This is to certify that the

dissertation entitled

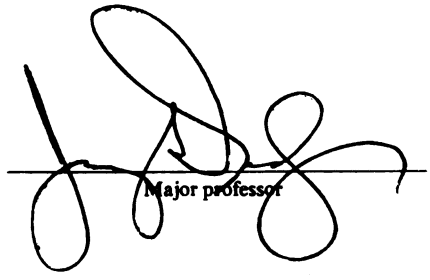
SOMATIC DISRUPTION OF CHICKEN HMG-17 AND HMG-14A GENES

presented by

YI LI

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in MICROBIOLOGY



Major professor

Date March 22, 1996

SOMATIC DISRUPTION OF CHICKEN *HMG-17* AND *HMG-14A* GENES

By

Yi Li

A DISSERTATION

Submitted To
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1996

ABSTRACT

SOMATIC DISRUPTION OF CHICKEN *HMG-17* AND *HMG-14A* GENES

By

Yi Li

HMG-14 and HMG-17 are ubiquitous non-histone chromosomal proteins that bind to nucleosome core particles. They 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* and *HMG-14a* genes, a targeted disruption (gene knockout) technique was used to generate *HMG-17* doubly disrupted cell lines (D2, D20, D98-7, and D108-1), one *HMG-14a* doubly disrupted mutant cell line (8/bsr8), and quadruply disrupted cell lines (Bp5, Bp39, Nh43, and Nh52) lacking both HMG-17 and HMG-14a from a chicken B-lymphoid cell line, DT40. As expected, doubly disrupted *HMG-17* mutant lines (D2, D20, D98-7, and D108-1) produced no detectable HMG-17 protein, the doubly disrupted *HMG-14a* mutant line (8/bsr8) produced no detectable HMG-14a, and quadruply disrupted cell lines (Bp5, Bp39, Nh43, and Nh52) produced neither HMG-17 nor HMG-14a. No significant changes in proliferation rate or other cell phenotypes were detected in all these cell lines with respect to the parental DT40, and no

compensatory changes in HMG-14b or histone protein levels were observed. It is concluded that HMG-14a and HMG-17 proteins are not required for normal growth of avian cell lines in vitro, nor does the absence of *HMG-17* and *HMG-14a* lead to any major changes in cellular phenotype, at least in lymphoid cells.

DEDICATIONS

To Shixia, Danting, Suzhen, Shaoqin

ACKNOWLEDGMENTS

I am greatly indebted to my mentor, Jerry B. Dodgson for his guidance, encouragement, and during my graduate program and also for his advice in my academic carrier planing and his generous help in my personal life. I am also indebted to Dr. Lucy Lee for her financial support in my first year of graduate studies, her encouragement, and friendship. I am very grateful to my committee members, Drs. Donald Salter, Richard Schwartz, Donald Jump, and Patrick Venta for their guidance and time.

I wish to express my gratitude to my fellow lab-mates Kyoung-Eun Kim, Huei-Min Lin, Ronald Okimoto, Natalie Brown, William Payne, and Steven Suchyta for their friendship and invaluable assistance. I would also like to thank Drs. Henry Hunt, Susan Conrad, Ronald Patterson, and Louis King for their advice and help and the excellent support staff of Microbiology for their assistance.

TABLE OF CONTENTS

LIST OF FIGURES vi

LIST OF TABLES.....viii

INTRODUCTION.....1

CHAPTER I
THE CHICKEN HMG-17 GENE IS DISPENSABLE FOR
CELL GROWTH IN VITRO 24
ABSTRACT 24
INTRODUCTION 25
MATERIALS AND METHODS 27
RESULTS AND DISCUSSION 33

CHAPTER II
NULL MUTANT CHICKEN CELL LINE UNABLE TO EXPRESS EITHER
HMG-14A AND *HMG-17* SHOW NO PHENOTYPIC DIFFERENCES AS A
RESULT 57
ABSTRACT 57
INTRODUCTION.....58
MATERIALS AND METHODS.....60
RESULTS AND DISCUSSION.....64

SUMMARY 85

APPENDIX
UNPUBLISHED RESULTS.....87

BIBLIOGRAPHY 98

LIST OF FIGURES

Introduction

Figure 1. Structure of the HMG-14/-17 genes in human and chickens 3
Figure 2. Sequence similarity between regions of HMG-14/-17 cDNAs.....5
Figure 3. Alignment of various HMG-14 and HMG-17 molecules. 7
Figure 4. Model of the location of HMG-14/-17 on a nucleosome core.....11
Figure 5. Diagram illustrating the generation of homozygous null mutants by two different approaches.....21

Chapter 1

Figure 1. Schematic diagram of the targeting vectors and wild-type and disrupted *HMG-17* alleles 28
Figure 2. Outline of a two-step transfection procedure to generate homozygous HMG-17 null mutants 35
Figure 3. Southern blotting analysis of representative colonies 36
Figure 4. Northern blotting analysis of RNA from representative clones.....41
Figure 5. SDS-PAGE analysis of 5% PCA extracts from representative clones 44
Figure 6. Western blot analysis of representative clones 46
Figure 7. Growth curves of representative cell lines....49
Figure 8. Histone profile of DT40 and null mutant cell lines 51
Figure 9. Electrophoretic analysis of nucleosomes from representative cell lines.....53

Chapter 2

Figure 1. Schematic diagram of the targeting vectors and wild-type and disrupted *HMG-14a* alleles. 61
Figure 2. Outline of the transfection procedures to generate targeted disruptions. 65
Figure 3. Southern blotting analysis of representative clones. 66
Figure 4. Northern blotting analysis of RNA from representative clones. 71

Figure 2. Outline of the transfection procedures to generate targeted disruptions. 65
Figure 3. Southern blotting analysis of representative clones. 66
Figure 4. Northern blotting analysis of RNA from representative clones. 71
Figure 5. Western blotting analysis of representative clones.....74
Figure 6. Growth curves of representative cell lines....75
Figure 7. Histone profiles of DT40 and quadruply disrupted cell lines.....78
Figure 8. Electrophoretic analysis of nucleosomes from representative cell lines.....80
Figure 9. DNase I sensitivity analysis of representative cell lines.....82

Appendix

Figure 1. The aberrant transcript from *HMG-17* null mutants does not contain exon 1.....88
Figure 2. The side-scattering profile does not correlate with *HMG-17 g*ene activity.....90
Figure 3. The hypersensitive profile is similar in the HMG-17 null mutants and DT40.....91
Figure 4. The side-scattering profile does not correlate with the *HMG-14a* and the *HMG-17* gene activity.....93
Figure 5. Surface IgM expression is similar in the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* and the parental DT40 cells.....95

LIST OF TABLES

Table 1. The cell cycle distribution does not correlate with the presence of a functional *HMG-17* gene.....89
Table 2. The colony-forming efficiency in soft-agar does not correlate with *HMG-17* gene activity.....89
Table 3. The cell cycle distributions of the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* do not show consistent differences compared with that of DT40...92
Table 4. Targeting frequencies of various vectors used...97

INTRODUCTION

In eukaryotes, chromatin consists of DNA, histones, and a variety of non-histone proteins. Among the non-histone proteins are the high mobility group (HMG) proteins. These proteins were named "HMG" because they consist of a group of proteins which could be extracted from chromatin by 0.35M NaCl and solublized in 2% TCA and which migrated with high mobility in acrylamide gel electrophoresis (41). Due to this arbitrary definition, the members of this family do not necessarily play a similar role in cell nuclei, nor do they necessarily have close structural relationships. As molecular characterization of these proteins continued and more have been learned of their functions, the HMG proteins are now further classified into three sub-families: HMG-14 and HMG-17, HMG-1 and HMG-2, and HMG-I and its isoform HMG-Y (12). HMG proteins within each family are structurally and functionally related, yet proteins between families demonstrate little, if any, relatedness. This thesis is focused on HMG-14 and HMG-17 only.

The genes and transcripts

HMG-14 and HMG-17 comprise a family of nucleosome-binding proteins with molecular weights of 10,000 to 12,000 (12). In fact, they are the only nuclear proteins identified to date that bind specifically to nucleosomes and bind nucleosomes more tightly than free DNA (79). HMG-14 and HMG-17 are ubiquitously expressed in all vertebrates examined to date (57). Mammals appear to express single HMG-17 and HMG-14 proteins (12). In chickens, two rather different types of HMG-14 (HMG-14a and HMG-14b) have been identified (26, 88). HMG-14a is the dominant form in all cell types examined, but HMG-14b is more closely related to mammalian HMG-14s in amino acid sequence (26, 88).

The human genes encoding HMG-17 (47) and HMG-14 (46, 49) and the chicken genes encoding HMG-17 (48), HMG-14a (26), and HMG-14b (88, 89) have been cloned and characterized. The human and chicken HMG-17 genes and the human HMG-14 gene each contain 6 exons and 5 introns (Figure 1). In the chicken HMG-14b gene, the exon-intron organization is very similar to HMG-17 or human HMG-14 except that intron 2 is absent producing a fusion at the GT/AG boundary of exon 2 and exon 3 resulting in an insertion of a GTA codon (valine). The chicken HMG-14a

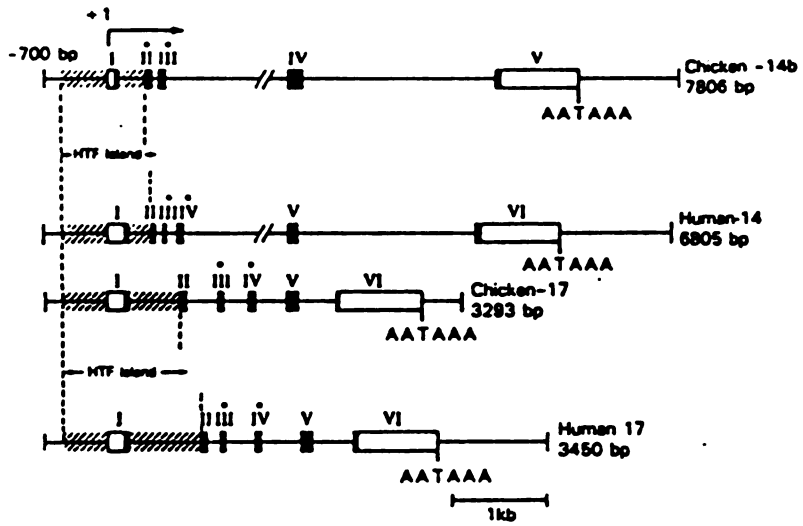


Figure 1. Structure of the HMG-14/-17 genes in human and chickens. The position of introns and exons are drawn to scale and aligned at the start of transcription. Arrows indicates direction of transcription. Exons are numbered by roman numerals. The black regions identify coding regions of the genes. Exons marked with asterisks are coding for the nucleosome-binding domain. The "HTF islands" starting from residues -500 and ending at the start of exon 2 are shaded. The length of the gene, from the start of transcription (nucleotide +1) to the poly(A) signal (AATAAA) is indicated under its name. This figure is copied from reference (12).

consists of 7 exons and 6 introns. The presence of an extra exon (exon 0) in chicken HMG-14b is due to alternative splicing in this gene (10). Exons 1, 3, and 4 are well conserved among all genes studied in this family while other exons are either not conserved or weakly so (12).

The promoter regions of this gene family have a very high GC content (75%) and contain multiple CpG dinucleotides; both are expected for a typical housekeeping gene. The HMG-17 genes contain two TATA boxes, whereas the HMG-14 genes lack a TATA box (12). This difference suggests that these two genes may be regulated differently. Indeed, when HMG-14 is overexpressed from transfected plasmid DNA, the level of endogenous HMG-17 does not change (70).

In chickens, a single-copy gene codes for each of the three genes in this family (26, 89). The human genome contains 60-80 gene copy equivalents for HMG-14 (44) and 30-50 for HMG-17 (47, 87), and the genes copies are not in close proximity and are probably located on different chromosomes (87). Most of the copies do not encode transcripts. Sequence analysis indicates that these non-functional genes hybridizing to the HMG-17 probe are processed retropseudogenes which arose around ten million years ago from a common ancestor (87). Northern blotting analysis using a cDNA probe indicates that only one or at most a few copies of either the *HMG-14* or *HMG-17* gene is functional in human cells (47, 49).

The cDNA clones of HMG-14 and HMG-17 from human (47,

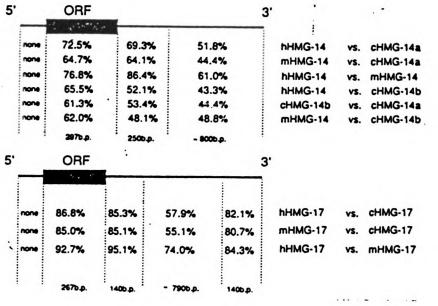


Figure 2. Sequence similarity between regions of HMG-14/-17 cDNAs. The open reading frames (ORF) are identified by large solid blocks, and the 5'a and 3' untranslated regions are represented by solid lines. The boundaries of the regions which have been compared are identified by vertical dotted lines. The percentage of identity is shown. The cDNAs that are compared are human HMG-17 (hHMG-17), mouse HMG-17 (mHMG-17), chicken HMG-17 (cHMG-17), human HMG-14 (hHMG-14), mouse HMG-14 (mHMG-14), and chicken HMG-14b (cHMG-14b). This figure is copied from reference (12) .

comprise most (approximately 75%) of the transcripts, is enriched in AT nucleotides. The nucleotide sequences between the HMG-14 and HMG-17 sub-families are only distantly related. Within each subfamily, the sequence in the open-reading-frame is evolutionarily conserved. But what is interesting is the remarkable sequence conservation in the 3' UTR (especially true in HMG-17) (Figure 2). Whether this non-coding region plays a role in mRNA processing and/or stability remains to be identified, but a recent report (9) suggests that it may enhance transcript degradation.

The peptides

The peptide sequences of HMG-17 and HMG-14 from several species including human (47, 49), mice (45, 50), and chickens (26, 88, 103) have been determined. In addition, HMG-17 sequences from swine (8), bovine (102), and rats (67) and HMG-14 from bovine (102a) have been determined. The alignment of HMG-14 and HMG-17 sequences identified several interesting features of this family of proteins (Figure 3). They all have a mildly basic N-terminus, a very basic central domain enriched in proline and lysine, and an acidic tail. The central domain is highly conserved in all members of this family. This extremely high conservation suggests that this region is important either functionally or structurally. In vitro mutagenesis (22, 72a) studies

Figure 3. Alignment of various HMG-14 and HMG-17 molecules. Note: part of this figure is modified from reference (12). Beside porcine HMG-17 sequence (90), all other sequences are from reference (12).

demonstrated that it mediates nucleosome binding. Neither the N-terminal nor the C-terminal region is well conserved between HMG-14 groups and HMG-17 groups except for the first four amino acids at the N-terminus and a stretch of 8 amino acids in the C-terminus. When any member of the HMG-17 group is compared to that of HMG-14, the sequence identity is only 35-55% identical (88). However, when amino acid sequences among HMG-17s from various species are compared, the similarity is high (>90% identical). The human and chicken HMG-17 genes diverged approximately 300 million years ago, yet the HMG-17 proteins from these two species are 94% identical (26). And when porcine HMG-17 is compared to the sequence of HMG-17 in the human (47), mouse(50), rat (67), and chicken (26, 48), there are only one, two, three, and seven substitutions, respectively. The mammalian HMG-14 peptide sequences are very similar along the whole sequence. The human HMG-14 sequence is 94% identical to bovine HMG-14. However, the chicken HMG-14b (mammalian HMG-14 homologue) is quite dissimilar to mammalian HMG-14 sequences. Between chicken HMG-14b and the human homologue the identity is only 69%. In chickens, HMG-14a is nearly as similar to HMG-17 (44% identical) as it is to HMG-14b (55% identical); therefore, the avian HMG-14 and HMG-17 family should probably be considered to contain 3 relatively independent members rather than two sub-families, one of which (HMG-14) contains two genes. The differences in sequence conservation between the HMG-17 family and the HMG-14 family

indicates that the HMG-17 family has evolved more slowly than that of HMG-14.

The C-terminal region of HMG-14 and HMG-17 has been reported to activate transcription on chromatin templates in vitro (98). As discussed above, this C-terminal region is not well conserved between HMG-14 and HMG-17; therefore, perhaps, the acidic feature in this region rather than the sequence itself is important for the in vitro activation observed. A stretch of 8 amino acid residues are almost invariably conserved in all HMG-14 and HMG-17 peptides characterized to date, and the replacement of a glutamic acid at residue 76 in that conserved region in HMG-14 with glutamine resulted in an almost complete loss of the transcriptional activation capability of this domain in vitro (97). These 8 residues may form the core of the activation domain.

HMG-17 and HMG-14 proteins are post-translationally modified by acetylation, methylation, glycosylation, ADP-ribosylation, and phosphorylation (5, 6, 73, 100). The functional roles of these modifications are unknown, but they potentially increase the structural flexibility and variability and binding specificity. Recent data suggest that phosphorylation of HMG-14 at the N-terminus by mitogen- or anisomycin-stimulated kinases may induce immediate-early gene expression through modulating the high-order chromatin stucture (6). The phosphorylation of HMG-14 and/or HMG-17 may also be involved in nucleosome spacing (96).

When not associated with nucleosomes, HMG-14 and HMG-17 do not seem to possess any stable secondary or tertiary structure as shown by circular dichroism (16) and nuclear magnetic renaissance (NMR) analysis (16). This is not surprising since apolar residues are almost completely absent in their primary structure. It is believed that a more rigid structure is gained upon binding to nucleosomes.

Functions

HMG-14 and HMG-17 bind to core nucleosomes near the dyad axis and interact with the DNA on the inside of the superhelix near the entry/exit point (Figure 4) (4, 20, 79, 84). H2a is the primary histone with which the HMGs interact; other histones are contacted as well but weakly (21). Since the HMG protein is inserted between the histone core and the DNA, the binding to nucleosomes may alter the interaction between the octamer and the nucleosomal DNA.

Since identical footprints were observed for both HMG-17 and HMG-14 in reconstituted nucleosomes, these proteins have been presumed to bind nucleosomes interchangeably (4). Each nucleosome has two potential binding sites for either HMG-17 or HMG-14 (3, 55, 79), and nucleosomes in association with 2, 1 or 0 molecules of HMG-14 and/or HMG-17 have been shown to exist in chromatin as assayed in vitro (3). The two bound HMG molecules do not seem to interact with each

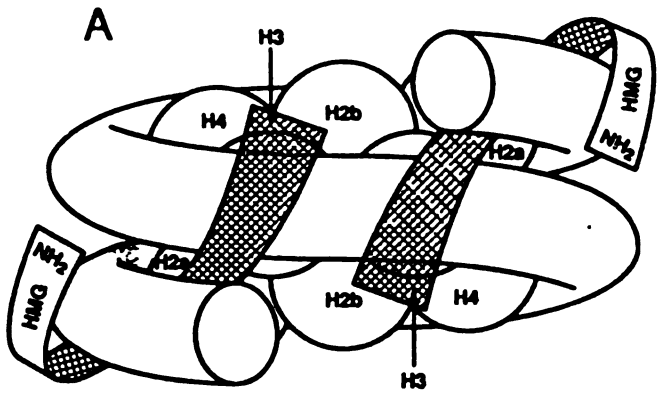


Figure 4. Model of the location of HMG-14/-17 on a nucleosome core. The nucleosome core particle binds up to two molecules of HMG-14/-17, which contact DNA near the entry and exit point. This figure is copied from reference (4).

other (21). The binding of the HMGs to nucleosomes stabilizes nucleosomes and protects the ends of the core particle DNA from DNase I digestion (55, 79). The HMG proteins bind specifically to core particles, but the binding does not seem to impede the binding of linker histones H1 or H5 (3, 4) and the formation of higher order chromatin structure (58). However, it must be noted that the linker DNAs may promote the binding of HMGs to chromatin since HMG-14/-17 bind in vitro with higher affinity to (92) and are enriched in (34, 91) nucleosomes with DNA fragments longer than the core DNA length (~146bp).

Although this family of proteins is ubiquitously present in all higher eukaryotic cells examined to date, the level of these proteins in nuclei is generally estimated to suffice for the binding of only about 10-20% of the total number of nucleosomes in the cell nucleus (assuming one molecule of HMG-14 or -17 per nucleosome) (80). It has been suggested that the subset of nucleosomes bound by these proteins is preferentially located in the active chromatin (29, 63,72, 79, 105-108). Active chromatin is generally more sensitive to DNase I than bulk chromatin (104). Reconstitution (105-107) experiments found that the increased DNase I sensitivity of some active genes (beta globin and nuclear RNA genes) is lost and regained upon elution and restoration, respectively, of HMG-14/-17 to mononucleosomes prepared from chick erythrocytes (105-107). When mononucleosome preparations from chicken erythrocyte

nuclei stripped of HMG proteins are partially titrated with HMG-14/-17, the nucleosome-HMG complex fraction is enriched in beta-globin gene sequences (79). Certain transcriptionally active gene sequences such as alpha-globin (erythrocytes), vitellogenin II gene (liver), ovalbumin, and lysozyme genes (oviduct) and acetylated histones (which preferentially associate with active DNA sequence) are preferentially retained when oligonucleosomes are fractionated on HMG-14/-17 affinity columns (29, 108, 109). In addition, transcription (as determined by uridine incorporation) is inhibited when antibodies against HMG-17 are microinjected into human fibroblasts (31), immunofluorescent-labeled anti-HMG-14 antibodies bind preferentially to transcriptionally active regions of polytene chromosomes *in Chironomus pallidivittatus* (110), and HMG-14 and HMG-17 are preferentially linked to the chick erythrocyte beta-globin gene in protein-DNA cross-linking experiments (72).

Despite these repeated observations of the association of HMG-14 and HMG-17 proteins with active chromatin, the structural basis for this association is unknown and, furthermore, a number of reports (7, 32, 52, 56, 74, 80) suggest HMG-14/-17 may not be solely associated with active chromatin domains. Seale et al. (80) reported that the enrichment of HMG-14/-17, relative to bulk chromatin, is less than 2-fold in the chromatin fraction enriched 6-fold in active sequences in HeLa cells and when the kinetics of

reconstitution of bulk versus coding sequences were measured with cDNA, there was no significant enrichment of active sequences in the HMG-containing mononucleosomes of HeLa. Similarly, Levinger et al. (7, 52) reported that the abundance of mononucleosomes containing HMG-14/-17 is not much different between the amplified, transcriptionally-activated dihydrofolate reductase gene and satellite chromatin regions. Moreover, some reports (33, 35, 66, 82) suggest that HMG-14/-17 may not be responsible for DNase I sensitivity or hypersensitivity in active chromatin. It has been observed that mononucleosomes from chick oviduct exhibit little difference between nuclease susceptibility of the ovalbumin gene and that of the globin gene (33, 82). Goodwin et al. (35) reported that when HMG-14/-17 were extracted from chromatin by low pH, a developmentally regulated DNase I hypersensitive site in the beta globin domain was not perturbed and the overall DNase I sensitive conformation of the beta globin gene (relative to the ovalbumin gene) was only minimally affected. Additionally, Nicolas et al. (66) observed that HMG-14/-17 containing chick erythrocyte mononucleosomes separated by electrophoresis are not sensitive to DNase I cleavage and contain both transcriptionally active and inactive sequences.

These discrepancies in the literature can partly be explained by the common extrapolation of results obtained from erythrocyte mononucleosomes and in vitro systems to

more complex systems and by the fact that different nucleosome and chromatin preparations have been employed. For example, the apparent affinity of HMG-17 to mononucleosomes increases more than 100-fold when the length of the nucleosomal DNA is increased by 3-5 bp over the core DNA length (~146 bp) (92); therefore, the in vitro preferential binding of HMG-14/-17 to active chromatin may be due to the greater affinity of these proteins for nucleosomes with longer nucleosomal DNAs released from active chromatin rather than indicating an in vivo preferential association.

The cellular levels of HMG-14 and HMG-17 mRNAs are higher in dividing cells than in non-dividing cells and their transcripts are made throughout the cell cycle with an increase in late S-phase (13). Rapidly growing cells and tissues also contain more HMG-14 and HMG-17 proteins than non-proliferating cells and tissues (71). The expression of this gene family is down-regulated during cellular differentiation (83) and, recently, Pash et al. (70) reported that aberrant expression of human HMG-14 inhibits the differentiation of mouse myoblast cells, suggesting that these proteins may modulate cell differentiation.

HMG-14 and HMG-17 have recently been reported to directly affect gene transcription on chromatin templates as assayed in vitro (23, 25, 69, 97). HMG-14 enhances transcription of genes by RNA polymerase II from in vivo assembled Simian Virus 40 minichromosomes (25), HMG-17

enhances activated but not basal level transcription of in vitro assembled chromatin templates (69), and both proteins enhance transcription of 5S rRNA by RNA polymerase III in a *Xenopus* egg extract assembly system (23, 97). The literature is conflicting regarding whether these transcriptional effects in vitro require the deposition of the HMGs during chromatin assembly (25, 97).

As discussed above, HMG-14 and HMG-17 are similar in molecular size, amino acid sequence, charge distribution along the sequence, functional domain structure, and interaction with nucleosomes. For this reason, these two proteins are generally assumed to play similar, if not identical, roles in cells. However, the facts that both proteins are present in all cell types examined to date and that the two proteins have evolved at different rates suggest that a unique function(s) may be possessed by one member but not the other. It has recently been reported that phosphorylation of HMG-14, but not HMG-17, was detected upon mitogenic induction of immediate-early genes in a mouse fibroblast cell line (6).

Targeted disruption (knockout)

Although a great amount of information has been generated on the biochemistry of HMG-14 and HMG-17 proteins, the functions of these proteins in vivo remain obscure. This is primarily due to the inherent limitation of the in

vitro assays used in most of these reports and the fact that, as discussed above, many of the reports are either not conclusive or are conflicting. To address the question of in vivo function more thoroughly we have taken a genetic approach as have a few other laboratories.

One genetic approach as reported by Pash et al. (70) is to over-express HMG-14 in cultured cells and study the biological consequences. Their results demonstrated that overexpression inhibits cellular differentiation in mouse myoblasts. Natalie Brown in our laboratory (9) performed similar studies in an avian fibroblast system without obvious phenotypic effects. However, these studies have been hampered by regulatory mechanisms, which seems to limit the level of overexpression, and the presence of the endogenous HMG genes in the transfected cells . Therefore, we became interested in generating null mutant cell lines, if possible, which were unable to express one or more HMG genes.

Most gene disruption experiments in vertebrates have been accomplished by using mouse embryonic stem (ES) cells as a vector to generate null mutant transgenic animals (15, 95). By transfecting a replacement vector that contains homologous sequences to the target gene sequence but with a drug-selectable marker inserted in the middle of the homologous sequence (or more frequently with the marker replacing a portion of the homologous sequences) (Figure 5), a mutant cell line can be selected in which the functional

gene sequence has been replaced by the vector sequence through homologous recombination. This results in the physical disruption of one copy of the target gene and, presumably, the loss of its function. Since ES cells are pluripotent, when reintroduced into mouse blastocysts, they can contribute efficiently to the formation of chimeric transgenic mice, including germ line chimeras. Therefore, using ES cells containing a singly disrupted target gene, mice heterozygous for the null mutant allele can be generated, and their offspring can be bred to provide mice homozygous for the null mutant allele (assuming it is not a recessive lethal).

Because of the precise replacement due to homologous recombination, gene targeting (knockout), as described above, allows for the introduction of defined mutations into a mouse genome and, in addition, any confounding effect of the endogenous gene is eliminated. Knockout mice have been generated for a number of important genes including the cystic fibrosis (*cftr*) gene (19), muscle development genes (*Myo*-D and *Myo*-5) (76,77), proto-oncogenes *N-myc* (17), *c-fos* (42), *c-src* (86), *c-abl* (99), *p53* (28) and *rb-1* (51), and genes that encode transforming growth factor (TGF) type ß1 (85), tenascin (78), and retinoic acid receptor alpha (53) and beta (59). Developmental, physiological, and immunological analyses of these mice have provided increased understanding of the in vivo function and regulation of these genes.

It is currently difficult to produce knockout mice for the *HMG-14* and *HMG-17* gene family for the following reasons. Mouse genomic sequences for neither *HMG-14* nor *HMG-17* have been characterized. We may assume that their general features are similar to those of their human homologues. As discussed above, more than one functional copy may exist per haploid mammalian genome at different chromosomal locations (44, 87). With the current technology, it is very difficult to disrupt multiple genes not located in close proximity. In addition, the existence of a large number of processed retropseudogenes complicates the difficulties in designing PCR screening primers and in finding diagnostic Southern blotting probes.

In chicken cells, both *HMG-17* and *HMG-14* are present as a single copy gene per haploid genome (26, 48, 88, 89); therefore, chickens and chicken cells are ideal for gene targeting studies of this family of proteins. Furthermore, chicken chromatin (especially chick erythrocyte chromatin) has been one of the major systems of choice for the biochemical analyses described above. However, an ES cell line has not yet been established from chickens. Since the HMG-14 and HMG-17 family is ubiquitously expressed, suggesting that its members may function in any and all cell types, and since a chicken lymphoid cell line, DT40, was shown to exhibit high levels of homologous recombination, we therefore chose to disrupt HMG-17 and HMG-14a genes in DT40 cells.

Currently, there are two methods to generate a doubly disrupted null mutant cell line. The first approach (60, 62, 75, 94) involves consecutive disruptions of two copies of the target gene by homologous recombination. The method for disruption of one copy of the target gene has been described above. To inactivate the second copy, a second drug-selectable marker must be cloned into the homologous sequences in the vector since the heterozygous null mutant cells are already resistant to the drug used in the first round of transfection and selection. Other than this difference in vector construction, the second round of disruption is identical (Figure 5).

The other approach to generate a doubly disrupted mutant cell line is based on the observation that several heterozygous mutant cell lines produced homozygous sublines at low frequency (14, 36, 64). (The mechanism for this has not been well studied, but a recent report (37) suggests that homogenization is probably due to duplication of the targeted chromosome in combination with loss of the untargeted chromosome via mitotic nondisjunction.) Therefore, starting with a heterozygous targeted mutant one can increase the selection intensity in hope of enriching for those cells whose gene of interest has become homozygous for the disrupted copy (homogenization of the disrupted copy would result in the duplication of the marker genes; therefore, it would be more resistant to the elevated concentrations of the antibiotic) (38, 39, 61). To date, a

Fi
H i
th
si
su
re

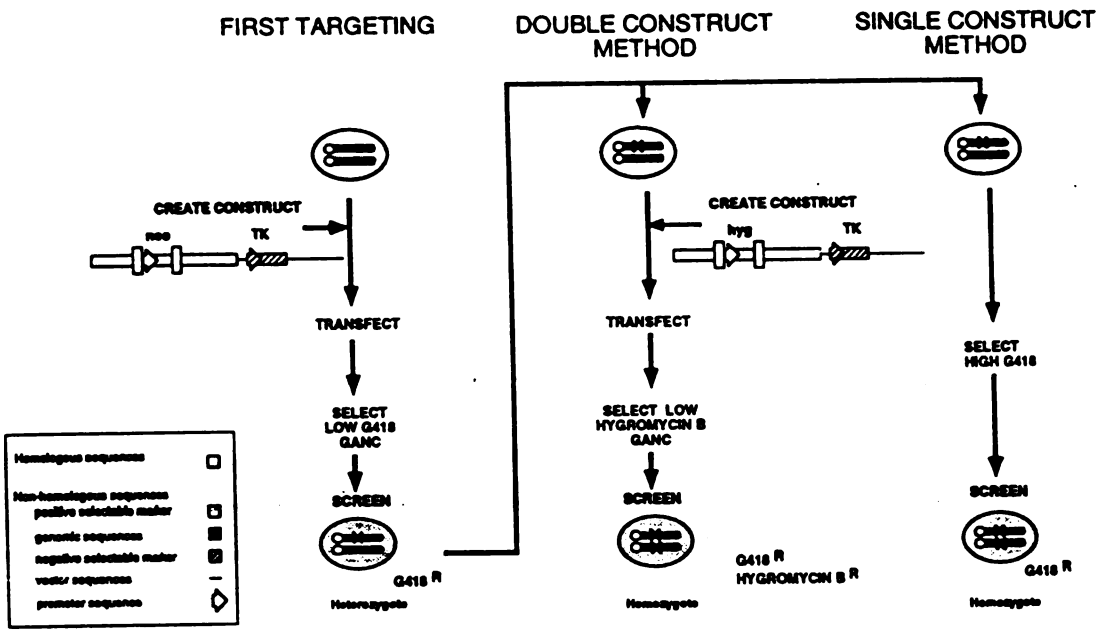


Figure 5. Diagram illustrating the generation of homozygous null mutants by two different approaches. The double construct method involves consecutive inactivation of two alleles. The single construct method depends upon the use of high concentration of G418 to enrich for the mutant that become homozygous at the targeted allele. TK, herpes simplex virus thymidine kinase gene (HSV-TK); GANC, gancyclovir; superscript R, resistance to the antibiotic. This figure is copied from reference (60).

mutated *neo* encoding a less active phosphotransferase that confers resistance to only low concentrations of G418 is the only marker that has been successful for this purpose (38, 39, 61).

The principal impediment in the gene disruption technique is that homologous recombination often occurs at a frequency of 3 to 5 orders of magnitude lower than that of nonhomologous recombination (15). Experiments in the last few years have shown that the frequency of homologous recombination is affected by cell line, isogenicity (24, 101) and length of flanking homologous sequences (24, 95), terminal nonhomologies of the transforming DNA (40), and the transcription state of the target locus (65, 66b). Various methods have been developed to increase the ratio of homologous vs. random recombination and/or to improve the screening efficiency for homologous recombinants; these include positive-negative selection (15, 54, 111), promoter trap (a promoterless selectable marker) (18, 27, 30), and poly(A) trap (no poly(A) in the selectable marker) (68). In chicken cells, some B-lymphoid cell lines (DT40, RP9, and 27L2) have been found to exhibit a remarkably high frequency of integration of transfected DNA via homologous recombination (up to 50%)(11). This high frequency has been hypothesized to be due to the ongoing active gene conversion in the immunoglobulin locus of these cell lines (11), although the apparent mechanism of immunoglobulin gene conversion differs substantially from simple homologous

recombination. We therefore chose DT40 for our attempts to disrupt *HMG-17* and *HMG-14* genes.

As discussed above, although HMG-17 and HMG-14 share great similarities in gene structure and in predicted protein structure, especially in nucleosome-binding domain, HMG-17 has been much more highly conserved during evolution than HMG-14. For this reason, we chose first to disrupt *HMG-17* gene in DT40 cells, and then the major *HMG-14* gene (*HMG-14a*) in parallel with an attempt to disrupt the *HMG-14a* gene in a doubly disrupted HMG-17 cell line. A potential problem for this line of research was that lack of *HMG-17*, *HMG-14a*, or both could have been lethal, or, at least, very deleterious to cell growth. More complex experimental designs were prepared, which involved covering the lethal phenotype by an additional regulatable HMG transgene. As will be demonstrated in subsequent chapters, it became clear that this was not something about which we had to be concerned. Indeed, the experience of other laboratories (28, 59, 76, 78, 81, 85, 86, 93) has been that homozygous null mutants in a surprisingly wide array of presumably critical genes are viable, even in the whole animal, let alone in a cell line grown in vitro.

Chapter I

THE CHICKEN *HMG-17* GENE IS DISPENSABLE FOR CELL GROWTH IN VITRO*

Yi Li and Jerry B. Dodgson

ABSTRACT

HMG-17 is a highly conserved and ubiquitous non-histone 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

* This chapter has been published in *Molecular and Cellular Biology*, 15: 5516-5523, 1995.

absence of HMG-17 protein lead to any major changes in cellular phenotype, at least in lymphoid cells.

INTRODUCTION

The high mobility group 17 (HMG) 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% CO_2 in DMEM supplemented with 10% fetal calf serum, 5% chicken serum, 0.01mM 2-mercaptoethanol, 10 µg gentamicin per ml, and 2.5 µg amphotericin B (fungizone) per ml.

Plasmid constructs. TFANEO (16), which contains the neomycin phosphotransferase gene under the control of the chicken ß-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 ß-actin promoter was provided by Jean-Marie Buerstedde, Basel Institute of Immunology, Basel, Switzerland. An *Eco*RI fragment (9.7 kb) containing the chicken *HMG-17* genomic sequence was excised from the lambda phage clone H7-1 (D. Browne and JBD, unpublished) and cloned into a pBSIIKSvSX vector, which was made from pBluescriptIIKS(+) (Stratagene) by removing part of the multiple cloning site (from *Xba*I to *Sma*I). The resulting construct was named pBSH17 (Figure 1). Gene cassettes for *neo* and *hisD* were used to replace a 755 bp *Bam*HI-*Sma*I 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 homologous sequence on

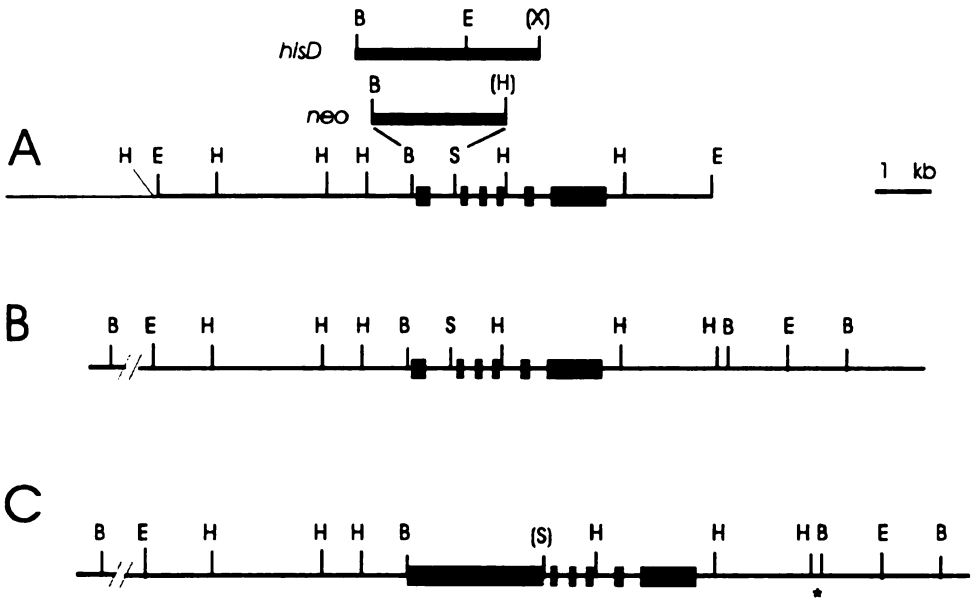


Figure 1. Schematic diagram of the targeting vectors and wild-type and disrupted HMG-17 alleles. Solid boxes represent the exons of the HMG-17 gene. Hatched boxes represent cassettes for drug selectable markers. E, B, H, and X represent the cleavage sites for EcoRI, BamHI, HindIII, and XhoI, respectively. Parenthesis represents sites blunt-ended by Klenow fill-in. The EcoRI 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 (HindIII-BamHI fragment, 0.2 kb) is shown by an asterisk. (A) Diagram of pBSH17 linearized at an EcoRI site. Above the line are shown the selectable cassettes inserted in the BamHI and SmaI sites of pBSH17 to generate pBSH17his (hisD) and pBSH17neo (neo), respectively. The thin line represents pBS vector sequences. (B) Map of the wild type HMG-17 gene as found in DT40. (C) Map of a HMG-17 disrupted mutant gene generated by homologous recombination of an insert from pBSH17neo.

either side of the selection marker gene cassettes, were designated pBSH17neo and pBSH17his, respectively (Figure 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-μg 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 250V and 960 μf 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 3 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 complete digestion with *Eco*RI or *Bam*HI, 15 μg 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-*Hin*dIII template (3' to the *HMG-17* gene [Figure 1]) by random primer extension (34). Blots were washed with 0.2x SSC (1x 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 on a 15% polyacrylamide gel (PAGE) 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 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 Institute 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 three times, 10 min each, 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 that 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 μg sample of RNA was fractionated on a 1.2% agarose gel containing 1x MOPS (morpholinepropanesulfonic acid), 0.66 M formaldehyde, and 1 μg of ethidium bromide per ml using 1x MOPS buffer, and blotted to a Magna charge membrane by the downward transfer method (10) in 10x 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 (JBD, unpublished). 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, CTCTCCCTCCTCGCACAACA) and exon 4 (primer E4, TCCGAAGCCAGAGCCTAAAC); one antisense primer (primer E6, ACTTGGCATCACCAGCACCT) was made to a region in exon 6. First-strand cDNA was synthesized from 5 μg of total RNA by reverse transcription in a 40 μl reaction mixture containing 20 μg of oligo(dT) (Pharmacia), 800 U of Moloney murine leukemia virus reverse transcriptase (Promega), 40 U of RNAguard (Pharmacia), and 20 pmol of each deoxynucleoside triphosphate (dNTP). After 1.5 h of incubation, 1 μl of the reaction mixture was transferred to 99 μl of PCR mixture containing 8 μl of dNTPs (10 mM each), 10 μl of 10X 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 μl 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-μg/ml propidium iodide in PBS that contained 5 U 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 CCCCACCATCACCCTCTT and GCACTCGGACCTCTTCAG 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-*Eco*RI genomic fragment containing the cloned *HMG-17* gene (D. Browne and JBD, unpublished) 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 Figure 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 histidinol-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 (Figure 2).

Disruption of one copy of the *HMG-17* gene. The *neo* targeting vector pBSH17neo (Figure 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 (Figure 1) is expected to generate a 12.9-kb band, and, similarly, a new 7.2- 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

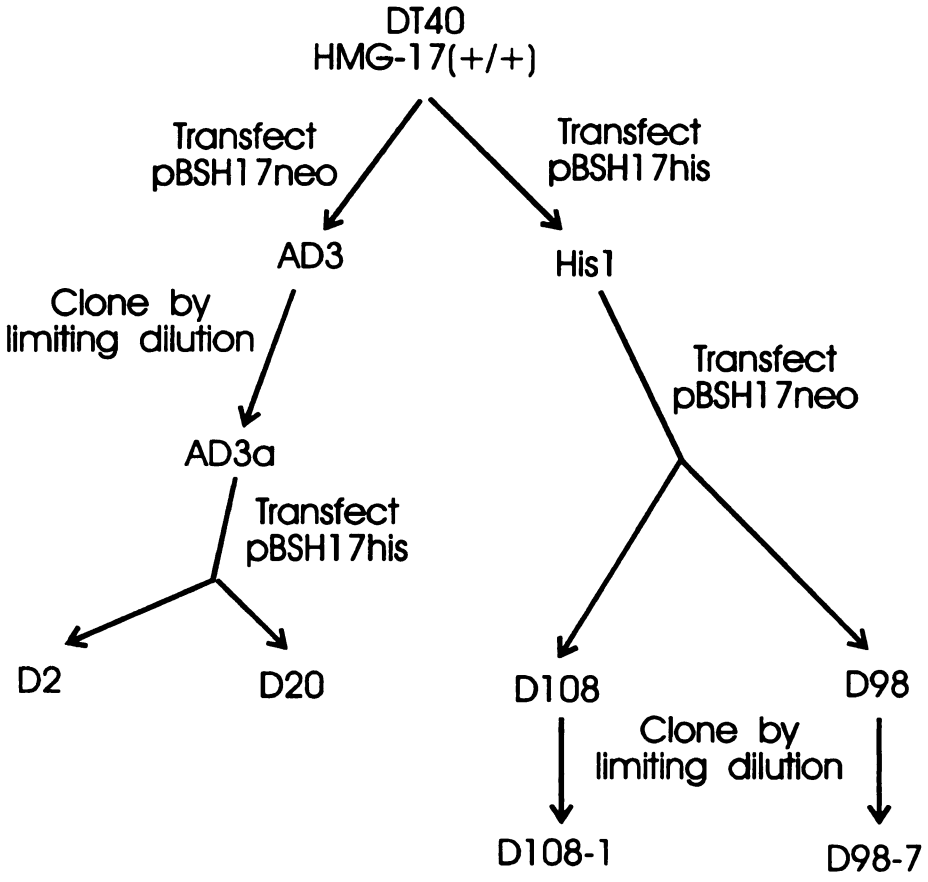


Figure 2. Outline of a two-step transfection procedure to generate homozygous HMG-17 null mutants. AD3, His1, and AD3a are derivatives of DT40 with single disruptions of the HMG-17 gene. D2, D20, D108, D108-1, and D98-7 are derivatives of AD3a and His1 with disruptions of both copies of the HMG-17 gene.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

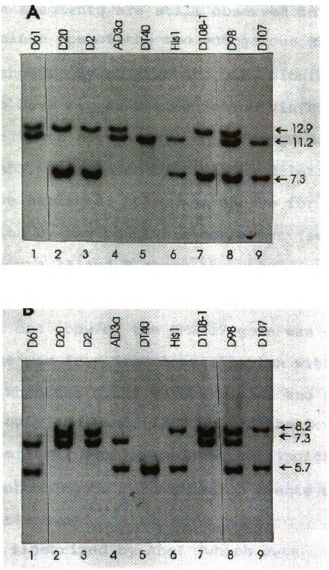


Figure 3. Southern blotting analysis of representative colonies. Genomic DNAs from DT40, AD3a and its derivatives (D2, D20, and D61), and His1 and its derivatives (D107, D108-1, and D98) were extracted, digested with EcoRI (A) and BamHI (B), fractionated on agarose gels, blotted, and hybridized to a 32P-labeled 0.2-kb diagnostic probe (Figure 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. 7.3 kb (A) and 8.2 kb (B) indicate those expected to be generated by homologous recombination of pBSH17his.

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 6 in Figure 3A and B shows 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 7.2 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, a 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 Figure 3, lanes 8. As withAD3a, 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 (Figure 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 (Figure 3B, lanes 2, 3, and 7). By this assay 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 produce 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 20mg/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 a *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

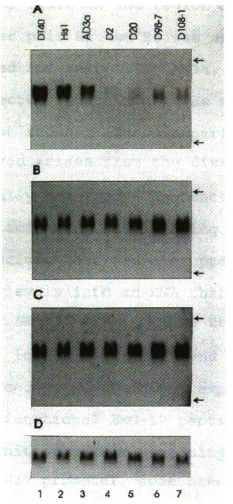


Figure 4. Northern blotting analysis of RNA from representative clones. (A) Total RNA (30 μg per lane) extracted from parental DT40 cells, singly mutant His1 and AD3a cells, and doubly mutant D2, D20, D98-7, and D108-1 cells was fractionated on a 1.2% agarose-formaldehyde gel, blotted to a membrane, and probed with 32P-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.

sho
cor
howe
313
indi
muta
that
and
geno
type
tran
exon
init
know
tran
tran
port
prom
was
for
and
ther
norm
reas
targe
Phosp

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 (Figure 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-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 (Figure 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 two-fold) 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

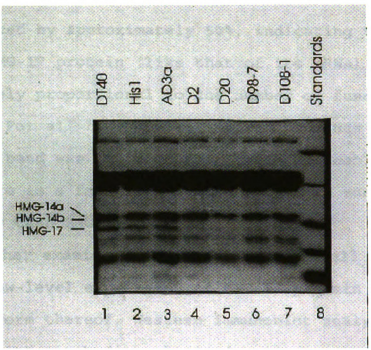


Figure 5. SDS-PAGE analysis of 5% PCA extracts from representative clones. The 5% PCA extracts from 107 cells were separated by SDS-PAGE (15% polyacrylamide) and stained with 0.1% Coomassie blue R250 as described in Materials and Methods. Cell lines tested include DT40 (lane 1), singly disrupted mutants His1 and AD3a (lanes 2 and 3, respectively), and doubly disrupted mutants D2, D20, D98-7 and D108-1 (lanes 4 to 7, respectatively). Electrophoretic standards included 43, 29, 18.4, 14.3, and 6.2 kDa.

mass of 18 kDa. As shown in Figure 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 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 Figure 6, lanes 11-14, HMG-17 protein was not detected even when the blot was heavily overexposed. A minimum of 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

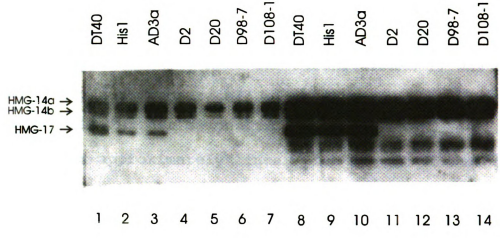


Figure 6. Western blot analysis of representative clones. The 5% PCA extracts from 106 cells (lanes 1 to 7) and 107 cells (lanes 8 to 14) were loaded onto SDS-PAGE gel (15% polyacrylamide). After electrophoresis, the gel was blotted to a polyvinylidene diflurodie membrane, which was probed with antibody 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 5 and 13), and D108-1 (lanes 7 and 14).

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 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 Figure 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-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 results of the same assays done before the extended culture (Figure 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 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 Figure 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 11.5 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

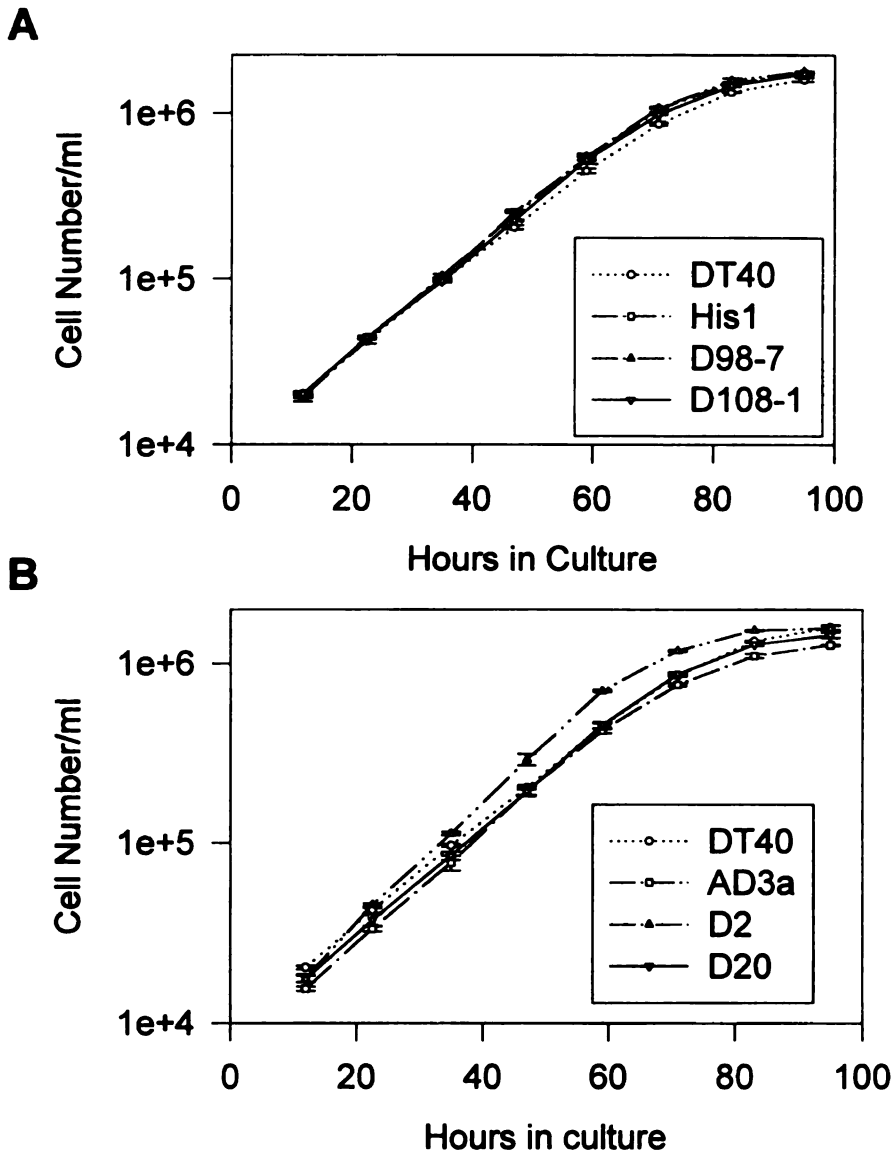


Figure 7. Growth curves of representative cell lines. (A) DT40, singly mutant His1, and its doubly mutant derivatives D98-7 and D108-1 were plated out at 10^4 per ml at -10 h and counted at 10- to 15-h intervals. Triplicate samples were counted at each time point, and the standard deviation values were used to plot the error bars (not visible for every time point). (B) Identical analysis as in Panel A. for DT40, singly disrupted AD3a, and its doubly mutant derivatives D2 and D20.

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 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).

Results discussed above (Figure 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 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%

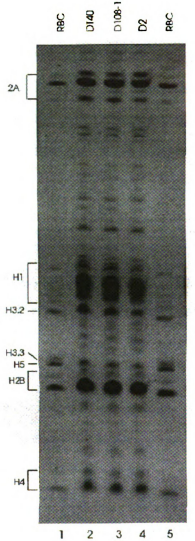


Figure 8. Histone profile of DT40 and null mutant cell lines. Total histones were extracted from chicken erythrocytes (RBC, lanes 1 and 5), DT40 (lane 2), D108-1 (lane 3), and D2 (lane 4) with 0.2 M sulfuric acid and separated on a Triton-acid-urea gel by the method of Lennox and Cohen (24). Lanes 1 and 5 contain 15 *g of protein, and lanes 2 to 4 contain 25 *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).

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 and 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 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 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 in the pattern observed between 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

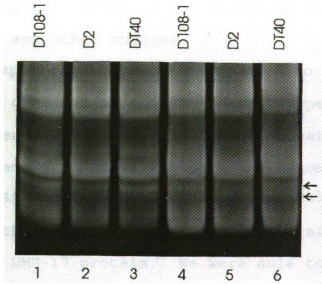


Figure 9. Electrophoretic analysis of nucleosomes from representative cell lines. Nuclear preparations from DT40 (lanes 3 and 6) and doubly mutant derivatives D2 (lanes 2 and 4) and D108-1 (lanes 1 and 4) were incubated with micrococcal nuclease at 20 U/mg of DNA equivalent (lanes 1 to 3) and 40 U/mg (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 1 volume of glycerol and loaded onto a 3.5% polyacrylamide-0.5% agarose gel containing 30% glycerol (21). The gel was run at 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).

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 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.

Chapter 2

NULL MUTANT CHICKEN CELL LINES UNABLE TO EXPRESS EITHER *HMG14A* AND *HMG-17* SHOW NO PHENOTYPIC DIFFERENCES AS A RESULT*

YI LI and JERRY B. DODGSON

ABSTRACT

HMG-14 and HMG-17 are ubiquitous non-histone chromosomal proteins that bind to nucleosome core particles. They form a family of chromosomal proteins that have been reported to bind preferentially to regions of active chromatin structure. Our previous studies demonstrated that the chicken HMG-17 gene was dispensable for normal cell growth of the DT40 chicken lymphoid cell line. Herein we report that the major chicken *HMG-14* gene, *HMG-14a*, is also dispensable and, moreover, that DT40-derived cells lacking both *HMG-17* and *HMG-14a* show no obvious change in phenotype with respect to the parental DT40 cells. Furthermore, no compensatory changes in HMG-14b or histone protein levels were observed in cells lacking both *HMG-14a* and *HMG-17*. It is concluded that HMG-14a and HMG-17 proteins are not

* This chapter is a manuscript to be submitted.

required for normal growth of avian cell lines in vitro, nor does the absence of HMG-17 and HMG-14a proteins lead to any major changes in cellular phenotype, at least in lymphoid cells.

INTRODUCTION

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 (1, 7, 21, 24), and that binding has been found to stabilize core particles (19). The limited amount of these proteins confines them to only a subset of nucleosomes (6), and a number of experiments including nuclease digestion (31), protein-DNA cross-linking (20), and immunofractionation (9) have suggested that HMG-14 and HMG-17 are preferentially associated with nucleosomes of regions of transcribed chromatin. 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. (18) found that aberrant overexpression 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 (6) with HMG-14a being

the dominant form in all tissues examined to date. 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% (6). HMG-17 is highly conserved among birds and mammals (human and chicken HMG-17 are 94% identical; [8, 13]), whereas HMG-14 proteins are less well conserved (approximately 50% identical, depending on the variant in question; [8, 25]).

The abundance, wide distribution, and evolutionary stability of HMG-14 and HMG-17 suggest that these proteins play an important role in chromatin, but their exact functions remain unknown. Previously, we reported that targeted disruption of HMG-17 in the chicken cell line DT40 does not affect cell growth and that no major changes in phenotype and chromatin structure occurred in such knockout cells (15). A possible explanation for this result could be that HMG-17 and HMG-14 are functionally redundant, although no increase in HMG-14 expression occurred in the cells lacking *HMG-17*. To test this possibility, we disrupted both copies of *HMG-14a* in both the DT40 cell line and in D108-1, which is a homozygous *HMG-17* null mutant. Herein we demonstrate that cells lacking *HMG-14a* and cells lacking both *HMG-14a* and *HMG-17* genes show no obvious phenotypic differences from the parental DT40 line.

MATERIALS AND METHODS

Cells and cell cycle analysis. The DT40 cell line (2) was obtained from Craig Thompson, University of Chicago, Chicago, IL. This cell line and all its derivatives were cultured at 40 °C under 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% chicken serum, 0.01 mM 2-mercaptoethanol, 10 µg of gentamicin per ml, and 2.5 µg of amphotericin B (fungizone) per ml. Cell cycle distribution analysis was performed as described (15).

Plasmid constructs. Gene cassettes containing either puromycin acetyltransferase (puro) and blasticidin S acetyltransferase (bsr) genes were kindly provided by Jean-Marie Buerstedde, Basel Institute of Immunology, Basel, Switzerland. Both genes are under the control of the chicken ß-actin promoter and end in a SV40 poly(A) signal. pHM1.9HB and pHM1.5HP are genomic clones from the *HMG-14a* locus (4). The 1.9-kb *Hin*dIII-*Bam*HI insert from pHM1.9HB was subcloned into the *Hin*dIII and *Bam*HI sites of the 3.9-kb pCRII vector (Invitrogen). The resulting construct, designated pCRHM1.9, was digested with *Xho*I and *Xba*I and ligated to the 1.3kb *Xho*I-*Xba*I insert from pHM1.5HP. This new construct was named pT14a and used as the recipient for insertion of the drug selectable cassettes. The *puro* gene cassette was cloned into pT14a using the *Bam*HI and *Xho*I sites; the *bsr* cassette was cloned into pT14a using the

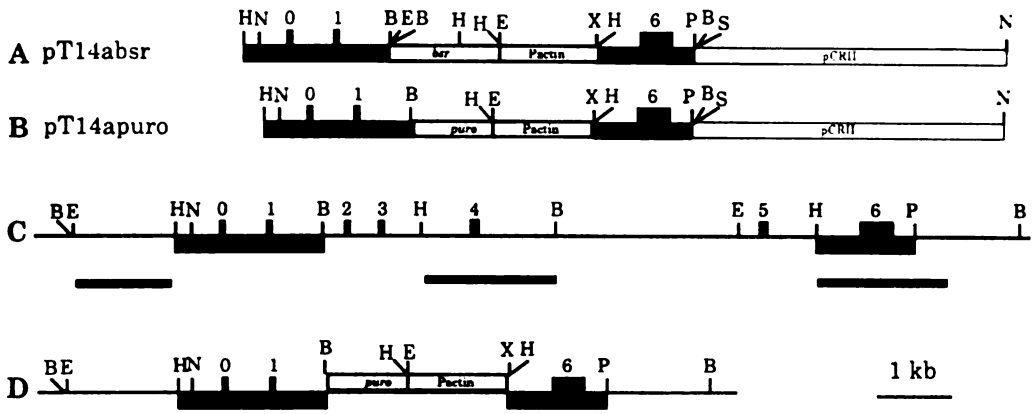


Figure 1. Schematic diagram of the targeting vectors and wild-type and disrupted HMG-14a alleles. Solid boxes represent the exons of the HMG-14 gene. Hatched boxes represent the homologous sequences present in the targeting vectors. Gray boxes represent the cassettes for drug-selectable markers. E, B, H, X, P, N, and S represent the cleavage sites for EcoRI, BamHI, HindIII, XhoI, PstI, NsiI, and SpeI, respectively. (A) Diagram of pT14absr. (B) Diagram of pT14apuro. (C) Map of the wild-type HMG-14a gene as found in DT40. Below the map are shown the three hybridization probes (black bars). (D) Map of a HMG-14a disrupted mutant gene generated by homologous recombination of an insert form pT14apuro.

*Eco*RI and *Xho*I sites. The resulting replacement constructs, each containing 1.9 kb of the *HMG-14a* gene on the left and 1.4 kb on the right, were designated pT14apuro and pT14absr, respectively. The *HMG-17* gene constructs pBSH17neo and pBSH17his have been described (15).

Electroporation and Screening. The targeting vector pT14absr was linearized by *Nsi*I digestion before use in electroporation, and pT14apuro was digested by *Nsi*I or *Nsi*I and *Spe*I before electroporation of DT40 and Bsr18 (see below). Electroporation was carried out as described previously (15). Cells were allowed to recover overnight before being subjected to selection by one of the following drugs: blasticidin S (ICN, 30 μg per ml), puromycin (Sigma, 0.5 μg per ml), G418 (Gibco-BRL, 2 mg per ml), and/or L-histidinol (Sigma, 1.5 mg per ml). Resistant colonies were expanded in drug-free media. DNA extraction and Southern blotting analysis were described previously (15). The diagnostic probes used were a 1.4 -kb *Bam*HI-*Hin*dIII fragment (5′ to the 1.9-kb fragment in pHM1.9HB, Figure 1) and a 1.8-kb *Hin*dIII-*Acc*I fragment (3′ of the *HMG-14a* gene, Figure 1). Those colonies whose DNA yielded the expected bands were further screened by probing the stripped blots with selectable markers, and the 1.8-kb *Hin*dIII-*Bam*HI fragment deleted in making the *HMG-14a* targeting constructs. Probes used in screening HMG-17 targeted mutants have been described previously (15).

Blotting analysis. Cells were washed with PBS and

lysed in 1x sample buffer (50 mM Tris-Cl, pH6.8, 2% SDS, 5% ß-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) by boiling for 5 min. A total of 74 μg of protein per lane were fractionated on a 15% polyacrylamide gel containing sodium dodecyl sulfate (SDS) and then transferred to a Biotrace polyvinylidene difluoride (Gelman) membrane using 29 mM Tris-179 mM glycine-20% ethanol as blotting solution in a Bio-Rad Trans-blot apparatus according to the manufacturer's recommendation except that the membrane was placed on the cathode side of the gel. Northern blotting analysis was carried out as described previously (15). The template for making the *HMG-14a* probe was the cDNA fragment (900-bp) excised from pLM3B by *Eco*RI (8). The membrane was subsequently stripped and reprobed with chicken *HMG-14b*, HMG-17, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization probes. The *HMG-14b* template was a 1.7 kb *Eco*RI fragment excised from a cDNA clone designated E32 (JBD, unpublished). The HMG-17 cDNA template was a 1.2-kb *Bstu*UI-*Eco*RI fragment from pLG1A (8). The GAPDH template was a *Pst*I fragment excised from pGAD28 (a gift of R.J. Schwartz, Baylor College of Medicine, [10]).

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 (12). 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 (14, 29). The gel was stained with Coomassie blue R250 in 35% methanol-10% acetic acid. Nuclei were isolated and digested with DNase I by previously described methods (3). DNA was isolated from nuclei, digested with *Sal*I and *Xho*I, gel electrophoresed, blotted and hybridized to a 295-bp PCR fragment amplified from the constant region of the chicken lambda light-chain gene as described previously (15).

RESULTS AND DISCUSSION

Disruption of the *HMG-14a* gene. The general scheme used to prepare cell lines lacking functional *HMG-14a* genes is shown in Figure 2a. The *puro* targeting vector pT14apuro (Figure 1) was used in DT40 electroporation to generate 34 resistant colonies. For colonies with integration by homologous recombination at the *HMG-14a* gene, hybridization of genomic DNA cut by *Eco*RI with the 5' 1.4-kb probe (Figure 1) is expected to generate a 4.35-kb band (Figure 3a, lane 6) and, similarly, a new 5.15-kb band would be expected in a *Bam*HI blot probed by the 1.8-kb *Hin*dIII-*Acc*I fragment (Figure 1 and Fig 3b, lane 6). The endogenous *HMG-14a* gene will give rise to an *Eco*RI fragment of 8.8-kb (Figure 3a, lane 5) and a *Bam*HI fragment of 6.2-kb (Figure 3b, lane 5) when hybridized, respectively, to these probes. These fragments are still observed in the desired cell lines,

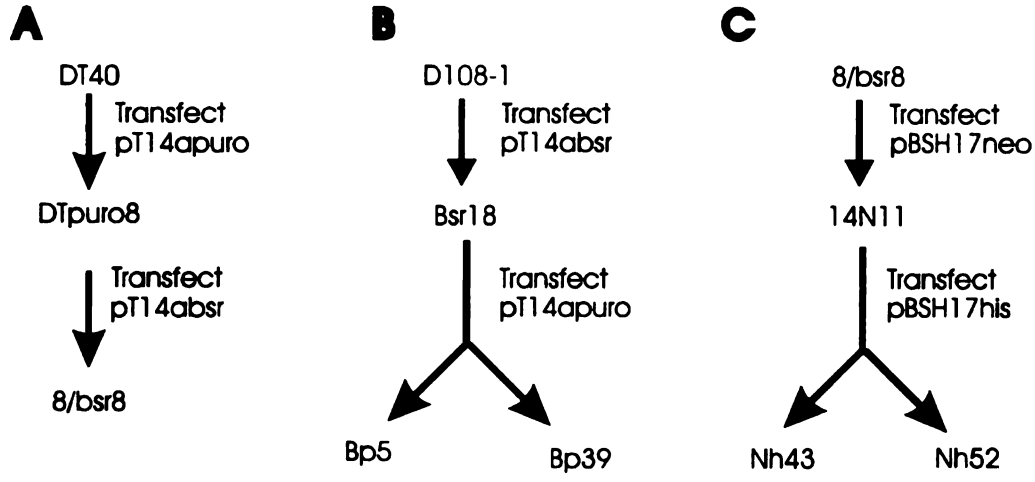


Figure 2. Outline of the transfection procedures to generate targeted disruptions. (A) Transfection scheme to obtain HMG-14a null mutant. DTpuro8 is a derivative of DT40 with a single disruption of the HMG-14a gene, and 8/bsr8 is a derivative of DTpuro8 with disruption of both copies of HMG-14a. (B) Transfection scheme to obtain the quadruple disruptions of both HMG-14a and HMG-17 genes. Bsr18 is a derivative of D108-1 (15) with one copy of the HMG-14a gene disrupted, and Bp5 and Bp39 are derivatives of Bsr18 with both copies of HMG-14a disrupted. D108-1 is a HMG-17 double knockout cell line (15). (C) Alternative transfection scheme to obtain the quadruple disruptions of both HMG-14a and HMG-17 genes. 14N11 is a derivative of 8/bsr8 with one copy of HMG-17 disrupted, and Nh43 and Nh52 are derivatives of 14N11 with both copies of HMG-17 disrupted.

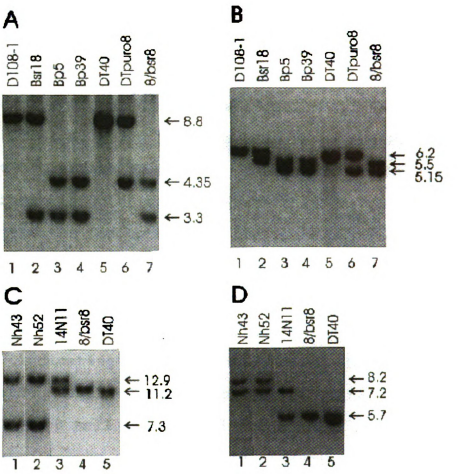


Figure 3. Southern blotting analysis of representative clones. Genomic DNAs from the indicated clones were extracted, digested with EcoRI (A and C) or BamHI (B and C), fractionated on agarose gels, blotted, and hybridized to 32P-labeled 1.4-kb (A), 1.8-kb (B), or 0.2-kb (C and D) diagnostic probes (see Figure 1 and (15) for the locations of the probes). The molecular sizes in kilobases are indicated. The notations 8.8 kb (A), 6.2 kb (B), 11.2 kb (C), and 5.7 kb (D) indicate the bands expected from the wild-type DT40 cell line, 4.35 kb (A) and 5.15 (B) indicate those expected after homologous recombination mediated by pT14apuro, 3.3 kb (A) and 5.5 kb (B) indicate those expected after homologous recombination mediated by pT14absr, 12.9 kb (C) and 7.2 kb (D) indicate those expected after homologous recombination mediated by PBSH17neo (15), and 7.3 kb (C) and 8.2 kb (D) indicate those expected after homologous recombination mediated by pBSH17his (15).

since one of the two endogenous *HMG-14a* genes remains unchanged. By this assay, 2 colonies were determined to have integration via homologous recombination (data not shown), for a frequency of 11%. One colony, DTpuro8, was chosen for a second round of transfection by the pT14absr construct (see below). Lane 6 in Figure 3a and Figure 3b shows the hybridization profile of DTpuro8. The two equally intense bands in both lanes indicate that one copy of the *HMG-14a* gene was disrupted, and the clone was free from contamination with parental DT40 cells. When the blots were stripped and reprobed with a *puro* probe, only one band (4.35 kb for the *Eco*RI blot and 5.15 kb for the *Bam*HI blot) was detected, indicating that no additional non-homologous recombination events occurred in DTpuro8 (data not shown).

The DTpuro8 cell line was electroporated with pT14absr and selected in the presence of blasticidin S. A total of 38 colonies survived the selection. When the *HMG-14a* gene is disrupted in both endogenous copies by pT14apuro and pT14absr, 4.35-kb and 3.3-kb bands are expected to completely replace the endogenous 8.8-kb band upon *Eco*RI cleavage and Southern hybridization with the 1.4-kb probe (Figure 3a, lane 7). Similarly, the 5.15-kb and 5.5-kb bands would be expected to completely replace the endogenous 6.2-kb band in a blot of *Bam*HI-cleaved DNA probed by the 1.8-kb probe (Figure 3b, lane 7). By this assay, one colony, designated 8/bsr8, was determined to have both copies disrupted by homologous recombination. The blot of

its DNA cleaved by *Bam*HI was additionally hybridized with the 1.8-kb *Hin*dIII-*Bam*HI genomic fragment deleted in making the targeting constructs and the bsr probe. As expected, no bands hybridized to the 1.8-kb probe; while the 5.5-kb band hybridized to the *bsr* probe (results not shown). When a blot of 8/bsr8 DNA cleaved by *Eco*RI was hybridized to the *puro* probe, one band of 4.35 kb hybridized to the probe, again as expected.

Isolation of cell lines lacking both *HMG-17* and *HMG-14a* genes. The general scheme for the isolation of cell lines lacking both *HMG-17* and *HMG-14a* was illustrated in Fig 2b. First, the D108-1 cell line was transfected with pT14absr and selected in the presence of blasticidin S. Resistant colonies were analyzed for homologous recombination by Southern hybridization as described. One of 33 resistant colonies was determined to have one copy of *HMG-14a* disrupted. This triply disrupted cell line was designated Bsr18 and used for another round of transfection by pT14apuro to inactivate the second *HMG-14a* gene. A total of 45 colonies survived puromycin selection. Among them, 2 (Bp5 and Bp39) were determined to have both copies of *HMG-14a* disrupted as shown by Southern blotting analysis using the 5 probes as stated above in the analysis of 8/bsr8 (Figure 3a and 3b).

An alternative strategy to obtain cells lacking both *HMG-14a* and *HMG-17*. As a control against fortuitous mutations that could compensate for the complete loss of

both HMG-14a and HMG-17, we also disrupted the *HMG-17* gene in the HMG-14a null mutant cell line, 8/bsr8 (see Figure 2c for the strategy).

The *neo* targeting vector pBSH17neo (15) was used to disrupt one copy of *HMG-17* in 8/bsr8. For colonies with targeted integration, hybridization of genomic DNA cut by *Eco*RI with the 3' 0.2 kb probe (15) is expected to generate a 12.9 kb band, and, similarly, a new 7.2 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, 23 of the 44 resistant colonies were determined to have targeted integration via homologous recombination (data not shown), for a frequency of 52%. One colony, 14N11, was chosen for a second round of transfection by the pBSH17his construct (15). Lanes 3 in Figures 3C and D show the hybridization profile of 14N11. The two equally intense bands in both lanes indicate that one copy of the HMG-17 gene was disrupted, and the clone was free from contamination with parental 8/bsr8 cells. When the blots were stripped and reprobed with a *neo* probe, only one band (7.2 kb for the *Bam*HI blot) was detected, indicating that no additional non-homologous recombination events occurred in 14N11 (data not shown).

14N11 was then electroporated by pBSH17his and selected

in the presence of L-histidinol. A total of 14 clones survived the selection. When the *HMG-17* gene is disrupted in both copies by pBSH17neo and pBSH17his, 12.9 kb 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 (Figure 3c, lanes 1-2). Similarly, the 7.2 kb and 8.2 kb bands should completely replace the endogenous 5.7 kb band in a blot of *Bam*HI-cleaved DNA (Figure 3d, lanes 1-2). By this assay, 11 colonies were determined to have both copies disrupted by homologous recombination, yielding a targeting efficiency of 79%. Two clones, Nh43 and Nh52 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 and the *his* probe. As expected, no bands hybridized to the 0.75 kb probe; while 8.2 kb-bands hybridized to the *his* probe (results not shown). The high frequencies of integration by homologous recombination obtained in generated Nh43 and Nh52 argue strongly against the possibility that cell lines were selected with mutations in other genes that compensated for the loss of both HMG-14a and HMG-17.

Null mutants do not express *HMG-14a* but do make low levels of an aberrant HMG-17 transcript. Both *HMG-14a* targeting vectors were designed to replace four of the six *HMG-14a* exons with drug-selectable markers; therefore, the *HMG-14a* doubly disrupted cell line 8/bsr8 and the quadruply

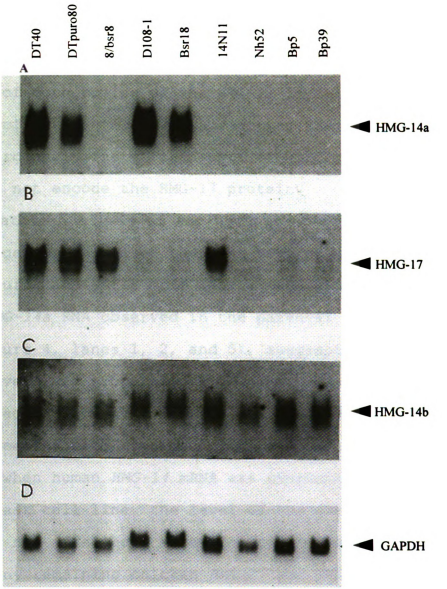


Figure 4. Northern blotting analysis of RNA from representative clones. (A) Total RNA (15 μg per lane) extracted from the indicated cells lines was fractionated on a 1.2% agarose-formaldehyde gel, blotted to a membrane, and probed with 32P-labelled HMG-14a cDNA. (B to D) The membrane was then stripped and reprobed sequentially with HMG-17 (B), HMG-14b (C) and GAPDH (D) hybridization probes. (See Materials and Methods for a description of the probes).

disrupted *HMG-14a/HMG-17* cell lines were not expected to make any *HMG-14a* message. Indeed, there was no transcript detected when total RNAs of these cells were analyzed by northern hybridization. As reported previously (15), the disruption of both copies of the HMG-17 in cells resulted in low levels of aberrant *HMG-17*-hybridizing RNAs with a mobility marginally faster than that of wild type, which, however, do not encode the HMG-17 protein.

Quantitative analysis of the northern blots by a PhosphorImager demonstrates that the heterozygous null mutants DTpuro8 and Bsr18 make approximately 50% of the level of *HMG-14a* RNA observed in the parental DT40 cells and D108-1 (Figure 4, lanes 1, 2, and 5), suggesting that *HMG-14a* mRNA levels are proportional to gene copy number as we have reported previously for the *HMG-17* gene (15). This is also in agreement with the results of Pash et al. (18), who found that when human *HMG-14* mRNA was overexpressed in a mouse myoblast cell line, the level of the endogenous *HMG-14* message was not affected. Furthermore, the expression levels of any remaining chicken *HMG-17* and *HMG-14b* genes in both the *HMG-14a* doubly disrupted 8/bsr8 and the quadruply disrupted Bp5, Bp39, Nh43, and Nh52 were not markedly altered (Figure 4b and 4c), indicating that the *HMG-14* and *HMG-17* genes are not coordinately regulated at the transcriptional level.

Quadruple disruptions do not make detectable HMG-14a or HMG-17 protein. HMG proteins extracted from cells by 5% PCA

were resolved by SDS-PAGE and transferred to a PVDF membrane. Western blot analysis was performed with antibodies elicited against a peptide that is common to both HMG-14 and HMG-17 (5, 13). As shown in Figure 5, HMG-14a protein was not detected in the *HMG-14a* doubly disrupted cell line 8/bsr8 (lane 7) or in the quadruply disrupted cells (lanes 1, 2, 9, 10). Because the epitope recognized by this antibody is located in the most conserved, nucleosome-binding domain, common to both HMG-17 and HMG-14, any truncated form of HMG-17 that might retain normal function should have been detected, if present.

Figure 5 shows that *HMG-14a* gene expression was approximately linear with the functional gene copy number (lanes 6 and 7) in accordance with our previous observations on the *HMG-17* gene (15). This gene copy number-protein level relationship is not affected by the presence or absence of a functional *HMG-17* gene (lanes 3-6). HMG-17 expression is also unaffected by the presence or absence of HMG-14a (lanes 5-8). And the total absence of both *HMG-14a* and *HMG-17* did not affect the cellular level of the HMG-14b protein (lanes 1-10). Thus, all three members of the HMG-14 and HMG-17 family are not coordinately regulated.

Null-mutation effects on phenotype and chromatin structure. All four quadruply disrupted cell lines divided normally in standard growth media, without any observable difference in phenotype from DT40. No change in morphology was found among any of these cell lines when they were

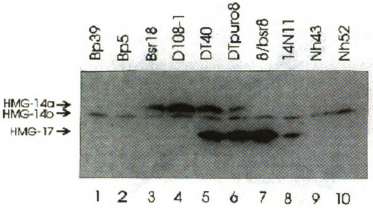


Figure 5. Western blotting analysis of representative clones. The total cell lysates (74 μg per lane) were separated by SDS-PAGE (15% polyacrylamide) and blotted to a polyvinylidene difluoride membrane. The membrane was probed with antibody against the DNA-binding domain of HMG-14/17 (5). ECL was used to located the bands after incubation in horseradish peroxidase conjugate. Cell lines tested are shown at the top, and the locations of the HMG proteins are indicated by arrows on the left.

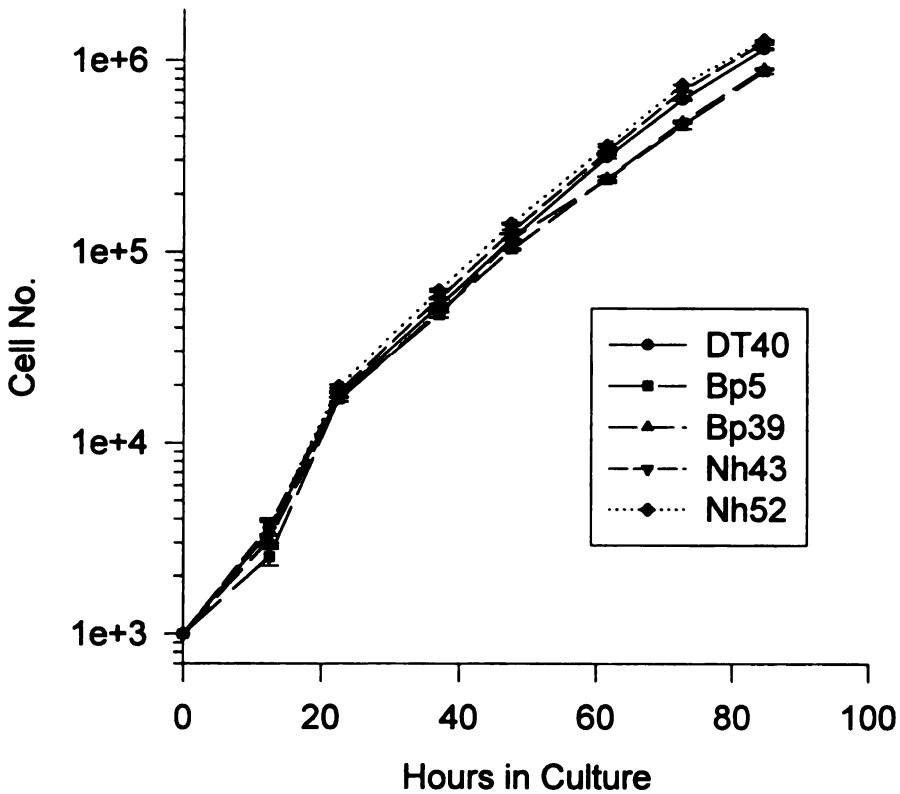


Figure 6. Growth curves of representative cell lines. Cell lines as indicated in the insert were plated out as 103 cells per ml and counted at 12-h intervals. Triplicate samples were counted at each time point, and the standard deviation values were used to plot the error bars.

examined by phase contrast microscopy. Growth rates of the mutant lines and DT40 were determined following plating each line at 10^4 cells/ml in 24-well plates. At about 12 hour intervals, three plates per cell line were counted. The results are presented in Figure 6. It is clear that the quadruply disrupted cells grow equally as well as the DT40 parental cell line. 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 the mutant cell lines in the percentage of cells in G1, S and G2 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 (23), and over-expression of HMG-14 inhibits differentiation of mouse myoblast cells in vitro (18). To assess whether the inactivation of the *HMG-17* and *HMG-14a* genes affected the differentiation of DT40 cells, we examined the targeted cell lines with Giemsa staining, light-scattering profile analysis, and IgM fluorescence staining. 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 later stages of

their normal differentiation, which would be to become mature B cells and then plasma cells. Both Giemsa and light-scattering analysis did not detect any significant difference between mutant cell lines and the DT40 parental cells. Surface IgM is a differentiation marker of B-cells. It is present on immature B-cells and mature B-cells but not on plasma cells. When DT40 cells and the quadruply disrupted cells (Bp5, Bp39, Nh43, and Nh52) were examined for surface IgM expression by flow cytometry, no difference was detected (results not shown).

The results discussed above (Figure 5, 6) indicated that no change in HMG-14b levels occurred in response to inactivation of the *HMG-17* and *HMG-14a* genes. We also were unable to discern any alteration in the pattern of total histone proteins present in bulk chromatin of the quadruply disrupted cells versus DT40, as detected by electrophoresis on a 15% Triton-acid-urea gel (Figure 7). This is not surprising, since only about 10% of the nucleosomes would be expected to bind HMG-14 and HMG-17 proteins in normal cells.

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, mono-nucleosomes of different sizes are released depending upon the number of HMG-17 and -14 and histone H1 molecules bound. These different forms of nucleoprotein complexes can be partially resolved in a polyacrylamide-agarose gel (12). We

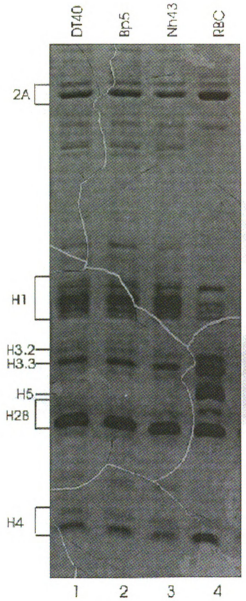


Figure 7. Histone profiles of DT40 and quadruply disrupted cell lines. Total histones were extracted from the indicated cell lines with 0.2 M sulfuric acid and separated on a Triton-acid-urea gel (30 μg per lane) by the method of Lennox and Cohen (14). The gel was stained by Coomassie blue R250. Various classes of chicken histones were identified as indicated by comparing the erythrocyte pattern with those previously described by Urban et al. (29).

previously reported that in the region of the mammalian MII band, two bands are discerned and the intensities of both bands (especially the faster-migrating one) are decreased in *HMG-17* null mutants (15). When the *HMG-14a* doubly disrupted 8/bsr8 and the quadruply disrupted Bp5 and Nh43 were examined by this gel, the lower MII band was absent in both Bp5 and Nh43. Since the MII particle consists of core nucleosomes bound to one molecule of HMG-17 or HMG-14, it appears that the low levels of the remaining HMG-14b are insufficient to form detectable MII-type nucleosomes in quadruply disrupted cells.

It has been repeatedly reported that the increased sensitivity of active chromatin domains to DNase I correlates with the presence of HMG-14 and/or HMG-17 (16, 30, 31). However, whether HMG-17 and HMG-14 are directly responsible for the increased DNase I sensitivity is controversial (11, 17, 22, 26, 27). As an immature B-cell, DT40 expresses large amounts of immunoglobulin light-chain proteins from the rearranged copy of its lambda light-chain gene. It has been shown that DNase I hypersensitivity exists in both the rearranged and the germline loci with some sites specific for each type of locus (28). We have previously shown that the disruption of *HMG-17* in DT40 does not affect the preferential DNase I sensitivity and the hypersensitive sites (15) in both the rearranged and the germline loci. To determine whether the complete absence of both *HMG-14a* and *HMG-17* affect the DNase I sensitivity,

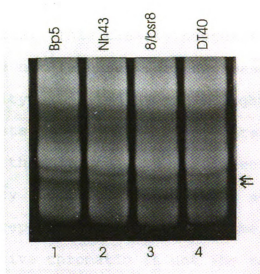


Figure 8. Electrophoretic analysis of nucleosomes from representative cell lines. Nuclear preparations from the indicated cell lines were incubated with micrococcal nuclease at 40 U/mg of DNA equivalent for 10 min at 37oC. After 10 min on ice and subsequent centrifugation, the supernatant (S1) was removed and the pellet was resuspended in 2 mM EDTA (pH7.4) and incubated on ice for another 10 min. After centrifugation, the supernatant (S2) was collected and quantitated for DNA content. A total of 30 μg was loaded, after mixing with 1 volume of glycerol, onto a 3.5% polyacrylamide-0.5% agarose gel containing 30% glycerol (12). The gel was run at constant current of 9 mA for 12h at 4oC. Arrows indicate two different bands in the region corresponding to the mammalian MII band (12).

nuclei from Nh43, 8/bsr8, and DT40 were digested with limited amounts of DNase I. As shown in Figure 9, the 7- and 5- kb bands were about equally sensitive in all cell lines tested and no difference in number and intensity were seen for the smaller bands produced due to cleavage at hypersensitive sites. This clearly suggests that the HMG-14a and HMG-17 are not required for the DNase I sensitivity and hypersensitivity exhibited at the λ light-chain locus. Since HMG-14b exists in very limited amounts in these cells, it seems unlikely that it could be sufficient to induce the DNase I sensitivity observed. Our results are consistent with a number of reports suggesting that the DNase I sensitivity of active chromatin is not the result of the presence of HMG proteins but rather is due to an altered higher order chromatin configuration (11, 17, 22). However, our data do not argue against the evidence that HMG-17 and HMG-14 associate preferentially with active genes, nor can we rule out the possibility that these proteins are involved in the DNase I sensitive chromatin structures observed in parental cells, but that in the knockout cells they are replaced by other proteins that can also confer DNase I sensitivity.

Cellular function of HMG-17 and HMG-14. In vitro assays on isolated chromatin and more recent studies involving the over-expression of exogenous HMG-14 in cultured cells have suggested that these HMGs may act to modulate gene expression and thereby influence cell proliferation and

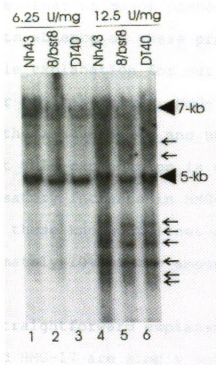


Figure 9. DNase I sensitivity analysis of representative cell lines. Nuclei from Nh43 (lanes 1, 4, and 7), 8/bsr8 (lanes 2, 5, and 8), and DT40 (lanes 3, 6, and 9) were prepared and incubated in the presence of DNase I at 0 (lanes 1, 2, and 3), 25x¼ (lanes 4, 5, and 6), and 25x½ (lanes 7, 8, and 9) U per mg of DNA equivalent for 20 min at room temperature. The partially digested DNAs were extracted and doubly digested by Sal I and XhoI, gel electrophoresed, and blotted as described previously (15). The blot was hybridized to a 295-bp fragment amplified from the constant region of the chicken 8 light-chain gene (15). The intact Sal I-XhoI fragment (approximately 5-kb) is shown by an arrow on the right.

dif

act

la

and

fro

re

re

ce

si

wa

ac

By

th

n

c

t

e

o

r

k

i

a

p

a

a

differentiation through their regulated expression or activity. The results reported herein demonstrate that HMG-14a and HMG-17 are not required for cell growth in culture and, furthermore, that no major phenotypic changes result from the complete absence of these proteins in DT40 cells.

One possible explanation for our results is that the remaining member of this family, HMG-14b, is functionally reconstituting the lost HMG-14a and HMG-17 in these knockout cell lines. But this explanation is unlikely to be true since no compensatory increase in HMG-14b protein expression was detected in these knockout lines and HMG-14b is present at only approximately 10% of the amount of either HMG-14a or HMG-17.

The most straightforward explanation for our results is that HMG-14a and HMG-17 are simply not required for the normal growth of, at least, chicken lymphoid cells in culture. This may seem surprising considering the fact that these two proteins (especially HMG-17) are well conserved in evolution. But recent reports have shown that the knockout of histones genes *H2b-v* (27a) and *01H1* (21a) in DT40 also resulted in no obvious phenotypic change and studies on knockout mice (10a) have shown that homozygous null mutants in a surprisingly wide array of presumably critical genes are viable, even in a whole animal. It is certainly possible that HMG-14a and HMG-17 are required for the growth and/or differentiation of other cell types, even though they appear to be expressed ubiquitously in all cells. It is

also possible that the dispensable character of these proteins is peculiar to a transformed cell line such as DT40. Perhaps the simplest explanation for our results is that HMG-14a and HMG-17 are not required for the growth or gross phenotypic properties of any individual cell type, but rather function in some unknown way(s) in the development of the animal. It is also possible that their function has 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.

SUMMARY

1. *HMG-17* doubly disrupted cell lines were generated by targeted gene disruption. When these lines were examined for morphology, proliferation rate, cell-cycle distribution, steady-state levels of histones, and DNase I hypersensitivity at the lambda light-chain gene, no difference was detected with respect to the parental DT40 cells. Furthermore, no compensatory increase in HMG-14 protein levels was observed.
2. One *HMG-14a* doubly disrupted cell line was generated by targeted gene disruption. No major phenotypic changes appeared to have resulted from this disruption when the tests listed in 1 were performed for this cell line. The levels of HMG-14a and HMG-17 did not rise in compensation for the loss of HMG-14a.
3. Quadruply disrupted cell lines lacking both *HMG-14a* and *HMG-17* were generated by targeted gene disruption. No major phenotypic changes resulted from the absence of both *HMG-14a* and *HMG-17* when examined similarly as in 1. This proves that the observations made in 1 and 2 do not

result from functional redundancy between *HMG-14a* and *HMG-17*. Furthermore, typical DNase I sensitivity of active chromatin was retained in these cells despite the absence of both *HMG-17* and *HMG-14a*. In addition, the level of HMG-14b did not show a compensatory increase in these quadruply disrupted mutants, demonstrating, in combination with the results in 1 and 2, that this family of proteins is not coordinately regulated.

4. It is concluded that neither *HMG-17* nor *HMG-14a is* required for cell growth in culture and the absence of these proteins does not cause phenotypic differences with respect to the parental DT40 cells.

APPENDIX

APPENDIX

Unpublished Results

1. The aberrant transcript from *HMG-17* null mutants does not contain exon 1 (Figure 1).
2. The cell cycle distribution does not correlate with the presence of a functional *HMG-17* gene (Table 1).
3. The colony-forming efficiency in soft-agar does not correlate with *HMG-17* gene activity (Table 2).
4. The side-scattering profile does not correlate with *HMG-17* gene activity (Figure 2).
5. The hypersensitive profile is similar in the HMG-17 null mutants and DT40 (Figure 3).
6. The cell cycle distributions of the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* do not show consistent differences compared with that of DT40 (Table 3).
7. The side-scattering profile does not correlate with *HMG-14a* and *HMG-17* gene activity (Figure 4).
8. Surface IgM expression is similar in the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* and the parental DT40 cells (Figure 5).
9. Targeting frequencies of various vectors used (Table 5).

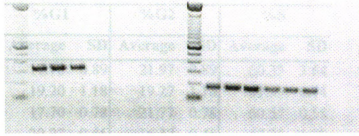


Figure 1. The aberrant transcript from HMG-17 null mutants does not contain exon 1. After PCR amplification of the first-strand cDNA reverse-transcribed from the mRNA extracted from DT40 (lanes 2 and 11), His1(lanes 3 and 12), AD3a (lanes 4 and 13), D2 (lanes 5 and 14), D20 (lanes 6 and 15), and D98-7 (lanes 7 and 16) as described in MATERIAL AND METHODS in Chapter 1, the product was electrophoresed on a 3% agarose gel and stained. Reverse transcriptase control (lanes 8 and 17) and no-DNA control (lanes 9 and 18) were included. Markers were 100-bp ladders (lanes 1 and 10). The bands were stained by ethidium bromide. The primer pairs used were E1-E6 (lanes 2-9) and E4-E6 (lanes 11-18). Only the 170-bp (but not the 313-bp) band was amplified from the double knockouts, indicating that the aberrant transcript seen in the northern blotting analysis was made from the HMG-17 locus but lacked the exon 1.

Table 1. The cell cycle distribution does not correlate with the presence of a functional *HMG-17* gene. Cells were prepared and analyzed as described in MATERIALS AND METHODS IN Chapter 1.

	%G1		%G2		%S	
Cell line	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
DT40	17.70	0.89	21.97	0.89	60.33	3.64
His1	19.20	1.18	19.27	1.18	61.53	0.74
AD3a	17.70	0.78	21.77	0.78	60.57	0.55
D2	20.27	0.46	16.47	0.46	63.23	1.70
D20	23.53	1.17	16.83	1.17	59.67	1.45
D98-7	20.53	0.06	16.57	0.06	62.90	1.28
D108-1	20.80	0.70	15.87	0.70	63.30	1.35

Table 2. The colony-forming efficiency in soft-agar does not correlate with *HMG-17* gene activity.

	Colony No.*				
Cell line	**Dish 1**	**Dish 2**	**Dish 3**	**Dish 4**	**Average**
DT40	72	47	70	54	61
His1	72	64	70	77	71
AD3a	9	12	17	5	11
D2	7	14	15	20	14
D20	0	0	1	0	0
D98-7	96	90	85	105	94
D108-1	65	67	46	45	56

*Colony numbers were determined 8-days post-plating (250 cells per dish).

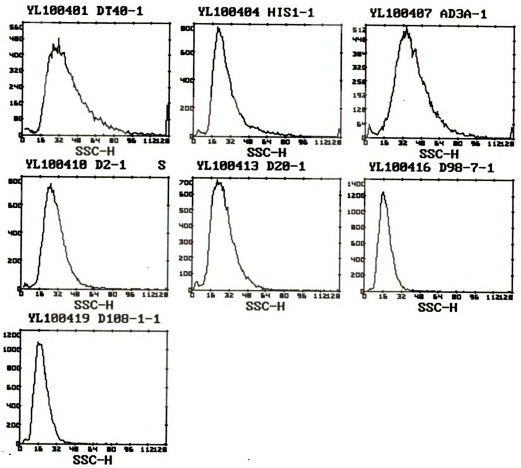


Figure 2. The side-scattering profile does not correlate with *HMG-17* gene activity. Cells (10^4) in logarithmic growth were analyzed in FACS Vantage (Becton Dickinson). Side scatter profile is shown here for the indicated cell lines. X-axis shows the channel numbers, and Y-axis indicates the cell numbers.

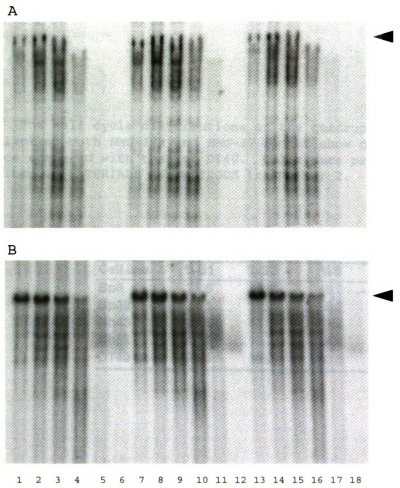
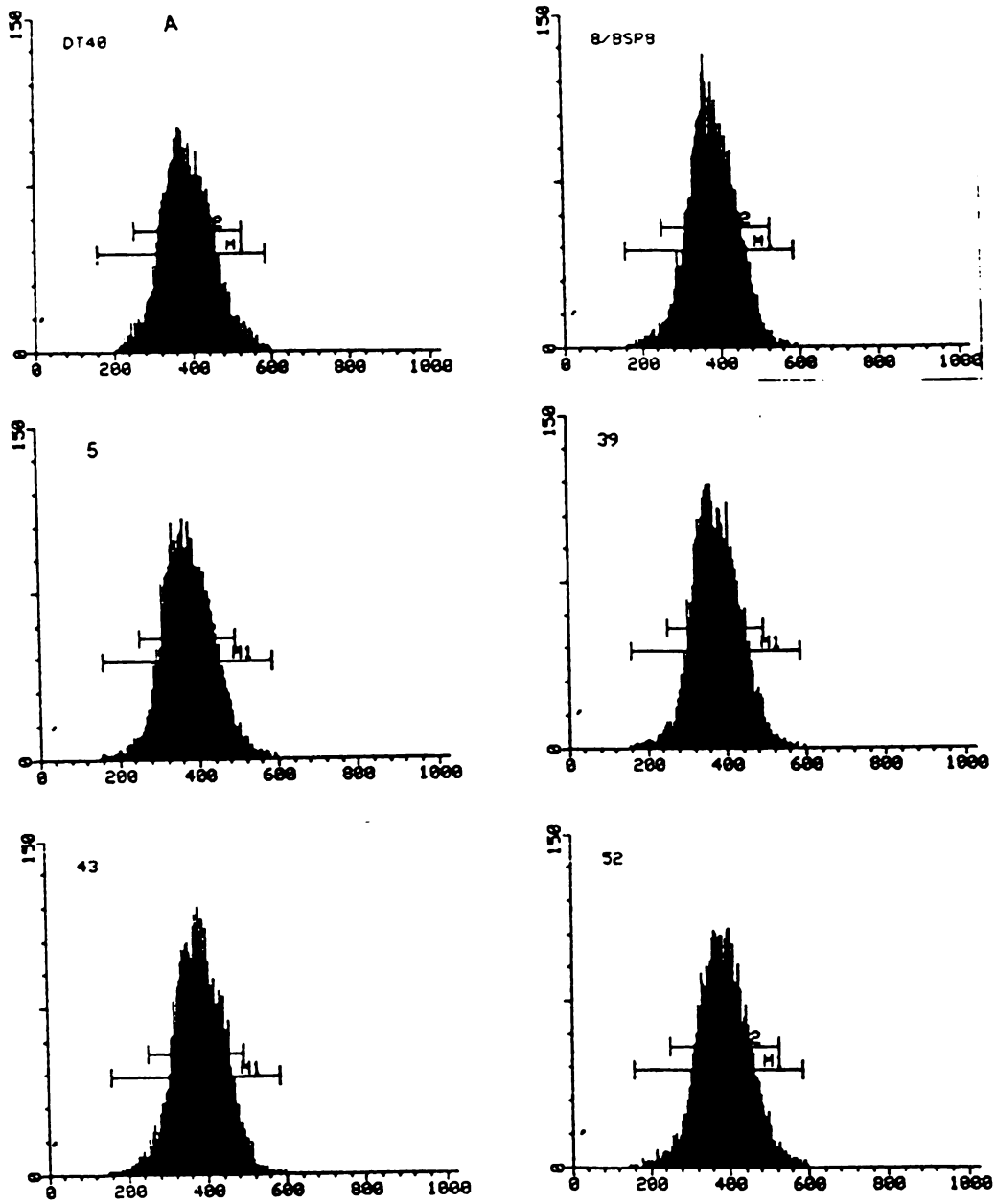


Figure 3. The hypersensitive profile is similar in the HMG-17 null mutants and DT40. Nuclei from DT40 (lanes 1-6), D108-1 (lanes 7-12), D2 (lanes 13-18) were digested by DNase I for 20 min (room temperature) at 0 (lanes 1, 7, and 13), 25x1/16 (lanes 2, 8, and 14), 25X1/8 (lanes 3, 9, and 15), 25x1/4 (lanes 4, 10, and 16), 25x1/2 (lanes 5, 11, and 17), and 25 (lanes 6, 12, and 18) U/mg DNA equivalent. The DNA was extracted and digested separately by SalI (A)and ScaI (B), electrophoresed, blotted to a membrane, and probed by 32P labeled 295-bp fragment amplified from the chicken lambda light-chain by PCR as described in MATERIALS AND METHODS in Chapter I. Arrows indicate the intact bands.

Table 3. The cell cycle distributions of the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* do not show consistent difference compared with that of DT40. Cells were prepared and analyzed as described in MATERIALS AND METHODS in Chapter 2.

Cell line	%G1	%G2	%S
Bp5	27.5	23.6	49.0
Bp39	24.1	17.7	58.2
Nh43	20.5	18.1	61.4
Nh52	19.0	16.7	64.4
DT40	20.5	16.0	63.5

Figure 4. The side-scattering profile does not correlate with the *HMG-14a* and the *HMG-17* gene activity. Cells (10^4) in logarithmic growth were analyzed in FACS Vantage (Becton Dickinson). (A) The histograms show the side-scattering profile of the indicated cell lines (5, 39, 43, and 52 represent Bp5, Bp39, Nh43, and Nh52, respectively). The X-axis indicates the channel numbers, and the Y-axis indicates the cell numbers. (B) The table lists the peak, mean, and median channels in the M1 region in (A).



B

	DT40	8/bsr8	Bp5	Bp39	Nh43	Nh52
PkChl	362.00	364.00	362.00	356.00	383.00	399.00
Mean	385.62	379.26	372.73	374.12	378.22	383.88
Median	382.00	378.00	371.00	372.00	378.00	382.00

Figure 4

Figure 5. Surface IgM expression is similar in the quadruply disrupted mutants lacking both *HMG-14a* and *HMG-17* and the parental DT40 cells. Cells in logarithmic growth were incubated with goat anti-chicken IgM FITC conjugate diluted in FA buffer (DMEM-1% BSA-0.1% NaN_3) for 20 min on ice. After 3 washes with the FA buffer, the cells were resuspended in FA buffer and analyzed by FACScan (Becton-Dickinson). The X-axis represents the fluorescence channels, and the Y-axis indicates the number of cells. The cell lines tested were DT40, Nh43, Nh52, Bp39, and Bp5 (clockwise from the top-left).

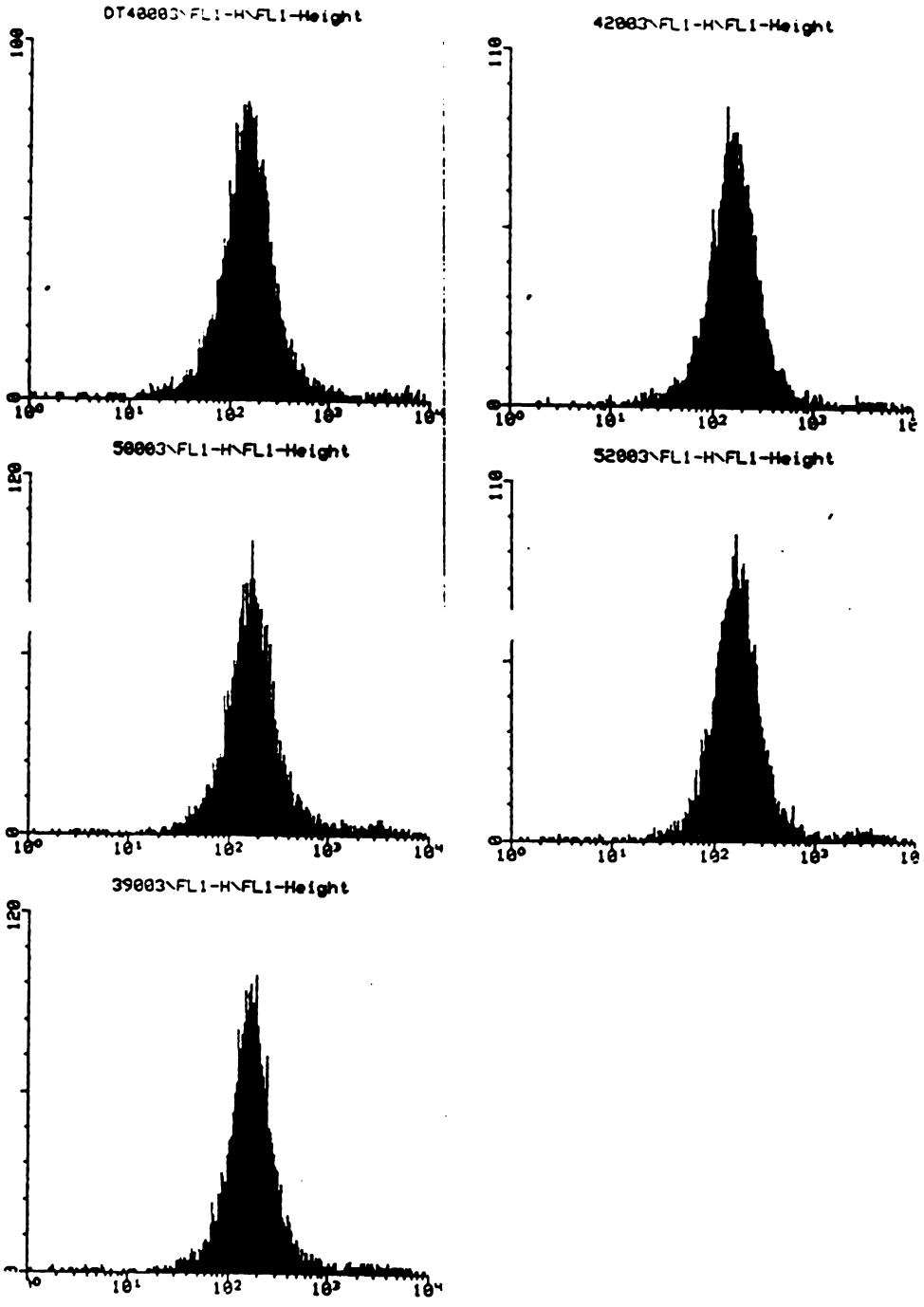

DT40003\FL1-H\FL1-Height
42003\FL1-H\FL1-Height
50003\FL1-H\FL1-Height
52003\FL1-H\FL1-Height
39003\FL1-H\FL1-Height

Figure 5

Table 4. Targeting frequencies of various vectors used.

Constructs	Cell line targeted	No. colonies screened	No. colonies targeted	% targeted vs. screened
pBSH17-neo	DT40	110	12	10.9
PBSH17-puro	DT40	5	0	0
pBSH17-puro	DT40	58	0	0
pBSH17-hygro	DT40	18	0	0
pBSH17-hygro	DT40	34	0	0
pBSH17-his	DT40	7	2	29
pBSH17-his	AD3a	42	11	26.2
pBSH17-neo	His1	88	2	2.2
pT14a-his	DT40	25	2	8.0
pT14a-puro	DT40	34	2	5.9
pT14a-bsr	DT40	15	0	0.0
pT14a-hygro	DT40	2	0	0.0
pT14a-gpt	DT40	11	0	0.0
pT14a-his	DTpuro8	23	1	4.3
pT14a-hygro	DTpuro8	2	0	0.0
pT14a-bsr	DTpuro8	38	1	2.6
pT14a-zeo	DTpuro8	34	1	2.9
pT14a-puro	D108-1	28	1	3.6
pT14a-bsr	D108-1	33	1	3.0
pT14a-gpt	D108-1	18	2	11.1
pT14azeo	D108-1	29	0	0.0
pT14a-gpt	Bsr18	37	0	0.0
pT14a-puro	Bsr18	45	2	4.4
pT14a-bsr	Gpt8	95	0	0.0
pBS17-his	14N11	14	11	78.6
pBS17-neo	8/bsr8	44	23	52.3

BIBLIOGRAPHY

BIBLIOGRAPHY

INTRODUCTION.

1. Deleted

2. Deleted

3. Albright, S. C., J. M. Wiseman, R. A. Lange, and W. T. Garrard. 1980. Subunit structures of different electrophoretic forms of nucleosomes. J. Biol. Chem. 255:3673-3684.

4. 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 [published erratum appears in J Mol Biol 1994 Jun 10;239(3):436]. J. Mol. Biol. 236:189-198.

5. Allfrey, V. W. 1982. The HMG Chromosomal Proteins. Academic Press, London.

6. Barratt, M. J., C. A. Hazzalin, N. Zhelev, and L. C. Mahadevan. 1994. A mitogen- and anisomycin-stimulated kinase phosphorylates HMG-14 in its basic amino-terminal domain in vivo and on isolated mononucleosomes. EMBO J. 13:4524-4535.

7. Barsoum, J., L. Levinger, and A. Varshavsky. 1982. On the chromatin structure of the amplified, transcriptionally active gene for dihydrofolate reductase in mouse cells. J. Biol. Chem. 257:5274-5282.

8. Boumba, V. A., O. Tsolas, D. Choli Papadopoulou, and K. Seferiadis. 1993. Isolation by a new method and sequence analysis of chromosomal HMG-17 protein from porcine thymus. Arch. Biochem. Biophys. 303:436-442.

9. Brown, N. S. 1995. Ectopic Expression of Chicken HMG14a and HMG17 Chromatin Binding Proteins. Ph.D. Dissertation, Michigan State University.

10. 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.

11. Buerstedde, J. M. and S. Takeda. 1991. Increased ratio of targeted to random integration after transfection of

10. 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.

11. 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.

12. 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.

13. Bustin, M., N. Soares, D. Landsman, T. Srikantha, and J. M. Collins. 1987. Cell cycle regulated synthesis of an abundant transcript for human chromosomal protein HMG-17. Nucleic Acids Res. 15:3549-3561.

14. 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.

15. Capecchi, M. R. 1989. Altering the genome by homologous recombination. Science 244:1288-1292.

16. Cary, P. D., D. S. King, C. Crane Robinson, E. M. Bradbury, A. Rabbani, G. H. Goodwin, and E. W. Johns. 1980. Structural studies on two high-mobility-group proteins from calf thymus, HMG-14 and HMG-20 (ubiquitin), and their interaction with DNA. Eur. J. Biochem. 112:577-580.

17. Charron, J., B. A. Malynn, P. Fisher, V. Stewart, L. Jeannotte, S. P. Goff, E. J. Robertson, and F. W. Alt. 1992. Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. Genes Dev. 6:2248-2257.

18. Charron, J., B. A. Malynn, E. J. Robertson, S. P. Goff, and F. W. Alt. 1990. High-frequency disruption of the N-myc gene in embryonic stem and pre-B cell lines by homologous recombination. Mol. Cell. Biol. 10:1799-1804.

19. Clarke, L. L., B. R. Grubb, S. E. Gabriel, O. Smithies, B. H. Koller, and R. C. Boucher. 1992. Defective epithelial chloride transport in a gene-targeted mouse model of cystic

B. H. Koller, and R. C. Boucher. 1992. Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. Science 257:1125-1128.

20. Cook, G. R., M. Minch, G. P. Schroth, and E. M. Bradbury. 1989. Analysis of the binding of high mobility group protein 17 to the nucleosome core particle by super(1)H NMR spectroscopy. J. Biol. Chem. 264:1799-1803.

21. Cook, G. R., P. Yau, H. Yasuda, R. R. Traut, and E. M. Bradbury. 1986. High mobility group protein 17 cross-links primarily to histone H2A in the reconstituted HMG 17-nucleosome core particle complex. J. Biol. Chem. 261:16185-16190.

22. Crippa, M. P., P. J. Alfonso, and M. Bustin. 1992. Nucleosome Core Binding Region of Chromosomal Protein HMG-17 Acts as an Independent Functional Domain. J. Mol. Biol. 228:442-449.

23. 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.

24. Deng, C. and M. R. Capecchi. 1992. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol. Cell Biochem. 12:3365-3371.

25. 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.

26. 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.

27. Doetschaman, T., N. Maeda, and O. Smithies. 1988. Targeted mutation of the Hprt gene in mouse embryonic stem cells. Proc. Natl. Acad. Sci. USA 85:8583-8587.

28. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery,Jr., J. S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature (London) 356:215-221.

29. Dorbic, T. and B. Wittig. 1986. Isolation of oligonucleosomes from active chromatin using HMG17-specific monoclonal antibodies. Nucleic Acids Res. 14:3363-3376.

30. Dorin, J. R., J. D. Inglis, and D. J. Porteous. 1989. Selection for precise chromosomal targeting of a dominant marker by homologous recombination. Science 243:1357-1360.

31. Einck, L. and M. Bustin. 1983. Inhibition of transcription in somatic cells by microinjection of antibodies to chromosomal proteins. Proc. Natl. Acad. Sci. USA 80:6735-6739.

32. Gabrielli, F., R. Hancock, and A. J. Faber. 1981. Characterization of a chromatin fraction bearing pulse-labeled RNA. 2. Quantification of histones and high-mobility-group proteins. Eur. J. Biochem. 120:363-369.

33. Garel, A. and R. Axel. 1976. Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. Proc. Natl. Acad. Sci. USA 73:3960-3971.

34. Goodwin, G. H., C. G. Mathew, C. A. Wright, C. D. Venkov, and E. W. Johns. 1979. Analysis of the high mobility group proteins associated with salt-soluble nucleosomes. Nucleic Acids Res. 7:1815-1835.

35. Goodwin, G. H., R. H. Nicolas, P. N. Cockerill, S. Zavou, and C. A. Wright. 1985. The effect of salt extraction on the structure of transcriptionally active genes; evidence for a DNAseI-sensitive structure which could be dependent on chromatin structure at levels higher than the 30 nm fibre. Nucleic Acids Res. 13:3561-3579.

36. 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.

37. Hanson, K. D. and J. M. Sedivy. 1995. Analysis of biological selections for high-efficiency gene targeting. Mol. Cell. Biol. 15:45-51.

38. 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.

39. 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.

40. Jeongyu, S. and D. Carroll. 1992. Effect of Terminal Nonhomologies on Homologous Recombination in Xenopus-Laevis Oocytes. Mol. Cell. Biol. 12:5426-5437.

41. Johns, E. W. 1982. The HMG Chromosomal Proteins. Academic Press, New York.

42. Johnson, R. S., B. M. Spiegelman, and V. Papaioannou. 1992. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell 71:577-586.

43. Deleted.

44. Landsman, D. and M. Bustin. 1986. Chromosomal proteins HMG-14 and HMG-17. Distinct multigene families coding for similar types of transcripts [published erratum appears in J Biol Chem 1987 Jun 15;262(17):8438]. J. Biol. Chem. 261:16087-16091.

45. Landsman, D. and M. Bustin. 1990. Mouse non-histone chromosomal protein HMG-14 cDNA sequence. Nucleic Acids Res. 18:5311.

46. Landsman, D., O. W. McBride, N. Soares, M. P. Crippa, T. Srikantha, and M. Bustin. 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.

47. Landsman, D., N. Soares, F. J. Gonzalez, and M. Bustin. 1986. Chromosomal protein HMG-17. Complete human cDNA sequence and evidence for a multigene family [published erratum appears in J Biol Chem 1988 Nov 5;263(31):16512]. J. Biol. Chem. 261:7479-7484.

48. 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.

49. Landsman, D., T. Srikantha, R. Westermann, and M. Bustin. 1986. Chromosomal protein HMG-14. Complete human cDNA sequence and evidence for a multigene family [published erratum appears in J Biol Chem 1988 Nov 5;263(31):16512]. J. Biol. Chem. 261:16082-16086.

50. Landsman, D., S. Zavou, N. Soares, G. H. Goodwin, and M. Bustin. 1988. Mouse non-histone chromosomal protein HMG-17 cDNA sequence. Nucleic Acids Res. 16:10386.

51. Lee, E. Y. H. P., C. Y. Chang, N. Hu, Y. C. J. Wang, C. C. Lai, K. Herrup, W. H. Lee, and A. Bradley. 1992. Mice deficient in Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature (London) 359:288-294.

52. Levinger, L., J. Barsoum, and A. Varshavsky. 1981. Two-dimensional hybridization mapping of nucleosomes. comparison of DNA and protein patterns. J. Mol. Biol. 146:287-304.

53. Lufkin, T., D. Lohnes, M. Mark, A. Dierich, P. Gorry, M. P. Gaub, M. LeMeur, and P. Chambon. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc. Natl. Acad. Sci. USA 90:7225-7229.

54. Mansour, S. L., K. R. Thomas, and M. R. Capecchi. 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (London) 336:348-352.

55. Mardian, J. K., 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.

56. Mathew, C. G., G. H. Goodwin, T. Igo Kemenes, and E. W. Johns. 1981. The protein composition of rat satellite chromatin. FEBS Lett. 125:25-29.

57. Mayes, E. L. 1982. Species and tissue specificity, p. 9-40. In E. W. Johns (ed.), The HMG Chromosomal Proteins. Acedmic Press, London.

58. McGhee, J. D., D. C. Rau, and G. Felsenfeld. 1982. The high mobility group proteins HMG 14 and 17, do not prevent the formation of chromatin higher order structure. Nucleic Acids Res. 10:2007-2016.

59. Mendelsohn, C., M. Mark, P. Dolle, A. Dierich, M. P. Gaub, A. Krust, C. Lampron, and P. Chambon. 1994. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. Dev. Biol. 166:246-258.

60. Mortensen, R. M. 1993. Double knockouts. Production of mutant cell lines in cardiovascular research. Hypertension 22:646-651.

61. 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.

62. Mortensen, R. M., M. Zubiaur, E. J. Neer, and J. G. Seidman. 1991. Embryonic stem cells lacking a functional inhibitory G-protein subunit (alpha i2) produced by gene targeting of both alleles. Proc. Natl. Acad. Sci. USA 88:7036-7040.

63. Nakayama, K., I. Negishi, K. Kuida, Y. Shinkai, M. C. Louie, L. E. fields, P. J. Lucas, V. Stewart, F. W. Alt, and D. Y. Loh. 1993. Disappearence of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science 261:1584-1588.

64. 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.

65. Nickoloff, J. A. and R. J. Reynolds. 1990. Transcription stimulates homologous recombination in mammalian cells. Mol. Cell. Biol. 10:4837-4845.

66. Nicolas, R. H., C. A. Wright, P. N. Cockerill, J. A. Wyke, and G. H. Goodwin. 1983. The nuclease sensitivity of active genes. Nucleic Acids Res. 11:753-772.

66b. Nickoloff,J.A. 1992. Transcription enhances intrachromosomal homologous recombination in mammalian cells. Mol. Cell. Biol. 12:5311-5318.

67. Nielsen, E., B. Welinder, and O. D. Madsen. 1990. Protein HMG-17 is hyper-expressed in rat glucagonoma. Single-step isolation and sequencing. Eur. J. Biochem. 192:81-86.

68. Niwa, H., K. Araki, S. Kimura, S. Taniguchi, S. Wakasugi, and K. Yamamura. 1993. An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. J. Biochem. Tokyo. 113:343-349.

69. Paranjape, S. M., A. Krumm, and J. T. Kadonaga. 1995. HMG17 is a chromatin-specific transcriptional coactivator that increases the efficiency of transcription initiation. Genes Dev. 9:1978-1991.

70. 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.

71. Pedrini, M., H. Grunicke, and A. Csordas. 1992. Tissue-specific pattern of nonhistone high mobility group proteins in various organs of the chicken. Electrophoresis 13:397-399.

72. Postnikov, Y. V., V. V. Shick, A. V. Belyavsky, K. R. Khrapko, K. L. Brodolin, T. A. Nikolskaya, and A. D.

Mirzabekov. 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.

72a. Postnikov, Y. V., A. D. Lehn, R. C. Robinson, F. K. Friedman, J. Shiloach, and M. Bustin. 1994. The cooperative binding of chromosomal protein HMG-14 to nucleosome cores is reduced by single point mutations in the nucleosomal binding domain. Nucleic Acids Res. 22:4520-4526.

73. Reeves, R., D. Chang, and S. C. Chung. 1981. Carbohydrate modifications of the high mobility group proteins. Proc. Natl. Acad. Sci. USA 78:6704-6708.

74. Reudelhuber, T. L., D. J. Ball, A. H. Davis, and W. T. Garrard. 1982. Transferring DNA from electrophoretically resolved nucleosomes to diazobenzyloxymethyl cellulose: properties of nucleosomes along mouse satellite DNA. Nucleic Acids Res. 10:1311-1325.

75. Riele, H., E. R. Maandag, A. Clarke, M. Hooper, and A. Berns. 1990. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. Nature (London) 348:649-651.

76. Rudnicki, M. A., T. Braun, S. Hinuma, and R. Jaenisch. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71:383-390.

77. Rudnicki, M. A., P. N. Schnegelsberg, R. H. Stead, T. Braun, H. H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75:1351-1359.

78. Saga, Y., T. Yagi, Y. Ikawa, T. Sakakura, and S. Aizawa. 1992. Mice Develop Normally Without Tenascin. Genes. Dev. 6:1821-1831.

79. Sandeen, G., W. I. Wood, and G. Felsenfeld. 1980. The interaction of high mobility proteins HMG14 and 17 with nucleosomes. Nucleic Acids Res. 8:3757-3778.

80. Seale, R. L., A. T. Annunziato, and R. D. Smith. 1983. High mobility group proteins: abundance, turnover, and relationship to transcriptionally active chromatin. Biochemistry 22:5008-5015.

81. Seguchi, K., Y. Takami, and T. Nakayama. 1995. Targeted disruption of 01H1 encoding a particular H1 histone variant causes changes in protein patterns in the DT40 chicken B cell line. J. Mol. Biol. 254:869-880.

82. Senear, A. W. and R. D. Palmiter. 1981. Multiple structural features are responsible for the nuclease sensitivity of the active ovalbumin gene. J. Biol. Chem. 256:1191-1198.

83. 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.

84. Shick, V. V., A. V. Belyavsky, and A. D. Mirzabekov. 1985. Primary organization of nucleosomes: Interaction of non-histone high mobility group proteins 14 and 17 with nucleosomes, as revealed by DNA-protein crosslinking and immunoaffinity isolation. J. Mol. Biol. 185:329-339.

85. Shull, M. M, I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, and T. Doetschman. 1992. Targeted disruption of the mouse transforming growth factor-ß1 gene results in multifocal inflammatory disease. Nature (London) 359:693-699.

86. Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693-702.

87. Srikantha, T., D. Landsman, and M. Bustin. 1987. Retropseudogenes for human chromosomal protein HMG-17. J. Mol. Biol. 197:405-413.

88. 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 [published erratum appears in J Biol Chem 1989 Jul 25;264(21):12744]. J. Biol. Chem. 263:13500-13503.

89. Srikantha, T., D. Landsman, and M. Bustin. 1990. A single copy gene for chicken chromosomal protein HMG-14b has evolutionarily conserved features, has lost one of its introns and codes for a rapidly evolving protein. J. Mol. Biol. 211:49-61.

90. Stanton, B. R., A. S. Perkins, L. Tessarollo, D. A. Sassoon, and L. F. Parada. 1992. Loss of N-myc Function Results in Embryonic Lethality and Failure of the Epithelial Component of the Embryo to Develop. Genes Dev. 6:2235-2247.

91. Stein, A. and T. Townsend. 1983. HMG 14/17 binding affinities and DNAase I sensitivities of nucleoprotein particles. Nucleic Acids Res. 11:6803-6819.

92. Swerdlow, P. S. and A. Varshavsky. 1983. Affinity of HMG17 for a mononucleosome is not influenced by the presence of ubiquitin-H2A semihistone but strongly depends on DNA fragment size. Nucleic Acids Res. 11:387-401.

93. Takami, Y., S. Takeda, and T. Nakayama. 1995. Targeted disruption of H2B-V encoding a particular H2B hsitone variant causes changes in protein patterns on two-dimensional polyacrylamide gel electrophoresis in the DT40 chicken B cell line. J. Biol. Chem. 270:30664-30670.

94. te Riele, H., E. R. Maandag, and A. Berns. 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. USA 89:5128-5132.

95. Thomas, K. R. and M. R. Capecchi. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51:503-572.

96. 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.

97. Trieschmann, L., P. J. Alfonso, M. P. Crippa, A. P. Wolffe, and M. Bustin. 1995. Incorporation of chromosomal proteins HMG-14/HMG-17 into nascent nucleosomes induces an extended chromatin conformation and enhances the utilization of active transcription complexes. EMBO J. 14:1478-1489.

98. Trieschmann, L., Y. V. Postnikov, A. Rickers, and M. Bustin. 1995. Modular structure of chromosomal proteins HMG-14 and HMG-17: definition of a transcriptional enhancement domain distinct form the nucleosomal binding domain. Mol. Cell. Biol. 15:6663-6669.

99. Tybulewicz, V. L., C. E. Crawford, P. K. Jackson, R. T. Bronson, and R. C. Mulligan. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell 65:1153-1163.

100. van Holde, K. E. 1989. Chromatin. Springer-verlag, berlin.

101. Vandeursen, J. and B. Wieringa. 1992. Targeting of the Creatine Kinase-M Gene in Embryonic Stem Cells Using Isogenic and Nonisogenic Vectors. Nucleic Acids Res. 20:3815-3820.

102. Walker, J. M., J. R. Hastings, and E. W. Johns. 1977. The primary structure of a non-histone chromosomal protein. Eur. J. Biochem. 76:461-468.

102a. Walker,J.M.; G. H. Goodwin, and E. W. Johns. 1979. The primary structure of the nucleosome-associated chromosomal protein HMG 14. FEBS Lett. 100:394-398.

103. Walker, J. M., C. Stearn, and E. W. Johns. 1980. The primary structure of non-histone chromosomal protein HMG17 form chicken erythrocyte nuclei. FEBS Lett. 112:207-210.

104. Weintraub, H. and M. Groudine. 1976. Chromosomal subunits in active genes have an altered conformation. Science 193:848-856.

105. Weisbrod, S. 1982. Active chromatin. Nature (London) 297:289-295.

106. Weisbrod, S., M. Groudine, and H. Weintraub. 1980. Interaction of HMG 14 and 17 with actively transcribed genes. Cell 19:289-301.

107. Weisbrod, S. and H. Weintraub. 1979. Isolation of subclass of nuclear proteins responsible for confering a DNAse I-sensitive structure on globin chromatin. Proc. Natl. Acad. Sci. USA 76:630-634.

108. Weisbrod, S. and H. Weintraub. 1981. Isolation of actively transcribed nucleosomes using immobilized HMG 14 and 17 and an analysis of alpha-globin chromatin. Cell 23:391-400.

109. Weisbrod, S. T. 1982. Properties of active nucleosomes as revealed by HMG 14 and 17 chromatography. Nucleic Acids Res. 10:2017-2042.

110. Westermann, R. and U. Grossbach. 1984. Localization of nuclear proteins related to high mobility group protein 14 (HMG 14) in polytene chromosomes. Chromosoma 90:355-365.

111. Yagi, T., Y. Ikawa, K. Yoshida, Y. Shigetani, N. Takeda, I. Mabuchi, T. Yamamoto, and S. Aizawa. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. Proc. Natl. Acad. Sci. USA 87:9918-9922.

Chapter 1.

1. 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.

2. 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.

3. Bellard, M., G. Dretzen, A. Giangrande, and P. Ramain. 1989. Nuclease digestion of transcriptionally active chromatin. Methods Enzymol. 170:317-346.

4. 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.

5. 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.

6. 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.

7. Bustin, M., M. P. Crippa, and J. M. Pash. 1990. Immunochemical analysis of the exposure of high mobility group protein 14 and 17 surfaces in chromatin. J. Biol. Chem. 265:20077-20080.

8. 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.

9. 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.

10. Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. 201:134-139.

11. 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.

12. 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.

13. 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.

14. 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.

15. Dugaiczyk, 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. Rülicke, 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, NY

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 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.

Chapter 2

1. 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.

2. 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.

3. Bellard, M., G. Dretzen, A. Giangrande, and P. Ramain. 1989. Nuclease digestion of transcriptionally active chromatin. Methods Enzymol. 170:317-346.

4. 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.

5. Bustin, M., M. P. Crippa, and J. M. Pash. 1990. Immunochemical analysis of the exposure of high mobility group protein 14 and 17 surfaces in chromatin. J. Biol. Chem. 265:20077-20080.

6. 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.

7. Cook, G. R., M. Minch, G. P. Schroth, and E. M. Bradbury. 1989. Analysis of the binding of high mobility group protein 17 to the nucleosome core particle by super(1)H NMR spectroscopy. J. Biol. Chem. 264:1799-1803.

8. 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.

9. 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.

10. Dugaiczyk, 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 messanger ribonucleic acid isolated from chicken muscle. Biochemistry 22:1605-1613.
10a. Gatherer, D. 1993. Gene knockouts and murine development. Dev. Growth. Differ. 35:365-370.
11. Goodwin, G. H., R. H. Nicolas, P. N. Cockerill, S. Zavou, and C. A. Wright. 1985. The effect of salt extraction on the structure of transcriptionally active genes; evidence for a DNAseI-sensitive structure which could be dependent on chromatin structure at levels higher than the 30 nm fibre. Nucleic Acids Res. 13:3561-3579.

12. Huang, S. Y. and W. T. Garrard. 1989. Electrophoretic analyses of nucleosomes and other protein-DNA complexes. Methods Enzymol. 170:116-142.

13. 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.

14. 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.

15. Li, Y. and J. B. Dodgson. 1995. The chicken HMG-17 gene is dispensable for cell growth in vitro. Mol. Cell. Biol. 15:5516-5523.

16. McGhee, J. D. and G. Felsenfeld. 1980. Nucleosome structure. Annu. Rev. Biochem. 49:1115-1156.

17. Nicolas, R. H., C. A. Wright, P. N. Cockerill, J. A. Wyke, and G. H. Goodwin. 1983. The nuclease sensitivity of active genes. Nucleic Acids Res. 11:753-772.

18. 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.

19. 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.

20. Postnikov, Y. V., V. V. Shick, A. V. Belyavsky, K. R. Khrapko, K. L. Brodolin, T. A. Nikolskaya, and A. D. Mirzabekov. 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.

21. Sandeen, G., W. I. Wood, and G. Felsenfeld. 1980. The interaction of high mobility proteins HMG14 and 17 with nucleosomes. Nucleic Acids Res. 8:3757-3778.

21a. Seguchi, K., Y. Takami, and T. Nakayama. 1995. Targeted disruption of 01H1 encoding a particular H1 histone variant causes changes in protein patterns in the DT40 chicken B cell line. J. Mol. Biol. 254:869-880.

22. Seale, R. L., A. T. Annunziato, and R. D. Smith. 1983. High mobility group proteins: abundance, turnover, and relationship to transcriptionally active chromatin. Biochemistry 22:5008-5015.

23. 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.

24. Shick, V. V., A. V. Belyavsky, and A. D. Mirzabekov. 1985. Primary organization of nucleosomes: Interaction of

non-histone high mobility group proteins 14 and 17 with nucleosomes, as revealed by DNA-protein crosslinking and immunoaffinity isolation. J. Mol. Biol. 185:329-339.

25. 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 [published erratum appears in J Biol Chem 1989 Jul 25;264(21):12744]. J. Biol. Chem. 263:13500-13503.

26. Stein, A. and T. Townsend. 1983. HMG 14/17 binding affinities and DNAase I sensitivities of nucleoprotein particles. Nucleic Acids Res. 11:6803-6819.

27. Swerdlow, P. S. and A. Varshavsky. 1983. Affinity of HMG17 for a mononucleosome is not influenced by the presence of ubiquitin-H2A semihistone but strongly depends on DNA fragment size. Nucleic Acids Res. 11:387-401.

27a. Takami, Y., S. Takeda, and T. Nakayama. 1995. Targeted dusruption of H2B-V encoding a particular H2B histone variant causes changes in protein patterns on two-dimensional polyacrylamide gen electrophoresis in the DT40 chicken B cell line. J. Biol. Sci. 270:30664-30670.

28. 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.

29. Urban, M. K., S. G. Franklin, and A. Zweidler. 1979. Isolation and characterization of the histone variants in chicken erythrocytes. Biochemistry 18:3952-3960.

30. Weisbrod, S. 1982. Active chromatin. Nature (London) 297:289-295.

31. Weisbrod, S., M. Groudine, and H. Weintraub. 1980. Interaction of HMG 14 and 17 with actively transcribed genes. Cell 19:289-301.