Expression of Replication-Dependent Histone Genes in Avian Spermatids Involves an Alternate Pathway of mRNA 3'-End Formation

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Received 28 September 1988/Accepted 5 December 1988

In somatic cells the expression of replication-dependent histone genes is coupled to the S phase of the cell cycle. However, we have found a number of novel H2a, H2b, and H3 poly(A)⁺ RNA species in avian haploid round spermatids. The spermatid-specific H2a and H2b 0.8-kilobase RNAs are transcribed from a subset of the replication-dependent H2a and H2b gene families. Two cDNAs derived from the spermatid-specific H2b transcripts were isolated and sequenced. The structures of these cDNAs reveal that the spermatid-specific RNAs are identical to the 0.5-kilobase poly(A)⁻ H2b mRNAs expressed in proliferating somatic cells, except for the addition of poly(A) at the 3' ends. The site of poly(A) addition in the spermatid-specific RNAs is located 26 to 28 nucleotides 3' of the poly(A)⁻ H2b mRNA terminus. Thus, the hairpin structures and purine-rich elements required for the U7 small nuclear ribonucleoprotein-mediated cleavage reaction that generates the 3' ends of poly(A)⁻ H2b mRNAs are not utilized in spermatids and are retained in the poly(A)⁺ H2b RNAs.

In higher eucaryotes the predominant class of histone proteins consists of replication-dependent variants that are synthesized during the S phase of the mitotic cell cycle. The expression of mRNAs encoding these proteins is coupled to DNA replication by a variety of transcriptional and posttranscriptional regulatory mechanisms. First, cells undergoing DNA replication sustain a three- to fivefold increase in the rate of histone transcription (18, 29). This increase occurs through the interaction of several promoter-proximal sequences with *trans*-acting regulatory factors (16, 21, 50). Second, recent experiments suggest that the 3' processing of histone primary transcripts can contribute to the cell cycle regulation of the mature mRNAs. During the S phase the poly(A)⁻ 3' termini of histone mRNAs are produced by an endonucleolytic cleavage reaction involving an Sm subtype small nuclear ribonucleoprotein (snRNP) containing the U7 small nuclear RNA (15, 40, 51). This reaction requires a hairpin structure formed by a conserved hyphenated symmetrical dyad element immediately 5' of the cleavage site in the histone pre-mRNA, as well as a more distal purine-rich sequence (3-5). If cells are arrested in G1, the amount of U7 snRNP remains relatively constant, but histone mRNA precursors accumulate as a heat-labile regulatory factor necessary for 3' processing becomes limiting (22, 35). Third, there is an increase in the rate of histone mRNA degradation when cells progress from S to G2 or when DNA synthesis is inhibited (48, 49). This specific degradation of histone mRNA requires the hairpin structure at the 3' terminus of the molecule (33, 41); consequently, degradation initiates at the 3' end of the histone mRNA and proceeds toward the 5' end (45).

The less prevalent replacement and tissue-specific variant histones are not subject to the cell cycle regulation that governs the expression of replication-dependent variants. Since steady-state levels of replacement variant transcripts typically do not fluctuate throughout the cell cycle or decrease significantly during cellular differentiation, these var-

iants tend to accumulate in quiescent cells and replace replication-dependent histones. There are several structural features that distinguish replication-dependent genes from replacement genes. Promoter elements that are necessary for the increase in transcription during the S phase, such as AAGAAACACA for H1 replication-dependent genes, are not found in replacement variant promoters. Replacement variant transcripts are polyadenylated and lack the terminal hairpin structure that is required for both the 3' processing and regulated degradation of replication-dependent mRNAs (20, 27, 32). Introns occur in replacement variant genes (9, 27, 32), but have never been observed in replication-dependent genes. Finally, replication-dependent genes are clustered together in the genome (17, 23), while replacement variant genes are dispersed.

During the process of spermatogenesis, both replication-dependent and tissue-specific histone variants are expressed. In the spermatogenic lineage of mammals, the synthesis of replication-dependent variants has not been detected later than the pachytene stage of meiosis (8). Most testis-specific variants are synthesized in spermatogonia and spermatocytes and ultimately replace replication-dependent variants in the chromatin of spermatocytes. The DNA sequences of two testis-specific variants from rats (an H1t gene and a TH2b cDNA) have been determined (14, 30). Although H1t and TH2b are expressed at high levels in the absence of DNA replication in pachytene spermatocytes, these genes have many of the structural features implicated in the cell cycle regulation of replication-dependent variants. For example, the H1t and TH2b transcripts have a 3'-terminal hairpin structure, and the H1t gene contains all of the conserved 5' sequence elements found in replication-dependent H1 variants, including the AAGAAACACA motif. Thus, *cis*-acting sequences, such as the 3'-terminal hairpin, that restrict the expression of replication-dependent variants to the S phase in somatic cells do not necessarily function as negative regulatory elements in the postmitotic stages of spermatogenesis.

In this paper we describe a class of replication-dependent

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TABLE 1. Oligonucleotide probes used in this study

Gene ^a	Oligonucleotide sequence	Source of sequence	
		Reference(s)	Plasmid(s) ^b
H2b-I	GTCGAAGTCTCCACGATCCCTTC	26	
H2b-II	AGTGGCACAACGCGC	24, 26	
H2b-III	TGCTGAACACCGAACGAGCTCGCA	23	
H2b-IV	AGCTACAATGAGCTCGAATCAAC	23	
H2b-V	TGCTGAACACCGAACGAGCTCGCA	17	pCH11.5E
H2b-VI	TTCGAACACGTTTCGCTCCTAACCAC	23	
H2b-VII	CCTTAAAAGTTTCTTTCCAAGCTCT	23	
H2b-VIII 5' ^c	CGCGCACGCCGCTACTGCCTGTAC	This study	pStH2B-16
H2b-VIII 3' ^c	GTGAACCACTCTTTTACTGAAAGT	This study	pStH2B-1, pStH2B-16
H2a-II	GCGAGCGACTGAACACTCAGAGAG	26	
H2a-III	CGCAGCGACCGAACGTCCAA	23	pRR3c-3.5
H2a-IV	GGCAGTAACGACAGATTCAAAGCG	17	pCH11.5E
H2a-V	CGCAGCGACCGAACGTCCAA	17	pCH11.5E
H2a-VII	TCAGTCGCTCAGAAGCTCAA	55	
H2a-VIII	CACTTCGCCTGCAAGTCA	17	pCH22.0B
H2a-IX	TCAGTCGCTCAGAAGCTCAA	55	

^a H2b gene nomenclature is based on reference 23; H2a gene nomenclature is described in the legend to Fig. 5.

^b Where a plasmid is listed, the 5' or 3' untranslated sequence for that gene had not been published previously and was determined in this study from the plasmid or a subclone of a portion of the plasmid insert. In these cases the reference given describes the structure of the plasmid. Unpublished 5' and 3' sequences are available upon request.

^c 5' and 3' refer to the 5' H2b and 3' H2b oligonucleotide probes used in Fig. 4.

variant mRNAs that are transcribed and translated in avian spermatids. These haploid cells use a novel posttranscriptional processing pathway in which the hairpin structure and purine-rich element in the histone pre-mRNA do not function as signals for endonucleolytic cleavage. Instead, replication-dependent variant mRNAs expressed in spermatids retain both of these sequences and are polyadenylated at a more distal site. Our results suggest that the processing of replication-dependent transcripts by an alternative pathway can supersede the cell cycle regulation of these genes.

MATERIALS AND METHODS

Preparation of cell suspensions. For the isolation of an unfractionated testicular cell population, testes were removed from adult (24- to 52-week) White Leghorn chickens and decapsulated in Krebs buffer. The testes were minced and incubated with a solution containing 1 mg of trypsin per ml and 1 µg of DNase 1 per ml at 33°C for 20 min. After the cells were filtered through 80-µm-mesh Nytex, they were collected by centrifugation at $2,500 \times g$. For the isolation of a round spermatid cell population, these unfractionated cells were suspended in Krebs buffer containing 5% fetal calf serum and loaded at 10 ml/min into a Beckman JE-10X counterflow centrifuge rotor spinning at 1,600 rpm. Round spermatid cells were collected by increasing the flow rate to 45 ml/min, and a population enriched in spermatocytes was collected at 80 to 100 ml/min. Thymocyte cell suspensions were prepared as previously described (53).

Plasmids and hybridization probes. For histone protein-coding region probes, plasmid inserts were isolated by glass elution from agarose gels (54) and ³²P labeled by random oligonucleotide priming. The following plasmids were used: pCH42Δ4, with a 1.5-kilobase (kb) *EcoRI*-to-*AccI* insert subcloned from λCH5e (52) that includes the H1.01 gene; pCH70, with a 0.7-kb *XhoI* insert from λCH5e that contains the H2a-II gene; pH2bΔ4, with a 0.65-kb *XhoI*-to-*DraI* insert subcloned from pSP6H2B (31) that includes the H2b-II gene; pCH3dR1 (52), with a 1.3-kb *EcoRI* insert containing the H3 gene from λCH3d; and pCH22.0B (17), where a 1.0-kb *HindIII*-to-*PvuII* fragment that includes the H4 gene

nearest H2a-VIII was isolated from the plasmid. Oligonucleotide probes were ³²P end labeled with T4 polynucleotide kinase. Oligonucleotides used as hybridization probes in this study are listed in Table 1.

RNA extraction and Northern (RNA) blot analysis. RNA was prepared from cell suspensions or tissues by lysis in 5 M guanidinium isothiocyanate–25 mM sodium citrate (pH 7.2)–0.5% sarcosyl–0.1 M 2-mercaptoethanol and centrifugation through a 5.7 M CsCl–100 mM EDTA cushion in an SW55 Ti rotor at 35,000 rpm overnight (11). The RNA was suspended in H₂O, sequentially extracted with 1:1 phenol-chloroform and 24:1 chloroform-isoamyl alcohol, precipitated, and re-suspended in H₂O. Oligo(dT) chromatography was used to separate total cellular RNA into poly(A)⁺ and poly(A)[−] fractions as described previously (36). RNA samples were electrophoresed in 1.2% agarose-formaldehyde gels, transferred to nitrocellulose in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and baked at 80°C. Filters that were hybridized with ³²P-labeled restriction fragments were prehybridized at 42°C in Stark buffer containing 1% sodium dodecyl sulfate (SDS) and 0.5% powdered milk. Hybridization was performed overnight at 42°C, using 10⁶ cpm/ml in Stark buffer containing 10% dextran sulfate, 1% SDS, and 0.5% powdered milk. Filters were washed at 55°C in 0.1% SSC–0.1% SDS. If a ³²P-labeled oligonucleotide probe was used, the filters were prehybridized at the hybridization (T_H) of the oligonucleotide [$T_H = 2^\circ\text{C}(\text{A-T bp}) + 4^\circ\text{C}(\text{G-C bp}) - 5^\circ\text{C}$, where bp is base pairs] in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO₄ [pH 7.7], and 1 mM EDTA)–0.1% SDS–5× Denhardt solution. A total of 10⁶ cpm of the ³²P-oligonucleotide per ml in 6× SSPE–0.1% SDS–1× Denhardt solution–200 µg of tRNA per ml was hybridized at the T_H overnight. Filters were washed in 6× SSPE–0.1% SDS at room temperature.

Nuclear runoff transcription assays. Nuclei were isolated from either spermatid cells or exponentially growing chicken embryo fibroblasts (CEF) as previously described (25). Nuclear runoff transcription reactions, using spermatid or CEF nuclei, were performed as described by Linial et al. (34). Nitrocellulose filters with 5 µg each of the plasmids of

interest were prepared with a Schleicher & Schuell slot blot apparatus, using the procedure recommended by the manufacturer. An equal number of counts per minute from each type of nuclei was hybridized to the filters at 10^7 cpm/ml in 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]–1% SDS–10 mM EDTA–250 μ g of tRNA per ml–300 mM NaCl–1 $\times$ Denhardt solution–0.25% powdered milk at 65°C for 48 h. After hybridization the filters were washed in 0.1 $\times$ SSC–0.1% SDS at 65°C before autoradiography.

Polyribosome analysis. Polyribosomes were prepared and analyzed as described previously (6, 7), with minor modifications. Spermatids isolated by counterflow centrifugation were lysed by Dounce homogenization in 10 mM Tris hydrochloride (pH 7.5)–10 mM KCl–1.5 mM MgCl₂–0.2% Triton X-100–20 mM ribonucleoside-vanadyl complexes. The lysate was centrifuged at $10,000 \times g$ to remove nuclei. Polyribosomes were pelleted through 50 mM Tris hydrochloride (pH 7.4)–25 mM KCl–5 mM MgCl₂ with 1.3 and 2.0 M sucrose steps by centrifugation at 35,000 rpm overnight in an SW41 rotor. The polyribosome pellet was suspended in 50 mM Tris hydrochloride (pH 7.4)–250 mM KCl–25 mM MgCl₂, layered over a 10 to 50% continuous sucrose gradient with 50 mM Tris hydrochloride (pH 7.4)–250 mM KCl–25 mM MgCl₂, and centrifuged at 47,000 rpm in an SW55 Ti rotor for 1 h. *A*₂₅₄ was monitored, and fractions were collected directly into guanidinium isothiocyanate–25 mM sodium citrate (pH 7.2)–0.5% sarcosyl–100 mM 2-mercaptoethanol. RNA was prepared from the fractions as described previously (11). For puromycin dissociation of polyribosomes, the pellet from the step gradient was first suspended in 50 mM Tris hydrochloride (pH 7.4)–250 mM KCl–25 mM MgCl₂, then EDTA was added to a final concentration of 15 mM, and the KCl concentration was brought to 1 M. After the addition of 2 mM puromycin, the solution was incubated at 4°C for 15 min, followed by 37°C for 10 min. Subsequent treatment of the puromycin sample was identical to that used for intact polyribosomes.

DNA sequencing. The dideoxy-chain termination technique (47) was used, with denatured plasmid DNA as the template (28). A subclone containing the 5.4-kb *Eco*RI fragment from pCH22.0B (17) was the source of the H2b-VIII genomic sequence.

RESULTS

Novel poly(A)⁺ histone RNAs in spermatids. To examine histone gene expression during the haploid stages of avian spermatogenesis, we used counterflow centrifugation to isolate a cell population consisting of round spermatids from adult rooster testes. The purity of spermatid cells obtained by this method was assayed by morphological examination and fluorescence-activated cell sorter (FACS) analysis of the DNA staining profile of nuclei treated with propidium iodide (Fig. 1). Contamination of the spermatid fraction by tetraploid primary spermatocytes and all diploid cell types was less than 5%.

Total cellular RNA was isolated from the spermatid fraction, unfractionated adult testes, immature 5-week testes that have no postmeiotic cells, somatic thymocytes, and liver. These five RNA samples were separated on agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized separately to probes from the conserved protein-coding regions of H1, H2a, H2b, H3, and H4 (Fig. 2). For each RNA sample the steady-state levels of the poly(A)⁺ replication-dependent variant histone mRNAs that comprise the

FIG. 1. Representative cell volume and nuclear DNA content of unfractionated testes and spermatid cell populations. A cell suspension was prepared from adult rooster testes, and counterflow centrifugation was used to isolate a fraction highly enriched in round spermatids from the unfractionated cells. The cell volume in the two populations was measured with an electronic cell counter-sizer. Nuclei were prepared by lysing cells with 0.5% Nonidet P-40 in Krebs buffer, and the nuclear DNA content was determined by FACS analysis of nuclei stained with propidium iodide. Unfractionated testes contain tetraploid (4N) primary spermatocytes and a variety of diploid (2N) cells, including spermatogonia, Sertoli cells, erythrocytes, and interstitial cells. The purity of the spermatid fractions was consistently greater than 95%.

major histone transcripts of somatic cells are a function of the percentage of proliferating somatic cells in each population. Somatic replication-dependent histone mRNAs cannot be detected in spermatids (Fig. 2, lane 5), demonstrating that these transcripts are not synthesized after meiosis in the spermatogenic lineage. Similarly, minor somatic replacement variant histone mRNAs, such as the 1.3-kb H3 transcript in thymocytes (Fig. 2, lane 1), are also not found in spermatids. However, there are a number of larger histone RNAs in spermatids that hybridize to the H2a, H2b, and H3 probes. Each of these three probes detects a diffuse band at approximately 0.8 kb, or 300 nucleotides larger than the corresponding somatic mRNAs. In addition, spermatids also contain 1.8- and 2.5-kb H2a RNAs and a 1.4-kb H3 RNA.

To determine whether the novel spermatid histone RNAs are synthesized earlier in spermatogenesis, we attempted to purify primary spermatocytes by counterflow centrifugation. Although the spermatocyte fraction invariably includes multinucleated cells formed from the fusion of syncytial spermatids, the amount of spermatid histone RNAs observed in partially purified spermatocyte populations always correlated with the amount of contaminating spermatids (data not shown). Furthermore, two results shown in Fig. 2 demonstrate that the larger histone RNAs are expressed predominantly in spermatids. First, the spermatid histone RNAs are not found in immature 5-week testes (Fig. 2, lane 3) that contain spermatogonia and spermatocytes but not spermatids. Second, the steady-state levels of spermatid histone RNAs in the mixed cell populations from unfractionated adult testes (Fig. 2, lane 4) are consistently a function of the number of spermatids in the populations.

Unlike somatic replication-dependent variant histone mRNAs, the expression of spermatid histone RNAs is not coupled to DNA replication. Since the transcripts of several replication-independent replacement and tissue-specific variant histone genes in somatic cells are polyadenylated (20, 27, 32), we wanted to determine whether spermatid histone RNAs are similarly polyadenylated. Total cellular

FIG. 2. Novel histone RNAs in spermatids. Total cellular RNA was isolated using the guanidinium isothiocyanate-CsCl procedure (11), electrophoresed on a 1.2% agarose-formaldehyde gel, and transferred to nitrocellulose. 32 P-labeled hybridization probes were conserved protein-coding regions from H1, H2a, H2b, H3, and H4 genes (see Materials and Methods). Lane 1, Thymocyte cells; lane 2, liver tissue; lane 3, immature 5-week testis tissue; lane 4, unfractionated adult testis cells as shown in Fig. 1; lane 5, spermatid cells as shown in Fig. 1; lane 6, poly(A)⁻ RNA from unfractionated adult testis cells; lane 7, poly(A)⁺ RNA from unfractionated adult testis cells. Lane 7 was loaded with 1 μ g of RNA; all other lanes were loaded with 10 μ g. The integrity of all RNA samples was confirmed by hybridizing the filters with a 32 P-labeled glyceraldehyde-3-phosphate dehydrogenase probe from pGAD-28 (19). Autoradiograms of lanes 6 and 7 were exposed two times longer than autoradiograms of lanes 1 to 5. Size estimates of RNAs are given in kilobases.

RNA from adult testes was separated into poly(A)⁺ and poly(A)⁻ fractions by oligo(dT)-cellulose chromatography. Northern blot hybridizations of those RNA samples to the H1, H2a, H2b, H3, and H4 probes are shown in Fig. 2, lanes 6 and 7. Without exception, the spermatid histone RNAs are found in the poly(A)⁺ fraction, while the somatic histone mRNAs are restricted to the poly(A)⁻ fraction.

H2b mRNA is transcribed and translated in spermatids. To simplify the initial study of the structure, function, and regulation of spermatid histone RNAs, we focused on the single 0.8-kb H2b transcript, rather than the several H2a or H3 RNAs. The Northern blot experiments described above cannot exclude the possibility that spermatid H2b RNA is synthesized during the later stages of meiosis and is not the product of transcription in spermatids. Thus, we performed nuclear runoff transcription assays on spermatid nuclei to determine whether H2b RNA is synthesized in these post-meiotic cells. Total transcriptional activity, expressed as the incorporation of [32 P]UTP into RNA per haploid genome, is three- to fivefold lower in spermatids than in proliferating CEF. However, if the spermatid and CEF runoff products are normalized according to incorporated [32 P]UTP, the

FIG. 3. H2b transcription and translation in spermatids. (A) Runoff transcription assays performed on exponentially growing CEF and a population of spermatid cells. Nuclei were isolated, and 32 P-nuclear runoff products were synthesized as described in Materials and Methods. A total of 10^7 cpm from each runoff reaction was hybridized to identical nitrocellulose filters containing plasmids specific for the following genes: H2b, pH2B Δ 4 (see Materials and Methods); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pGAD-28 (19); β -actin, pA2 (13); β -tubulin, pT2 (13). (B) Localization of spermatid H2b mRNA on polyribosomes. Polyribosomes were isolated from spermatids and fractionated on a continuous 10 to 50% sucrose gradient as described in Materials and Methods (-P). Alternatively, the polyribosomes were dissociated by treatment with 2 mM puromycin before centrifugation on the continuous gradient (+P). The profile of the optical density at 254 nm (OD_{254}) of these gradients shows the locations of the polyribosome peaks in the +P sample and the dissociated ribosomes in the -P sample. RNA was prepared from fractions 1 to 12 by the guanidinium isothiocyanate-CsCl procedure (11) and blotted to nitrocellulose. This filter was hybridized to the 32 P-labeled H2b probe used in Fig. 2.

level of H2b transcription is the same in spermatids and the somatic cell control (Fig. 3A). Thus, although total transcriptional activity is lower in spermatids than in proliferating somatic cells, the proportional rate of H2b synthesis is similar.

To confirm that the 0.8-kb spermatid H2b transcript functions as an mRNA, polyribosomes were isolated by sedimentation through 2.0 M sucrose. These polyribosomes were centrifuged over a continuous 10 to 50% sucrose gradient, and total RNA was prepared from fractions collected across the gradient. Figure 3B shows the profile of the optical

A

ala lys ala lys ala arg ala lys gly gly lys gly arg gly ser met
GGCT CTT GGC CTT GGC GCG CCG CTT CCC GCC CTG CTT CCC GCG GCC CGA CAT
CACTTCGCCCTGCAAGTCAGCCCAAAAACAATCGAGAGACAGACTGAAGG 101
:λClI-01 H2A

CCAGAAGACTCGAAGAAATCCAGCAGTAACTCAGACACCGGCCCTTTTATGCTTCTGCTGGGGTCTGTCGGCACATTTGATTGGCGGAGACGGAGTAGTCGGGGTTAAACAATGGGAAC

AGCGGTAGAAATACCGAATTTGAACAGCGCGAGTAGTTTGCAGAGATGTCCTAATTGGGAGGAAACGACCTCGAGTCTCCTAATTGGCATACCGCCCTGTATAAGTACA 339

GGGGGGGGCCATTGTACCTGGTCGATCTGGGTACAGGCAGTACGGGGCGTGGCGG
 met pro glu pro ala lys ser ala pro ala pro lys lys gly ser
 ATG CCA GAA CCA CCG AAG TCG GCT CCT GCG CCT AAG AAG GGC TCC 443
 H2B consensus: --- --C --G --G --- --C --G --- --G --- ---
 pStH2B-16

lys lys ala val thr lys thr gln lys lys arg arg arg thr arg lys glu ser tyr ser ile tyr val tyr lys val
AAG AAG GCG GTC ACT AAG ACC CAA AAG AAG GGC GAC AAG AAA CGT AGA AAG ACG AGG AAA GAG AGC TAC TCG ATC TAC GTG TAC AAG GTG 533

▲-----C-----G-----G-----G-C-A-G---GC-C-C---G----- ----
 lys ser
pstH2B-1

leu lys gln val his pro asp thr gly ile ser ser lys ala met gly ile met asn ser phe val asn asp ile phe glu arg ile ala
CTG AAG CAG GTG CAC CCC GAC CCC ACG GGC ATC TCG TCC AAG GCC ATG GGC ATC ATG AAC GAC ATC TTC GAG CCC ATC GCC GGC GTC

gly glu ala ser arg leu ala his tyr asn lys arg ser thr ile thr ser arg glu ile gln thr ala val arg leu leu pro gly
GGC GAG GCG TCG CGC CGC TAC AAC AAG CGC TCG ACC ATC ACG TCG CGG GAG ATC CAG ACG GCC GTG CGG CTG CTG CCC GGC 713

glu leu ala lys his ala val ser glu gly thr lys tyr thr ser ser lys stop
GAG CTG GCC AAG CAC GCG GTC TCC GAG GGC ACC AAG CCG GTC ACC AGC TCC AAG TAG AGCGGTGGCATTACTCGATTTCACCCAAAG 810

symmetrical dyad purine-rich box
 GCTCTTTTCAGAGCCACCTACACCTTTTCAGTAAAGAGTGGTTCACAGATGCGTGTGGTGAGTGGCTATTTCATACTGCTCTTTGGTTTTTCAGAGTAGGTCGATCTGTTAAGGATGG 929
 * pStH2B-1 pStH2B-16

FIG. 4. Structure of a spermatid-expressed H2b gene. (A) Nucleotide sequences of the cDNA inserts in pStH2B-1 and pStH2B-16 and the genomic locus encoding those cDNAs, H2b-VIII. The H2b-VIII sequence was determined from subclones of the insert in pCH22.0B (17). For simplicity, only the genomic sequence is shown, and the extents of the pStH2B-1 and pStH2B-16 cDNA inserts are indicated by arrowheads. pStH2B-1 and pStH2B-16 sequences are identical to the genomic H2b-VIII sequence, except for a T at nucleotide 737 in pStH2B-16. The 3' ends of the pStH2B-1 and pStH2B-16 inserts (at nucleotides 855 and 857) have poly(A) tracts of 77 and 115 nucleotides, respectively. The H2b-VIII protein-coding region is compared with an H2b consensus sequence based on the seven H2b genes described in reference 23. Similarly, the H2a-VIII gene at this locus (nucleotides 1 to 51) is compared with H2a-II, the H2a gene in the genomic clone λ CH-01 as described in reference 26. All 5' and 3' conserved elements that are shared with the seven sequenced replication-dependent H2b genes are boxed and named. The position of the hyphenated symmetrical dyad and purine-rich box predicts that the 3' end of a poly(A)⁻ H2b mRNA transcribed from this locus would occur at nucleotide 829 (indicated by an asterisk). Regions homologous to oligonucleotide probes described in Table 1 and used in panel C and Fig. 5 are underlined. (B) Schematic illustration of the sequences shown in panel A. Somatic mRNA refers to the poly(A)⁻ transcript that would result from cleavage at the 3' end of the hairpin structure. Only the cDNA inserts from pStH2B-1 and pStH2B-16 are shown. Vertical lines in protein-coding regions indicate differences with the H2a-II or H2b consensus sequences. bp, Base pairs. (C) 5' and 3' H2b oligonucleotide hybridizations to somatic and spermatogenic cellular RNAs. The H2b-VIII 5' and 3' oligonucleotides shown in Table 1 and panels A and B were hybridized to Northern blots of the following RNAs: lane 1, thymocyte cells; lane 2, liver tissue; lane 3, immature 5-week testis tissue; lane 4, unfractionated adult testis cells; lane 5, spermatid cells. RNA size estimates are as in Fig. 2.

density at 254 nm of the gradient and the RNA fractions hybridized to the H2b coding sequence probe. Clearly, the majority of the spermatid H2b mRNA is associated with polyribosomes. This association is not due to H2b RNP particles that cosediment with the polyribosomes, since treatment with puromycin releases spermatid H2b mRNA from the polyribosome region of the gradient (Fig. 3B).

Spermatid H2b mRNA structure. The selection of spermatid H2b mRNA by oligo(dT)-cellulose suggested that inserts representing this transcript would be present in a cDNA library made by oligo(dT) priming of the first cDNA strand synthesis on the total adult testis poly(A)⁺ RNA template. When 10⁵ recombinant bacteriophage from such a library were screened by hybridization to the H2b coding sequence probe, more than 30 positive plaques were obtained. Inserts from these phage were subcloned into pGEM2. The sequences of the cDNA inserts from two of the plasmids, pStH2B-1 and pStH2B-16, were determined and are shown in Fig. 4A and B. Except for one nucleotide, the 412 nucleotides where the pStH2B-1 and pStH2B-16 sequences overlap are identical.

Based on Southern blot analysis, chickens have between 9 and 11 H2b genes (23, 46). Eight of these genes are located in the two large genomic clusters that include almost all of the replication-dependent variant H1 and core histone genes (17, 23; see Fig. 5). Seven of the eight clustered H2b genes have been sequenced (23), and none of these seven genes encodes the spermatid H2b cDNA. However, the protein-coding sequence of the spermatid H2b cDNA is very similar to the other H2b genes. Relative to a consensus of the other H2b coding sequences, the spermatid cDNA has 19 base substitutions, 2 of which result in conservative amino acid changes (Fig. 4A). All 19 of the substitutions are located in the first third of the coding sequence, including 8 in the first 10 codons.

The 3' untranslated regions of the seven sequenced H2b genes are not conserved, except for the two elements required for the posttranscriptional processing reaction that generates the 3' ends of mature H2b mRNAs (i.e., the 26 nucleotides, including the hairpin structure immediately 5' of the cleavage site, and the 10-nucleotide purine-rich box located eight bases 3' of the cleavage site). In contrast, the polyadenylation of other RNA polymerase II transcripts is dependent on an AATAAA hexamer upstream of the poly(A) addition site (43). Therefore, we were surprised to find that the 3' untranslated region of the poly(A)⁺ spermatid H2b mRNA includes a perfect hairpin and purine-rich box in the correct positions for normal H2b 3' cleavage. Furthermore, the poly(A) addition sites, located 9 and 11 nucleotides 3' of the purine-rich box in pStH2B-1 and pStH2B-16, respectively, are not preceded by an AATAAA sequence. Poly(A)⁺ spermatid H2b mRNA is the first example of an endogenous histone mRNA where both the hairpin and purine-rich box are retained in the mature processed transcript and do not direct 3' cleavage.

A single gene encodes both somatic and spermatid H2b mRNAs. As described above, the structure of spermatid H2b mRNA closely resembles the seven sequenced replication-dependent H2b genes, except that during posttranscriptional processing of the spermatid transcript poly(A) addition has been substituted for the conventional histone 3' cleavage. Thus, the gene encoding spermatid H2b mRNA might also be expressed in other germ line or somatic cells where poly(A)⁻ replication-dependent H2b transcripts are detected. To test this possibility, an oligonucleotide probe to a 25-nucleotide sequence in the 5' untranslated region of the

spermatid H2b mRNA was synthesized (Fig. 4 and Table 1). This probe hybridized to a single 5.4-kb band on a Southern blot of chicken genomic DNA digested with *EcoRI-HindIII* (data not shown). When the oligonucleotide was hybridized to a Northern blot of RNA isolated from spermatids, unfractionated adult testes, 5-week testes, thymocytes, and liver, the 0.8-kb band in spermatid RNA was detected, confirming that the pStH2B-16 cDNA sequence is representative of spermatid H2b mRNA (Fig. 4C). This 5' H2b oligonucleotide probe also detects an RNA in thymus, 5-week testes, and adult unfractionated testes that is 0.5 kb long, or the same size as somatic replication-dependent H2b mRNA. Therefore, while 0.8-kb poly(A)⁺ H2b mRNA is the only H2b mRNA synthesized in spermatids, 0.5-kb poly(A)⁻ transcripts from the same gene are made in a number of somatic and germ line cells.

To determine whether the 0.5-kb RNA transcribed from the "spermatid" H2b gene is generated by conventional histone 3' processing based on the position of the hairpin and purine-rich box, the RNAs described above were also hybridized to an oligonucleotide probe that begins three nucleotides 3' of the putative cleavage site in the 0.5-kb RNA and ends at the poly(A) addition site in the 0.8-kb spermatid mRNA (Fig. 4 and Table 1). Although this 3' H2b oligonucleotide includes the 10-nucleotide purine-rich box that is conserved in other H2b genes, it functions as a unique sequence probe when hybridized at stringent conditions based on the *T_H* of the complete 24-nucleotide sequence. The 3' oligonucleotide probe hybridizes to the 0.8-kb spermatid H2b mRNA, but does not detect 0.5-kb H2b transcripts in total RNA from somatic or spermatogenic cells (Fig. 4C). This confirms that the 0.5-kb poly(A)⁻ transcript does not extend beyond the 3' cleavage site predicted by the locations of the hairpin structure and purine-rich box.

Genomic organization of spermatid-expressed H2b genes. While the gene encoding spermatid H2b mRNA expresses a conventional H2b mRNA in somatic cells, it remains transcriptionally active in spermatids. The continued transcription of this gene in haploid cells could occur because the promoter structure is different from the seven sequenced replication-dependent H2b genes. However, the 5.4-kb genomic restriction fragment that contains the spermatid-expressed H2b gene (see above) is the same size as a fragment that includes the remaining unsequenced H2b gene located in one of the two clusters of replication-dependent histone genes (i.e., H2b-VIII in references 23 and Fig. 5). The H2b-VIII gene and flanking regions were originally isolated in a 22.0-kb *BamHI* genomic fragment in pCH22.0B (17). We subcloned the 5.4-kb fragment from pCH22.0B and found that the 5' H2b oligonucleotide probe hybridizes to a single 150-bp fragment from an *RsaI* digest of the subclone (data not shown). We sequenced the H2b-VIII gene and flanking regions (Fig. 4A) and observed that the cDNA inserts in pStH2B-1 and pStH2B-16 are in perfect agreement with the genomic H2b-VIII sequence, confirming that this locus is the source of spermatid mRNA. The 5' flanking region of H2b-VIII includes four CCAAT boxes, the octamer-binding transcription factor (OTF-1) binding element ATTTGCATA, and a TATAA box, all located at the same conserved positions previously described for the other replication-dependent H2b genes in the two histone clusters. No other repeated sequences or dyad symmetries occur in the region upstream of the gene.

The absence of any obvious structural features that distinguish the spermatid-expressed H2b-VIII gene from other members of the H2b gene family suggested that the other

FIG. 5. H2b and H2a gene-specific oligonucleotide hybridizations to somatic and spermatogenic RNAs. The oligonucleotide probes listed in Table 1 were hybridized to Northern blots of the following RNAs: lane 1, thymocyte cells; lane 2, unfractionated adult testis cells; lane 3, spermatid cells. Each Northern blot is joined by a line to the H2a or H2b gene that is recognized by the oligonucleotide probe. Open arrows show the location and direction of transcription for all H2a and H2b genes in the two clusters of replication-dependent histone genes. Closed arrows locate H1, H3, and H4 genes in these regions. Expression in spermatids (+) or the absence of expression in spermatids (–) is noted for each gene. *, Not determined. Genomic maps are from references 17 and 23. The nomenclature of H2b genes is from reference 23. H2a genes are assigned names that agree with the H2b gene in H2a/H2b pairs. For example, the H2a gene next to H2b-VIII is referred to as H2a-VIII.

H2b genes might also be transcribed in spermatids. Oligonucleotide probes specific for the 5' untranslated region of each H2b gene were synthesized (this region is identical in H2b-III and H2b-V, so an oligonucleotide probe cannot distinguish between expression from one or both of these genes), and each probe was hybridized to Northern blots of total cellular RNA from spermatids, unfractionated adult testes, and thymocytes. Although the somatic cells contain 0.5-kb transcripts from all seven genes, four of the seven (H2b-I, H2b-II, H2b-IV, and H2b-VII) do not produce detectable levels of H2b mRNA in spermatids (Fig. 5). However, the two probes specific for H2b-VI and H2b-III+H2b-V both hybridize to 0.8-kb spermatid H2b mRNA. Including H2b-VIII, three or four of the eight H2b genes in the histone clusters are expressed in spermatids. The genomic maps in Fig. 5 show that the spermatid-expressed H2b genes are not grouped together in one of the clusters. Furthermore, there are no conserved 5' sequence elements

that are restricted to either the subset of spermatid-expressed H2b genes or the subset of H2b genes that are silent in spermatids.

Coordinate expression of H2b/H2a pairs in spermatids. Although there are no obvious conserved structural features that define the subset of H2b genes expressed in spermatids, this subset is restricted to members of divergently transcribed, head-to-head H2b/H2a gene pairs (Fig. 5). Some of the regulatory elements in the approximately 250 base pairs that separate the transcription units in a pair are probably shared (26) and so affect the expression of both genes. Therefore, if transcriptional regulation is the mechanism by which a specific H2b gene is selected for expression in spermatids, the H2a gene in that pair may be coordinately transcribed in spermatids. To test this possibility, oligonucleotide probes to the 5' untranslated regions of seven of the nine H2a genes in the histone clusters were hybridized to Northern blots of spermatid, unfractionated adult testes, and

thymocyte RNA (Fig. 5). No transcripts were detected in spermatid RNA by probes for unpaired H2a genes (H2a-VII and H2a-IX) or for H2a genes in pairs where the H2b gene is not expressed in spermatids (H2a-II/H2b-II and H2a-IV/H2b-IV). However, in those pairs where the H2b gene is spermatid expressed (H2a-VIII/H2b-VIII and H2a-III+H2a-V/H2b-III+H2b-V), the H2a-specific probes hybridized to 0.8-kb spermatid H2a RNAs. The 1.8- and 2.5-kb spermatid H2a RNAs shown in Fig. 2 are not transcribed from any of the seven H2a genes that we examined. These larger RNAs are probably products of H2a genes that are not located in the two clusters of replication-dependent histone genes.

DISCUSSION

In this study we report that avian spermatids contain a number of novel poly(A)⁺ histone RNA species, and we present a detailed analysis of the 0.8-kb class of spermatid H2b mRNA. The haploid expression of H2b mRNA is unique in several respects. First, the source of spermatid H2b mRNA appears to be replication-dependent H2b genes. In somatic tissues this type of histone variant is only expressed at high levels in proliferating cells and is transcriptionally and posttranscriptionally regulated such that steady-state mRNA levels are greatest during the S phase of the mitotic cell cycle (29). Thus, each spermatid-expressed H2b gene also produces 0.5-kb poly(A)[−] transcripts in proliferating thymocytes (Fig. 5), but does not generate detectable mRNA in nonmitotic tissues such as liver (Fig. 2). While the expression of replication-dependent histone variants is coupled to DNA replication in somatic cells, the synthesis of spermatid H2b transcripts clearly does not require DNA replication. There are a number of possible conditions that would result in the efficient transcription of replication-dependent H2b genes in spermatids, including the presence of dominant spermatid-specific positive regulatory factors or the absence of somatic negative regulatory factors. Alternatively, these genes may simply be transcribed in spermatids at a basal level equivalent to that observed in somatic cells outside the S phase. A second component of the cell cycle regulation of replication-dependent histone variants in somatic cells is the U7 snRNP-mediated 3' processing of the pre-mRNAs (22, 35); this would not occur in spermatid cells, where the synthesis of poly(A)⁺ H2b mRNAs must involve a different processing pathway. A final point of regulation for replication-dependent histones in somatic cells is the rapid and specific degradation of the mRNA outside the S phase (49). Spermatid cells may lack this activity and consequently accumulate H2b mRNA, or since the cell cycle-specific degradation of histone mRNA begins at the 3' end of the transcript and requires the terminal hairpin structure (33, 41, 45), the polyadenylation of spermatid H2b mRNA could interfere with degradation.

Only three or four of the eight replication-dependent H2b genes in the chicken histone clusters are expressed in spermatids, and at least 60% of spermatid H2b mRNA consists of transcripts from a single gene, H2b-VIII. In contrast, all eight genes are expressed in proliferating thymocytes, and the level of steady-state mRNA from an H2b gene in thymocytes generally is not indicative of the amount of mRNA that the same gene will produce in spermatids. Is the expression of a subset of H2b genes in spermatids entirely a consequence of the selective transcription of those genes? Certainly the coordinate regulation of both members of the H2b/H2a gene pairs in spermatids (Fig. 5) is difficult to explain by invoking posttranscriptional mechanisms. How-

ever, the basis for the selective transcription of specific H2b genes is not evident in the structures of the 5' regions flanking the genes. Common sequence elements that could function as *cis*-acting regulators of transcription do not occur upstream of either the spermatid-expressed genes or those genes that are silent in spermatids. Another factor that could contribute to the relative representation of specific H2b transcripts in spermatids is the differential stability of individual H2b mRNAs. Since the only stable histone mRNAs found in spermatids are polyadenylated, a major determinant of the steady-state mRNA level for a particular H2b gene could be how efficiently the primary transcript is polyadenylated (see below).

The synthesis of H2b poly(A)⁺ mRNAs in spermatids requires a biosynthetic pathway that differs in several respects from the posttranscriptional processing of poly(A)[−] transcripts from the same genes in somatic cells. Most important, poly(A)[−] products of the U7 snRNP-mediated cleavage reaction are not observed in spermatids, even though spermatid H2b mRNA includes all of the *cis*-acting sequences necessary for this reaction. This could be due to a spermatid-specific polyadenylation activity that competes effectively with the U7 snRNP-mediated cleavage. In this context it is interesting that chimeric mouse H3 genes transfected into mouse L cells produce a minor poly(A)⁺ transcript that extends 250 nucleotides beyond the hairpin loop structure (1, 12). Since longer poly(A)⁺ mRNAs from the endogenous H3 gene have never been detected, it appears that a cryptic downstream polyadenylation site is occasionally utilized when the introduction of large numbers of chimeric H3 genes into the genome results in less efficient 3' cleavage. If a similar competition between 3' cleavage and polyadenylation exists in avian spermatids, the rate of the polyadenylation reaction must be much greater than the rate of the cleavage reaction to account for the fact that poly(A)[−] histone mRNAs can not be detected in these cells. Indeed, the simplest explanation for the total absence of any poly(A)[−] histone transcripts in spermatids is a deficiency in some component of the U7 snRNP-mediated cleavage activity during the postmeiotic stages of spermatogenesis.

The H2b-VIII cDNAs that we sequenced represent the products of histone pre-mRNA processing without 3' cleavage in spermatids. The termini of these cDNAs have a poly(A) tract beginning 26 or 28 nucleotides 3' of the predicted H2b-VIII poly(A)[−] mRNA terminus. This characteristic distinguishes avian spermatid poly(A)⁺ histone mRNAs from the poly(A)⁺ histone mRNAs that accumulate in amphibian oocytes (2, 10). The termini of histone transcripts in *Xenopus laevis* oocytes have up to 20 adenine residues added to the point where conventional 3' cleavage, based on the location of the hairpin structure, normally occurs. Thus, while posttranscriptional processing of histone mRNAs in the oocyte apparently consists of a U7 snRNP-mediated cleavage followed by *de novo* poly(A) synthesis, this cleavage reaction is not a prerequisite for the polyadenylation of histone transcripts in spermatids.

Although conventional polyadenylation of RNA polymerase II transcripts requires an AATAAA hexanucleotide 10 to 29 nucleotides 5' of the poly(A) addition site (37, 43), this sequence does not occur in any of the H2b genes that encode poly(A)⁺ mRNAs in spermatids. A change in the sequence requirements for polyadenylation during the postmeiotic stages of spermatogenesis could account for at least some of the genes that have been shown to produce spermatid-specific transcripts with lengths different from those of the corresponding somatic mRNAs, such as *c-mos*, *c-abl*, *N-*

TABLE 2. Polyadenylation signal sequences in H2b 3' untranslated regions

Gene	Spermatid expression	3' Untranslated sequence ^a	
		Purine box	Downstream YGTGTTY
H2b-VIII	+	CAGTAAAAGA	GTGGTTCACAGATGCGTGTGGTGA
H2b-VI	+	CAATGAAAGA	GCTTTAACACAGCAGTTC CGTGT
H2b-III	+ ^b	CAATGAAAGA	GCTTTAATTACCGTGTTTAGTGTT
H2b-V	+ ^b	CCATGGAAGG	GCTAAAGCACTCGGTG GTGATATTT
H2b-I	—	TAATAAAAGG	GCTGTATTACTTTTTTCTTTTTTT
H2b-II	—	CAGAGAAAGA	GCTGTAGTCATATCAGAAGAAAATA
H2b-IV	—	CAGTAAAAGA	GCTAAAGCGCTGATACTAGCCTGTA
H2b-VII	—	CTTACAAAGG	GCTGTAATTGCTTTTTTTTCTCTCC

^a Except for H2b-VIII, which was sequenced in this study, 3' untranslated sequences are from reference 23. AATAAA-like and YGTGTTY-like elements are underlined. The points of poly(A) addition from the pStH2B-1 and pStH2B-16 cDNAs are indicated by arrows. Gaps between the purine-rich boxes and downstream sequences indicate the 3' end of the boxes and do not represent a discontinuity in the sequences.

^b As stated in Results, it was not possible to determine whether one or both of the H2b-III and H2b-V genes are expressed in spermatids. The absence of an AATAAA-like element in the H2b-V purine-rich box predicts that transcripts from this gene would not be polyadenylated in spermatids. This would result in the spermatid-specific expression of H2b-III, and not H2b-V, poly(A)⁺ mRNA.

ras, and *pim-1* (reviewed in references 42 and 44). Specifically, the 4.0-kb RNA that is the predominant *c-abl* transcript in mouse spermatids is the result of polyadenylation at an alternative site 1.2 kb upstream of the poly(A) addition site that produces the 5.0- and 6.0-kb somatic *c-abl* mRNAs (38). The *c-abl* spermatid polyadenylation site is not preceded by an AATAAA element, but it does have a TGTGTTTT sequence that agrees with the YGTGTTY consensus that is found approximately 30 nucleotides downstream of the AATAAA sequence in a majority of mammalian genes (37).

Given that a perfect AATAAA element is not required for polyadenylation in spermatids, it is interesting that the consensus for the H2b purine-rich box, CAATGAAAGA, includes an AATGAA hexamer that differs from the AATAAA sequence by a single nucleotide. This implies that the same purine-rich sequence motif might function not only to bind U7 small nuclear RNA during 3' processing in somatic cells, but also to locate a more downstream endonucleolytic cleavage site (39) that defines the point of poly(A) addition in spermatids. For H2b-VIII the purine-rich box is CAGTAAAAGA; this places an AGTAAA element 12 or 14 nucleotides upstream of the spermatid poly(A) addition sites. H2b-VIII also has a CGTGTTGG sequence in the correct location to function as a YGTGTTY element. Similarly, the spermatid-expressed H2b-III and H2b-VI genes both have an AATGAA sequence in the purine-rich box and a downstream YGTGTTY-like element, while the H2b-II and H2b-VII genes, which are silent in spermatids, do not have these two elements (see reference 23 and Table 2 for a comparison of H2b 3' untranslated sequences). A hexamer in the purine-rich box that closely resembles the AATAAA sequence is not sufficient to predict which H2b gene will be expressed in spermatids; the H2b-I and H2b-IV purine-rich boxes include AATAAA and AGTAAA sequences, respectively, but these genes are not expressed postmeiotically. However, H2b-I and H2b-IV do not have a downstream YGTGTTY sequence, and of the eight H2b genes, only those that encode spermatid poly(A)⁺ mRNAs have both AATAAA-like and YGTGTTY-like sequences. Thus, it is possible that both elements are required for the efficient polyadenylation of H2b pre-mRNAs, indicating that the 3' untranslated region is a major factor in selecting the H2b genes that will ultimately be expressed as stable poly(A)⁺ mRNAs in spermatids.

By determining the structure of the 0.8-kb spermatid-specific H2b mRNA, we have defined an alternate 3' pro-

cessing pathway that can account for the synthesis of poly(A)⁺ transcripts from replication-dependent H2b genes in these quiescent cells. The generality of this pathway in spermatids is suggested both by the absence of any poly(A)[−] histone mRNAs and by the presence of 0.8-kb poly(A)⁺ H2a and H3 transcripts. In particular, the fact that 0.8-kb H2a RNAs are also transcribed from a subset of replication-dependent genes (Fig. 5) strongly suggests that the H2b 3' processing pathway is also used for the H2a transcripts. While the H2a and H2b (and probably H3) 0.8-kb RNAs are all products of replication-dependent genes, the source of the 1.8-kb H2a, 2.5-kb H2a, and 1.4-kb H3 spermatid-specific poly(A)⁺ RNAs is not known. The larger H2a RNAs are not transcribed from any of the seven replication-dependent H2a genes that we examined (Fig. 5). Therefore, these H2a and H3 transcripts probably originate from dispersed genes and may represent true testis-specific variants. Recently, we have discovered a mouse H2b gene, with several features that are characteristic of tissue-specific histone variants, that is only expressed in spermatids (S. B. Moss, P. B. Challoner, and M. Groudine, submitted for publication). For example, transcripts from this gene lack the terminal hairpin structure and have an AATAAA hexamer 16 nucleotides upstream of a poly(A) tract. Although a number of possible functions can be envisioned for both replication-dependent and tissue-specific histone proteins in spermatids, an understanding of the biological significance of histone synthesis during the postmeiotic stages of spermatogenesis awaits further study.

ACKNOWLEDGMENTS

We thank J. Dodgson and J. Wells for contributing the avian genomic histone clones; A. Hein, K. Hudson, and M. Peretz for valuable technical assistance; and H. Eisen, A. Kirsh, N. Hutchinson, and H. Sive for useful discussions and a critical reading of the manuscript.

This work was supported by National Science Foundation grant DCB 8502591. P.B.C. is a special fellow of the Leukemia Society of America. S.B.M. is a recipient of National Institutes of Health New Investigator Research Award 1R23 HD20460.

ADDENDUM IN PROOF

Recently, Sturm et al. (R. A. Sturm, S. Dalton, and J. R. E. Wells, *Nucleic Acids Res.* 16:8571–8586, 1988) have determined the sequence of 198 nucleotides between the H2b-VIII and H2a-VIII genes. Their sequence corresponds

to, and is in agreement with, nucleotides 140 to 338 in our Fig. 4A.

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