DNA AND CELL BIOLOGY
Volume 14, Number 12, 1995
Mary Ann Liebert, Inc.
Pp. 997–1005

Characterization of Transgenic Mice with an Increased Content of Chromosomal Protein HMG-14 in Their Chromatin

MICHAEL BUSTIN,[1] PEDRO J. ALFONSO,[1] JAMES M. PASH,[1] JERROLD M. WARD,[2] JOHN D. GEARHART,[3] and ROGER H. REEVES[4]

ABSTRACT

Chromosomal protein HMG-14 is a ubiquitous nuclear protein that may modulate the chromatin structure of transcriptionally active genes. To gain insights into the cellular function of the HMG-14 protein, we generated two transgenic mouse lines carrying either two or six copies of the human HMG-14 gene. The transgenic mice express human HMG-14 mRNA and protein in all tissues examined at a level reflecting the increased gene dosage, suggesting that the *HMG14* transgene contains all the control regions necessary for regulated gene expression. Expression of the human HMG-14 protein does not alter the expression of the endogenous mouse HMG-14 protein or its close homolog, protein HMG-17. The intracellular distribution of the exogenous human protein is indistinguishable from that of the endogenous mouse protein, resulting in a three-fold increase in the level of the chromatin-bound HMG-14. The transgenic mice had a higher incidence of epithelial cysts in their thymus than did control animals. We conclude that the cellular levels of HMG-14/-17 are determined by gene copy number, that the DNA fragment containing the gene and about 1,000 bp flanking its 5′ and 3′ ends contain most of the elements necessary for gene expression, that the upper limits of HMG-14 in chromatin are not stringently regulated, and that a three-fold increase in chromatin-bound protein cause only mild phenotypic changes in the transgenic mice.

INTRODUCTION

REGULATED EXPRESSION OF THE GENETIC INFORMATION encoded in DNA, which is essential for proper differentiation and development, is dependent on precise interaction between proteins and DNA in the chromatin fiber. Because chromosomal proteins HMG-14 and HMG-17 are intimately associated with nucleosomes, they may modify the structure of the chromatin fiber and affect the transcriptional process. Indeed, recent experiments with SV40 minichromosomes indicate that elevated levels of chromosomal protein HMG-14 stimulate the rate of transcriptional elongation by RNA polymerase II (Ding *et al.*, 1994). The enhancement of transcriptional elongation occurs only on chromatin templates and not on DNA templates. Likewise, HMG-14 and HMG-17 proteins enhance the transcriptional potential of chromatin assembled in *Xenopus laevis* egg extracts (Crippa *et al.*, 1993). Structural analysis of the chromatin assembled in these extracts suggests that the transcriptional potential of the templates is increased because the binding of HMG-14/-17 proteins to nucleosomes unfolds the chromatin template (Trieschmann *et al.*, 1995). These findings are in agreement with previous data indicating that the chromatin structure of active genes is less compact than that of transcriptionally inactive regions (reviewed in Gross and Garrard, 1988; Paranjape *et al.*, 1994), and also with experiments suggesting that HMG-14/-17 proteins are involved in the generation, or maintenance, of features that are specific to the chromatin structure of transcriptionally active genes (reviewed in Bustin *et al.*, 1990b). Thus, antibodies against HMG-14 preferentially bind to transcriptionally active Balbiani rings of poly-

[1]Laboratory of Molecular Carcinogenesis, NCI, NIH, Bethesda, MD 20892.
[2]Veterinary and Tumor Pathology Section, Office of Laboratory Animal Science, NCI, Frederick, MD 21702-1201.
[3]Department of Obstetrics and Gynecology and [4]Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

tene chromosomes of *Chironomus pallidivittatus* (Westermann and Grossbach, 1984), and microinjection of antibodies to HMG-17 into human fibroblast nuclei inhibits transcription (Einck and Bustin, 1983). Affinity columns containing anti-HMG-17 antibodies bind nucleosome core particles that contain elevated levels of acetylated histones (Malik *et al.*, 1984), and chromatin fractions enriched in HMG-14 and HMG-17 proteins are also enriched in transcribable sequences (Druckmann *et al.*, 1986; Dorbic and Wittig, 1987; Postnikov *et al.*, 1991). Thus, results from various experiments are consistent with the possibility that the HMG-14/-17 chromosomal protein family is an integral part of transcriptional regulation by the chromatin template.

The association of HMG-14/-17 proteins with transcriptionally active genes, and their ability to enhance the transcription potential of chromatin, raises the possibility that these proteins may also influence developmental processes. Experiments with cells in tissue culture indicate that the levels of HMG-14/-17 mRNA fluctuate during the cell cycle and are down-regulated during differentiation (reviewed in Bustin *et al.*, 1992). It has been suggested that the rapid and transient phosphorylation of HMG-14 observed upon mitogenic stimulation of resting cells may mediate changes in chromatin structure and induce the transcription of immediate early genes (Barratt *et al.*, 1994). In cultured myoblasts, the aberrant expression of HMG-14 inhibits myogenic differentiation (Pash *et al.*, 1993), suggesting that cellular differentiation may require regulated expression of HMG-14. Additional interest in the cellular function of HMG-14 stems from the finding that the human HMG-14 gene is located in region 21q22.3 (Pash *et al.*, 1991), a region associated with several anomalies of Down Syndrome (DS), which is one of the most common birth defects and is associated with multiple developmental abnormalities (Epstein, 1989; Korenberg *et al.*, 1994). Cultured cells obtained from human trisomy 21 patients and tissues obtained from individuals with DS contain elevated levels of HMG-14 protein (Pash *et al.*, 1991), indicating that the protein is expressed according to its gene dosage.

In view of the putative role of HMG-14 in transcriptional regulation and cellular differentiation, we produced transgenic mice expressing elevated levels of human HMG-14 protein.

The main goal of this study is to determine whether the cellular levels of HMG-14 protein are stringently regulated and whether the aberrant expression of this protein has significant phenotypic consequences. We found that the human protein is expressed in every tissue examined and that the content of HMG-14 is significantly elevated in the chromatin purified from these tissues. The transgenic mice display mild histological abnormalities in the thymus.

MATERIALS AND METHODS

Generation of transgenic mice

An 8.8-kb human genomic *Bam* HI fragment containing the entire *HMG14* gene was isolated from plasmid pH14g (Landsman *et al.*, 1989), resuspended at 300 copies per picoliter in 10 m*M* TRIS, 0.1 m*M* EDTA pH 7.2, and 2 pl were injected into (B6A × CD-1)F_1 × C57BL/6 pronuclear-stage embryos. Injected embryos were cultured overnight and introduced into pseudopregnant females. DNA from putative transgenic offspring was digested with *Pvu* II and subjected to Southern blot analysis (Reeves *et al.*, 1987) using as a probe a 700-bp *Pst* I fragment from the first intron of the human *HMG14* gene. This probe does not hybridize with mouse DNA (Landsman *et al.*, 1989). Four founders were identified and crossed to C57BL/6 for two generations before inbreeding to establish the transgenic lines.

Preparation of RNA and RNase protection analysis

Total RNA was extracted from tissues or whole embryos either by the guanidinium thiocyanate technique (Ausubel *et al.*, 1993) or by the hot phenol/NaDodSO$_4$ method (Hsuing *et al.*, 1982). RNase protection analysis was done with an RPAII kit (Ambion), as recommended by the manufacturer. Riboprobes were prepared from human and mouse HMG-14 cDNAs into Bluescript SK$^+$ plasmid (Stratagene). The mouse HMG-14 riboprobe hybridized to a 165-bp *Pst* I–*Xba* I cDNA fragment, while the human HMG-14 probe hybridized to a 271-bp fragment starting from the *Sty* I site of the cDNA (Pash *et al.*, 1993). The [α-^{32}P]CTP radiolabeled riboprobes were synthesized using the appropriate T7 or T3 RNA polymerase.

Protein isolation, electrophoresis in polyacrylamide gels, and Western analysis

For protein isolation, tissues were homogenized in six volumes of 5% perchloric acid. The suspension was stirred at 4°C for 30 min and centrifuged for 20 min. at 10,000 × *g*. The supernatant was collected and made 25% trichloroacetic acid by the addition of 3 volumes of 100% trichloroacetic acid. The suspension was incubated 2 hr at 4°C and centrifuged at 10,000 × *g* for 15 min. The pellet was dissolved in 0.3 *M* HCl, and insoluble material was removed by centrifugation. The HMGs were precipitated by the addition of 6.5 volumes of acetone and overnight incubation at −20°C. The pellet was collected by centrifugation, and washed three times: once in a large volume of 90% acetone in 0.3 *M* HCl, once in 90% acetone, and once in 100% acetone. For one-dimensional polyacrylamide gels, proteins were fractionated in 15% or 18% polyacrylamide gels run in the presence of sodium dodecyl or in 20% acid urea gels. In the two-dimensional gel electrophoresis system, the first dimension consisted of 20% acid urea gel electrophoresis and the second dimension was a 15% NaDodSO$_4$-polyacrylamide gel electrophoresis. Gels were visualized by Coomassie blue staining. HMG-14 and HMG-17 were identified by Western blot analysis using a mixture of antibodies to both proteins, each diluted 1:3,000 in 2% milk (Bustin, 1989; Bustin *et al.*, 1990a). The location of the bound antibodies was visualized with alkaline phosphatase-labeled goat anti-rabbit IgG. For quantitation, the gels were analyzed with a Molecular Dynamics Computing Densitometer.

Chromatin preparation and fractionation

Approximately 0.5 gram of tissue was minced with scissors in 5 ml of HB buffer [0.34 *M* sucrose, 15 m*M* Tris-Cl pH 8, 15 m*M* NaCl, 60 m*M* KCl, 10 m*M* dithiothreitol (DTT)] containing 0.5 m*M* spermidine, 0.15 m*M* spermine, 0.1% Triton X-100, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and homogenized by 15 strokes of a glass tissue grinder. Nuclei were

collected by centrifugation at 800 × *g*. The nuclei were washed once with HB buffer and suspended in 1 m*M* PMSF, 1 m*M* Tris-Cl, 5 m*M* EDTA pH 8. The nuclei were allowed to swell in this solution for 20 min at 4°C, disrupted by homogenization, and pelleted by centrifugation at 6,000 × *g*. The pellet was washed once with 10 m*M* Tris-Cl, 5 m*M* EDTA pH 8, and 1 m*M* PMSF, and the chromatin was purified by sedimentation through 1.7 *M* sucrose in the 5 m*M* Tris-Cl, 5 m*M* EDTA pH 8. The pelleted chromatin was suspended in 10 m*M* Tris, 5 m*M* EDTA pH 8, and 1 m*M* PMSF, and the HMG protein fraction was extracted with 5% perchloric acid as described above. To obtain the S1, S2, and P1 chromatin fractions, purified nuclei were prepared and digested with micrococcal nuclease as described by Huang and Garrard (1989). HMG proteins were extracted from these fractions with 5% perchloric acid.

Histological analysis

A complete necropsy was performed on each normal control or *HMG14* transgenic mouse. The numbers of mice necropsied were: five C57BL/6N 1–2 months old; line 25, 4 males, 5 females, 1–7 months old; line 29, 3 males, 9 females, 1–7 months old. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4–6 microns. Hematoxylin and eosin-stained sections were reviewed.

Phenotype analysis

Flow cytometry and immunofluorescence studies to determine the number of thymocyte cells that were positive for T-cell differentiation markers were done essentially as described by Fowles and Pardoll (1989). All the experiments involving T-cell screening were done by Dr. A. Singer and Ms. T. Guinter (Laboratory of Experimental Immunology, NCI, NIH).

RESULTS

Production of transgenic mice

An 8.8-kb fragment containing the entire human *HMG14* gene (Landsman *et al.*, 1989) was injected into pronuclear-stage embryos. Because the *HMG14* coding sequences of the mouse and human are very similar (Bustin *et al.*, 1990b) and because the mouse genome contains more than a dozen pseudogenes that cross-hybridize with either probe (Landsman *et al.*, 1986), a unique human-specific fragment from the first intron of the human *HMG14* gene (Landsman *et al.*, 1989) was used to identify transgenic animals. Four transgenic mice were identified among the 60 pups born. From these, two founders were crossed with C57BL/6J mice to generate two founder lines (line 25 and line 29). These animals were mated with C57BL/6J mice for an additional generation, and obligate heterozygous F_3 progeny from each line were brother × sister mated to produce animals homozygous for the transgene. DNA from progeny of these crosses was probed sequentially with the human HMG-14 intron probe and a probe for the mouse S100b gene, and the relative intensities were used to identify homozygous animals (data not shown). Putative homozygotes identified in this manner were outcrossed to produce eight, or more, progeny. A male and two females that produced only transgenic offspring with these outcrosses were selected to establish the homozygous lines used in this study.

Transgene conformation and copy number

The complexity of the endogenous Hmg14 gene family in mice precluded use of coding sequences in the analysis of the transgenes in lines 25 and 29. To assess integration sites, DNA from each of the transgenic lines was digested with several restriction endonucleases and examined by Southern analysis with a probe specific for the human *HMG14* gene (Fig. 1B). Line 29 mice displayed a single band, indicating that one copy of the human gene was integrated at a single site in the mouse genome. Analysis of the DNA from line 25 mice indicated that three copies of the human gene were randomly integrated in the haploid mouse genome. Thus, the genome of homozygous lines 25 and 29 mice analyzed here contain respectively, six and two copies of the human HMG-14 gene.

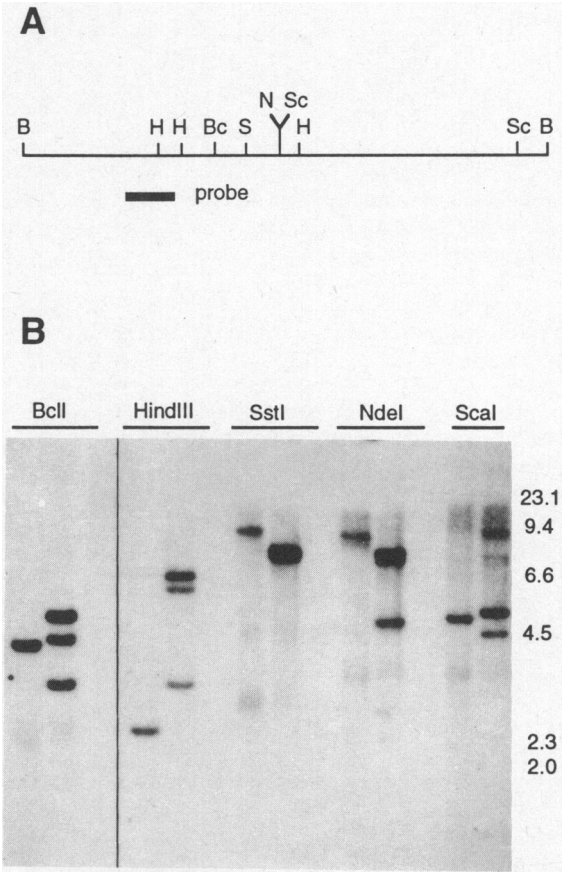

FIG. 1. HMG14 transgenic mice. **A.** Restriction map of the 8.8-kb human genomic fragment containing the HMG-14 gene. The position of the intron-specific probe is indicated by the heavy line. B, *Bam* HI; H, *Hind* III; Bc, *Bcl* I; S, *Sst* I; N, *Nde* I, Sc, *Sca* I. **B.** Line 29 mice, shown to the left of each set of digests, contain a single copy of the transgene per haploid genome, while line 25 mice, shown in the center of each set, contain three copies. DNA from a nontransgenic control mouse does not hybridize to the human intron probe (right lane in the *Bcl* I, *Hind* III, *Sst* I, and *Nde* I sets).

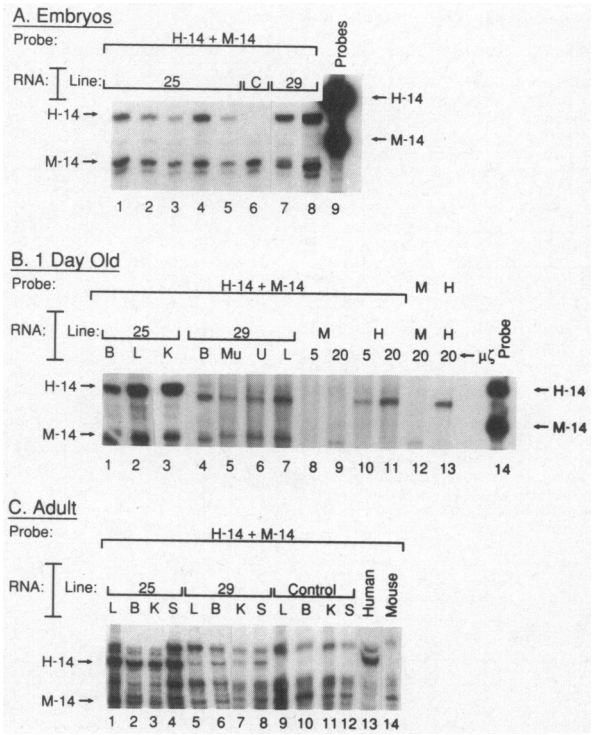

FIG. 2. Expression of human HMG-14 mRNA in transgenic mice. RNA was extracted from embryos by the CsCl method and from postnatal day 1 and adult (3-month) tissues by the hot phenol method. Human and mouse HMG-14 mRNA was detected by RNase protection. H-14, human HMG-14; M-14, mouse HMG-14. The arrows on the left point to the position of the fragments protected by the cellular mRNA. The arrows on the right point to the position of the probes used. The RNA in panel A, lanes 1–8, in panel B, lanes 1–11, and in panel C, lanes 1–14 was probed with a mixture of the human and mouse HMG-14 probes (*i.e.*, H-14 + M-14). A. RNA extracted from either line 25 (lanes 1–5), control (lane 6), or line 29 (lanes 7 and 8) whole embryos. In B, mouse control RNA was present in lanes 8, 9, and 12; human RNA controls in lanes 10, 11, and 13. The amount of RNA added in micrograms is indicated. The RNA in lane 12 was probed only with the mouse probe whereas the RNA in lane 13 was probed only with the human probe. In B and C, the RNA was extracted from the following tissues: B, brain; L, liver; K, kidney; Mu, muscle; U, lung; S, spleen. Note the absence of the human mRNA in the RNA obtained from control animals (C, lanes 9–12).

Dose-dependent expression of human HMG14 *transcripts*

An RNase protection assay that distinguishes between the mouse and human HMG-14 mRNAs (Pash *et al.*, 1993) detected human mRNA in all embryonic, 1-day-old, and adult mice from both transgenic lines. In 18-day-old embryos that were heterozygous for the human gene, the ratio of human to mouse HMG-14 mRNA varied from 0.6 to 1.5. RNA from control, nontransgenic embryos (lane 6 in Fig. 2A) does not contain human message. In the homozygous 1-day-old and adult mice, the ratio of human to mouse HMG-14 mRNA is approximately three-fold higher in the tissues derived from line 25 than in tissues derived from line 29 mice. Human HMG-14 mRNA is expressed in several tissues obtained either from 1-day-old or adult (about 3 months old) transgenic mice (Fig. 2B,C). Within each transgenic line, there was very little tissue-specific variation in the ratios of human to mouse HMG-14 mRNA. These results suggest that the expression of the human *HMG14* transgene is under the control of the same regulatory mechanism as that of the endogenous mouse *Hmg14* gene. Thus, the 8.8-kb insert of human DNA in the pH14g clone, which includes 847-bp 5′ to the start of transcription and 1,228 bp 3′ to the polyadenylation site, contains most of the regulatory elements necessary for expression of the human HMG-14 gene product in all tissues examined.

Expression of human HMG-14 protein in transgenic mice increases the cellular level of the HMG-14/-17 protein pool

Previous studies with transfected cells indicated that elevated levels of HMG-14/-17 mRNA do not always bring about a concomitant increase in the amount of cellular proteins (Giri *et al.*, 1987; Pash *et al.*, 1993). Therefore, we examined whether the human HMG-14 mRNA expressed in the transgenic mice is translated into protein. HMG proteins were partially purified by extraction with 5% perchloric acid, fractionated by electrophoresis in 18% polyacrylamide gels containing $NaDodSO_4$, and then the HMG-14/-17 proteins were identified by Western blot analysis. In this analytical system, the human HMG-14 protein has a lower electrophoretic mobility than the mouse HMG-14 protein that co-migrates with mouse HMG-17 protein. Western analysis reveals the presence of human HMG-14 in all the tissues obtained from mice derived from either line 25 or line 29 (Fig. 3A). The amounts of human HMG-14 protein in line 25 are two- to five-fold higher than in line 29, as estimated by comparing the intensity of the human HMG-14 band to the mouse HMG-14 + HMG-17 bands. The relative levels of human HMG-14 protein reflect the levels of the mRNA and the number of human transgenes in each transgenic line. We conclude, therefore, that the human HMG-14 protein is present in most tissues of the transgenic mice.

Next, we tested whether expression of human HMG-14 affects the cellular levels of the endogenous mouse HMG-14 and HMG-17 proteins. Two-dimensional gel electrophoresis resolved the three HMG-14/-17 molecular species (*i.e.*, human HMG-14 and mouse HMG-14 and -17) present in the transgenes. Western analysis of the two-dimensional gels (Fig. 3B) indicates that expression of human HMG-14 did not affect the expression of the endogenous mouse HMG-14 or HMG-17 proteins. From panels 1–3, it is evident that the kidney of both line 25 and line 29 transgenic lines contain significant amounts of human HMG-14 that is not present in the kidney of control animals. Human HMG-14 is also present in the brain and spleen (panels 4 and 5) and in the thymus and muscle (not shown) of every transgenic mouse tested. We conclude, therefore, that the transgenic animals contained elevated levels of HMG-14 and that the cellular levels of the HMG-14 and HMG-17 protein are not interdependent.

Human HMG-14 protein is associated with mouse chromatin

The question arises whether the cellular human HMG-14 protein present in the *HMG14* transgenes translocates into the nucleus and is associated with chromatin. Chromatin prepared

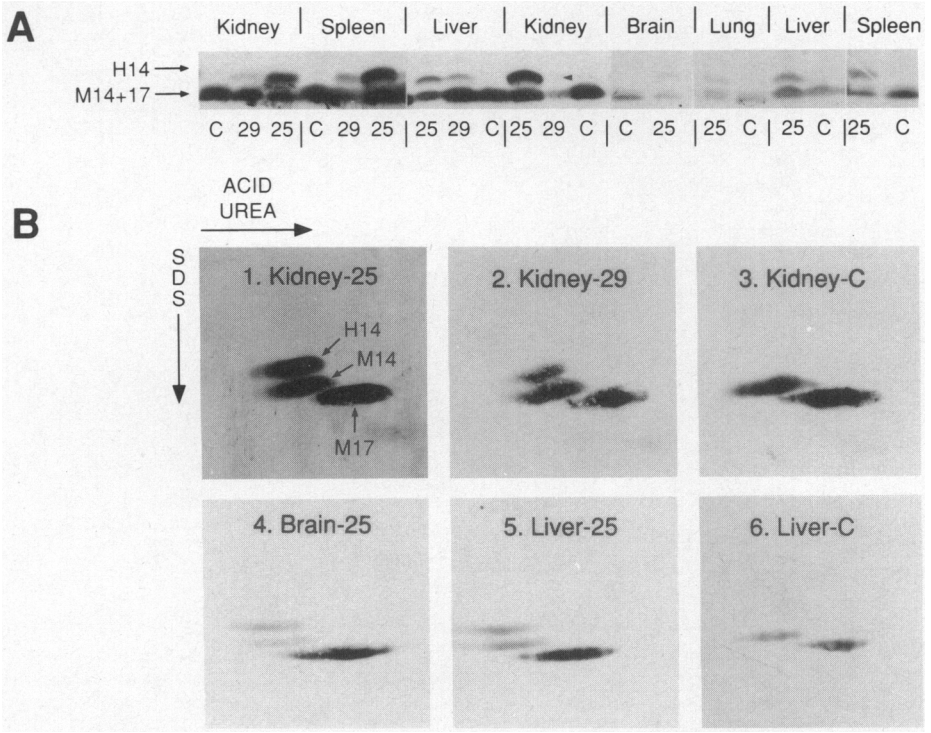

FIG. 3. Expression of human HMG-14 protein in transgenic mice. **A.** Western blot analysis of 5% perchloric acid extracts from whole tissue. The tissue from which the protein was extracted is indicated above the lanes. The line from which the tissue was obtained is indicated under each lane. C, Control, nontransgenic animals; 29 and 25 indicate the respective transgenic lines. **B.** Two-dimensional protein analysis. The tissue and line from which the protein samples were prepared are indicated: C, control; −25 and −29, transgenic lines; H-14, human HMG-14; M-14 and M-17, mouse HMG-14 or -17.

from kidney or spleen was purified by sucrose density gradient centrifugation. HMG proteins were extracted from this chromatin and analyzed by two-dimensional polyacrylamide gel analysis and immunoblotting (Fig. 4). The relative amounts of mouse HMG-14/-17 proteins were the same in control and transgenic mice (Fig. 4A,B). The level of human HMG-14 in purified chromatin is indistinguishable from that extracted from unfractionated tissue (Fig. 4B,C) and thus represents additional chromatin-associated protein. Human protein is also present in the chromatin from spleen (Fig. 4D), liver, and thymus (not shown). As an additional way to compare the cellular distribution of the exogenous human HMG-14 to that of the endogenous mouse HMG-14/-17 proteins, we examined the distribution of the protein in the S1 chromatin subfraction. The S1 chromatin subfraction, which is obtained by short digestion of intact nuclei with micrococcal nuclease (Huang and Garrard, 1989), is enriched in HMG-14/-17 proteins. The HMG protein fraction extracted from the S1 chromatin subfraction was applied to 10–20% polyacrylamide gradient containing $NaDodSO_4$. In these gradient polyacrylamide gels, it is possible to resolve human HMG-14, mouse HMG-14, and mouse HMG-17 by one-dimensional electrophoresis. The proteins were visualized by Coomassie blue stain and the intensity of the bands was quantitated by densitometric scanning. The densitometric scans in Fig. 5 indicate that human HMG-14 is present in the S1 chromatin subfraction extracted from the thymus and kidney of the transgenes but not from the same fractions obtained from control animals. In agreement with the results obtained by two-dimensional polyacrylamide gel analysis, these scans indicate that the expression of the human protein did not alter the ratio of the endogenous HMG-14 to HMG-17 mouse proteins.

In summary, human HMG-14 protein is expressed in a dose-related manner in adult mice from both lines 25 and 29. The human protein is present in all the tissues analyzed. The human protein is associated with the chromatin fiber and its ratio to the endogenous HMG-14/-17 proteins is the same as that obtained in extracts prepared from whole tissues or purified nuclei. Expression of the human protein does not significantly alter the amount or relative ratio of the endogenous mouse HMG-14 and HMG-17 proteins. Thus, the total amount of HMG-14 protein associated with chromatin is increased in all tissues of the transgenic mouse lines.

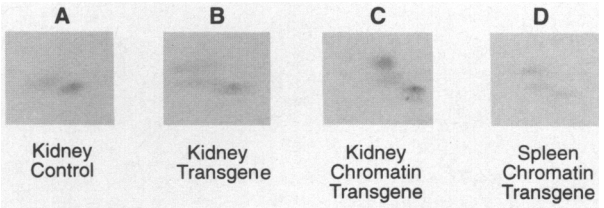

FIG. 4. Human HMG-14 protein is present in the chromatin of the transgenic mice. Western analysis of two-dimensional protein gels identifies human HMG-14 and mouse HMG-14/-17 (see Fig. 3) and indicates that the proteins are present at similar levels in whole tissues and in purified chromatin. The source of the HMG protein is indicated at the bottom of each panel.

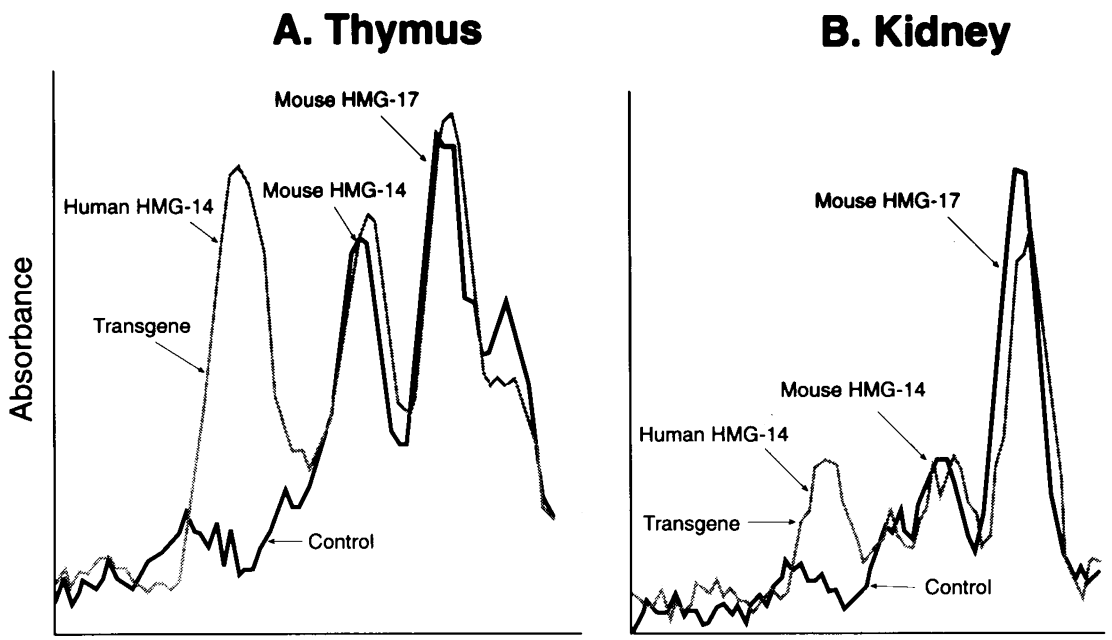

FIG. 5. Human HMG-14 is present in the S1 chromatin fraction of transgenic mice. Densitometric scans of Coomassie blue-stained polyacrylamide gels. Note that the ratio of the endogenous mouse HMG-14 to HMG-17 protein is not affected by the expression of the exogenous human protein.

Pathological anomalities in the thymus of HMG-14 transgenic mice

In mice, the gene coding for HMG-14 is located on chromosome 16 (Johnson *et al.*, 1992). Mice trisomic for chromosome 16, which has been used as a model for DS (Epstein, 1989), express elevated levels of HMG-14 protein (Pash *et al.*, 1991). Therefore, we tested whether the *HMG14* transgenes display phenotypic features resembling those of mouse trisomy. Phenotypically, mice from both lines 25 and 29 appeared clinically normal from birth to approximately 6 months of age. Only a few mice were observed for longer periods and these also did not display any clinical abnormalities. Histological analysis did not reveal overt changes in most of the tissues tested. However, mild lesions were detected in the thymus of both transgenic lines (Table 1). Dilatation of medullary epithelium produced small thymic cysts in one or more thymic lobules. Some of these cysts contained ciliated cells (Fig. 6A), while others were lined by hypertrophied epithelium and their lumens contained cellular debris (Fig. 6B). Several mice showed focal or diffuse thickening of the connective tissue in the capsule (Fig. 6C), sometimes in association with thymic cysts.

Thymic cysts are embryonic remnants of brachial pouch endoderm, active in the transport of lymphocytes and secretions into the circulation, and may provide a proper environment for lymphocyte differentiation (Khosla and Ovalle, 1986). Therefore, we tested by immunofluorescence and flow cytometry whether the pathological abnormalities in the thymus of the transgenic mice are associated with irregularities in thymocyte differentiation (not shown). The proportion of CD4, CD8, and T-cell receptor-positive thymocyte populations in the thymus and in mature peripheral cells of the transgenic lines was indistinguishable from that of normal controls. Therefore, we conclude that the pathological abnormalities in the thymus did not produce a noticeable effect on thymocyte differentiation.

TABLE 1. HISTOLOGICAL ANALYSIS OF HUMAN HMG-14 TRANSGENIC MICE

Line	*Number of mice examined*	*Sex M/F*	*Age (days)*	*Number of mice with thymic lesions*
25	9	4/5	60–90	6
29	12	3/9	30–60	10
Control	5	1/4	30–90	0

Histological abnormalities found only in the thymus. All other tissues examined were normal.

DISCUSSION

The major finding reported in this manuscript is that human HMG-14 is expressed in all tissues of *HMG14* transgenes, without altering the expression of the endogenous HMG-14/-17 proteins, thereby producing a three-fold increase in the level of chromatin-bound HMG-14 protein. The elevated level of HMG-14 protein in the chromatin is associated with the occurrence of only minor phenotypic changes in the thymus of two independently derived clones of transgenic mice. Thus, in spite of the putative role of HMG-14 in chromatin structure and transcriptional processes, the cellular levels of this protein are not stringently regulated.

HMG-14 and the closely related protein HMG-17 are expressed in all cells of higher eukaryotes, suggesting that both

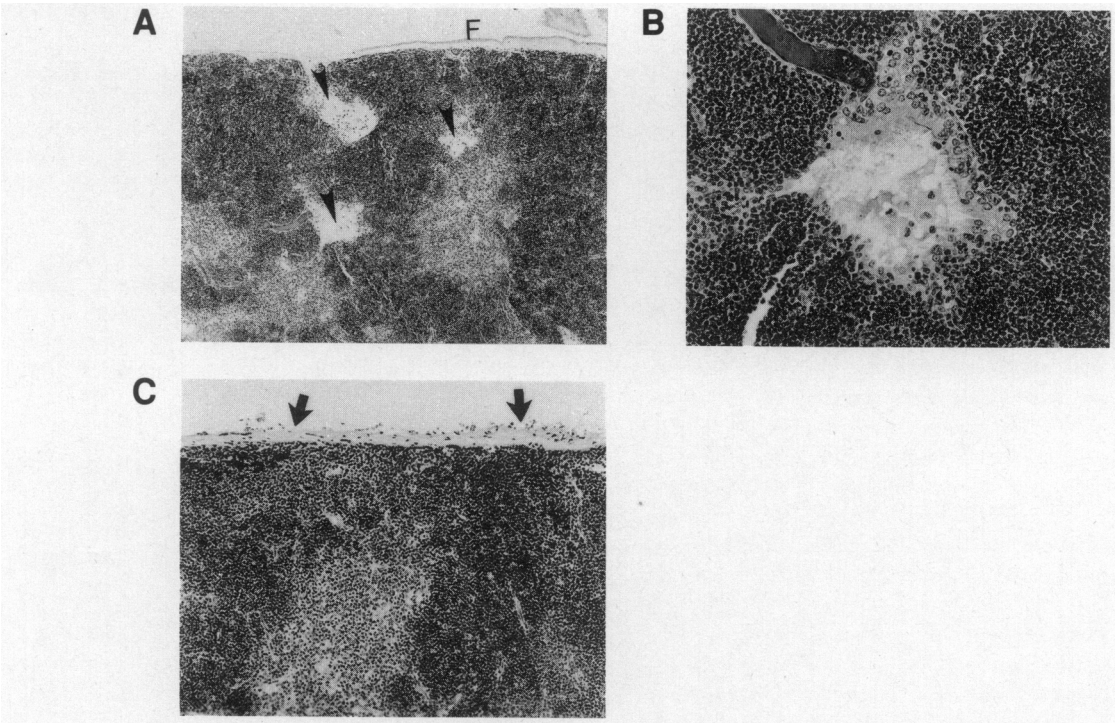

FIG. 6. Cysts in the thymus of human HMG-14 transgenic mice. A. Thymus showing three cystic medullary areas (arrows) and focal capsular fibrosis (F). H&E stain; magnification, 100×. B. Cystic thymic medulla showing tall columnar cells, some with cilia lining cystic space and accumulation of debris in the lumen of the cyst. H&E; magnification, 400×. C. Focal fibrosis (arrows) in capsule of thymus. H&E; magnification, 200×.

proteins are necessary for proper cellular function (for review, see Bustin *et al.*, 1990b). Previous data on the association of HMG-14 with transcriptionally active regions in chromatin together with recent findings supporting a role for HMG-14/-17 in transcriptional processes (Crippa *et al.*, 1993; Barratt *et al.*, 1994; Ding *et al.*, 1994; Trieschmann *et al.*, 1995), and studies on HMG-14/-17 expression during cell cycle and differentiation (for review, see Bustin *et al.*, 1992), have raised the possibility that these proteins may affect developmental processes. Although the regulation of HMG-14/-17 gene expression has not been studied in detail, it is known that in all the members of this gene family, the 700-bp region 5′ to the start of transcription is extremely rich in GC residues and contains HpaII tiny fragments (HTF) islands, multiple SP1 binding sites, and several evolutionarily conserved motifs (Bustin *et al.*, 1990b; Lehn and Bustin, 1993). These structural features are characteristic of genes coding for "housekeeping" proteins. The *HMG14* transgene used in this study contains 847 bp 5′ to the transcription start site and 1,228 bp 3′ to the polyadenylation site. Transgenic mice expressed human HMG-14 in all tissues examined and transcript levels were correlated with gene copy number. These results suggest that this human *HMG14* transgene contains not only the promoter elements, but also the boundary elements and locus control regions necessary for regulated gene expression in all the tissues of the transgenic mice.

In all tissues examined, expression of human HMG-14 led to an overall increase in levels of HMG-14 protein without compensation (down-regulation) in the production of the endogenous mouse HMG-14 or HMG-17 proteins. Thus, the level of HMG transcription is determined by gene copy number, rather than through a feedback regulatory system. The cellular distribution of the transfected human HMG-14 protein is indistinguishable from that of the endogenous murine HMG-14/-17 proteins. The human HMG-14 protein translocates into the nucleus and significantly elevates the total amount of chromatin-bound HMG-14. Therefore, we conclude that the normal complement of chromatin-bound HMG-14/-17 protein does not preclude binding of additional HMG protein and that the amount of HMG-14/-17 in chromatin is dependent on the total cellular amount of HMG protein synthesized in the cell.

Elevated levels of HMG-14 protein in the chromatin do not produce dramatic phenotypic effects in transgenic mice. This result is somewhat surprising, given previous results showing that HMG-14 enhances the transcriptional potential of chromatin (Crippa *et al.*, 1993; Ding *et al.*, 1994) and that aberrant expression of HMG-14 disrupts myogenic differentiation (Pash *et al.*, 1993). *In vitro* studies of chromatin assembly and transcriptional regulation using *Xenopus* egg extracts demonstrate that incorporation of HMG proteins during, but not after, chromatin assembly on replicating DNA affects the structure of the chromatin template as well as the transcriptional potential of pol III-transcribed genes (Crippa *et al.*, 1993). The protein enhances the rate of transcriptional elongation by polymerase II on SV40 chromatin templates (Ding *et al.*, 1994). Thus, it might have been predicted that increased levels of HMG-14 protein in chromatin would result in a general disruption of transcriptional processes that would have significant phenotypic consequences.

We note, however, that the effect of HMG-14 on myogenic differentiation was obtained with transfected cells grown in tissue culture, where *HMG14* transcription was regulated by an inducible mouse mammary tumor virus (MMTV) promoter and the protein levels were held at an artificially high level throughout the cell cycle (Pash *et al.*, 1993). The results obtained with *Xenopus* egg extracts indicating that HMG-14 is properly assembled into nucleosomes only during chromatin assembly on replicating DNA (Crippa *et al.*, 1993; Trieschmann *et al.*, 1995), and the observation that HMG-14 levels normally vary throughout the cell cycle (Bustin *et al.*, 1987), raise the possibility that proper deposition of this protein into chromatin is linked to cell cycle events. Thus, the abnormal timing of HMG-14 expression in myoblasts may be deleterious, possibly due to inappropriate timing of HMG-14 incorporation into chromatin. In the *HMG14* transgenes, however, the expression of the protein driven by its own promoter may resemble that of the endogenous protein (Bustin *et al.*, 1990b; Lehn and Bustin, 1993), and therefore may be less deleterious. Alternatively, the effect of HMG-14 on myogenesis in tissue culture reflects the fact that myogenic differentiation in tissue culture is dependent on very stringent growth conditions and can be easily disrupted by a variety of treatments (for references, see Pash *et al.*, 1993).

The gene encoding HMG-14 is located on human chromosome 21q22.3 and thus is present in three copies in individuals with DS (Pash *et al.*, 1991). The present studies suggest that the excess HMG-14 protein, previously detected in tissues from DS patients (Pash *et al.*, 1991) is incorporated into chromatin. Because altered incorporation of HMG-14 affects transcription and cellular differentiation, it is reasonable to expect that overexpression of HMG-14 in DS and in the trisomy 16 mouse model of this syndrome would have pleiotropic effects on gene expression and perhaps be involved in the etiology of DS. However, the phenotypic anomalies observed in both transgenic lines to date are relatively mild. Histological screening indicated consistent pathological changes only in the thymus, where a majority of animals developed cysts and exhibited other minor anomalies. T-cell maturation is affected in DS individuals who exhibit varying degrees of immune system compromise (Murphy and Epstein, 1992; Murphy *et al.*, 1993). However, the ratios of CD4, CD8, and T-cell receptor positive cells were unchanged in *HMG14* transgenic mice. Thus, the immediate consequences of increased *HMG14* dosage alone are not dramatic. It is still possible, however, that HMG-14 protein acts in synergy with yet another factor coded by chromosome 21 and that such synergistic interactions would bring about more significant phenotypic changes. Thus, HMG-14 dosage imbalance may make an important additive contribution to the complex dosage imbalance of individuals trisomic for all of chromosome 21.

ACKNOWLEDGMENTS

Michael Citron provided excellent technical assistance in husbandry and characterization of transgenic mice. The experiments involving T-cell differentiation were done by Dr. A. Singer and Ms. T. Guinter, Laboratory of Experimental Immunology (NCI, NIH). This work was supported in part by PHS grant #HD24605 (J.D.G. and R.H.R).

REFERENCES

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A., and STRUHL, K. eds. (1993). *Current protocols in Molecular Biology.* (New York, Wiley).

BARRATT, M.J., HAZZALIN, C.A., ZHELEV, N., and MAHADEVAN, L.C. (1994). A mitogen- and anisomycn-stimulated kinase phosphorylates HMG-14 in its basic amino-terminal domain in vivo and on isolated mononucleosomes. EMBO J. **13,** 4524–4535.

BUSTIN, M. (1989). Preparation and application of immunological probes for nucleosomes. Methods Enzymol. **170,** 214–251.

BUSTIN, M., SOARES, N., LANDSMAN, D., SRIKANTHA, T., and COLLINS, J.M. (1987). Cell cycle regulated synthesis of an abundant transcript for human chromosomal protein HMG-17. Nucleic Acids Res. **15,** 3549–3561.

BUSTIN, M., CRIPPA, M.P., and PASH, J.M. (1990a). Immunochemical analysis of the exposure of high mobility group protein 14 and 17 surfaces in chromatin. J. Biol. Chem. **265,** 20077–20080.

BUSTIN, M., LEHN, D.A., and LANDSMAN, D. (1990b). Structural features of the HMG chromosomal proteins and their genes. Biochim. Biophys. Acta **1049,** 231–243.

BUSTIN, M., CRIPPA, M.P., and PASH, J.M. (1992). Expression of HMG chromosomal proteins during cell cycle and differentiation. Crit. Rev. Eukaryotic Gene Expr. **2,** 137–143.

CRIPPA, M.P., TRIESCHMANN, L., ALFONSO, P.J., WOLFFE, A.P., and BUSTIN, M. (1993). Deposition of chromosomal protein HMG-17 during replication affects the nucleosomal ladder and transcriptional potential of nascent chromatin. EMBO J. **12,** 3855–3864.

DING, H.F., RIMSKY, S., BATSON, S.C., BUSTIN, M., and HANSEN, U. (1994). Stimulation of RNA polymerase II elongation by chromosomal protein HMG-14. Science **265,** 796–799.

DORBIC, T., and WITTIG, B. (1987). Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. EMBO J. **6,** 2393–2399.

DRUCKMANN, S., MENDELSON, E., LANDSMAN, D., and BUSTIN, M. (1986). Immunofractionation of DNA sequences associated with HMG-17 in chromatin. Exp. Cell Res. **166,** 486–496.

EINCK, L., and BUSTIN, M. (1983). Inhibition of transcription in somatic cells by microinjection of antibodies to chromosomal proteins. Proc. Natl. Acad. Sci. **80,** 6735–6739.

EPSTEIN, C.J. (1989). Down Syndrome. In *The Metabolic Basis of Inherited Disease*, vol. 6. (McGraw-Hill, New York) pp. 291–326.

FOWLES, B.J., and PARDOLL, D.M. (1989). Molecular and cellular events of T cell development. Adv. Enzymol. 44, 207–265.

GIRI, C., LANDSMAN, D., SOARES, N., and BUSTIN, M. (1987). Modulation of the cellular ratio of chromosomal high mobility group proteins 14 to 17 in transfected cells. J. Biol. Chem. **262,** 9839–9843.

GROSS, D.S., and GARRARD, W.T. (1988). Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. **57,** 159–197.

HSUING, N., ROGINSKI, R.S., HENTHORN, P., SMITHIES, O., KUCHERLAPATI, R., and SKOULTCHI, A.I. (1982). Induction and expression of a fetal human globin gene in mouse fibroblasts. Mol. Cell. Biol. **2,** 401–411.

HUANG, S.Y., and GARRARD, W.T. (1989). Electrophoretic analysis of nucleosomes and other protein-DNA complexes. Methods Enzymol. **170,** 116–141.

JOHNSON, K.R., COOK, S.A., BUSTIN, M., and DAVISSON, M.T. (1992). Genetic mapping of the murine gene and 14 related sequences encoding chromosomal protein HMG-14. Mamm. Genome **3,** 625–632.

KHOSLA, S., and OVALLE, W.K. (1986). Morphology and distribution of cystic cavities in the normal murine thymus. Cell Tissue Res. **246,** 531–542.

KORENBERG, J.R., CHEN, X.N., SCHIPPER, R., SUN, Z., GONSDKY, R., GERWEHR, S., CARPENTER, N., DAUMER, C.,

DIGNAN, P., DISTECHE, C., GRAHAM, J.M., HUDGINS, L., McGILLIVRAY, B., MYAZAKI, K., OGASAWARA, N., PARK, J.P., PAGON, R., PUESCHEL, S., SACK, G., SAY, B., SCHUFFENHAUER, S., SOUKUP, S., and YAMAKANA, T. (1994). Down Syndrome Phenotypes: The consequences of chromosomal imbalance. Proc. Natl. Acad. Sci. USA **91,** 4997–5001.

LANDSMAN, D., SRIKANTHA, T., WESTERMANN, R., and BUSTIN, M. (1986). Chromosomal protein HMG-14. Complete human cDNA sequence and evidence for a multigene family [published erratum appears in J. Biol. Chem. **263,** 16512, 1988]. J. Biol. Chem. **261,** 16082–16086.

LANDSMAN, D., McBRIDE, O.W., SOARES, N., CRIPPA, M.P., SRIKANTHA, T., and BUSTIN, M. (1989). Chromosomal protein HMG-14. Identification, characterization, and chromosome localization of a functional gene from the large human multigene family. J. Biol. Chem. **264,** 3421–3427.

LEHN, D.A., and BUSTIN, M. (1993). Evolutionarily conserved motifs and protein binding elements in the 5′ region of the chromosomal protein HMG-14 gene. DNA Cell Biol. **12,** 753–761.

MALIK, N., SMULSON, M., and BUSTIN, M. (1984). Enrichment of acetylated histones in polynucleosomes containing HMG-17 revealed by immunoaffinity chromatography. J. Biol. Chem. **259,** 699–702.

MURPHY, M., and EPSTEIN, L.B. (1992). Down Syndrome peripheral blood cells: Evidence for an inefficient release of mature T cells by the DS thymus. Clin. Immunol. Immunopathol. **62,** 245–251.

MURPHY, M., INSOFT, R.M., PIKE-NOBILE, I., DERBIN, K.S., and EPSTEIN, L.B. (1993). Over expression of LFA-1 and ICAM-1 in Down syndrome thymus. Implication for abnormal thymocy maturation. J. Immunol. **150,** 5696–5703.

PARANJAPE, S.M., KAMAKAKA, R.T., and KADONAGA, J.T. (1994). Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63,** 265–297.

PASH, J., SMITHGALL, T., and BUSTIN, M. (1991). Chromosomal protein HMG-14 is overexpressed in Down syndrome. Exp. Cell Res. **193,** 232–235.

PASH, J.M., ALFONSO, P.J., and BUSTIN, M. (1993). Aberrant expression of high mobility group chromosomal protein 14 affects cellular differentiation. J. Biol. Chem. **268,** 13632–13638.

POSTNIKOV, Y.V., SHICK, V.V., BELYAVSKY, A.V., KHRAPKO, K.R., BRODOLIN, K.L., NIKOLSKAYA, T.A., and MIRZABEKOV, A.D. (1991). Distribution of high mobility group proteins 1/2, E and 14/17 and linker histones H1 and H5 on transcribed and non-transcribed regions of chicken erythrocyte chromatin. Nucleic Acids Res. **19,** 717–725.

REEVES, R.H., GALLAHAN, D., O'HARA, B.F., CALLAHAN, R., and GEARHART, J.D. (1987). Genetic mapping of Prm-1, Smst, Mtv-6, Sod-1, and Ets-2 and localization of the DS region on mouse chromosome 16. Cytogenet. Cell Genet. **44,** 76–81.

TRIESCHMANN, L., ALFONSO, P.J., CRIPPA, M.P., WOLFFE, A.P., and BUSTIN, M. (1995). Incorporation of chromosomal proteins HMG-14/-17 into nascent nucleosomes induces an extended chromatin conformation and enhances the utilization of active transcription complexes. EMBO J. **14,** 1478–1489.

WESTERMANN, R., and GROSSBACH, U. (1984). Localization of nuclear proteins related to HMG-14 in polytene chromosomes. Chromosoma **90,** 355–365.

Address reprint requests to:
Dr. Michael Bustin
Laboratory of Molecular Carcinogenesis
Bldg. 37, Room 3D-12
National Cancer Institute
National Institutes of Health
Bethesda, MA 20892

Received for publication June 15, 1995; revised August 23, 1995; accepted August 26, 1995.