The Chicken *HMG-17* Gene Is Dispensable for Cell Growth In Vitro

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HMG-17 is a highly conserved and ubiquitous nonhistone chromosomal protein that binds to nucleosome core particles. HMG-17 and HMG-14 form a family of chromosomal proteins that have been reported to bind preferentially to regions of active chromatin structure. To study the functional role of the single-copy chicken *HMG-17* gene, null mutants were generated by targeted gene disruption in a chicken lymphoid cell line, DT40. Heterozygous and homozygous null mutant cell lines were generated by two independent selection strategies. Heterozygous null mutant lines produced about half the normal level of HMG-17 protein, and homozygous null lines produced no detectable HMG-17. No significant changes in cell phenotype were observed in cells harboring either singly or doubly disrupted *HMG-17* genes, and no compensatory changes in HMG-14 or histone protein levels were observed. It is concluded that HMG-17 protein is not required for normal growth of avian cell lines in vitro, nor does the absence of HMG-17 protein lead to any major changes in cellular phenotype, at least in lymphoid cells.

The high-mobility-group 17 (HMG-17) and HMG-14 non-histone chromosomal proteins are found in all higher eucaryotic species. They bind to nucleosomes in chromatin, and that binding has been found to stabilize core particles (31) and to regularly space nucleosomes (39) when assayed in vitro. Nuclease digestion (41, 42) and immunofractionation (14) experiments have suggested that HMG-14 and HMG-17 are preferentially associated with nucleosomes of regions of transcribed chromatin, and reconstitution experiments (41) have indicated that HMG-14 and/or HMG-17 is needed to maintain the increased nuclease sensitivity of transcribed chromatin in vitro. This hypothesis is supported by more recent findings that HMG-14 enhances transcription of genes by RNA polymerase II from in vivo assembled simian virus 40 minichromosomes (12) and by RNA polymerase III in a *Xenopus* egg extract assembly system (11). The possible role of HMG-17 and HMG-14 in regulating gene expression has also been examined in studies involving ectopic expression of exogenous genes; in these studies, Pash et al. (30) found that aberrant expression of human HMG-14 inhibits the differentiation of mouse myoblast cells.

HMG-17 and HMG-14 are expressed in virtually all avian and mammalian cell types that have been examined. Unlike mammals, however, chickens express two rather different types of HMG-14, HMG-14a and HMG-14b (8). HMG-17 and HMG-14 proteins appear to compete for the same binding sites in vitro (1), although the homology between the two proteins is less than 50% (8). HMG-17 is highly conserved among birds and mammals (human and chicken HMG-17 are 94% identical [13, 23]), whereas HMG-14 proteins are much less well conserved (approximately 50% identical, depending on the variant in question [13, 36]).

The abundance, wide distribution, and evolutionary stability of HMG-17 suggest that this protein plays an important role in chromatin, but its exact function remains unknown. We have taken a genetic approach in which null mutations of the

HMG-17 gene have been generated by targeted disruption. The chicken *HMG-17* gene was chosen because it is highly conserved and is represented by a single copy per haploid chicken genome (13, 23) and because chicken lymphoid cell lines, such as DT40, have been shown to exhibit quite high levels of homologous recombination (5). While existing technology does not yet permit the generation of null mutant transgenic birds, the fact that *HMG-17* is a ubiquitously expressed housekeeping gene suggested that the phenotypic effect of an *HMG-17* null mutation in chicken lymphoid cells might not differ greatly from analogous effects in other avian cells.

MATERIALS AND METHODS

Cells. The DT40 cell line (2) was obtained from Craig Thompson, University of Chicago, Chicago, Ill. This cell line and all its derivatives were cultured at 40°C under 10% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% chicken serum, 1 mM 2-mercaptoethanol, 10 mg of gentamicin per ml, and 2.5 mg of amphotericin B (Fungizone) per ml.

Plasmid constructs. TFANEO (16), which contains the neomycin phosphotransferase gene under the control of the chicken β -actin promoter, was obtained from Steve Hughes, National Cancer Institute Frederick Cancer Research Center. A gene cassette encoding histidinol dehydrogenase (19, 37) under the control of the chicken β -actin promoter was provided by Jean-Marie Buerstedt, Basel Institute of Immunology, Basel, Switzerland. An *EcoRI* fragment (9.7 kb) containing the chicken *HMG-17* genomic sequence was excised from the lambda phage clone H7-1 (3a) and cloned into a pBSIIKSvSX vector, which was made from pBluescriptIIKS(+) (Stratagene) by removing part of the multiple cloning site (from *XbaI* to *SmaI*). The resulting construct was named pBSH17 (Fig. 1). Gene cassettes for *neo* and *hisD* were used to replace a 755-bp *BamHI-SmaI* fragment in pBSH17, which contains both TATA boxes and exon 1 (encoding the initial 4 amino acid residues of HMG-17 [23]). The resulting replacement targeting vectors, each containing about 4.5 kb of homologous sequence on either side of the selection marker gene cassettes, were designated pBSH17neo and pBSH17his, respectively (Fig. 1).

Electroporation. Cells (10^7) were washed with cold phosphate-buffered saline (PBS) twice and resuspended in 0.5 ml of PBS in a 0.4-cm Bio-Rad Gene Pulser cuvette. A 10-mg portion of targeting plasmid DNA was added, and the mixture was placed on ice for 10 min. The electroporation was performed at room temperature at 250 V and 960 μ F with a Bio-Rad Gene Pulser. After electroporation and another 10 min on ice, the cells were resuspended in 50 to 100 ml of growth medium and plated in four 24-well plates. After 24 h of recovery, incubation was begun with growth medium containing appropriate concentrations of drugs (2 mg of Geneticin [GIBCO BRL] per ml and/or 1.5 mg of histidinol [Sigma] per ml). Resistant colonies typically develop in 10 days.

Screening. Genomic DNA samples were extracted from individual colonies by digestion with proteinase K and extraction with phenol-chloroform. After com-

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FIG. 1. Schematic diagram of the targeting vectors and wild-type and disrupted *HMG-17* alleles. Solid boxes represent the exons of the *HMG-17* gene. Hatched boxes represent cassettes for drug-selectable markers. E, B, H, and X represent the cleavage sites for *Eco*RI, *Bam*HI, *Hind*III, and *Xho*I, respectively. The *Eco*RI site at the 3' end of pBSH17 originates from a synthetic linker used in cloning the genomic DNA. The location of a diagnostic 3' external hybridization probe (*Hind*III-*Bam*HI fragment, 0.2 kb) is shown by an asterisk. (A) Diagram of pBSH17 linearized at an *Eco*RI site. Above the line are shown the selectable cassettes inserted in the *Bam*HI and *Sma*I sites of pBSH17 to generate pBSH17his (*hisD*) and pBSH17neo (*neo*), respectively. The thin line represents pBS17 vector sequences. (B) Map of the wild-type *HMG-17* gene as found in DT40. (C) Map of an *HMG-17* disrupted mutant gene generated by homologous recombination of an insert from pBSH17neo.

plete digestion with *Eco*RI or *Bam*HI, 15 mg of DNA was fractionated on a 0.7% agarose gel, blotted onto a Magna Charge (Micron Separations Inc.) membrane by the downward alkaline method (10), and hybridized to a 32 P-labeled probe made from a 0.2-kb *Bam*HI-*Hind*III template (3' to the *HMG-17* gene [Fig. 1]) by random primer extension (34). Blots were washed with $0.2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C. Colonies whose DNA yielded the expected band were further screened by probing the stripped blots with selectable marker cassette DNAs and the 0.75-kb *Bam*HI-*Sma*I fragment deleted in making the targeting construct.

Protein analysis. HMG proteins were extracted from exponentially growing cultures with 5% perchloric acid (PCA) as described previously (6). The extract, which contains HMG proteins and histone H1, was subjected to polyacrylamide gel electrophoresis (PAGE) on a 15% polyacrylamide gel containing SDS. For direct staining of the proteins on the gel, 0.1% Coomassie blue R250 in 35% ethanol–10% acetic acid was used. For Western immunoblotting, proteins on the gel were transferred to a Biotrace polyvinylidene difluoride (Gelman) membrane with a 29 mM Tris–179 mM glycine–20% ethanol blotting solution in a Bio-Rad Trans-blot apparatus as recommended by the manufacturer. The membrane was blocked with 5% nonfat milk–0.1% Tween 20 in PBS for 2 h and then incubated with rabbit antibody against the DNA-binding domain of HMG-14/17 (gift of Michael Bustin, National Institutes of Health [7]) for 1 h at room temperature. The blot was then washed three times for 10 min each with PBS containing 0.1% Tween 20 and incubated with goat anti-rabbit horseradish peroxidase conjugate. It was washed as above, and Renaissance chemiluminescence (ECL) reagent (DuPont-NEN) was used to detect the location of immune complexes.

Analysis of nucleosomes and nucleosomal proteins. Micrococcal nuclease digestion of isolated nuclei and gel electrophoresis on a 3.5% polyacrylamide–0.5% agarose gel containing 30% glycerol were performed as described previously (21). Total histones and HMGs were extracted with 0.2 M sulfuric acid and fractionated on a 12% polyacrylamide gel containing 0.375% Triton X-100, 5% acetic acid, and 7.5 M urea as described previously (24, 40). The gel was stained with Coomassie blue R250.

Northern (RNA) blot and reverse transcriptase PCR analysis. Total RNA was extracted from cells by the RNazol B method as described by the manufacturer (Tel-Test Inc.). A 30-mg sample of RNA was fractionated on a 1.2% agarose gel containing $1 \times$ MOPS (morpholinepropanesulfonic acid), 0.66 M formaldehyde, and 1 mg of ethidium bromide per ml, using $1 \times$ MOPS buffer, and blotted to a Magna charge membrane by the downward-transfer method (10) in $10 \times$ SSC. The RNA was hybridized to a 32 P-labeled *HMG-17* cDNA probe as for Southern blot analysis (34). The template for making the probe was the *HMG-17* cDNA fragment (1.2 kb) excised from pLG1A (13) by *Bst*UI-*Eco*RI digestion. The membrane was then stripped and reprobed with chicken *HMG-14a*, *HMG-14b*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization probes. The *HMG-14a* template was a 900-bp *Eco*RI insert from pLM3B (13). The *HMG-14b* template was a 1.7-kb *Eco*RI fragment excised from a cDNA clone designated E32 (12a). The GAPDH template was a *Pst*I fragment excised from pGAD28 (a gift of R. J. Schwartz, Baylor College of Medicine [15]). Three primers were synthesized for reverse transcriptase PCR analysis. Two sense

primers were made to anneal to exon 1 (primer E1; CTCTCCCTCTCGCAC AACA) and exon 4 (primer E4; TCCGAAGCCAGAGCCTAAAC); one anti-sense primer (primer E6, ACTTGGCATCACCAGCACCT) was made to a region in exon 6. First-strand cDNA was synthesized from 5 μ g of total RNA by reverse transcription in a 40- μ l reaction mixture containing 20 μ g of oligo(dT) (Pharmacia), 800 U of Moloney murine leukemia virus reverse transcriptase (Promega), 40 U of RNase H (Pharmacia), and 20 pmol of each deoxynucleoside triphosphate (dNTP). After 1.5 h of incubation, 1 μ l of the reaction mixture was transferred to 99 μ l of PCR mixture containing 8 μ l of dNTPs (10 mM each), 10 μ l of $10 \times$ PCR buffer with 15 mM $MgCl_2$ (Promega), 50 pmol each of sense and antisense primers, and 2.5 U of *Taq* polymerase. PCR was performed in a Programmable Thermal Controller (MJ Research, Inc.) with 35 cycles of 1 min at 94°C, 2 min at 47°C, and 2 min at 72°C. Then, 20 μ l of the product was analyzed on a 1.5% agarose gel.

Cell cycle distribution. A total of 10^6 logarithmic cells were washed in cold PBS three times. After resuspension in 0.4 ml of PBS-fetal bovine serum (1:1), the cells were fixed by addition of 1.2 ml of 50% ethanol. The fixed cells were then stained for 1 h at room temperature with 1 ml of 50-mg/ml propidium iodide in PBS that contained 5 U of RNase per ml, 0.1% Triton X-100, and 0.1 mM EDTA. A minimum of 10^4 cells were used for flow cytometry analysis in a FACS Vantage apparatus (Becton Dickinson). The cell cycle distribution of the population was analyzed by the Multiplus Cell Cycle program.

DNase I-hypersensitive-site analysis. Nuclei were isolated and digested with DNase I generally by previously described methods (3). DNA was isolated from treated nuclei, digested with either *Sal*I or *Sca*I in separate experiments, gel electrophoresed, and blotted as described above. Blots were hybridized to a 295-bp fragment of the constant region of the chicken λ light-chain gene (32) obtained by PCR amplification with the sequences CCCACCATCACCTCTT and GCACTCGGACCTCTTCTAG as primers.

RESULTS AND DISCUSSION

The DT40 cell line is diploid at the *HMG-17* locus. Southern blot hybridization of DT40 genomic DNA digested with *Bam*HI, *Eco*RI, and *Cla*I and hybridized to a probe prepared from the 11.2-kb *Eco*RI genomic fragment containing the cloned *HMG-17* gene (3a) indicated that the DT40 cell line remains diploid at the *HMG-17* locus (data not shown). Both band intensity and band number corresponded to those expected for a cell with two homologous copies of the chicken *HMG-17* gene (13, 23). This conclusion was confirmed by analysis of the singly disrupted cell lines described below.

Strategy for the isolation of null mutant cells. The general scheme used to prepare cell lines lacking functional *HMG-17* genes is shown in Fig. 2. First, one of the two *HMG-17* genes in DT40 was inactivated by homologous recombination with either the G418-resistant construct (pBSH17neo) or the his-

FIG. 2. Outline of a two-step transfection procedure to generate homozygous *HMG-17* null mutants. AD3, His1, and AD3a are derivatives of DT40 with single disruptions of the *HMG-17* gene. D2, D20, D108, D108-1, and D98-7 are derivatives of AD3a and His1 with disruptions of both copies of the *HMG-17* gene. D98 is a mixed population containing both homozygous mutant cells and heterozygous mutant cells with a copy of the pBSH17neo targeting vector inserted nonspecifically.

tidinol-resistant construct (pBSH17his). Resistant colonies were analyzed for homologous recombination by Southern blot assay and, if necessary, cloned at limiting dilution to eliminate any potential contaminating resistant lines. The singly resistant cell lines were then targeted with the alternate selectable construct to inactivate the second endogenous *HMG-17* gene. Again, homologous recombinants were identified by Southern blot assay and cloned, as necessary, by limiting dilution. Null mutant lines were obtained with the two selectable markers in either order (Fig. 2).

Disruption of one copy of the *HMG-17* gene. The *neo* targeting vector pBSH17neo (Fig. 1) was used in DT40 electroporation experiments as described in Materials and Methods to generate 110 resistant colonies. For colonies with targeted integration (i.e., homologous recombination of the transfecting *HMG-17* gene), hybridization of genomic DNA cut by *Eco*RI with the 3' 0.2-kb probe (Fig. 1) is expected to generate a 12.9-kb band, and, similarly, a new 7.3-kb band would be expected in a *Bam*HI blot. The endogenous *HMG-17* gene will give rise to an *Eco*RI fragment of 11.2 kb and a *Bam*HI fragment of 5.7 kb when hybridized to the diagnostic probe. These fragments are still observed in the desired cell lines, since one of the two endogenous *HMG-17* genes remains unchanged. By this assay, 12 colonies were determined to have targeted integration via homologous recombination (data not shown), for a frequency of 11%. One colony, AD3, was cloned by limiting dilution, and one of its derivative sublines, AD3a, was chosen for a second round of transfection by the pBSH17his construct (see below). Lanes 4 in Fig. 3A and B show the hybridization profile of AD3a. The two equally intense bands in both lanes indicate that one copy of the *HMG-17* gene was disrupted and that the clone was free from contamination with parental DT40 cells. When the blots were stripped and reprobed with a *neo* probe, only one band (12.9 kb for the *Eco*RI blot and 7.3 kb for the *Bam*HI blot) was detected, indicating that no additional nonhomologous recombination events occurred in AD3a (data not shown).

pBSH17his linearized by *Xho*I (which cuts in the polylinker region of the vector) was also used to transfect DT40 cells. For clones with a targeted integration, a 7.3-kb band would be expected when the 0.2-kb diagnostic probe was used to hybridize to an *Eco*RI blot. Similarly, an 8.2-kb band would be expected in the *Bam*HI blot. Among seven colonies selected for histidinol resistance, two were found to have the bands expected from integration by homologous recombination. This represents a targeting frequency of 30%. One colony, His1, was chosen for further investigation (see below). The hybridization profile of this colony is shown in Fig. 3, lanes 6. As with AD3a, the equal intensities of the two bands in both lanes indicate that His1 is not contaminated with parental cells. His1 was also found not to have any random integrations of additional exogenous DNAs when a *his* probe was hybridized to these blots (data not shown).

Disruption of the second copy of the *HMG-17* gene in AD3a and His1. AD3a and His1 cell lines were electroporated by pBSH17his and pBSH17neo, respectively, and selected in the presence of both G418 and histidinol. A total of 130 clones survived this dual-drug selection; 42 of these were derived from AD3a, and 88 were from His1. When the *HMG-17* gene is disrupted in both copies by pBSH17neo and pBSH17his, 12.9- and 7.3-kb bands are expected to completely replace the endogenous 11.2-kb band upon *Eco*RI cleavage and Southern hybridization with the 0.2-kb probe (Fig. 3A, lanes 2, 3, and 7). Similarly, the 7.3- and 8.2-kb bands would be expected to completely replace the endogenous 5.7-kb band in a blot of *Bam*HI-cleaved DNA (Fig. 3B, lanes 2, 3, and 7). By this assay,

FIG. 3. Southern blotting analysis of representative colonies. Genomic DNAs from DT40, AD3a and its derivatives (D2, D20, and D61), and His1 and its derivatives (D107, D108-1, and D98) were extracted, digested with *Eco*RI (A) and *Bam*HI (B), fractionated on agarose gels, blotted, and hybridized to a ³²P-labeled 0.2-kb diagnostic probe (Fig. 1). The molecular sizes (in kilobases) are indicated. The notations 11.2 kb (A) and 5.7 kb (B) indicate the bands expected from the wild-type DT40 cell line, 12.9 kb (A) and 7.3 kb (B) indicate the expected bands after homologous recombination mediated by pBSH17neo, and 7.3 kb (A) and 8.2 kb (B) indicate those expected to be generated by homologous recombination of pBSH17his.

11 colonies were determined to have both copies disrupted by homologous recombination. Besides these 11 clones, there were 2 further colonies (D98 and D28) which not only had the new bands but also retained an endogenous band, indicating that they were not clonally pure. D98 was cloned by limiting dilution. As expected, both doubly homologous recombinants and nontargeted transfectants were recovered as determined by Southern hybridization. Four homozygous null mutants due to double homologous recombination events (D2 and D20 were derivatives of AD3a, and D98-7 and D108-1 were derivatives of His1) were chosen for further studies. Blots of their DNA cleaved by *Bam*HI were additionally hybridized with the 0.75-kb fragment replaced in the targeting constructs, the *neo* probe, and the *his* probe, as described above. As expected, no bands hybridized to the 0.75-kb probe, while 7.3- and 8.2-kb bands hybridized to the *neo* and *his* probes, respectively (results not shown).

Several higher eucaryotic cells lines have been shown to produce homozygous doubly disrupted mutants from originally heterozygous singly disrupted cells at low frequency in cell culture (9, 18, 29), presumably via some sort of gene conversion event. By raising the selectable drug concentration in the culture media to enrich for cells with two copies of the selectable marker allele, several laboratories have been able to pro-

duce homozygous mutant cell lines after initially disrupting a single gene copy (20, 22, 27). In the course of our studies, an attempt was made to select for such a mitotic recombination in the AD3a cell line. Higher concentrations of geneticin (2, 4, 6, 8, 10, 15, and 20 mg/ml) were added to the growth media. Cells survived 20 mg of Geneticin per ml (10-fold increase over the primary screening concentration) without showing any detectable generation of homozygous null mutant colonies as demonstrated by Southern hybridization (data not shown). This may be because we used a wild-type *neo* gene in our original transfections, since it has been suggested that a mutant *neo* gene that makes a less active phosphotransferase is conducive to the selection of doubly disrupted mutants (26).

Null mutants express low levels of an aberrant *HMG-17* transcript. Both targeting vectors were designed to replace the majority of the promoter sequence and exon 1 (which contains the translation initiation codon and four additional highly conserved codons) with drug-selectable cassettes; therefore, the null mutants were not expected to make an *HMG-17* transcript. In fact, low levels of *HMG-17*-hybridizing RNAs (5 to 28% of that in DT40 in various experiments) with a mobility marginally higher than that of the wild type were detected by Northern blot analysis in total cellular RNA from all four homozygous null mutants (Fig. 4). To determine whether the faint bands observed were due to nonspecific cross-hybridization or diminished transcription at the disrupted *HMG-17* loci, a reverse transcriptase PCR assay was used. Two sense primers were made complementary to exons 1 (E1 primer) and 4 (E4 primer), and one antisense primer was made complementary to a region in exon 6 (E6 primer). When the cDNA made from total RNA samples was used as a template for PCR, a fragment of 170 bp was amplified with primer pair E4 and E6 for all four null mutants as well as for DT40, AD3a, and His1 (data not shown), indicating that the targeted gene is transcribed and correctly spliced at least in the region of exons 4 to 6; however, when primer pair E1 and E6 was used, a fragment of 313 bp was amplified for only DT40, AD3a, and His1, indicating, as expected, that exon 1 was absent in the null mutant RNA (data not shown). These experiments indicate that the RNA observed arises from the disrupted *HMG-17* genes and not from partially homologous sequences elsewhere in the genome or from contaminating cells having at least one wild-type locus. We conclude that the disrupted gene is transcribed at low levels into an RNA that lacks at least exon 1 of wild-type *HMG-17* mRNA. Since the translation initiation codon is located in exon 1 and there is no other known in-frame ATG codon, we would not expect the truncated transcript to make functional HMG-17 peptide. Whether the transcription was initiated from remaining (upstream) portions of the *HMG-17* promoter, some other cryptic promoter, or a promoter in the selectable marker cassette was not investigated. As has been demonstrated previously for the *HMG-14a* gene (4), the use of alternative promoters and splicing patterns is not unusual among *HMG* genes; therefore, the use of a cryptic promoter upstream from the normal *HMG-17* promoter in the disrupted genes is a reasonable possibility. Aberrant transcription from a targeted gene has also been observed by Müller et al. (28).

Quantitative analysis of Northern blots by a PhosphorImager demonstrates that heterozygous null mutants AD3a and His1 make approximately 50% of the level of *HMG-17* RNA observed in parental DT40 cells (Fig. 4), suggesting that *HMG-17* mRNA levels are proportional to gene copy number. (His1 and doubly disrupted lines derived from His1 show consistently higher levels of *HMG-17* RNA than do AD3a and its doubly disrupted lines, respectively. This may be a chance effect of the selection of the His1 transfectant line.) Dose-

FIG. 4. Northern blotting analysis of RNA from representative clones. (A) Total RNA (30 mg per lane) extracted from parental DT40 cells, singly mutant His1 and AD3a cells, and doubly mutant D2, D20, D98-7, and D108-1 cells was fractionated on a 1.2% agarose-formaldehyde gel, blotted to a membrane, and probed with 32 P-labeled *HMG-17* cDNA. (B to D) The membrane was then stripped and reprobed sequentially with *HMG-14a* (B), *HMG-14b* (C), and GAPDH (D) hybridization probes. (See Materials and Methods for a description of the probes.) Arrows indicate molecular sizes of 2.37 and 0.24 kb.

dependent transcription has also been observed for *HMG-14* by Pash et al. (30), who found that when human *HMG-14* mRNA was overexpressed in a mouse myoblast cell line, the level of the endogenous *HMG-14* mRNA was not affected. Furthermore, the expression levels of chicken *HMG-14a* and *HMG-14b* genes did not seem to be markedly altered in the D2 and D20 null mutant cell lines (Fig. 4), suggesting that the *HMG-14* and *HMG-17* genes are not coordinately regulated at the transcriptional level. This is consistent with a previous observation in COS cells (17) of mammalian *HMG* gene expression. However, the two doubly disrupted lines derived from His1, D98-7 and D108-1, do appear to contain somewhat (about twofold) elevated levels of *HMG-14a* and *HMG-14b* RNAs. This is not reflected in the *HMG-14* protein levels found in those cells (see below), and it is unclear whether it is in any way a response to the inactivation of the *HMG-17* gene.

Null mutants do not make detectable *HMG-17* protein. HMG proteins extracted from cells by 5% PCA were resolved by SDS-PAGE. Chicken *HMG-17* migrates at an apparent molecular mass of 18 kDa. As shown in Fig. 5, a band of about 18 kDa is clearly visible in the DT40, AD3a, and His1 lanes. After correction for loading variation (by comparison with the total amount of histone H1 loaded per lane), the intensity of

FIG. 5. SDS-PAGE analysis of 5% PCA extracts from representative clones. The 5% PCA extracts from 10^7 cells were separated by SDS-PAGE (15% polyacrylamide) and stained with 0.1% Coomassie blue R250 as described in Materials and Methods. Cell lines tested include DT40 (lane 1), singly disrupted mutants His1 and AD3a (lanes 2 and 3, respectively), and doubly disrupted mutants D2, D20, D98-7 and D108-1 (lanes 4 to 7, respectively). Electrophoretic standards included 43, 29, 18.4, 14.3, and 6.2 kDa.

the HMG-17 band in AD3a and His1 was estimated to be reduced by approximately 50%, indicating that the level of HMG-17 protein (like that of its mRNA) is approximately proportional to the number of functional genes per cell. For all four double-knockout mutants examined, the 18-kDa band was not discernible at the resolution of our gel. (There is a faint background band that runs slightly more slowly than HMG-17.)

To further examine these doubly mutant cell lines for possible low-level expression of HMG-17 protein or some truncated form thereof, Western immunoblot analysis was performed with antibodies elicited against a peptide that is common to HMG-14 and HMG-17 (7, 23). As shown in Fig. 6, lanes 11 to 14, HMG-17 protein was not detected even when the blot was heavily overexposed. A minimum 40-fold reduction in the level of HMG-17 protein must have occurred in these doubly mutant cell lines compared with AD3a or His1 (or >80-fold compared with DT40), since 1/4 of the HMG-17 band in lane 3 (approximately the intensity of the HMG-14b band in lane 5) should be visible and since lane 3 is loaded with 1/10 of the total protein loaded onto lane 11 (in which we still cannot observe any HMG-17). Because the epitope recognized by this antibody is located in the most highly conserved, common DNA-binding domain of HMG-17 and HMG-14, any truncated form of HMG-17 that might retain normal function should have been detected, if present. However, we cannot rule out the possibility that truncated or fused protein that happens to comigrate with HMG-14a or HMG-14b is made in these mutant cell lines.

Quantitative analysis of the autoradiogram in Fig. 6 shows that approximately half the amount of HMG-17 was made in the singly null mutant cell lines AD3a and His1 as that in DT40. This further demonstrates that *HMG-17* gene expression is approximately linear with the functional gene copy number (at least for zero to two copies). Figure 6 also shows that the levels of HMG-14a and HMG-14b were not markedly affected upon the disruption of one or both copies of the *HMG-17* gene. This is in agreement with earlier findings that the levels of these two proteins are not coordinately regulated in mammalian cells (17, 30).

Null-mutation effects on phenotype and chromatin structure. All four doubly disrupted mutant cell lines divided normally in standard growth media, without any observable difference in phenotype between them, AD3a, His1, and DT40.

No change in morphology was found among any of these cell lines when they were examined by phase-contrast microscopy. To test whether the mutant lines were stable for extended periods in cell culture, they were grown in vitro for 1 month without drug selection. At the end of the period, genomic DNA and HMG proteins were extracted from each of the lines and examined by Southern hybridization and Western blotting. As expected, no differences were detected from the results of the same assays done before the extended culture (Fig. 3 and 6), indicating that the mutations created are stable and that no reversions or genomic rearrangements occurred at the *HMG-17* locus during long-term growth in the absence of selection (results not shown).

Growth rates of the mutant lines and DT40 were determined following plating of each line at 10^4 cells per ml in 60-mm plates. At about 12-h intervals, three plates per cell line were counted. The results are presented in Fig. 7. It is clear that the singly or doubly mutant cells grow just as well as the DT40 parental cell line (the doubling time was 10 h under these growth conditions). Since there was no detectable difference in growth rate, we next examined the distribution of these lines in different stages of the cell cycle by flow cytometry analysis. Again, there was no consistent difference between DT40 and singly or doubly mutant cell lines in the percentage of cells in G_1 , S, and G_2 phases of the cell cycle (results not shown).

Normal cell proliferation and differentiation have been suggested to require the regulated expression of HMG-14 and HMG-17. Their expression is down-regulated during differentiation of rat osteoblasts and promyelocytic leukemia cells (35), and overexpression of HMG-14 inhibits differentiation of mouse myoblast cells (30). To assess whether the inactivation of the *HMG-17* gene advanced the cell differentiation of DT40 cells, we examined the targeted cell lines by Giemsa staining, light-scattering profile analysis, and the soft agar assay. Giemsa staining and light-scattering analysis with flow cytometry detect the complexity of the cytoplasmic structure. A more complex cytoplasmic structure would be expected if DT40 cells proceeded toward the next stage of their normal differentiation, which would be to become plasma cells. Neither Giemsa staining nor light-scattering analysis detected any significant difference between *HMG-17* mutant cell lines and the DT40 parental cells. Furthermore, in a soft agar assay, all mutant and parental cell lines were found to form foci after a week of culturing, without obvious differences in colony-forming efficiency (results not shown).

FIG. 6. Western blot analysis of representative clones. The 5% PCA extracts from 10^6 cells (lanes 1 to 7) and 10^7 cells (lanes 8 to 14) were loaded onto an SDS-PAGE gel (15% polyacrylamide). After electrophoresis, the gel was blotted to a polyvinylidene difluoride membrane, which was probed with antibody against the DNA-binding domain of HMG-17/14 (7). ECL was used to locate the bands after incubation in horseradish peroxidase conjugate. Cell lines tested include DT40 (lanes 1 and 8), singly disrupted mutants His1 (lanes 2 and 9) and AD3a (lanes 3 and 10), and doubly disrupted mutants D2 (lanes 4 and 11), D20 (lanes 5 and 12), D98-7 (lanes 6 and 13), and D108-1 (lanes 7 and 14).

FIG. 7. Growth curves of representative cell lines. (A) DT40 ($\circ$), singly mutant His1 ($\bullet$), and its doubly mutant derivatives D98-7 ($\square$) and D108-1 ($\blacksquare$) were plated out at 10^4 cells per ml at 0 h and counted at 10- to 15-h intervals. Triplicate samples were counted at each time point, and the standard deviation values were used to plot the error bars (not visible for every time point). (B) Analysis identical to that in panel A for DT40 ($\circ$), singly disrupted AD3a ($\bullet$), and its doubly mutant derivatives D2 ($\square$) and D20 ($\blacksquare$).

Results discussed above (Fig. 5 and 6) indicated that no change in HMG-14 levels occurred in response to inactivation of the *HMG-17* gene. We also were unable to discern any alteration in the pattern of total histone proteins present in bulk chromatin of *HMG-17* mutant cells with respect to that in DT40, as detected by electrophoresis on Triton-acid-urea gels run under various conditions (24). Figure 8 shows one example of such a Triton-acid-urea gel in which the histone protein complements of two doubly mutant *HMG-17* cell lines are compared with that of DT40 and with erythrocyte histones as markers. The absence of HMG-17 was not expected to have a major effect on the levels of histone variants or their modified forms, since only about 5 to 10% of the nucleosomes, at most, would be expected to bind HMG-17 protein in normal cells (25).

We also wished to examine the structure of mutant cell line chromatin at the level of individual nucleosomes. When chromatin is digested by micrococcal nuclease, mononucleosomes of different sizes are released depending upon the number of HMG-17, HMG-14, and histone H1 molecules bound. These different forms of nucleoprotein complexes can be partially resolved in a polyacrylamide-agarose gel (21). Figure 9 is an example of the mononucleosome (and dinucleosome) profile of DT40 and the null mutant D2 and D108-1 cell lines in which the chromatin has been treated with two different concentrations of micrococcal nuclease. It is interesting that two bands are clearly discernible for all these cell lines in the region of the

FIG. 8. Histone profile of DT40 and null mutant cell lines. Total histones were extracted from chicken erythrocytes (RBC; lanes 1 and 5), DT40 (lane 2), D108-1 (lane 3), and D2 (lane 4) with 0.2 M sulfuric acid and separated on a Triton-acid-urea gel by the method of Lennox and Cohen (24). Lanes 1 and 5 contain 15 μ g of protein, and lanes 2 to 4 contain 25 μ g. The gel was stained with 0.1% Coomassie blue R250 in 35% methanol-10% acetic acid. Various classes of chicken histones were identified as indicated by comparing the erythrocyte pattern with those previously described by Urban et al. (40).

single mammalian MII particle (for nomenclature, see reference 21). Whether this is due to the presence of both HMG-14a and HMG-14b (compared with only a single HMG-14 protein in mammalian cells), differential modification of the HMG proteins, or some other unknown complex present in the chromatin of DT40 is not clear. The differences between the patterns of DT40 and the null mutant lines are not dramatic, but there does appear to be a consistent decrease in both MII bands (especially the faster-migrating one) in the null mutant lines from those observed in DT40. This is not surprising, since MII consists of core nucleosomes bound to one molecule of HMG-17 or HMG-14 and it was shown above that HMG-14 levels do not increase in response to the absence of HMG-17. There may also be some difference in the intensity of more slowly migrating nucleosome complexes, but the resolution of this nondenaturing gel system is not enough to clearly demonstrate any other changes.

As a lymphoid cell, DT40 is expected to express its λ light-chain gene and to contain DNase I-hypersensitive sites near this gene that are specific to the rearranged, active locus, as demonstrated by Thompson and Neiman (38). We examined DT40 and two *HMG-17* null mutant cell lines for changes in

FIG. 9. Electrophoretic analysis of nucleosomes from representative cell lines. Nuclear preparations from DT40 (lanes 3 and 6) and doubly mutant derivatives D2 (lanes 2 and 5) and D108-1 (lanes 1 and 4) were incubated with micrococcal nuclease at 20 U/mg of DNA equivalent (lanes 1 to 3) and 40 U/mg (lanes 4 to 6) for 10 min at 37°C. After 10 min on ice and subsequent centrifugation, the supernatant (S1) was removed and the pellet was resuspended in 2 mM EDTA (pH 7.4) and incubated on ice for another 10 min. After centrifugation, the supernatant (S2) was mixed with 0.5 volume of glycerol and loaded onto a 3.5% polyacrylamide–0.5% agarose gel containing 30% glycerol (21). The gel was run at a constant current of 9 mA for 11 h at 4°C. Arrows indicate two different bands in the region corresponding to the mammalian MII band (21).

the number or location of such sites due to the loss of the HMG-17 protein. We were able to detect those hypersensitive sites previously described (38) but observed no differences in the hypersensitive site pattern between DT40, D108-1, and D2 (results not shown).

Cellular function of HMG-17. In vitro assays on isolated chromatin and more recent studies involving the overexpression of exogenous HMG-17 or HMG-14 in vivo have suggested that these HMGs may act to modulate gene expression and thereby influence cell proliferation and differentiation through their regulated expression or activity. The results reported herein demonstrate that HMG-17 is not required for cell growth in culture and, furthermore, that no major phenotypic changes result from the complete absence of HMG-17 in the DT40 cells. There are several possible explanations for this result. A trivial explanation would be that, in the gene disruption process, we have selected for additional mutations outside the *HMG-17* gene that somehow compensate for the loss of *HMG-17* gene function. This seems unlikely, since we have been able to obtain doubly mutant cells via two distinct protocols. Furthermore, the efficiency with which we obtained doubly mutant colonies from a singly mutant parental cell was not significantly different from the efficiency with which we obtained initial homologous recombinants from DT40 (nor is the frequency of targeted integration into the *HMG-17* gene significantly different from that for other gene constructs [5, 37]). This argues that generation of the completely null genotype did not require additional compensatory mutations elsewhere in the genome which would presumably have decreased the frequency at which such colonies appeared.

The most straightforward explanation for our results is that HMG-17 and HMG-14 are functionally redundant and that HMG-14 alone is sufficient for normal function. This is certainly possible, although no change in HMG-14a or HMG-14b level was observed in response to the absence of HMG-17. Complete redundancy of HMG-14 and HMG-17 also contrasts with the facts that HMG-14 proteins differ considerably in amino acid sequence from HMG-17 sequences and that the HMG-17 (but not HMG-14) sequence has been very highly conserved (8) in the approximately 300 million years of evolutionary difference between birds and mammals (33). This same

evolutionary conservation argues strongly that HMG-17 (and the HMG-14 and HMG-17 family in general) does play a significant role in vertebrate development. While our results prove that HMG-17 is not required for the growth of, at least, chicken lymphoid cells in culture, it is certainly possible that HMG-17 is required for the growth and/or differentiation of other cell types, even though it appears to be expressed ubiquitously in all cells. It is also possible that the dispensable character of HMG-17 is peculiar to a transformed cell line such as DT40. Perhaps the simplest explanation for our results is that HMG-17 is not required for the growth or gross phenotypic properties of any individual cell type but, rather, functions in some unknown way(s) in the development of the animal or that HMG-17 function has a only a small effect in any individual cell, one which is significant enough to be evolutionarily selected but which is negligible when examined in short-term culture in growth media. Further genetic and cell physiological experiments are required before these alternatives can be clarified.

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REFERENCES

- Alfonso, P. J., M. P. Crippa, J. J. Hayes, and M. Bustin. 1994. The footprint of chromosomal proteins HMG-14 and HMG-17 on chromatin subunits. *J. Mol. Biol.* **236**:189–198.
- Baba, T. W., B. P. Giroir, and E. H. Humphries. 1985. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. *Virology* **144**:139–151.
- Bellard, M., G. Dretzen, A. Giangrande, and P. Romain. 1989. Nuclease digestion of transcriptionally active chromatin. *Methods Enzymol.* **170**:317–346.
- Browne, D., and J. B. Dodgson. Unpublished data.
- Browne, D. L., and J. B. Dodgson. 1993. The gene encoding chicken chromosomal protein HMG-14a is transcribed into multiple mRNAs. *Gene* **124**:199–206.
- Buerstedde, J.-M., and S. Takeda. 1991. Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* **67**:179–188.
- Bustin, M., P. S. Becerra, M. P. Crippa, D. A. Lehn, J. M. Pash, and J. Shiloach. 1991. Recombinant human chromosomal proteins HMG-14 and HMG-17. *Nucleic Acids Res.* **19**:3115–3121.
- Bustin, M., M. P. Crippa, and J. M. Pash. 1990. Immunochromatin analysis of the exposure of high mobility group protein 14 and 17 surfaces in chromatin. *J. Biol. Chem.* **265**:20077–20080.
- Bustin, M., D. A. Lehn, and D. Landsman. 1990. Structural features of the HMG chromosomal proteins and their genes. *Biochim. Biophys. Acta* **1049**:231–243.
- Campbell, C. E., and R. G. Worton. 1981. Segregation of recessive phenotypes in somatic cell hybrids: role of mitotic recombination, gene inactivation, and chromosome nondisjunction. *Mol. Cell. Biol.* **1**:336–346.
- Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* **201**:134–139.
- Crippa, M. P., L. Trieschmann, P. J. Alfonso, A. P. Wolffe, and M. Bustin. 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., S. Rimsky, S. C. Batson, M. Bustin, and U. Hansen. 1994. Stimulation of RNA polymerase II elongation by chromosomal protein HMG-14. *Science* **265**:796–799.
- Dodgson, J. B. Unpublished data.
- Dodgson, J. B., D. L. Browne, and A. J. Black. 1988. Chicken chromosomal protein HMG-14 and HMG-17 cDNA clones: isolation, characterization and sequence comparison. *Gene* **63**:287–295.
- Druckmann, S., E. Mendelson, D. Landsman, and M. Bustin. 1986. Immunofractionation of DNA sequences associated with HMG-17 in chromatin. *Exp. Cell Res.* **166**:486–497.
- Dugaiczky, A., J. A. Haron, E. M. Stone, O. E. Dennison, K. N. Rothblum,

- and R. J. Schwartz. 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochemistry* **22**:1605–1613.
16. Federspiel, M. J., L. B. Crittenden, and S. H. Hughes. 1989. Expression of avian reticuloendotheliosis virus envelope confers host resistance. *Virology* **173**:167–177.
 17. Giri, C., D. Landsman, N. Soares, and M. Bustin. 1987. Modulation of the cellular ratio of chromosomal high mobility group proteins 14 to 17 in transfected cells. *J. Biol. Chem.* **262**:9839–9843.
 18. Groden, J., Y. Nakamura, and J. German. 1990. Molecular evidence that homologous recombination occurs in proliferating human somatic cells. *Proc. Natl. Acad. Sci. USA* **87**:4315–4319.
 19. Hartman, S. C., and R. C. Mulligan. 1988. Two dominant-acting selectable markers for gene transfer studies in mammalian cells. *Proc. Natl. Acad. Sci. USA* **85**:8047–8051.
 20. Hilberg, F., A. Aguzzi, N. Howells, and E. F. Wagner. 1993. c-jun is essential for normal mouse development and hepatogenesis. *Nature (London)* **365**:179–181.
 21. Huang, S. Y., and W. T. Garrard. 1989. Electrophoretic analyses of nucleosomes and other protein-DNA complexes. *Methods Enzymol.* **170**:116–142.
 22. Jakobovits, A., G. J. Vergara, J. L. Kennedy, J. F. Hales, R. P. McGuinness, D. E. Casentini-Borocz, D. G. Brenner, and G. R. Otten. 1993. Analysis of homozygous mutant chimeric mice: deletion of the immunoglobulin heavy-chain joining region blocks B-cell development and antibody production. *Proc. Natl. Acad. Sci. USA* **90**:2551–2555.
 23. Landsman, D., T. Srikantha, and M. Bustin. 1988. Single copy gene for the chicken non-histone chromosomal protein HMG-17. *J. Biol. Chem.* **263**:3917–3923.
 24. Lennox, R. W., and L. H. Cohen. 1989. Analysis of histone subtypes and their modified forms by polyacrylamide gel electrophoresis. *Methods Enzymol.* **170**:532–549.
 25. Mardian, J. K. W., A. E. Paton, G. J. Bunick, and D. E. Olins. 1980. Nucleosome cores have two specific binding sites for nonhistone chromosomal proteins HMG 14 and HMG 17. *Science* **209**:1534–1536.
 26. Mortensen, R. M. 1993. Double knockouts: production of mutant cell lines in cardiovascular research. *Hypertension* **22**:646–651.
 27. Mortensen, R. M., D. A. Conner, S. Chao, A. A. T. Geisterfer-Lowrance, and J. G. Seidman. 1992. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12**:2391–2395.
 28. Müller, U., N. Cristina, Z. W. Li, D. P. Wolfer, H. P. Lipp, T. Rülcke, S. Brandner, A. Aguzzi, and C. Weissmann. 1994. Behavioral and anatomical deficits in mice homozygous for a modified β -amyloid precursor protein gene. *Cell* **79**:755–765.
 29. Nelson, F. K., W. Frankel, and T. V. Rajan. 1989. Mitotic recombination is responsible for the loss of heterozygosity in cultured murine cell lines. *Mol. Cell. Biol.* **9**:1284–1288.
 30. Pash, J. M., P. J. Alfonso, and M. Bustin. 1993. Aberrant expression of high mobility group chromosomal protein 14 affects cellular differentiation. *J. Biol. Chem.* **268**:13632–13638.
 31. Paton, A. E., E. Wilkinson-Singley, and D. E. Olins. 1983. Nonhistone nuclear high mobility group proteins 14 and 17 stabilize nucleosome core particles. *J. Biol. Chem.* **258**:13221–13229.
 32. Reynaud, C.-A., A. Dahan, and J.-C. Weill. 1983. Complete sequence of a chicken λ light chain immunoglobulin derived from the nucleotide sequence of its mRNA. *Proc. Natl. Acad. Sci. USA* **80**:4099–4103.
 33. Romer, A. S. 1966. Vertebrate paleontology. University of Chicago Press, Chicago.
 34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 35. Shakoori, A. R., T. A. Owen, V. Shalhoub, J. L. Stein, M. Bustin, G. S. Stein, and J. B. Lian. 1993. Differential expression of the chromosomal high mobility group proteins 14 and 17 during the onset of differentiation in mammalian osteoblasts and promyelocytic leukemia cells. *J. Cell. Biochem.* **51**:479–487.
 36. Srikantha, T., D. Landsman, and M. Bustin. 1988. Cloning of the chicken chromosomal protein HMG-14 cDNA reveals a unique protein with a conserved DNA binding domain. *J. Biol. Chem.* **263**:13500–13503.
 37. Takeda, S., E. L. Masteller, C. B. Thompson, and J.-M. Buerstedde. 1992. RAG-2 expression is not essential for chicken immunoglobulin gene conversion. *Proc. Natl. Acad. Sci. USA* **89**:4023–4027.
 38. Thompson, C. B., and P. E. Neiman. 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell* **48**:369–378.
 39. Tremethick, D. J., and H. R. Drew. 1993. High mobility group proteins 14 and 17 can space nucleosomes in vitro. *J. Biol. Chem.* **268**:11389–11393.
 40. Urban, M. K., S. G. Franklin, and A. Zweidler. 1979. Isolation and characterization of the histone variants in chicken erythrocytes. *Biochemistry* **18**:3952–3960.
 41. Weisbrod, S. 1982. Active chromatin. *Nature (London)* **297**:289–295.
 42. Weisbrod, S., M. Groudine, and H. Weintraub. 1980. Interaction of HMG 14 and 17 with actively transcribed genes. *Cell* **19**:289–301.