan, N. J. (1985) *J. Cell Biol. 101*, 852−861.

MacDonald, R. J., Swift, G. H., Przybyla, A. E., & Chirgwin, J. M. (1987) *Methods Enzymol. 152*, 219−227.

Makiguchi, K., Chida, Y., Yoshida, M., & Shimura, K. (1984) *J. Biochem. (Tokyo) 95*, 423−429.

Ohashi, M., Aizawa, S., Ooka, H., Ohsawa, T., Kaji, K., Kondo, H., Kobayashi, T., Noumura, T., Matsuo, M., Mitsui, Y., Murota, S., Yamamoto, K., Ito, H., Shimada, H., & Utakoji, T. (1980) *Exp. Gerontol. 15*, 121−133.

Reeck, G. R., Isackson, P. J., & Teller, D. C. (1982) *Nature 300*, 76−78.

Rosenthal, N. (1987) *Methods Enzymol. 152*, 704−720.

Sambroook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp 1.21−1.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sawada, Y., Raska, K., & Shenk, T. (1988) *Virology 166*, 281−284.

Seyedin, S. M., & Kistler, W. S. (1979) *J. Biol. Chem. 254*, 11264−11271.

Shirakawa, H., & Yoshida, M. (1992) *J. Biol. Chem. 267*, 6641−6645.

Shirakawa, H., Tsuda, K., & Yoshida, M. (1990) *Biochemistry 29*, 4419−4423.

Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180−186.

Singh, J., & Dixon, G. H. (1990) *Biochemistry 29*, 6295−6302.

Thomas, P. S. (1983) *Methods Enzymol. 100*, 255−266.

Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M., & Sakiyama, S. (1986) *Nucleic Acids Res. 14*, 2829.

Tremethick, D. J., & Molloy, P. L. (1986) *J. Biol. Chem. 261*, 6986−6992.

Tremethick, D. J., & Molloy, P. L. (1988) *Nucleic Acids Res. 16*, 11107−11123.

Tsuda, K., Kikuchi, M., Mori, K., Waga, S., & Yoshida, M. (1988) *Biochemistry 27*, 6159−6163.

Tsutsui, K., Hara, E., Nakada, S., & Oda, K. (1990) *Virus Genes 4*, 239−256.

Waga, S., Mizuno, S., & Yoshida, M. (1988) *Biochem. Biophys. Res. Commun. 153*, 334−339.

Waga, S., Mizuno, S., & Yoshida, M. (1990) *J. Biol. Chem. 265*, 19424−19428.

Williams, M., Brys, A., Weiner, A. M., & Maizels, N. (1992) *Nucleic Acids Res. 20*, 4935−4936.

Yoshida, M., & Shimura, K. (1984) *J. Biochem. (Tokyo) 95*, 117−124.

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