Nuclear Accumulation of HMG2 Protein Is Mediated by Basic Regions Interspaced with a Long DNA-Binding Sequence, and Retention within the Nucleus Requires the Acidic Carboxyl Terminus†

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ABSTRACT: High mobility group 2 (HMG2) protein is ubiquitously distributed in the nucleus of higher eukaryotic cells. Accumulation of an HMG2−β-galactosidase fusion protein expressed in COS-7 cells suggested active transport of HMG2 protein into the nucleus after translation in the cytoplasm. Deletion analysis of the HMG2 sequence in the HMG2−β-galactosidase fusion protein indicated that basic regions interspaced with the long DNA-binding sequence in HMG2, called the HMG1/2 box, are necessary for the nuclear accumulation of HMG2. The close configuration of basic regions at both ends of the DNA-binding sequence in the tertiary structure may function as the nuclear localization signal. This novel nuclear localization signal structure is different from typical ones such as the single or bipartite basic cluster in many nuclear proteins. A portion of the HMG2 molecule remained in the cytoplasm after translation. Interspecies heterokaryon assay demonstrated that the acidic carboxyl terminus of HMG2 was necessary for retention of the protein in the nucleus.

High mobility group 2 (HMG2)[1] protein is an abundant chromosomal protein present in the cells of higher eukaryotes and may be distributed in active chromatin (Goodwin & Mathew, 1982). The amount of HMG2 protein parallels the proliferative activity of cells in various organs (Seyedin & Kistler, 1979). The level of HMG2 mRNA is enhanced in exponentially growing cells and cells transformed with various viral genes (Shirakawa & Yoshida, 1995; Yamazaki et al., 1995), and cell growth is repressed by expression of antisense HMG2 RNA (Yamazaki et al., 1995). These findings indicate that HMG2 protein must be closely related to cell proliferation. Several experiments have indicated that HMG2 participated in gene transcription (Stelzer et al., 1994; Shykind et al., 1995; Zwilling et al., 1995). HMG1, which shows marked structural similarity to HMG2, is also a ubiquitously distributed protein in the nucleus (Tsuda et al., 1988; Shirakawa et al., 1990). Several studies have indicated that HMG1 stimulates transcription *in vitro* (Tremethick & Molloy, 1986, 1988; Waga et al., 1988, 1990; Singh & Dixon, 1990) and in cultured cells (Aizawa et al., 1994; Ogawa et al., 1995), suggesting that its function is different from that of HMG2 protein, despite their structural similarity. If this is the case, the subcellular distributions and translocational mechanisms of the two proteins may differ.

The nuclear proteins are translocated into the nucleus through the nuclear pore complexes on the nuclear membrane after translation in the cytoplasm (Peter, 1986). Because the nuclear pore complexes function as aqueous channels with a diameter of about 10 nm, they must allow passive diffusion of proteins with molecular masses of up to 40−60 kDa (Peter, 1986). On the other hand, Breeuwer and Goldfarb (1990) have suggested that many nuclear proteins actively enter the nucleus regardless of their molecular size. The molecular mass of HMG2 protein deduced from its cDNA sequence is 24 kDa (Shirakawa et al., 1990). After microinjection into the cytoplasm, isotope-labeled HMG2 is translocated into the nucleus (Wu et al., 1981). However, it is unclear whether the nuclear accumulation of HMG2 is dependent on passive diffusion or on active transport mediated by a nuclear localization signal (NLS) present in the protein.

HMG2 protein consists of three structural regions (Reeck et al., 1982). Two-thirds of the sequence from the amino terminus has a DNA-binding region consisting of two similar, but nonidentical, repeats of about 75 amino acids, which are called the "HMG1/2 box", respectively. This novel DNA-binding motif is also known to be present in many nuclear transcription factors (Laudet et al., 1993). The carboxyl terminus of HMG2 is a unique acidic domain consisting of 23 continuous acidic amino acid residues. HMG1 and some HMG-related proteins containing the HMG1/2 box(es) have been shown to become localized in the nucleus by active transport (Tsuneoka et al., 1986; Maeda et al., 1992; Poulat et al., 1995). However, the NLS for HMG1 has not been identified (Tsuneoka et al., 1986). The NLS for UBF and SRY exists in each of their respective HMG1/2 boxes (Maeda et al., 1992; Poulat et al., 1995), and the NLSs for many DNA-binding proteins have been identified in their DNA-binding sequences and flanking regions (LaCasse &

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[1] Abbreviations: BSA, bovine serum albumin; β-gal, β-galactosidase; HMG, high mobility group; NLS, nuclear localization signal(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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Lefebvre, 1995). Thus, it seems likely that nuclear accumulation of HMG2 after translation may be similarly mediated by the NLS contained in the molecule, despite suggestions that HMG2 exists in both the nucleus and cytoplasm (Bustin & Neihart, 1979; Isackson et al., 1980; Wu et al., 1981; Kuehl et al., 1984; Mosevitsky et al., 1989).

In the present study, we examined the subcellular distribution of HMG2 by expressing the protein fused to β-galactosidase (β-gal). It was found that the HMG2−β-gal fusion protein was actively translocated into the nucleus and that basic regions interspaced with the long DNA-binding sequence were necessary for the nuclear accumulation of HMG2. The close configuration of basic regions at both ends of the sequence in the tertiary structure may function as the NLS. This novel NLS structure is different from typical ones such as the single or bipartite basic cluster in many nuclear proteins (Dingwall & Laskey, 1991). It was also indicated that part of HMG2 remained in the cytoplasm after translation, while the major portion was translocated into the nucleus. The acidic carboxyl-terminal region is important for retention of HMG2 in the nucleus.

MATERIALS AND METHODS

Cell Culture. COS-7, CV-1, TIG-3 (human fibroblast) (Matsuo et al., 1982), HeLa, and C127 (mouse epithelial-like) cells were grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal calf serum (GIBCO) under 5% CO_2 at 37 °C. TIG-3 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB).

Polymerase Chain Reaction. Polymerase chain reaction (PCR) was carried out in a total volume of 100 μL containing 40 pmol of 5′- and 3′-primers, 50 ng of pig thymus HMG2 cDNA, 160 μM each dNTP, *Pfu* DNA polymerase buffer (Stratagene), and 2 drops of mineral oil (Sigma). After being heated at 95 °C for 5 min to denature the template DNA, 2.5 units of *Pfu* DNA polymerase (Stratagene) was added, and the mixture was subjected to 25 cycles at 95 °C for 1.5 min, 55 °C for 1.5 min, and 72 °C for 3 min. The 5′-primers contained a *Hin*dIII site and Kozak's sequence (Kozak, 1989) upstream from the ATG codon. All of the 3′-primers had a *Kpn*I site.

Plasmid Construction. Several expression plasmids were constructed by ligating the whole (plasmid pCH-UF) or truncated sequences (plasmid pCH-U) of the cDNA encoding pig thymus HMG2 (Shirakawa et al., 1990) amplified by PCR between the *Hin*dIII and *Kpn*I sites upstream from the *lacZ* gene of the plasmid pCH110 (Pharmacia). The pCH-DF plasmid was constructed by ligating the full length of the HMG2 cDNA into the *Eco*RI site in the *lacZ* gene. The plasmids were prepared by the alkaline−SDS method and then purified by equilibrium centrifugation in cesium chloride (Sambrook et al., 1989).

DNA Transfection. Each respective expression plasmid (0.5 μg) was transfected into COS-7 cells by a cationic liposome method using Transfection reagent (Boehringer Mannheim). The DNA−liposome complex was added to semiconfluent COS-7 cells in Hepes-buffered saline (20 mM Hepes−NaOH, pH 7.4, containing 150 mM NaCl) on a coverslip in a 35-mm-diameter dish. After transfection for 4 h, the medium was exchanged with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and the cells were incubated for another 48 h.

Detection of HMG2−β-Galactosidase Fusion Proteins by Western Blotting Analyses. The COS-7 cells transfected with the expression plasmid were harvested and suspended in SDS gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% sucrose), followed by heating in boiling water for 5 min. The protein in the cell lysate was electrophoresed on 7.5% polyacrylamide gel by the method of Laemmli (1970) and then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) in 48 mM Tris, 39 mM glycine, and 20% methanol at 250 mA constant current for 3 h. Anti-HMG2 monoclonal antibody (final concentration 0.2 μg/mL) and anti-β-galactosidase antibody (0.2 μg/mL, Promega) were used as primary antibodies, followed by detection with an ECL assay system (Amersham).

Immunofluorescence Analysis of HMG2 Protein in Cells. Exponentially growing cells on coverslips were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 10 min. After treatment with 1% Nonidet P-40 in PBS for 5 min, the cells were incubated with anti-HMG2 monoclonal antibody (0.5 μg/mL) and with anti-mouse IgG−fluorescein (20 μg/mL) as the secondary antibody (Boehringer Mannheim).

Chromogenic Staining of HMG2−β-Gal Fusion Protein in Cells. COS-7 cells transfected with the expression plasmid were washed with PBS after 48 h twice and fixed in 2% formaldehyde containing 0.2% glutaraldehyde in PBS at 4 °C for 10 min. After being washed with PBS, the cells were incubated in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 0.2 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in PBS at 37 °C for 16 h. After immersion in cold acetone at −20 °C for 10 min, the cellular distribution of fusion protein was examined by microscopy.

DNA-Binding Assay for Fusion Proteins. The fusion proteins were isolated from the cell lysate by immunoprecipitation (Cullen, 1987). Briefly, the cells transfected with the plasmid so as to express the fusion protein were harvested and then suspended in RIPA buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride). Anti-β-gal antibody was added to each cell lysate, followed by incubation at 4 °C for 1 h. The complex of the fusion protein with the antibody was precipitated with protein A-conjugated beads and dissolved in SDS gel loading buffer. After electrophoresis on 7.5% polyacrylamide gel, the fusion proteins were transferred to a PVDF membrane and treated with Tris-buffered saline containing Tween-20 (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20), followed by Southwestern analysis. The protein on the membrane was incubated in binding buffer (10 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, and 0.1% BSA) for 30 min at room temperature and then in the binding buffer containing ^{32}P-labeled linearized pBR322 DNA for 1 h. The filter was washed three times with the binding buffer, removing NaCl, for 10 min and then processed for autoradiography. The radioactivity was determined with a BAS2000 imaging analyzer (Fuji Film).

Interspecies Heterokaryon Assay. The fusion protein was expressed in COS-7 cells on a coverslip by transfection. At 48 h after transfection, C127 cells were seeded on the same

coverslip for 3 h; then the cells, after once washing with calcium−magnesium-free Hank's solution, were fused according to Borer et al. (1989). After heterokaryon formation, the cells were incubated in a culture medium containing 50 μg/mL cycloheximide for 5 h. Indirect immunofluorescence assays were carried out after cell fixation with 4% paraformaldehyde in PBS. The cells were stained with anti-mouse IgG−rhodamine (Boehringer Mannheim) as a second antibody, followed by staining with Hoechst 33258 dye (0.5 μg/mL, Wako) to distinguish the nuclei.

RESULTS

Expression of HMG2−β-Galactosidase (HMG2−β-Gal) Fusion Protein in COS-7 Cells. In order to examine whether the HMG2−β-gal fusion protein accumulated in the cell nucleus, the fusion protein was expressed in *Escherichia coli* cells and purified to homogeneity. The protein was then microinjected into the cytoplasm of CV-1 cells. However, the cellular distribution of the protein could not be observed due to the rapid aggregation. Therefore, the HMG2−β-gal fusion protein was directly expressed in eukaryotic cells. The plasmids pCH-UF and pCH-DF, each containing HMG2 cDNA up- and downstream from the *lacZ* gene in the eukaryotic expression vector pCH110, were transfected into COS-7 cells. The cells were harvested after 48 h, and expression of the fusion proteins was examined by Western blotting analysis after SDS−polyacrylamide gel electrophoresis using anti-β-gal monoclonal antibody. A band of 120 kDa was observed in the lysate of cells transfected with pCH110 (data not shown). The molecular mass of the protein corresponded to that of β-gal. Molecules larger than β-gal (140 kDa) were detected in the lysates of cells transfected with pCH-UF and pCH-DF, respectively (data not shown). The molecular masses of the proteins were similar to those expected for β-gal fused with HMG2 (Shirakawa et al., 1990). The fusion proteins obtained from the cell lysates by immunoprecipitation with anti-β-gal monoclonal antibody were electrophoresed and subjected to the Western blotting analysis with anti-HMG2 monoclonal antibody. Bands in the corresponding positions were detected (data not shown). These results indicated that the HMG2−β-gal fusion protein was expressed in COS-7 cells.

Nuclear Accumulation of HMG2−β-Gal Fusion Protein. The cellular distribution of HMG2−β-gal fusion protein was examined by an indirect immunofluorescence method. Forty-eight hours after transfection with the plasmids pCH-UF and pCH-DF, the cells were fixed and incubated with anti-β-gal antibody and then with anti-mouse IgG−fluorescein. The fluorescent signals were detected exclusively in the nucleus, and not in the cytoplasm, of cells transfected with pCH-UF and pCH-DF and in the cytoplasm of cells transfected with pCH110, indicating that the HMG2−β-gal fusion protein accumulated in the nucleus (data not shown). Similar results were obtained for cell lines which stably express the fusion proteins (data not shown). Proteins with a molecular mass exceeding 60 kDa are unable to diffuse passively through the nuclear membrane (Peter, 1986). Therefore, accumulation of the fusion proteins in the nucleus was considered to be dependent on a pathway mediated by the NLS which is present in the HMG2 protein.

Identification of the NLS in HMG2 Protein. To identify the NLS in HMG2, we constructed various derivative plasmids containing truncated HMG2 cDNA upstream from the *lacZ* gene in pCH110. After confirmation of the entirety for HMG2 cDNA sequences introduced by the PCR reactions into plasmids, each plasmid was then transfected into COS-7 cells. The cellular distribution of the β-gal fusion protein expressed was investigated by staining the respective cells with the chromogenic substrate X-gal. Although the respective expression levels of fusion proteins in COS-7 cells were different, the nuclear accumulation of fusion proteins had no relation to the expression levels (data not shown). As shown in Figure 1, the fusion construct containing amino acids 1−91 of the HMG2 sequence was localized in the nucleus (Figure 1e). However, the relative accumulation was lower than that of the fusion protein containing the full HMG2 molecule (Figure 1b). HMG1/2 box A (amino acid residues 1−75), one of the DNA-binding domains in HMG2, is present in this part of the molecule. However, the fusion proteins containing amino acid residues 1−75 and 12−91 did not accumulate in the nucleus (Figure 1f,g), and those containing amino acid residues 74−164 and 88−179 were localized in the nucleus (Figure 1n,m). HMG1/2 box B (amino acid residues 88−164), another DNA-binding domain in HMG2 contained in these regions, did not show nuclear accumulation activity (Figure 1o). The fusion proteins containing one of the flanking sequences of HMG1/2 box B (amino acids 74−91 and 165−209) were localized in the cytoplasm (Figure 1r,l). The subcellular localizations of the various fusion proteins in Figure 1 are summarized in Figure 3, together with their DNA-binding properties. At least one of two DNA-binding domains (amino acids 1−75 or 88−164) was necessary, but not sufficient alone, for localization of the fusion proteins in the nucleus. A few amino acid clusters corresponding to the so-called bipartite motif of the NLS in both the HMG1/2 boxes (amino acids 42−58 and 126−132) were detected. However, these motifs did not seem to take part in nuclear accumulation of the fusion proteins. Instead, both the flanking amino acid sequences of the HMG1/2 box and HMG1/2 box itself were necessary. The nuclear accumulation of the fusion proteins containing two HMG1/2 boxes and the flanking sequences (Figure 1b,c,d,i) was more effective than that of proteins with a single HMG1/2 box and the respective flanking sequence (Figure 1e,j,k,m,n), suggesting an additional effect of the NLS.

DNA-Binding Property of Fusion Proteins. In order to examine whether the DNA-binding property of HMG2 protein is related to its nuclear accumulation, the DNA-binding activity of fusion proteins was examined. The fusion proteins partially purified by immunoprecipitation with anti-β-gal antibody were electrophoresed, followed by blotting onto a membrane. As shown in Figure 2a, the fusion constructs containing amino acid residues 1−91 (lane 2) and the full length of HMG2 (lane 3) showed DNA-binding activity. However, no DNA binding for the protein fused with HMG1/2 box A (1−75) was detected (lane 1), even though a substantial amount of the protein was loaded on the membrane. This may have resulted partly from the weaker DNA-binding property of the sole HMG1/2 box, weaker binding of DNA to the immobilized HMG1/2 box on the membrane, and/or partial shielding of the HMG1/2 box in the fusion protein. Therefore, the DNA-binding properties of the peptides which were expressed in *E. coli* and purified (Tanabe et al., in preparation) were also taken into consideration. Even the peptides containing a single

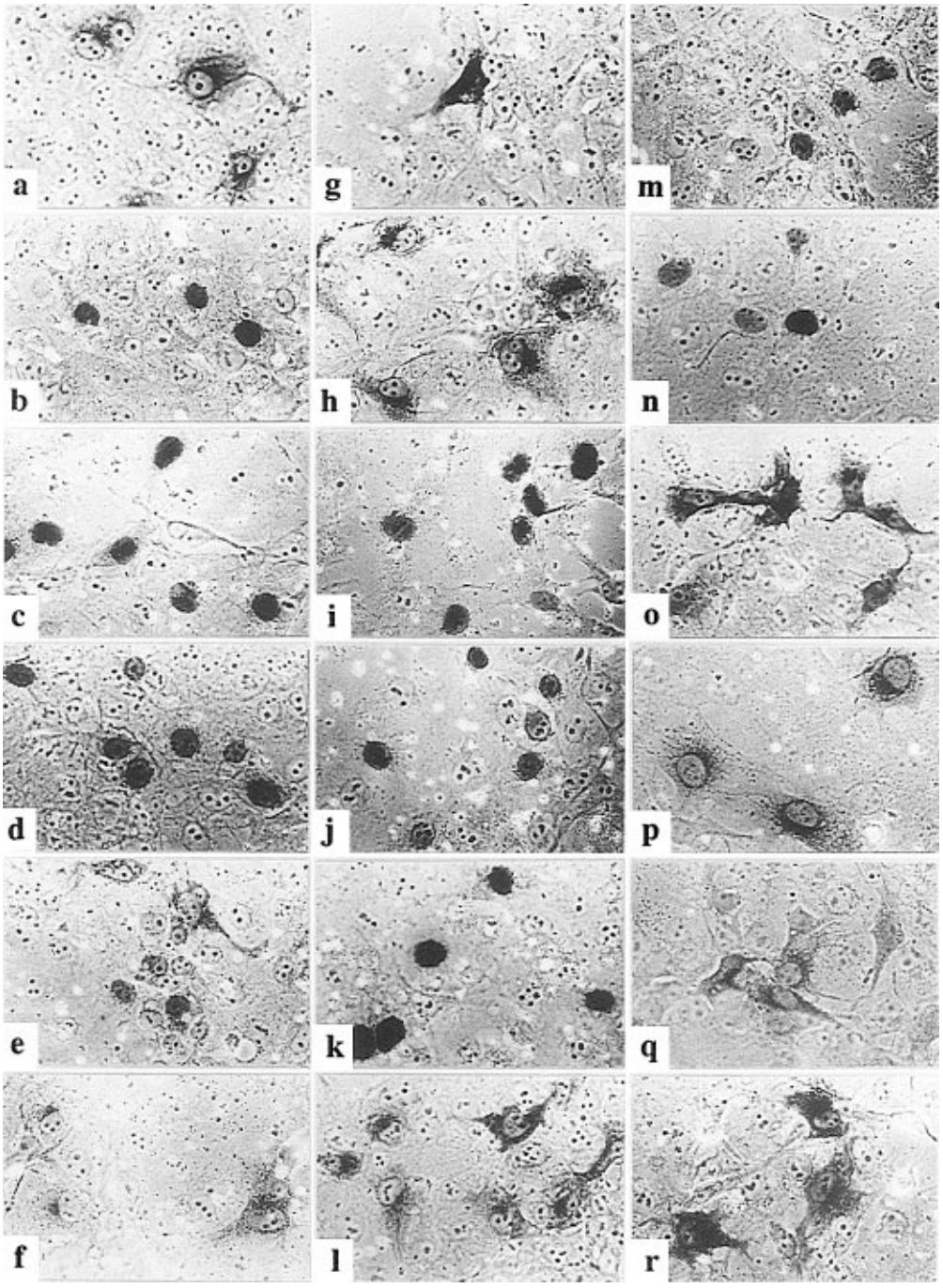

FIGURE 1: Chromogenic analysis of the subcellular distribution of β-gal fused with HMG2-truncated peptide. Cells fixed at 48 h after transfection with each respective plasmid were incubated with X-gal and examined by microscopy. The expression plasmids used for transfection were pCH110 (panel a), pCH-UF (panel b), pCH-U(1−179) (panel c), pCH-U(1−164) (panel d), pCH-U(1−91) (panel e), pCH-U(1−75) (panel f), pCH-U(12−91) (panel g), pCH-U(12−75) (panel h), pCH-U(12−209) (panel i), pCH-U(74−209) (panel j), pCH-U(88−209) (panel k), pCH-U(165−209) (panel l), pCH-U(88−179) (panel m), pCH-U(74−164) (panel n), pCH-U(88−164) (panel o), pCH-U(99−179) (panel p), pCH-U(74−155) (panel q), and pCH-U(74−91) (panel r). The numbers in parentheses show the amino acid residues in HMG2 fused to β-gal.

HMG1/2 box showed DNA-binding activity, as shown diagrammatically in Figure 3. These results indicate that the DNA-binding sequence is required, but insufficient alone for nuclear accumulation of HMG2 protein.

Subcellular Localization of HMG2 Protein. Several investigations have shown that HMG2 exists in the cytoplasm as well as the nucleus (Bustin & Neihart, 1979; Isackson et al., 1980; Wu et al., 1981; Kuehl et al., 1984; Mosevitsky et al., 1989). These results seemed to be inconsistent with the nuclear accumulation of HMG2 observed in the above experiments. In order to confirm the presence of HMG2 in the cytoplasm, immunological staining of the cytoplasm was conducted using the monoclonal antibody against HMG2. A lysate of the cytoplasmic and nuclear fractions from exponentially growing CV-1 cells was electrophoresed and analyzed for HMG2 by Western blotting with the monoclonal antibody against HMG2 of neither cross-reaction with HMG1 nor other proteins in cell lysate. HMG2 was observed in the both fractions (not shown). On the other hand, CV-1 cells in the exponential growth phase were fixed and subjected to immunofluorescence staining. The nuclei showed intense staining (Figure 4a). In addition, a weak

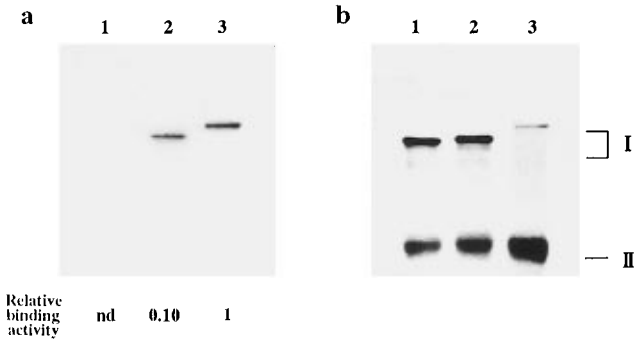

FIGURE 2: DNA-binding activity of β-gal fusion proteins. The partially purified β-gal fusion proteins were electrophoresed on 7.5% SDS−polyacrylamide gel, transferred to a PVDF membrane, and incubated with ^{32}P-labeled pBR322 DNA (panel a) and β-gal antibody (panel b). The fusion proteins were from lysates of cells transfected with pCH-U(1−75) (lane 1), pCH-U(1−91) (lane 2), and pCH-UF (lane 3). The DNA-binding activities at the bottom in panel a are expressed as values relative to that of the pCH-UF construct. nd indicates undetected DNA binding. Bands I and II in panel b correspond to fusion proteins and heavy chains of antibody, respectively.

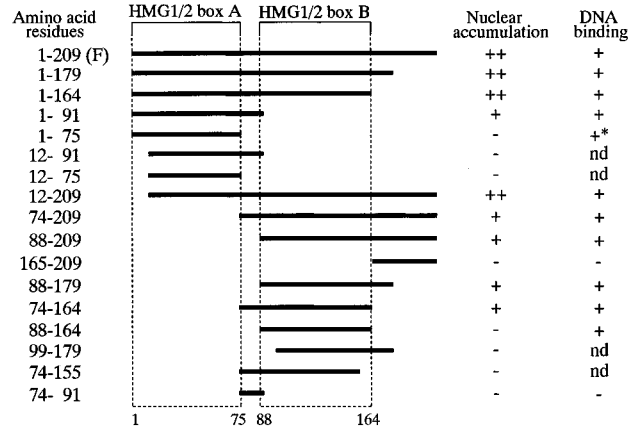

FIGURE 3: Summary of nuclear accumulation of β-gal fusion constructs containing HMG2-truncated peptides. HMG1/2 boxes A (1−75) and B (88−164) are boxed by dotted lines. The percentages of cells that exhibited nuclear accumulation of the fusion construct are shown in the right-hand column; ++, more than 80% of fusion proteins were accumulated in the nucleus of 100 expressing cells; +, 30−80%; −, less than 30%. DNA-binding ability is shown in the right-hand column; +, DNA binding shown by Southwestern analysis and/or electrophoresis mobility shift assay; +*, shown by electrophoresis mobility shift assay but not Southwestern analysis; nd, not determined.

fluorescence signal was observed in the cytoplasm. Similar results were obtained for COS-7, TIG-3 human fibroblasts, and HeLa cells (Figure 4b−d). Because neither cytoplasmic nor nuclear fluorescence could be observed using anti-HMG2 antibody which had preincubated with pig HMG2 protein, some HMG2 molecules may exist in the cytoplasm. There were two possible explanations for the presence of HMG2 in the cytoplasm: one is that a constant amount of HMG2 remains in the cytoplasm after translation, and the other is that some HMG2 molecules are exported from the nucleus by an unknown mechanism.

Export of Acidic Carboxyl-Terminal-Truncated HMG2 Fusion Proteins out of the Nucleus. To investigate whether HMG2 could be exported out of the nucleus, an interspecies heterokaryon assay was performed. The assay has been used previously to analyze nuclear−cytoplasmic shuttling proteins such as nucleolin (Borer et al., 1989; Schmidt-Zachmann et al., 1993) and hormone receptors (Madan & DeFranco, 1993; Guiochon-Mantel et al., 1994). An interspecies heterokaryon was formed by fusing COS-7 cells expressing HMG2−β-gal fusion protein with mouse C127 cells. The cells were then incubated in culture medium containing cycloheximide, fixed with formaldehyde, and stained with anti-β-gal antibody. The fusion protein in the nucleus of COS-7 did not translocate into the nucleus of C127 after 5 h (Figure 5a−c) and 10 h of incubation (not shown). These results suggested that the HMG2−β-gal fusion protein does not shuttle between the nucleus and the cytoplasm. The protein fused with carboxyl-terminal-truncated HMG2 (amino acid residues 1−179) translocated to the nucleus of C127 cells (Figure 5d−f), suggesting that the carboxyl terminus of HMG2 is necessary for its retention in the nucleus.

DISCUSSION

NLSs in several nuclear HMG-related proteins have been reported. The NLS in human SRY, which contains a single HMG1/2 box for DNA binding, is considered to be a typical bipartite sequence in the HMG1/2 box motif (Poulat et al., 1995), and nuclear localization of other nuclear HMG-related proteins may also be mediated by similar bipartite NLSs (Poulat et al., 1995). The NLS in mouse UBF has been shown to be a basic cluster in a region out of the HMG1/2 box (Maeda et al., 1992). In the present study, fusion proteins containing a single HMG1/2 box including amino acid residues 1−75 (box A) or 88−164 (box B) were not accumulated in the cell nucleus. Therefore, the nuclear localization mechanism of HMG2 may differ from those of SRY, UBF, and other HMG-related proteins.

The accumulation of β-gal fusion protein with HMG2 in the cell nucleus was considered to be dependent on the NLS contained in HMG2 (Figure 1). One of three amino acid sequences, residues 1−91, 74−164, or 88−179, was necessary for nuclear accumulation (Figures 1 and 3). The fusion constructs containing shorter sequences of residues 12−91, 99−179, or 74−155, as well as 1−75 (box A) and 88−164 (box B), did not accumulate in the nucleus. These results indicated that a sequence additional to a single HMG1/2 box is required for HMG2 accumulation in the nucleus.

The presence of HMG1/2 box B is common to two sequences (amino acid residues 74−164 and 88−179). Another sequence (1−91) contains HMG1/2 box A. The amino- and carboxyl-terminal regions of these sequences contain common basic-charged regions (Shirakawa et al., 1990). Because the fusion constructs from which one of the basic regions (12−91, 99−179, and 74−155) was deleted were not translocated into the nucleus, both of the two basic regions must be necessary for nuclear accumulation. How do two basic regions interspaced with a long DNA-binding sequence function as an NLS? The tertiary structure of a single HMG1/2 box suggests that the nuclear accumulation signal of this protein is determined by a higher order structure rather than the simple primary sequence. A hypothetical tertiary model structure of HMG2 protein derived from the HMG1 box B structure determined by NMR spectroscopy (Read et al., 1993; Weir et al., 1993) is illustrated in Figure 6. Each respective HMG1/2 box comprises three α-helices. The relative distance between the two HMG1/2 boxes may be less, considering the thermodynamic stability of the molecules. The acidic carboxyl-terminal region is expected

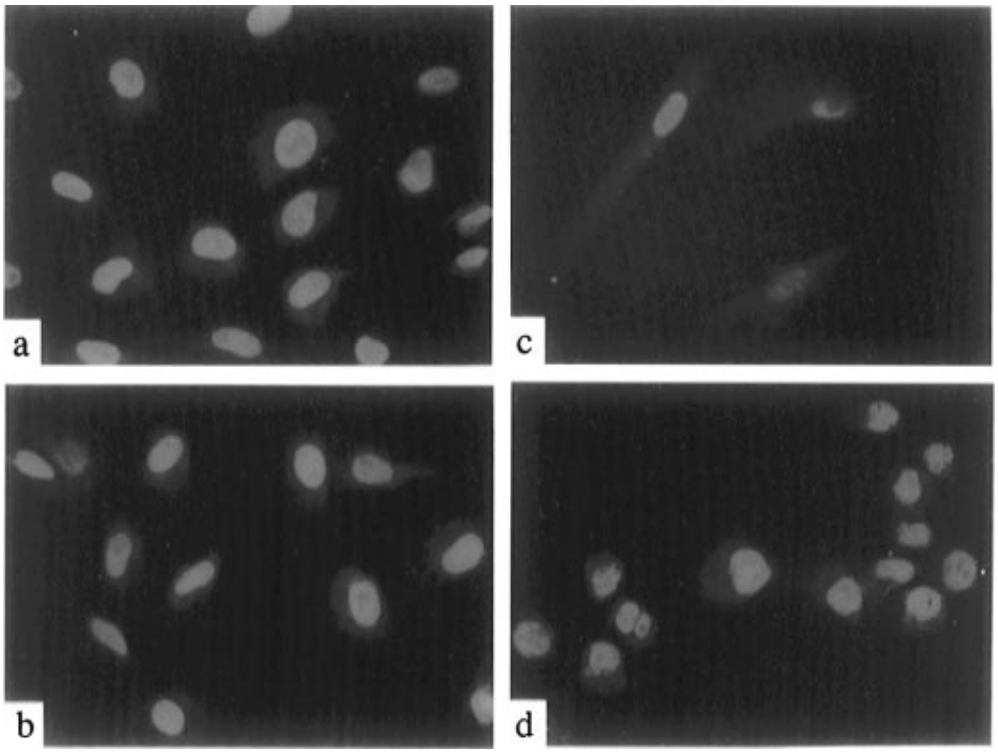

FIGURE 4: Indirect immunofluorescence detection of the subcellular distribution of HMG2. CV-1 (panel a), COS-7 (panel b), TIG-3 (panel c), and HeLa (panel d) in exponential growth phase were fixed and incubated with anti-HMG2 monoclonal antibody (0.5 μg/mL) and then with anti-mouse IgG−conjugated fluorescein (20 μg/mL).

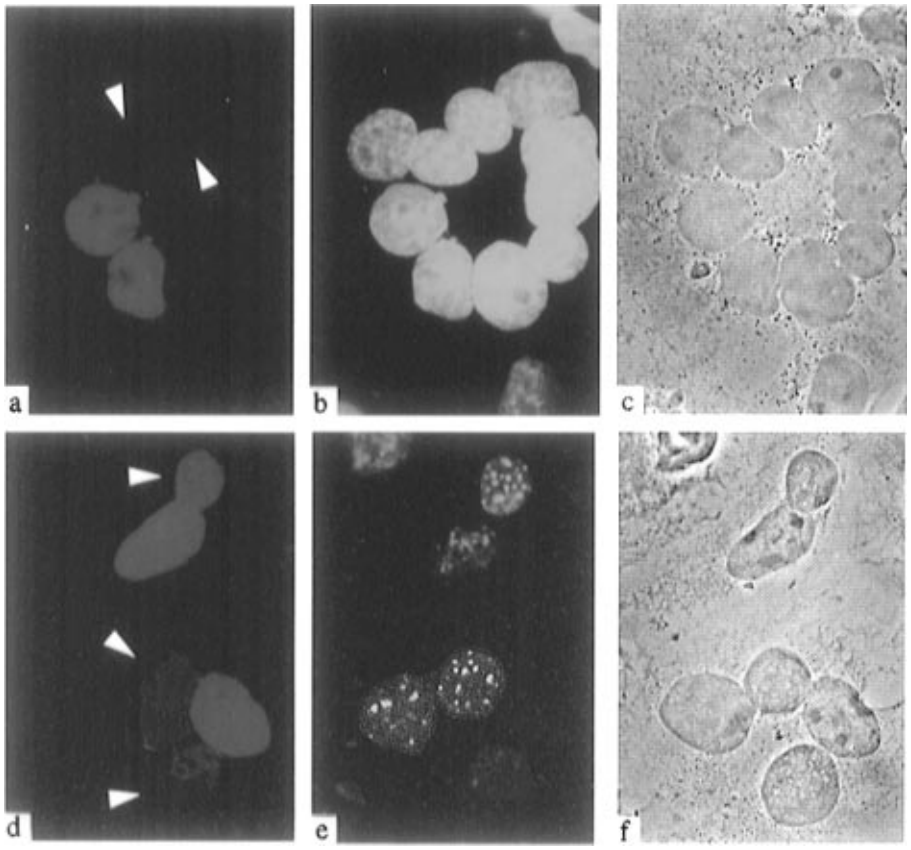

FIGURE 5: Interspecies heterokaryon assay to examine the export of fusion protein to the cytoplasm. COS-7 cells transfected with pCH-UF (panels a−c) and pCH-U(1−179) (panels d−f) were fused with mouse C127 cells and fixed at 5 h after fusion. The fusion proteins were stained with anti-β-gal antibody and then with anti-mouse IgG−rhodamine (panels a and d). Mouse nuclei (marked by arrows) could be distinguished by their morphology from COS-7 nuclei by staining of their DNA with Hoechst 33258 (panels b and e). The corresponding phase-contrast figures are shown in panels c and f.

to interact with the linker region between the two HMG1/2 boxes or the carboxyl-flanking sequence of HMG1/2 box B (Tanabe et al., in preparation). NMR spectroscopy showed that the amino and carboxyl termini in a single HMG1/2

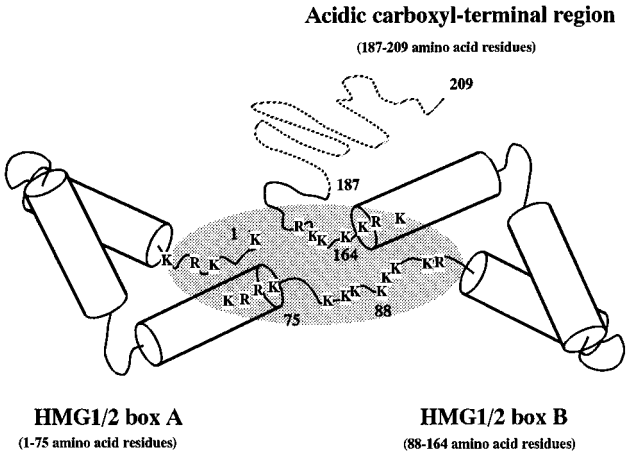

FIGURE 6: Hypothetical tertiary model structure of HMG2 protein. HMG2 consists of three structural domains: HMG1/2 box A and box B and an acidic carboxyl terminus. The simplified tertiary structures of both HMG1/2 boxes were deduced from NMR spectroscopy data for HMG1 (Read et al., 1993; Weir et al., 1993). Three α-helices comprising each HMG1/2 box are shown by tubes. The shaded ellipse shows the NLS region. The basic regions present in the amino- and carboxyl-terminal regions of the NLS are close to each other, and the four basic regions are also in close proximity though the linker region between the two HMG1/2 boxes.

box are located in close proximity to each other in the tertiary structure. Accordingly, both the basic regions in the nuclear accumulation sequences interspaced by more than 60 amino acid residues in the primary sequence may function intimately in nuclear localization of the HMG2 molecule. In a sense, the relationship between the neighboring clusters has the appearance of a bipartite NLS motif. However, the fundamental difference between the NLS in HMG2 and the general bipartite motif is that the former is composed of two basic regions that function by coming close to each other in the tertiary structure. The NLS activities of the fusion constructs containing two of the nuclear accumulation sequences (1−209, 1−179, or 1−164) were enhanced in comparison with those containing a single one (Figures 1 and 3). The four basic regions closed through the linker region between the two HMG1/2 boxes, as shown in Figure 6, may function more efficiently as the NLS.

The fusion constructs that accumulated in the nucleus showed DNA-binding properties (Figure 2). The peptides of HMG1/2 box A (1−75) and box B (88−164) bind with DNA (Tanabe et al., in preparation). On the other hand, β-gal fused with HMG1/2 box A did not show DNA-binding ability. In this fusion protein, the fused β-gal may have an inhibitory effect on DNA binding. Several nuclear localization sequences are considered to have DNA-binding ability inherent in their respective sequences (LaCasse & Lefebvre, 1995). However, the DNA-binding sequence of the HMG1/2 box seems necessary, but insufficient alone, for nuclear accumulation, and must function to organize the active, tertiary basic regions described above.

Analyses by immunofluorescence staining and protein fractionation showed that HMG2 was present in both the nucleus and cytoplasm (Figure 4), consistent with earlier observations (Bustin & Neihart, 1979; Isackson et al., 1980; Kuehl et al., 1984; Mosevitsky et al., 1989). The interspecies heterokaryon assay indicated that part of the HMG2 molecule remains in the cytoplasm after translation (Figure 5). On the other hand, HMG2−β-gal fusion protein expressed in the cytoplasm was observed exclusively in the nucleus, possibly due to its active transport. The NLS activities of some nuclear proteins are abolished by certain types of modification such as phosphorylation or by complex formation with NLS-inactivating proteins (Baeuerle & Baltimore, 1988). The translocation of phosphorylated insect (*Chironomus*) HMG1 protein into the nucleus has been reported to be slower than that of the nonphosphorylated molecule (Wisniewski et al., 1994). Pig thymus HMG2 is phosphorylated by DNA-dependent protein kinase in vitro (Watanabe et al., 1994). Thus, it is probable that the cytoplasmic HMG2 is modified, with the result that it remains in the cytoplasm. As suggested in our hypothetical model (Figure 6), the carboxyl-terminal region consisting of 23 continuous acidic amino acids may be in close proximity to the linker region between the two HMG1/2 boxes. If the basic regions responsible for the NLS could interact with the acidic carboxyl-terminal region in the cytoplasm, the NLS activity might be reduced, allowing some of the HMG2 molecule to remain in the cytoplasm. The acidic carboxyl terminus in HMG2−β-gal fusion constructs may be prevented from undergoing such NLS inactivation due to fusion with the large β-gal molecule. This may explain why the HMG2−β-gal fusion proteins were localized only in the nucleus. Cytoplasmic HMG2 was observed in serum-starved cells (data not shown), and the amount of HMG2 mRNA is enhanced in rapidly proliferating cells compared with that in arrested cells (Shirakawa & Yoshida, 1995; Yamazaki et al., 1995). Thus, HMG2 in the cytoplasm must have an important function such as cell proliferation or some unidentified cellular reaction.

It is noteworthy that the fusion construct containing acidic carboxyl-terminal-truncated HMG2 was exported to the cytoplasm from the nucleus (Figure 5). Recently, nuclear export signals have been identified in protein kinase A inhibitor and HIV Rev proteins (Wen et al., 1995; Fisher et al., 1995). These signals, which were not contained in HMG2, were rich in leucine and other hydrophobic amino acids. Schmidt-Zachmann et al. (1993) have suggested that shuttling nuclear protein has no positively acting export signal; instead, its ability is determined primarily by its intranuclear interactions. Lamin, containing both the NLS and membrane-interacting region, shows no shuttling, but a mutant protein in which the membrane-associating region had been deleted was converted into a shuttling protein (Schmidt-Zachmann et al., 1993). Cross-linking experiments have revealed that HMG2 interacts with histones in the nucleus (Bernués et al., 1986). Similar results were obtained in our study. On the other hand, the carboxyl-terminal-truncated molecule was found not to be cross-linked with histones (data not shown). Thus, HMG2 may be retained in the nucleus by interacting with histones in chromatin through its acidic carboxyl-terminal region in addition to the DNA-binding force acting through HMG1/2 boxes.

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