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Differential Expression of Individual Members of the Histone Multigene Family Due to Sequences in the 5′ and 3′ Regions of the Genes

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Histone proteins are encoded by a multigene family. The H3.2(614) and H2a(614) genes are present as single copies which are expressed at high levels, accounting for 30 to 40% of the H3 and H2a mRNAs, respectively, in different types of mouse cells. The other genes which have been isolated each contribute only a very small amount to the total type-specific mRNA pool. We demonstrate here that the differences in the level of expression of these genes are partly due to differences in their transcription rates. To investigate the sequences responsible for these differences in expression among the members of each family, we carried out DNA-mediated gene transfer experiments with both intact and chimeric histone genes. The 5′ region of a highly expressed gene [H3.2(614) or H2a(614)] was attached to the 3′ region of a histone gene which was expressed at low levels (H3-221 or H2a-291) and vice versa. The results show that sequences in both the 5′ and 3′ regions of the H3.2(614) and H2a(614) genes contribute to their high level of mRNA production by two independent mechanisms. The effect of the 3′ sequences on mRNA accumulation has been narrowed to a 65-base-pair region including the 3′-terminal palindrome and downstream signal implicated in mRNA processing.

The histone proteins are highly conserved in evolution. The five classes of histone proteins are encoded by multiple copies of genes in all species examined. In higher eucaryotes, there are a number of nonallelic variants of each histone protein which are encoded by distinct histone genes organized randomly in clusters (7, 12, 15, 27). Most histone genes and mRNAs from various organisms have common structural features, including the absence of intervening sequences and the presence of a 3′-terminal stem-loop structure in place of a polyadenylated terminus (3, 15). The expression of these genes is coordinately regulated at both the transcriptional and posttranscriptional levels (1, 8, 14, 24, 26, 29).

In mice there are about 10 genes coding for the replication-type H3 proteins (13, 17) and probably a similar number of genes coding for each of the other core histone proteins. These genes encode a small set of protein variants which differ by a few amino acids (33). The functional significance of these multiple copies of histone genes is not known (12). Graves and co-workers have isolated four H3 genes, four H2a genes, and three H2b genes (13). All but one of these genes are expressed in cultured mouse cells, the exception being an H2a pseudogene (13, 20). These genes are found on two clusters located on mouse chromosomes 3 and 13 (22).

The several genes which code for each type of histone protein are highly homologous in the coding region but differ significantly in the untranslated regions, producing mRNAs which can be distinguished by S1 nuclease analysis (13, 28). The single-copy H3.2(614) and H2a(614) genes located on chromosome 3 are expressed at high levels, accounting for 30 to 40% of the H3 and H2a mRNAs, respectively, in different mouse cells (13). The other isolated genes located on chromosome 13 contribute less than 5% each to the total type-specific mRNA pool (13).

To investigate the basis for the differences in the level of expression among the individual members of each family, we carried out DNA-mediated gene transfer experiments with cloned genes. Plasmids containing the highly and poorly expressed mouse histone genes with various amounts of 5′- and 3′-flanking sequences, as well as chimeric genes, were introduced into Chinese hamster ovary (CHO) cells, and the steady-state level of mRNA produced from the transferred genes was compared. The results show that sequences in the 5′ region, presumably affecting the transcription rate, as well as the 3′ region, involved in mRNA processing, contribute to the differences in mRNA accumulation.

MATERIALS AND METHODS

Cell culture and gene transfer. CHO cells were maintained in modified Eagle medium supplemented with 10% fetal bovine serum. CHO cells were cotransfected with 150 ng of pHSV2neo DNA (30) and 15 μg of plasmid DNA containing mouse histone genes by the Polybrene method of Chaney et al. (5). In each experiment, several pools of G418-resistant cells, each derived from an average of 30 G418-resistant clones, were selected in medium containing G418 (400 μg/ml; GIBCO Laboratories) and expanded for analysis.

Plasmid construction. pH614 is a 6-kilobase (kb) *Eco*RI fragment containing both the H2a(614) gene and the H3.2(614) gene from MM614 (Fig. 1) cloned into pUC9 (13, 32). Two subclones of pH614 were made that each contained only one of the histone genes. pH3.2-614(XbaI-BglII) is a subclone of pH614 containing the H3.2(614) gene with 520 base pairs (bp) of 5′-flanking sequences and 320 bp of 3′-flanking sequences; pH2a-614(SstII-XbaI) is a subclone of pH614 containing the H2a(614) gene with 270 bp of 5′-flanking sequences and 320 bp of 3′-flanking sequences; and pH2a-614(EcoRI-HpaII) is a subclone of pH614 containing the H2a(614) gene with only 40 bp of 3′-flanking sequence. pH3.1(221) is a 2.4-kb *Eco*RI fragment containing the entire H3.1(221) gene from MM221 (Fig. 1) cloned into pACYC184. This fragment contains 185 bp of the 5′-flanking sequences

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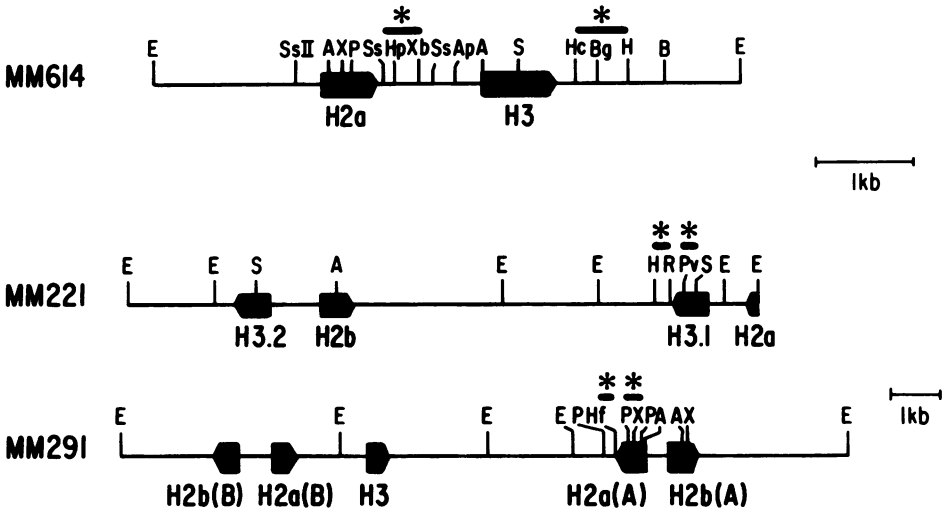

FIG. 1. Mouse histone gene clusters. The location and orientation of the genes on the three recombinant phages MM221, MM291, and MM614 are shown. The bars with asterisks above the maps indicate the coding region fragments and the flanking region fragments that were cloned in both orientations into M13 vectors. Single-stranded recombinant phage DNAs containing the flanking regions were used to measure transcription of the specific genes, while recombinant phage DNAs containing the coding regions were used to measure transcription from all histone genes of that class. Abbreviations: Ap, *Apa*I; A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; Hf, *Hin*fI; Hp, *Hpa*II; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sal*I; Ss, *Sst*I; SsII, *Sst*II; Xb, *Xba*I; X, *Xho*II.

and 1.7 kb of the 3'-flanking sequence (28). pH2a2b-291A is a 1.8-kb *Eco*RI-*Hin*dIII fragment containing the entire H2a(291A) gene and the entire H2b(291A) gene from MM291 (Fig. 1) cloned into pUC9 (13, 20). Chimeric H3 and H2a histone genes used in this study are shown in Fig. 5. pH3(614,221) was constructed by joining the *Pst*I-*Sal*I fragment of MM614 to the *Sal*I-*Hin*dIII fragment of MM221. The 5' region of the H3.2(614) gene and the 3' region of the H3.1(221) gene were joined at the common *Sal*I site at codon 58. The construction of this chimera was described previously (6). pH3(221,614) is a fusion of the 5' end of the H3.2(221) gene and the 3' end of the H3.2(614) gene. This chimeric gene was constructed by ligating the *Ava*I-*Sal*I fragment from MM221 [extending from codon 92 of the H2b(221) gene to the *Sal*I site at codon 58 of the H3.2(221) gene containing all the DNA between these genes] to the *Sal*I site at codon 58 of the H3.2(614) gene (the *Sal*I-*Hin*dIII fragment from MM614). A chimeric histone H2a gene, designated pH2a(614,291), was made by joining the 5' end of the H2a(614) gene and the 3' end of the H2a(291A) gene at the common *Ava*I site at codon 20. This chimera was constructed by ligating the *Eco*RI-*Ava*I fragment from MM614 [ending at the *Ava*I site at codon 20 of the H2a(614) gene] to the *Ava*I-*Eco*RI fragment from MM291, which contains the 3' end of the H2a(291A) gene and 420 bp of the 3'-flanking sequences. pH2a(291,614) is a fusion of the 5' end of the H2a(291A) gene and the 3' end of the H2a(614) gene. This chimeric H2a gene was constructed by ligating the *Ava*I-*Ava*I fragment from MM291 [extending from codon 92 of the H2b(291A) gene to the *Ava*I site at codon 20 of the H2a(291A) gene] to the *Ava*I-*Hin*dIII fragment from MM614 [containing the 3' end of the H2a(614) gene and the entire H3.2(614) gene]. pH2a(291,614SL) is a fusion of the entire coding region of the H2a(291A) gene and the stem-loop sequence of the H2a(614) gene. A 65-bp fragment (*Sst*I-*Hpa*II) containing the H2a(614) stem-loop and downstream signal (23) was attached to the 3' untranslated region of H2a(291A) at the *Hin*fI site.

RNA isolation and analysis. Total cellular RNA was isolated by phenol extraction at 60°C as described previously (16). S1 nuclease mapping with end-labeled probes was performed as described previously (13, 29). DNA was labeled at the 5' end with [γ-^{32}P]ATP and T4 polynucleotide kinase or at the 3' end with [α-^{32}P]dCTP and avian myeloblastosis virus reverse transcriptase. RNA was incubated with the probe at 58°C, and the S1-resistant DNA fragments were analyzed on 6% polyacrylamide gels in 8 M urea. The dried gels were then autoradiographed. The relative amount of each DNA fragment was determined by scanning the autoradiographs with a Quantimet analyzer.

Run-on transcription in isolated nuclei. Isolation of nuclei and the conditions for RNA synthesis and purification of nuclear RNA have been described previously (1, 29). [α-^{32}P]UTP-labeled RNA was hybridized at 42°C in a reaction mixture containing 0.3 M NaCl, 1 mM EDTA, 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.4), 0.2% sodium dodecyl sulfate (SDS), and 50% formamide to cloned single-stranded DNA that was immobilized on nitrocellulose. The filters were washed at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and then treated with RNase A at 37°C for 1 h as previously described (1, 29). The RNA-DNA hybrids were detected by autoradiography. The relative intensity of each band was determined by scanning the autoradiographs with a Quantimet analyzer. These values were normalized per 100 bp of the probe and corrected for uridine content to yield the relative transcription rate of each of the genes.

RESULTS

Differences in transcriptional activity of individual histone genes. The three clusters of mouse histone genes isolated by Marzluff and co-workers (22, 28) are shown in Fig. 1. The genes in these clusters were used to construct subclones in the pUC9 plasmid for DNA-mediated gene transfer. The genes contained in phages MM221 and MM291 are present on mouse chromosome 13 (28). MM221 contains two H3 genes, each of which codes for only a minor fraction of the H3 mRNA pool, an H2b gene, and part of an H2a gene (13). Phage MM291 contains two H2a genes, two H2b genes, and

an H3 gene (20). Each of these genes codes for a minor portion of their type-specific mRNA pools (13). The insert in phage MM614 mapped to mouse chromosome 3 and contained an H3 gene and an H2a gene. Both of these genes, designated H3.2(614) and H2a(614), respectively, were expressed at high levels, accounting for 30 to 40% of the total H3 and H2a mRNAs, respectively, in different mouse cell lines (4, 13, 20).

The large difference in the steady-state mRNA levels produced from the individual genes could be due to differences in several parameters, including rates of transcription, efficiencies of posttranscriptional processing and transport, and mRNA half-lives. To determine whether the differences in the steady-state mRNA levels were due to differences in transcription, we measured the relative transcription rate of several specific genes by nuclear run-on experiments in isolated nuclei (1, 6).

Since the coding regions of each class of histone genes are highly homologous, it was necessary to identify a unique transcribed region for each gene. Although the 5′ and 3′ untranslated mRNA sequences are quite divergent and can be used to distinguish the mRNAs in S1 nuclease protection experiments, they are too short to be used for transcription measurements. Earlier, we showed that transcription of the H3.1(221) gene normally extends at least 500 nucleotides (nt) past the 3′ end of the mRNA, and subsequently we have shown that several other mouse histone genes are transcribed several hundred nucleotides past the 3′ end of the mRNA (6; unpublished results). Since the 3′ transcribed spacer regions are unique to each gene, these regions were used as probes to measure the transcription of the individual gene. Figure 1 shows the restriction maps of the three histone gene clusters, indicating the 3′-flanking fragments of the H3.1(221), H3.2(614), H2a(291A), and H2a(614) genes used to measure their specific transcription as well as the coding region fragments used to measure the combined transcription from all of the H3 or H2a genes.

The specific transcription of the individual genes was measured relative to total H3 and H2a transcription. Run-on transcription reactions were carried out in nuclei isolated from exponentially growing mouse erythroleukemia (MEL) cells. Nuclei were incubated in the presence of [α-^{32}P]UTP, and the newly synthesized DNA was isolated and hybridized to M13 phage single-stranded recombinant DNAs bound to nitrocellulose filters (Fig. 2). As expected, most of the newly synthesized RNA was complementary to the anticoding strand of the DNA probes. The relative amount of hybridized radioactivity was determined by quantitative densitometry of the autoradiogram. These values were normalized for differences in the lengths of the mouse DNA inserts and expressed relative to the respective coding regions (Fig. 2). The transcription rates of the highly expressed H3.2(614) and H2a(614) genes constituted between 15 and 25% of total H3 and H2a transcription, respectively. Likewise, the transcription rates of the H3.1(221) and H2a(291) genes represented less than 5% of total H3 and H2a transcription, respectively. Thus, the difference in the steady-state mRNA levels among the two histone genes of each type in MEL cells was due in part to differences in the transcription rate of the individual genes. However, the observed differences in the transcription rates only accounted for a part of the difference in the steady-state mRNA levels, since the steady-state levels differed by about 20-fold, whereas the transcription rates differed by 3- to 8-fold. The transcription rates measurements may be subject to some error due to differences among the genes in the rate at which polymerase

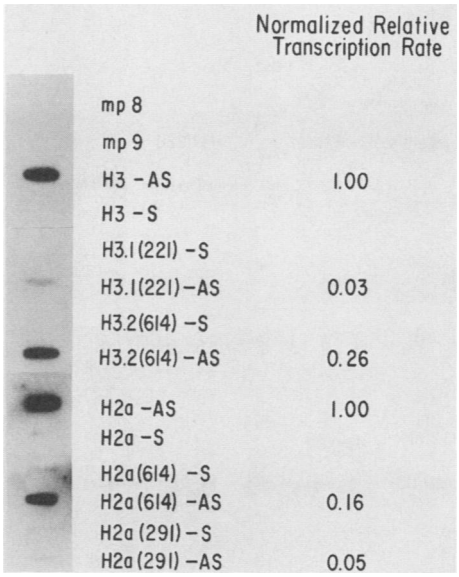

FIG. 2. Transcription of endogenous histone genes in isolated nuclei. Isolated MEL cell nuclei were incubated with [α-^{32}P]UTP, and the RNA synthesized in vitro was hybridized with M13 single-stranded recombinant DNAs (2 μg per slot) bound to nitrocellulose filters. The antisense strands of the H3 coding fragment (207-nt *Sal*I-*Pvu*II), and the H2a coding fragment (180-nt *Pst*I-*Pst*I) were cloned into M13mp8, while the antisense strands of fragments from H3.1(221) (291-nt *Rsa*I-*Hin*dIII), H3.2(614) (277-nt *Hin*cII-*Hin*dIII), H2a(614) (345-nt *Sst*I-*Xba*I), and H2a(291A) (240-nt *Hin*fI-*Pst*I) were cloned into M13mp9. RNA (10^7 cpm) was hybridized to the coding and flanking fragments separately. The relative transcription rate of each coding region was arbitrarily assigned a value of 1. AS, Antisense strand; S, sense strand.

molecules terminate beyond the 3′ end of the genes. However, the difference in transcription rate and mRNA level was very pronounced in the case of the H2a genes, and this suggests that there are also differences among the genes in the efficiency of some posttranscriptional process.

Expression of mouse H3 genes after gene transfer. To identify the regions within the mouse histone genes that were responsible for differences in mRNA production, we carried out DNA-mediated gene transfer experiments with the cloned genes and various constructs made from the 5′ and 3′ parts of the genes. These DNAs were introduced into CHO cells. Although the coding sequences of the mouse and hamster histone genes are highly homologous, the 5′ and 3′ untranslated mRNA regions are divergent. The mouse DNA probes used to detect mouse histone mRNAs also hybridized with the coding regions of the hamster histone mRNAs, producing smaller protected fragments that did not include the hamster untranslated region sequences (Fig. 3A, lanes 4 and 9, and Fig. 4A, lanes 3 and 6). These protected fragments served as a convenient internal control for the amounts of mouse histone mRNAs produced in independent transfection experiments.

CHO cells were cotransfected by a Polybrene transfection protocol (5) with one of several mouse H3 genes and the plasmid pSV2neo, conferring resistance to G418. In each transfection experiment, several large pools of transfected cells, each containing an average of 30 G418-resistant clones, were isolated. By using large pools of stable transfectants, we were able to measure expression of the transferred histone genes averaged over many integration sites, eliminating the potential effects of individual integration sites

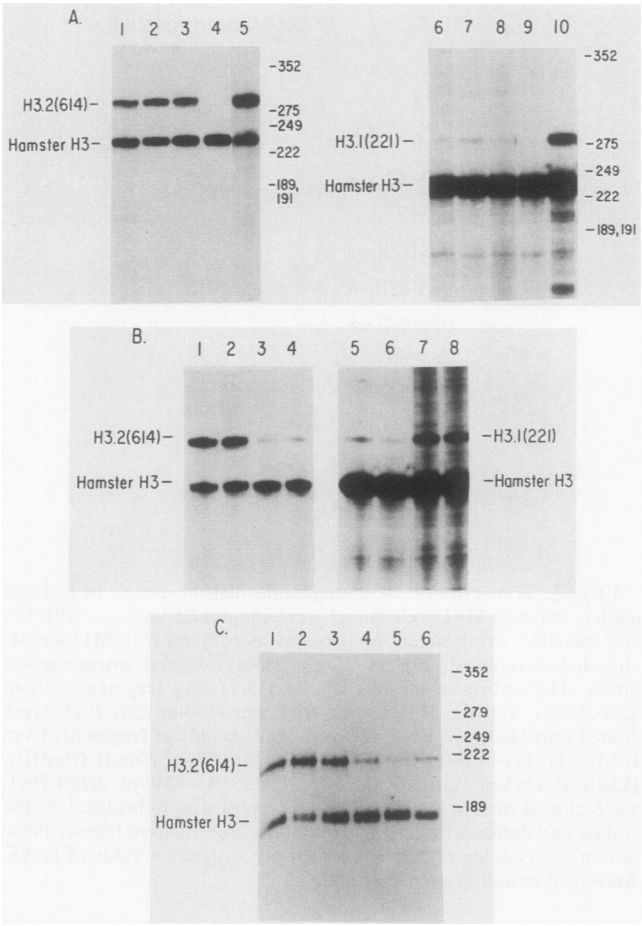

FIG. 3. S1 nuclease analysis of transcripts from mouse histone H3 genes transfected into CHO cells. (A) Transcripts from intact H3.2(614) and H3.1(221) genes. RNA was prepared from several mass cultures of CHO cells transfected with either the H3.2(614) gene (lanes 1 to 3) or the H3.1(221) gene (lanes 6 to 8). RNA was prepared from untransfected CHO cells (lanes 4 and 9) and from mouse Ltk$^-$ cells (lanes 5 and 10). RNA (20 μg) was hybridized with H3.2(614) DNA labeled at the 3′ end of the *Sal*I site at codon 58 (lanes 1 to 5) or with H3.1(221) DNA labeled at the 3′ end of the *Sal*I site at codon 58 (lanes 6 to 10). The hybrids were treated with S1 nuclease, and the resistant DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis and detected by autoradiography. The radioactive protected fragments observed were as follows: H3.2(614), protection to the end of the H3.2(614) mRNA; H3.1(221), protection to the end of the H3.1(221) mRNA; and hamster H3, protection to the termination codon of a number of different endogenous hamster H3 mRNAs. The 240-nt protected fragments in lanes 5 and 10 were due to protection to the TAA termination codon of mouse H3 mRNAs other than H3.2(614) and H3.1(221) respectively. (B and C) Comparison of mRNA produced from chimeric H3 constructs. The RNAs were hybridized with the appropriate probes, and the S1 resistant fragments were analyzed as above. (B) RNA (20 μg) was hybridized with H3.2(614) DNA 3′-end labeled at the *Sal*I site at codon 58 (lanes 1 to 4) or with H3.1(221) DNA 3′-end labeled at the *Sal*I site at codon 58 (lanes 5 to 8). The RNAs were isolated from cells transfected with the following DNA constructs: lanes 1 and 2, H3.2(614) contained on the *Eco*RI fragment; lanes 3 and 4, the chimeric H3(221,614) gene; lanes 5 and 6, H3.1(221); and lanes 7 and 8, the chimeric H3(614,221) gene. The protected fragments are as in panel A. (C) RNA (10 μg) was hybridized with H3.2(614) DNA labeled at the 5′ end of the *Sal*I site at codon 58. The RNAs were isolated from cells transfected with the following DNA constructs: lanes 1 to 3, H3.2(614); lanes 4 to 6, chimeric H3(614,221). The protected fragments were: H3.2(614), protection to the 5′ end of the mRNA from the transfected mouse gene; hamster H3, protection to the initiation codon of endogenous hamster H3 mRNAs. For each of the transfected genes, a minimum of four pools of transfected cells were analyzed, though only two or three of each are shown here.

on gene expression. Southern blot analysis was done on all of these pools and showed that each contained an average of 3 copies of the transferred gene per cell, with a variability of about 30% (data not shown).

Initially we transfected intact copies of the mouse histone genes to determine whether the differences in mRNA production measured from endogenous genes in mouse cells were also observed after transfection of cloned DNAs in CHO cells. Figure 3A shows the results of S1 nuclease protection experiments designed to measure the amount of mRNA produced from transfected copies of the highly expressed H3.2(614) gene and the poorly expressed H3.1(221) gene. In each case the 3′-end-labeled probe from the specific mouse gene produced two protected DNA fragments after hybridization with transfected cell RNA. The larger fragments terminated at the correct position at the 3′ end of the specific mouse histone mRNA (compare with mouse cell RNA, lanes 5 and 10), indicating that the mouse mRNAs were processed correctly in CHO cells. S1 nuclease protection experiments with 5′-end-labeled probes from the specific mouse genes also showed that the mRNAs were initiated at the same sequence as the genes in mouse cells (Fig. 3C). The smaller protected fragments were the result of hybridization with the coding regions of CHO H3 mRNAs (compare with CHO cell RNA, lanes 4 and 9). These experiments showed that the H3.2(614) gene (Fig. 3A, lanes 1 to 3) was expressed at much higher levels than the H3.1(221) gene (Fig. 3A, lanes 6 to 8) after transfection into CHO cells. Likewise, the level of mRNA produced from another poorly expressed MM221 H3 gene [H3.2(221)] after introduction into CHO cells was similar to the low level of expression observed from the H3.1(221) gene (data not shown). The difference in the relative activity of the transferred H3.2(614) gene compared with the two genes from MM221 was about 20-fold (Table 1), similar to the differences observed from these genes in mouse cells.

To determine whether the differences in the amount of mRNA produced from these three genes were due to sequences in the 5′ or 3′ region of the genes, we analyzed expression of two chimeric genes constructed from their 5′ and 3′ regions by fusing the genes at a common *Sal*I site at codon 58 in the coding region (Fig. 5). mRNA production from the intact, highly expressed H3.2(614) gene was compared with that of a chimeric gene, designated H3(221,614) in which the 5′ region of H3.2(614) was replaced with the 5′ region of H3.2(221). The mRNA produced from the two genes was assayed with a 3′-end-labeled probe from H3.2 (614), corresponding to a region which is shared by the two genes. Expression of the intact H3.2(614) gene (Fig. 3B, lanes 1 and 2) was about five times greater than that of the chimera H3(221,614) (Fig. 3B, lanes 3 and 4). A similar comparison was made between the intact, poorly expressed H3.1(221) gene and a chimera designated H3(614,221), in which the 5′ region of H3.1(221) was replaced with the 5′ region of H3.2(614). Expression of the chimera (Fig. 3B, lanes 7 and 8) was about five times greater than that of the intact H3.1(221) gene (lanes 5 and 6). We conclude from these comparisons that sequences 5′ of the *Sal*I site at codon 58 in the H3.2(614) gene are partially responsible for the high level of expression of this gene.

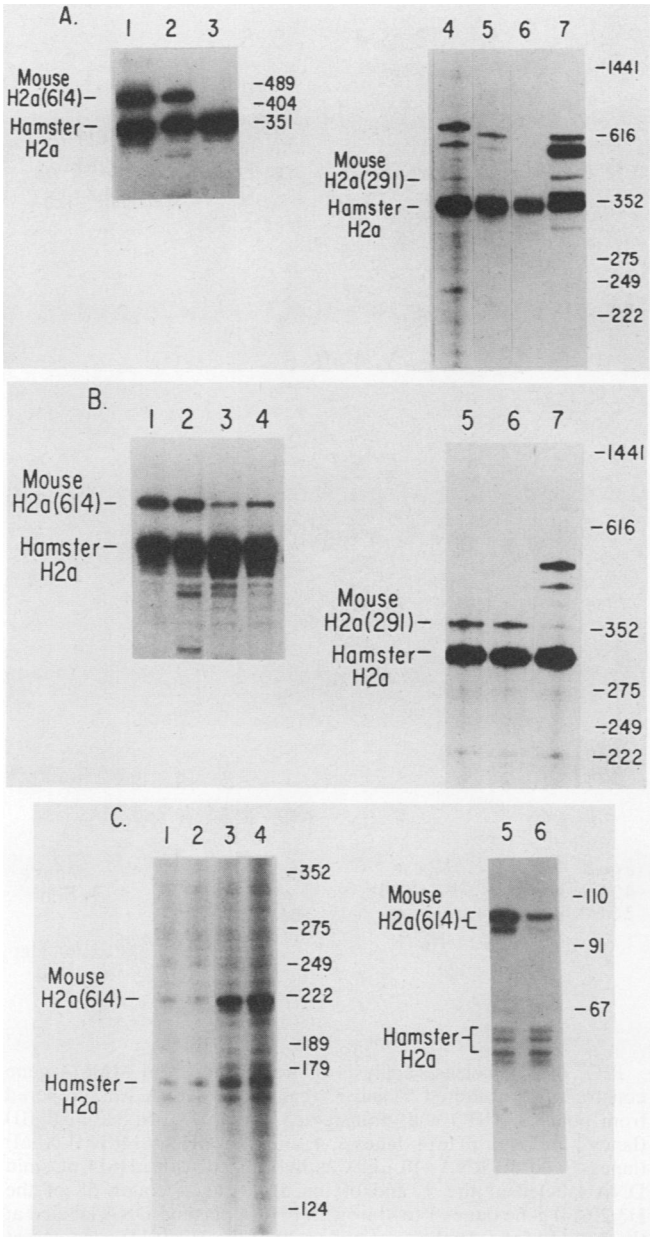

FIG. 4. S1 nuclease mapping of transcripts from transfected H2a genes. (A) Transcripts from the intact H2a(614) and H2a(291) genes. RNA was prepared from several mass cultures transfected with the H2a(614) gene (lanes 1 and 2) and the H2a(291A) gene (lanes 4 and 5). Control RNAs were prepared from untransfected CHO cells (lanes 3 and 6) and mouse Ltk$^-$ cells (lane 7). RNA (20 μg) was hybridized with pH614 plasmid DNA labeled at the 3′ end of the *Ava*I site at codon 20 of the H2a(614) gene (lanes 1 to 3) or with H2a(291A) DNA labeled at the 3′ end of the *Ava*I site at codon 20 (lanes 4 to 7). The S1-protected fragments were analyzed as described in the legend to Fig. 3. The radiolabeled protected fragments observed were as follows: mouse H2a(614), protection to the end of the H2a(614) mRNA; mouse H2a(291A), protection to the end of the H2a(291) mRNA; and hamster H2a, protection to the termination codon of a number of different endogenous hamster H2a mRNAs. The fragments greater than 500 nt in lanes 4, 5, and 7 (and panel B, lane 7) are renatured fragments of the H2a(291A) probe and were present in different amounts in different samples. Lane 4 is from a different gel in which the bands in the 600-bp range were separated better. (B and C) Comparison of mRNAs produced from intact and chimeric H2a genes. RNA from transfected CHO cells was hybridized with the appropriate probes, and the S1 nuclease-resistant fragments were analyzed as above. (B) RNA (20 μg) was hybridized with pH614 digested with *Ava*I and 3′-end labeled (lanes 1 to 4) or with H2a(291A) labeled at the 3′ end of the *Ava*I site (lanes 5 to 7). The RNAs were isolated from cells transfected with the following DNA constructs: lanes 1 and 2, the pH614 plasmid which contains both the H2a(614) and the H3.2(614) genes; lanes 3 and 4, the chimeric H2a(291,614) gene; lanes 5 and 6, the chimeric H2a(614,291) gene; and lane 7, the pH2a2b-291A plasmid. The protected fragments are as in panel A. (C) RNA (10 μg) was hybridized to H2a(614) DNA labeled at the 5′ end of the *Xho*II site at codon 61 (lanes 1 to 4) or with H2a(614) DNA labeled at the 5′ end of the *Ava*I site at codon 20 (lanes 5 and 6). The RNAs were isolated from cells transfected with the following DNA constructs: lanes 1 and 2, the chimeric H2a(614,291) gene; lanes 3 and 4, the pH614 plasmid; lane 5, the same pool of cells transfected with the pH614 plasmid as in lane 4; and lane 6, the same pool of cells transfected with pH2a(614,291) as in lane 1. The protected fragments are: mouse H2a, protection to the 5′ end of the mRNA from the transfected mouse gene; and hamster H2a, protection to the initiation codon of the endogenous hamster H2a mRNAs.

TABLE 1. Relative expression of H3 and H2a genes after gene transfer

Gene	No. of transfected pools analyzed	Relative mRNA production[a] (%)
H3.2(614)	8	100
H3(614,221)	6	25–27
H3(221,614)	4	19–21
H3.1(221) or H3.2(221)	8	5
H2a(614)	8	100
H2a(291,614)	4	26–34
H2a(614,291)	4	22–23
H2a(291A)	5	5

[a] Data were obtained by quantitation of autoradiographs like that shown in Fig. 3 and 4. The average mRNA production from the H3.2(614) and H2a(614) genes was set at 100%, and the range of values obtained from the indicated number of pools is shown.

The presence of the 5′ region of H3.2(614) in the chimeric H3(614,221) gene was not sufficient to obtain as high a level of expression as that of the intact H3.2(614) gene itself. Figure 3C shows an analysis of the mRNAs produced from transfectant pools containing either the intact H3.2(614) or the chimeric H3(614,221) gene with a 5′-end-labeled probe that is common to both genes. Expression of the intact gene (Fig. 3C, lanes 1 to 3) was about four times greater than that of the chimera containing the 3′ end of the H3.1(221) gene (lanes 4 to 6). This finding indicates that sequences 3′ of the *Sal*I site at codon 58 in the H3.2(614) gene also contribute to the high level of mRNA produced from this gene.

The relative activities of the three H3 genes and the two chimeras in transfectants are summarized in Table 1. The poorly expressed H3.1(221) and H3.2(221) genes each had about 5% of the activity of the H3.2(614) gene, similar to that observed from the endogenous genes in mouse cells. The two chimeras had activities between those of the intact genes. Thus, sequences both 5′ and 3′ of the *Sal*I site in the H3.2(614) gene are required for the high level of expression of this gene.

Expression of mouse H2a genes after gene transfer. Expression of two cloned mouse H2a histone genes was analyzed by a similar strategy. The highly expressed H2a(614) gene, the poorly expressed H2a(291A) gene, and two chimeric genes were cotransfected separately into CHO cells. The

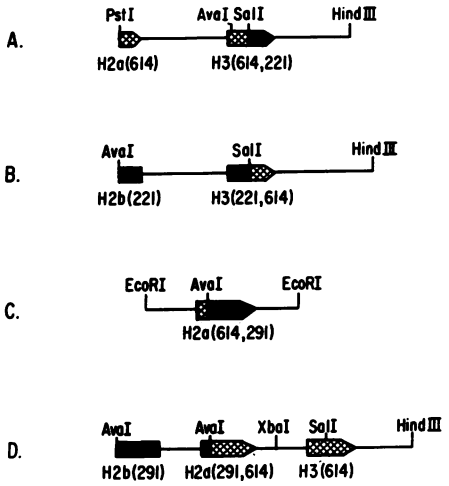

FIG. 5. Chimeric histone gene constructs. The figure shows the mouse DNA inserts cloned in plasmid vectors containing chimeric histone genes as follows: (A) H3(614,221); (B) H3(221,614); (C) H2a(614,291); and (D) H2a(291,614). These vectors were constructed as described in Materials and Methods. Hatched bars, specific histone mRNA sequences; straight line, flanking mouse DNA. The arrows indicate the direction of transcription of each gene.

two chimeric genes were constructed by joining the two different genes at a common *Ava*I site at codon 20 (Fig. 5). The results of S1 protection experiments with the mRNAs produced from these genes are shown in Fig. 4. The steady-state levels of mRNA expressed from the intact H2a(614) and H2a(291A) genes were measured by using specific 3′ probes end labeled at the *Ava*I site at codon 20 (Fig. 4A). The transfected H2a(614) gene (lanes 1 and 2) produced about 15 to 20 times more mRNA than the transfected H2a(291A) gene (lanes 4 and 5). As with the transfected H3 genes, this difference was similar to the difference observed between the two endogenous genes in cultured mouse cells (13).

To determine the contribution of sequences in the 5′ region of the highly expressed H2a(614) gene, we compared expression of the intact gene with that of a chimeric gene designated H2a(291,614) (Fig. 5). A direct comparison of the amounts of mRNA produced from these two genes was made by using a 3′-end-labeled probe from H2a(614) that lies in sequences shared by the two genes. Expression of the intact gene (Fig. 4B, lanes 1 and 2) was about three times greater than that of the chimera (lanes 3 and 4). The result of replacing the 5′ region of the highly expressed H2a(614) gene with that of the H2a(291A) gene was a reduction in expression. A similar comparison was made between the intact H2a(291A) gene and a chimera, H2a(614,291). Expression of the chimera (Fig. 4B, lanes 5 and 6) was about four times greater than that of the intact H2a(291A) gene (lane 7). These two sets of comparisons indicated that sequences 5′ of the *Ava*I site of the H2a(614) gene were involved in the high level of expression of this gene. However, like the H3.2(614) gene, these sequences were not sufficient to produce the highest level of mRNA accumulation. The effect of the sequences 3′ of the *Ava*I site in the H2a(614) was demonstrated directly by comparing other pairwise combinations in which 3′ substitutions were made. Figure 4C shows a comparison of the amount of mRNA produced from the transfected, intact H2a(614) gene (lanes 3 to 5) and the chimera, H2a(614,291) (lanes 1, 2, and 6), in which most of

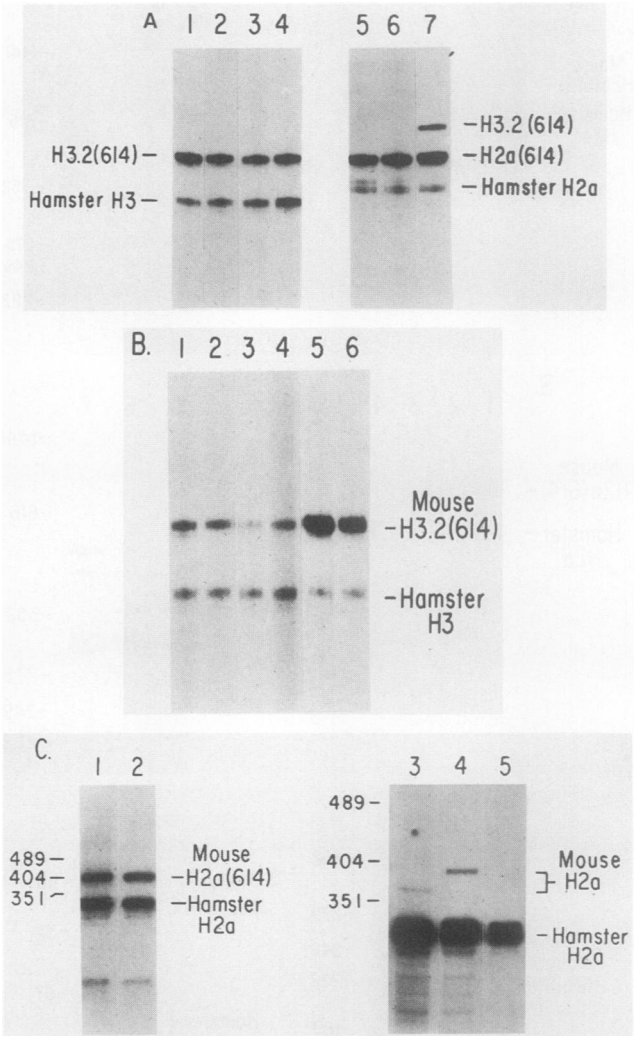

FIG. 6. S1 nuclease analysis of transcripts from MM614 gene constructs with altered 5′ and 3′ regions. (A) RNA was prepared from pools of CHO cells transfected with pH3.2-614(XbaI-BglII) (lanes 1 and 2) or pH614 (lanes 3, 4, and 7) or pH2a-614(SstII-XbaI) (lanes 5 and 6). RNA (10 μg) was hybridized with pH614 plasmid DNA labeled at the 3′ end of the *Sal*I site at codon 58 of the H3.2(614) gene (lanes 1 to 4) or with pH614 plasmid DNA labeled at the 3′ end of the *Ava*I site at codon 20 of the H2a(614) gene and at the 3′ end of the *Ava*I site at codon 1 of the H3(614) gene (lanes 5 to 7). The protected fragments are as described in the legends to Fig. 3 and 4. (B) RNA was prepared from pools of CHO cells transfected with pH3.2-614(SstI-HindIII) (lanes 1 and 2), pH3.2-614(ApaI-HindIII) (lanes 3 and 4), or pH614 (lanes 5 and 6). RNA (5 μg) was hybridized with pH614 plasmid DNA labeled at the 3′ end of the *Sal*I site at codon 58 of the H3.2(614) gene. The protected fragments are as described in the legend to Fig. 3. (C) RNA was prepared from CHO cells (lane 5) or from pools of CHO cells transfected with pH614 (lane 1), pH2a-614(EcoRI-HpaII) (lane 2), pH2a2b-291A (lane 3), or pH2a(291,614SL) (lane 4). RNA (10 μg) was hybridized with H2a(614) DNA labeled at the 3′ end of the *Ava*I site at codon 20 (lanes 1 and 2) or with H2a2b-(291A) DNA 3′-end labeled at the *Ava*I site (lane 3) or with pH2a(291,614SL) DNA 3′-end labeled at the *Ava*I site (lanes 4 and 5). The fragments labeled mouse H2a (lanes 3 and 4) correspond to protection by mRNA from the H2a(291A) and the H2a(291,614SL) genes, respectively. Other protected DNA fragments are as described in the legend to Fig. 4A.

the coding sequences and the 3′ region of the gene were replaced with the corresponding region from H2a(291A). Expression of the intact gene was approximately four times greater than the chimera. Thus, replacing the coding region and the 3′ end of the mRNA of the highly expressed H2a(614) with the corresponding region of the H2a(291A) gene resulted in a reduction in mRNA production. In other experiments, we showed that the chimeric H2a(291,614) gene was expressed about five times better than the H2a(291A) gene. These results imply that sequences 3′ of the *Ava*I site in the H2a(614) gene are also involved in the high level of expression of this gene.

To summarize the results with the H2a gene constructs, Table 1 shows the relative activity of the two H2a genes and the two chimeras. Similar to the comparisons made with H3 genes, the two H2a genes investigated here differed by 15- to 20-fold in their mRNA production, whereas both types of chimeras exhibited activities between those of the two intact genes. Here also, sequences both 5′ and 3′ of the *Ava*I site in the H2a(614) gene contributed to its high level of expression.

Further definition of the 5′ and 3′ sequences required for maximum expression. The proximity of the highly expressed H2a and H3 genes in the mouse genome (Fig. 1) suggested that the 6-kb *Eco*RI fragment in phage MM614 might contain a single enhancer sequence that stimulates transcription of both genes. Alternatively, each of these genes might contain a highly active promoter region. To distinguish between these possibilities, smaller fragments of the two genes containing 300 to 500 bp of 5′- and 3′-flanking sequences were cloned into separate plasmids. These plasmids were transfected into CHO cells, and expression of the genes was compared with their expression when transfected as part of the larger 6-kb *Eco*RI fragment in the pH614 plasmid. The H3.2(614) gene was expressed at the same level whether it was present on the 1.6-kb *Xba*I-*Bgl*II fragment (Fig. 1) or as part of the 6-kb *Eco*RI fragment containing both genes (Fig. 6A, lanes 1 to 4). A similar result was obtained with the H2a(614) gene. Its expression was the same whether it was present on the 1.4-kb *Sst*II-*Xba*I fragment (Fig. 1) or as part of the larger 6-kb *Eco*RI fragment in pH614 (Fig. 6A, lanes 5 to 7). We conclude that the possibility of a single enhancer sequence regulating expression of both genes is unlikely, although separate similar enhancers of each gene located within 500 bp on either side of the genes remains a possibility.

Two additional subclones of H3.2(614) with less 5′-flanking sequence from the start of transcription were constructed. pH3.2-614(SstI-HindIII) contains 283 nt of upstream sequence and pH3.2-614(ApaI-HindIII) contains 143 nt of upstream sequence. The relative activity of these genes was determined by S1 nuclease protection assays, measuring the amounts of mRNA produced from these constructs in at least five pools each of transfected cells. A decline in the level of expression of the H3.2(614) gene was apparent as its 5′ region was shortened. Removal of sequences between nt −500 and −283 [pH3.2-614(SstI-HindIII)] (Fig. 6B, lanes 1 and 2) and between nt −283 and −143 [pH3.2-614(ApaI-HindIII] (Fig. 6B, lanes 3 and 4) led to a reduction of expression to about 20 to 30% of the maximum level. The promoter region of the H3.2(614) gene contained a TATAA box at nt −68 and two CAAT boxes between nt −170 and nt −130 (32). Thus, it appears that the distal region upstream of the two CAAT boxes in the H3.2(614) gene is important for determining the high level of expression of this gene.

To define more precisely the sequences in the 3′ region required for maximum expression of the H2a(614) gene, two modified H2a genes were transfected into CHO cells, and mRNA production was analyzed in several pools of the transfected cells. pH2a-614(EcoRI-HpaII) is a subclone of H2a(614) with only 35 bp of sequence beyond the stem-loop at the 3′ end of the mRNA. mRNA production from this subclone was compared with that of the much larger H2a(614) gene fragment in pH614. Expression of these two H2a gene constructs was nearly the same (Fig. 6C, lanes 1 and 2), establishing the farthest 3′ boundary of the sequences required for high expression of H2a(614) at 35 bases beyond the end of the mRNA. To determine the 5′ border of the sequences required for high expression, we constructed the chimera pH2a(291,614SL). It contains the 5′ region and the entire coding region of H2a(291A) but with the H2a(291A) stem-loop replaced by a 65-bp fragment containing the last 30 bases of the 3′ untranslated region, including the stem-loop and 35 bases of the 3′-flanking sequences from H2a(614). Expression of H2a(291,614SL) was compared with the intact H2a(291A) gene. H2a(291,614SL) (Fig. 6C, lane 4) produced about four times as much mRNA as H2a(291A) (lane 3) relative to the internal standard provided by hamster H2a mRNA. Other results showed that the amount of mRNA produced from H2a(291,614SL) was similar to that from the H2a(291,614) chimera, in which the two genes were fused at the *Ava*I site at codon 20 (data not shown). This result shows that the activity of the relatively weak H2a(291A) gene can be increased simply by replacing its stem-loop region with a small region from the 3′ end of H2a(614). Thus, a 65-bp region (*Sst*I-*Hpa*II) from the H2a(614) gene, containing only the information necessary for 3′-end formation, provided the 3′ sequence information required for the high level of expression of this gene.

DISCUSSION

All the genes which have been examined in this study code for replication-type variants which are coordinately regulated during the cell cycle (8), yet these genes are expressed at very different levels (13). These differences are largely due to differences in rates of mRNA biosynthesis (11; unpublished results). The linked H3.2(614) and H2a(614) genes located on mouse chromosome 3 are expressed at much higher levels than the other H3 and H2a genes located on chromosome 13. We have shown here, by directly measuring nuclear run-on transcription, that the H3.2(614) and H2a (614) genes, located on the MM614 cluster within 1 kb of each other, are transcribed at higher rates than the other H3 and H2a genes located in the MM221 and MM291 clusters from chromosome 13.

To investigate the differences in mRNA production among the members of each family, we carried out DNA-mediated gene transfer experiments. The results showed that the H3.2(614) and H2a(614) genes contained on the 6-kb *Eco*RI fragment of MM614 were expressed at about 20 times the level of other H3 and H2a genes, comparable to the ratio observed from endogenous genes in mouse cells. However, we also demonstrated that the highly active H3.2(614) and H2a(614) genes were expressed equally well when transfected as individual genes with a few hundred base pairs of flanking regions. Thus, it seems unlikely that their activities are the result of a single enhancer sequence regulating the expression of both genes. Instead, it seems more plausible that *cis* control elements located close to each of these genes are responsible for their high level of mRNA production. In fact, the results of the transfection experiments with the chimeric H3 and H2a genes demonstrated that there were

control regions lying in both the 5′ and 3′ regions of these genes. The contributions of the 5′ and 3′ regions to their high level of expression are independent of one another, and the combination of the two effects accounts for the maximum level of expression observed with the genes on MM614.

The coding regions of the genes in each class of histone proteins are highly conserved, but the sequences in the flanking regions are extremely divergent (20, 28, 32; Marzluff, unpublished results). The 5′-flanking sequences of each of the genes located on chromosomes 3 and 13 revealed the existence of the typical RNA polymerase II consensus sequences, CCAAT and TATAA boxes. Otherwise, the 5′-flanking regions of the genes on chromosome 3 were dramatically different from the corresponding regions of the genes on chromosome 13. The sequence of the 5′ region of both H3.2(614) (32) and H2a(614) (unpublished results) genes shows the presence of multiple consensus binding sites for the Sp1 transcription factor (9, 18). These GC-rich sequences are not present in the 5′ region of any of the H3 and H2a genes located on chromosome 13 (20, 30). The H3.2(614) gene contains six of these GC boxes, four that have nine bases homologous to the decanucleotide consensus sequence and two that have 8 of 10 bases homologous. Deletion of the 5′ region of the H3.2(614) gene containing the GC boxes resulted in decreased mRNA production. Further experiments are in progress to determine whether one or more of these GC-rich sequences contribute to the high transcription rate of the H3.2(614) gene.

The sequence of the 3′ untranslated regions of all of these H3 and H2a mouse histone genes revealed little similarity except for the region containing the hairpin loop near the end of the mRNA and the conserved AAAGA sequence in the 3′-flanking region (20, 28, 32). Within this highly conserved region, present in all eucaryotic histone genes, is the site of processing that creates the 3′ end of histone mRNAs (3, 10, 25). The results of the gene transfer studies with chimeric H3 and H2a genes demonstrate that this region makes an important contribution to the level of mRNA accumulation. These sequences could act by stimulating the rate of transcription. However, because of the role of this region in mRNA processing and the fact that differences in transcription rate do not fully account for the observed differences in mRNA accumulation, we think it is more likely that the 3′ control sequences affect mRNA production by determining the efficiency of mRNA 3′-end formation. In the case of the H2a(614) gene, we have defined a 65-bp region including the stem-loop sequence as the essential 3′ element contributing to the high level of expression of this gene. This region also corresponds to the 3′ processing signal region in the mouse histone H4 gene, which Stauber et al. (31) have demonstrated to be the minimal sequences required for processing of the mRNA. More recently, Luscher and Schumperli (21) have shown that RNA 3′ processing is the posttranscriptional event responsible for the regulation of histone gene expression. Thus, the 65-bp region at the 3′ end of the histone gene is involved in three types of regulation: amount of expression of different histone genes, synthesis of mRNA during the cell cycle (21), and mRNA stability (19, 23).

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