Regulated Expression of a Chimeric Histone Gene Introduced into Mouse Fibroblasts

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The regulated expression of a mouse histone gene was studied by DNA-mediated gene transfer. A chimeric H3 histone gene was constructed by fusing the 5' and 3' portions of two different mouse H3 histone genes. Transfection of the chimeric gene into mouse fibroblasts resulted in the production of chimeric mRNA at levels nearly equal to that of the total endogenous H3 histone mRNAs. Most chimeric RNA transcripts had correct 5' and 3' termini, and the chimeric mRNA was translated into an H3.1 protein that accumulated in the nucleus of the transfected cells. Expression of the chimeric gene was studied under several conditions in which the rate of transcription and the stability of endogenous H3 transcripts change. Chimeric mRNA levels were regulated in parallel with endogenous H3 mRNAs, suggesting that *cis*-acting regulatory sequences lie within or near individual histone genes. In addition to correctly initiated and terminated chimeric mRNA, we also detected a novel H3 transcript containing an additional 250 bases at the 3' end. Surprisingly, the longer transcript is polyadenylated and accumulates in the cytoplasm.

Histone genes provide an attractive system to study the factors involved in the control of gene expression because they constitute a multigene family whose expression is regulated coordinately during the mitotic cell cycle. The histone genes from several organisms have been isolated and their sequences determined. With some exceptions, histone genes and mRNAs from many organisms share common structural features, including the absence of intervening sequences and the presence of a 3'-terminal stem-loop structure rather than the polyadenylate [poly(A)] tail present on most eucaryotic mRNAs (7, 13, 14). Recent evidence suggests that the 3' end of histone mRNAs is formed by a posttranscriptional cleavage mechanism in several nonmammalian organisms (2, 11, 19, 24). There are about 20 genes coding for the replication-type H3 histone proteins in mouse cells (9) and probably an equal number for each of the other core histone proteins as well. Graves and co-workers have isolated 10 histone genes, including 4 H3 genes, from three different genomic clusters and have shown that each of the genes is expressed in cultured mouse cells (5a). Thus, for each type of histone protein there is a family of coordinately regulated mRNAs which contains common coding region sequences. However, these RNAs differ in the 5' and 3' untranslated regions, and the sequences flanking the genes show little homology with one another (13).

The level of histone mRNAs in growing cells is regulated periodically with DNA synthesis during the mitotic cell cycle (1, 3, 6, 18). When cells are treated with drugs that inhibit DNA synthesis, the level of histone mRNAs declines rapidly (6, 23). Studies in several laboratories have shown that this coordinate regulation of histone mRNA levels occurs through a combination of transcriptional and posttranscriptional controls (1, 3, 6). Thus, very likely there are nucleotide sequences or structures in both the DNA and histone mRNA transcripts that are recognized by cellular factors

controlling histone gene expression. One approach to identifying these regulatory sequences is to modify cloned histone genes and introduce them into cells. We describe here experiments demonstrating that a histone gene introduced into cells by DNA-mediated gene transfer is subject to the same controls operating on endogenous histone genes. A novel mouse histone gene was constructed by fusing the 5' and 3' regions of two different H3 histone genes. Upon introduction into mouse fibroblasts, the H3 mRNA produced from the chimeric gene is readily distinguished from normal mouse histone mRNAs by S1 nuclease mapping. Expression of the chimeric gene was studied under several conditions in which the levels of endogenous histone mRNAs change. Chimeric mRNA production was regulated coordinately with endogenous histone gene expression during stimulation and inhibition of DNA synthesis. The results indicate that potential control sequences are located within or near an individual histone gene.

Although most transcripts of the chimeric gene contain correct 5' and 3' termini, we also detected transcripts which are terminated about 250 bases beyond the normal 3' end. Unexpectedly, these longer transcripts are polyadenylated and accumulate in the cytoplasm of the transfected cells.

MATERIALS AND METHODS

Cell culture. Ltk⁻ mouse fibroblasts (10) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Ltk⁻ cells were cotransfected with 10 or 100 ng of pHSV106 DNA (15) and 1 or 10 µg, respectively, of pH3-(2,1) DNA by the method of Wigler et al. (26). Calf thymus DNA was included so that a total of 20 µg of DNA was added per plate containing 10⁶ cells. tk⁺ colonies were selected in hypoxanthine-aminopterin-thymidine (HAT) medium (12), isolated with cloning rings, and expanded for analysis.

To synchronize cells by serum starvation, logarithmically growing cultures were plated at 10⁶ cells per ml in DMEM containing 10% FBS without the components of the HAT

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selection system. After incubation for 3 days the medium was exchanged with DMEM containing 1% FBS, and the cells were further incubated for 10 days. Cell growth was reinitiated by exchanging the medium with DMEM containing 10% FBS. DNA synthesis was measured by pulse labeling the cells for 30 min with [^3H]thymidine (5 $\mu\text{Ci/ml}$). Cells were washed twice with isotonic saline, and the incorporated radioactivity was determined after precipitation with 10% trichloroacetic acid. Treatments with aphidicolin (Natural Products Division, National Cancer Institute, Bethesda, Md.) and 5-fluorodeoxyuridine (FUDR; Sigma Chemical Co., St. Louis, Mo.) were performed at the indicated concentration on cultures that had grown to about 50% of final saturation density.

RNA isolation. Total cellular RNA was isolated by phenol extraction at 60°C as described previously (8). Cells were fractionated into nuclear and cytoplasmic fractions as described by Alterman et al. (1), and RNA was isolated by phenol extraction at 60°C. Polyadenylated [poly(A) $^+$] and nonpolyadenylated [poly(A) $^-$] RNA fractions were prepared by chromatography on oligodeoxythymidylate [oligo(dT)]-cellulose (type 2; Collaborative Research, Inc., Waltham, Mass.).

Construction of the H3-(2,1) gene. The H3-(2,1) gene was constructed from the H3-2 and H3-1 genes in clone MM221 (22) by ligating the two genes at the common *SalI* site at amino acid 58 (see Fig. 1). An *AvaI-SalI* fragment from MM221 containing the 5' end of an H2B-1 gene (amino acids 1 to 92) and the 5' end of the H3-2 gene (amino acids 1 to 58) was inserted into the *AvaI-SalI* site of pUC9 to form the plasmid pMH2B-H3-2. The 2.1-kilobase (kb) *SalI-EcoRI* fragment containing the 3' end of the H3-1 gene was digested with *HinfI*, and the 670-base-pair *SalI-HinfI* fragment containing the 3' end of the H3-1 gene was isolated. The free ends of the fragment were filled in with the Klenow fragment of DNA polymerase I, and the resulting blunt-ended fragment was ligated to the *SalI* site of pMH2B-H3-2 with T4 DNA ligase.

S1 analysis with a single-stranded H3-(2,1) probe. The *SmaI-HindIII* DNA fragment of pH3-(2,1) containing both chimeric H3 and H2B gene sequences was cloned into *SmaI-HindIII*-digested M13 mp8 bacteriophage (16) at an approximately eightfold molar excess of insert to vector DNA. Bacteriophage containing the anticoding strand of the chimeric histone gene was precipitated overnight from infected cultures in 0.5 M NaCl-4% polyethylene glycol, collected by centrifugation, dissolved in 10 mM Tris hydrochloride (pH 8)-10 mM NaCl-1 mM EDTA, and reprecipitated from 0.5 M NaCl-20% polyethylene glycol for 30 min at 4°C. Following centrifugation, the bacteriophage DNA was deproteinized by phenol-chloroform-isoamyl alcohol extraction and precipitated from ethanol.

Single-stranded DNA (0.5 μg) was annealed to 7.5 ng of M13 pentadecamer primer (New England BioLabs, Inc., Beverly, Mass.) as described by the manufacturer, and the primer was extended by incubation with DNA polymerase I-Klenow fragment (New England BioLabs) at 20°C for approximately 40 min in the presence of [α - ^{32}P]dCTP (specific activity, 400 Ci/mmol). Unlabeled dCTP was added to a final concentration of 10 μM , and the mixture was incubated for an additional 7 min. The reaction mixture was digested with *EcoRI*, and the 2.2-kb radiolabeled histone DNA insert was electroeluted from a denaturing agarose-formaldehyde gel (25) following identification by autoradiography. DNA (7×10^4 cpm) was mixed with 5 μg of RNA in 40 mM PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]; pH 6.4)-0.4 M

NaCl-1 mM EDTA-80% formamide, denatured for 15 min at 85°C, and hybridized for 3 to 4 h at 58°C. The reaction was brought to 30 mM sodium acetate (pH 4.5)-0.25 M NaCl-2 mM ZnSO_4 -*Escherichia coli* tRNA (20 $\mu\text{g/ml}$) and incubated with 100 U of S1 nuclease (Sigma) for 60 min at 37°C. To terminate digestion, the reaction was brought to 10 mM EDTA-0.8 M ammonium acetate, and nucleic acid was precipitated with ethanol. Precipitated nucleic acid was dissolved in 0.1 N NaOH-84% formamide-0.1% bromophenol blue-0.1% xylene cyanol and fractionated by electrophoresis on a 6% polyacrylamide-8 M urea sequencing gel in the Tris-borate buffer system.

S1 analysis with end-labeled probes. S1 nuclease mapping with end-labeled probes was performed as previously described (23). Analysis of 5' ends was performed by labeling the *SalI* site of the H3-1 gene or the H3-2 gene with [γ - ^{32}P]ATP and polynucleotide kinase. Analysis of 3' ends was done by labeling the *SalI* site with [α - ^{32}P]dCTP using the Klenow fragment of DNA polymerase I. For analysis of H2B 5' ends the H2B-1 gene was labeled at the *EcoRI* site with [γ - ^{32}P]ATP and polynucleotide kinase. The 3' ends of H2B truncated transcripts were analyzed by labeling pUC 9 at the *AvaI* site with [α - ^{32}P]dCTP and DNA polymerase. In some experiments a labeled *SalI-HindIII* fragment was prepared by further digestion of labeled plasmid DNA with *HindIII* followed by preparative gel electrophoresis. After incubation the mixture was treated with 100 U of S1 nuclease (Sigma) in 0.05 ml of 0.03 M sodium acetate (pH 4.5)-0.25 M NaCl-2 mM ZnSO_4 -*E. coli* tRNA (20 $\mu\text{g/ml}$) for 60 min at 37°C. S1-resistant DNA fragments were analyzed on 6% polyacrylamide gels in 8 M urea followed by autoradiography. The relative amounts of each DNA fragment were determined by scanning the autoradiograms with a Joyce-Loebl MK111 densitometer.

Isolation and electrophoresis of histone proteins. Nuclei were isolated from cells as previously described (1). Total nuclear protein was extracted in 0.4 M sulfuric acid for 1 h at 4°C, clarified by centrifugation, and precipitated by the addition of 4 volumes of ethanol. The protein pellet was collected by centrifugation and analyzed by polyacrylamide gel electrophoresis in the presence of 6 M Triton X-100 and 2.5 M urea as described by Zweidler (27).

RESULTS

Construction of a chimeric H3 gene. To study the expression of a mouse histone gene introduced into mouse cells, it was necessary to modify the gene structure so that the resulting mRNA could be readily distinguished from endogenous mouse histone mRNA. At the same time, it was desirable to maintain the protein coding sequence so as to avoid possible toxic effects due to the production of unusual histonelike proteins. To satisfy these requirements, a recombinant plasmid containing a chimeric H3 gene, designated H3-(2,1), was constructed (Fig. 1). Portions of two different H3 genes, H3-2 and H3-1, cloned in MM221 (22) were combined by joining a segment of DNA containing both the 5' region of H3-2 and a portion of an H2B gene with the 3' region of H3-1 at a shared *SalI* site within the H3 coding region. The resulting chimeric H3 gene would be expected to specify an mRNA containing the H3-2 5' untranslated region and the H3-1 3' untranslated region and should encode an H3.1 protein. In addition, the pH3-(2,1) plasmid construct contains approximately 350 nucleotides (nt) of mouse DNA 3' to the chimeric gene and retains the entire intergenic region between the H3-2 and H2B-1 genes as well as the first

FIG. 3. S1 nuclease gel analysis of H3 mRNA termini. (A) Levels of mRNA containing the 3' end of the H3-1 gene. One microgram of total RNA was assayed with a probe from the 3' end of the H3-1 gene labeled at the *SalI* site. Lanes: 1, mouse myeloma; 2, a tk⁺ transfectant which does not contain the chimeric histone gene; 3 to 9, pH3-(2,1) transfectants LH3-(2,1)-7A, -21B, -22A, -23B, -24A, -27A, and -30B. The arrow indicates the position of a novel protected DNA fragment resulting from extended transcripts described in the text. (B) Levels of mRNA containing the 5' ends of the H3-2 and H3-1 genes. One microgram of total RNA was assayed with probes from the 5' end of the H3-2 or H3-1 genes labeled at the *SalI* site. Lanes 1 to 4, H3-2 probe; lanes 5 to 8, H3-1 probe; lanes 1 and 5, mouse myeloma; lanes 2 and 6, a tk⁺ transfectant which does not contain the chimeric histone gene; lanes 3 and 7, LH3-(2,1)-21B; lanes 4 and 8, LH3-(2,1)-24A. (C) Description of fragments protected in the S1 nuclease assay using end-labeled probes. For details see the text.

of H3 mRNAs, indicating a very high level of expression of the chimeric gene.

To confirm the high level of expression of the chimeric gene in these transfectants, several types of S1 nuclease protection experiments for the 5' and 3' ends of the mRNA were performed. Since the mRNA derived from the chimeric gene has the same 3' end as H3-1 mRNA, cells producing large amounts of chimeric mRNA should have elevated levels of mRNA with this 3' end. Similarly, these cells should have elevated levels of the 5' end characteristic of the H3-2 mRNA. Conversely, however, there should be no increase in the levels of H3-2 3' ends or H3-1 5' ends.

Figure 3A shows the results of an S1 nuclease assay in which an end-labeled DNA fragment corresponding to the 3' end of the H3-1 gene was used as a probe. In both mouse myeloma cells (lane 1) and a tk⁺ cell line which was transfected with only the HSV tk gene (lane 2), there is a major protected fragment (H3, 231 nt) which extends to the

end of the H3 coding region (23) and a minor protected fragment (H3-1, 282 nt) which extends to the end of the H3-1 mRNA. The relatively low level of the 282-nt protected fragment is consistent with previous observations (13) indicating that H3-1 mRNA constitutes only 5 to 10% of the total H3 mRNA in mouse cells. In all but one of the transfectants tested, there was a significant increase in the proportion of the H3-1 mRNA 3' end relative to the H3 band. In some cases, the two bands were almost equally intense, indicating that chimeric mRNA was produced at levels approaching 50% of total H3 mRNA synthesis. Two other protected fragments were observed. One fragment (117 nt) is due to cleavage at amino acid 96 which differs in H3.1 and H3.2 proteins. Some of the H3-2 mRNAs have sufficient mismatch in this region for S1 nuclease to cleave the DNA-RNA hybrid (5a). The intensity of this band was reduced in tk⁺ lines expressing high levels of the chimeric mRNA consistent with the observation that a greater proportion of H3

mRNA in these cells contains the 3' end of the H3-1 gene. The second band is a novel fragment of about 530 nt which is present only in transfectants producing the chimeric mRNA. The origin of this band is discussed below.

To confirm that the increase in the amount of mRNA with H3-1 3' ends was due to expression of the chimeric gene, the level of mRNA with 5' ends like that of the H3-2 gene was determined. Figure 3B (lanes 1 to 4) shows that the levels of mRNA with H3-2 5' ends were greatly increased in the same transfectants that had increased levels of H3-1 3' ends. As expected, the levels of mRNA with H3-1 5' ends (Fig. 3B, lanes 5 to 8) and H3-2 3' ends (data not shown) were not increased in these transfectants, relative to a tk⁺ transfectant that did not receive chimeric histone DNA. These results confirm the analysis shown in Fig. 2 in which a full-length, single-stranded probe derived from the H3-(2,1) gene was used and indicate that in some transfectants the amount of chimeric H3 mRNA is about 50% of the total H3 mRNA content produced from all of the endogenous genes.

The pH3-(2,1) plasmid used for transfection also contains a truncated H2B-1 gene in addition to the chimeric H3 gene (Fig. 1). The 5' end of the gene is intact but it is truncated at amino acid 92 of the H2B protein. S1 nuclease mapping experiments with several types of 5'- and 3'-end-labeled probes showed that the truncated H2B-1 gene was also highly transcribed in transfected cells. The 5' end of these transcripts mapped to the expected position in the H2B-1 gene, but the 3' ends were very heterogeneous, extending 150 to more than 500 nt into the adjacent plasmid DNA sequences (data not shown). These H2B-1 transcripts were present almost exclusively in the nucleus.

Chimeric mRNA is translated in transfected cells. The H3.1 and H3.2 histone subtypes differ only at amino acid residue 96, which is either cysteine or serine in H3.1 and H3.2, respectively (4, 17). Since the H3-(2,1) gene was constructed by fusion of the two genes at residue 58, mRNA transcribed from the chimeric gene encodes an H3.1 protein. To determine whether the chimeric mRNA is translated in transfected cells, we analyzed the levels of the histone subtypes in chromatin from these cells by Triton X-100-urea gel electrophoresis (Fig. 4). Microdensitometry of the H3.1 and H3.2 bands from untransfected cells (Fig. 4, lane 2) showed that the H3.1 subtype constitutes about 25% of the total H3 protein in these cells, consistent with previous results on other mouse cells (4). As shown in Fig. 4 (lane 1), the amount of H3.1 protein relative to H3.2 was increased to about 50% of the total in a transfectant cell line producing large amounts of the chimeric mRNA, whereas the relative levels of the other histone subtypes in these cells was unchanged. This indicates that the chimeric mRNA is efficiently translated in transfected cells and that the H3.1 protein produced is incorporated into chromatin.

Extended, poly(A)⁺ transcripts of the chimeric gene. S1 nuclease mapping experiments, using a probe from the 3' end of the H3-1 gene, showed that in addition to correct mRNA 3' termini, RNA from all transfected cell lines expressing the chimeric gene also protected a novel DNA fragment 530 nt long (Fig. 3A). This fragment results from protection of the probe an additional 250 nt past the normal end of the mRNA. Presumably, the longer transcript arises by a failure to process or terminate the histone mRNA correctly. To determine whether the extended transcript was a nuclear precursor of chimeric mRNA, nuclear and cytoplasmic RNA fractions were prepared and analyzed by S1 nuclease mapping. The same relative proportion of the longer transcript was detected in both the nuclear and

FIG. 4. Analysis of histone proteins in cells expressing the H3-(2,1) gene. Histones were isolated by acid extraction of nuclei as described in the text, and the proteins were resolved by electrophoresis on Triton X-100-urea gels containing 2.5 M urea. The histone proteins are named according to the nomenclature of Zweidler (27). Histones were analyzed from a tk⁺ transfectant which does not contain any exogenous histone DNA (lane 2) and transfectant cell line LH3-(2,1)-22A (lane 1), expressing large amounts of chimeric mRNA (Fig. 3A, lane 5).

cytoplasmic fractions (Fig. 5A). This indicates that most of the extended transcripts are located in the cytoplasm.

Total RNA from transfected cells was fractionated on oligo(dT)-cellulose, and the poly(A)⁺ and poly(A)⁻ RNA fractions were analyzed by S1 nuclease mapping using the 3' end of the H3-1 gene as a probe. Most (95%) of the histone mRNA eluted in the poly(A)⁻ (unbound) fraction as expected (Fig. 5B, lanes 1 and 2). However, the novel extended transcript was detected only in the poly(A)⁺ (bound) fraction (lane 3). The transcript was also shown to contain authentic poly(A) by formamide gradient chromatography on polyuridylylate-Sepharose. The longer transcript eluted in the same fractions as pulse-labeled, poly(A)⁺ nuclear RNA (data not shown).

To determine whether the longer transcript was initiated at the same site as the chimeric mRNA, the same RNA fractions were analyzed by S1 nuclease mapping by using the 5' end of the H3-2 gene as a probe (Fig. 5C). The predominant 5' end in the poly(A)⁺ fraction (lane 3) was identical to the 5' end of the chimeric mRNA in the poly(A)⁻ fraction (lane 2). Thus, the long poly(A)⁺ H3 transcript starts at the same site as the H3-(2,1) mRNA but extends approximately 250 nt past the normal mRNA 3' terminus where it is polyadenylated.

Chimeric gene expression is regulated coordinately with endogenous histone genes. The levels of histone mRNAs in cultured mammalian cells are regulated coordinately with changes in the rate of DNA synthesis (1, 3, 6, 18). We investigated chimeric gene expression under two conditions in which the levels of endogenous histone mRNAs are altered: (i) when cells are synchronized by serum starvation

FIG. 5. Polyadenylation and cytoplasmic accumulation of the extended chimeric transcript. (A) Transfectant cells (line LH3-[2,1]-22A) were lysed with 0.3% Nonidet P-40, and RNA was prepared from nuclear and cytoplasmic fractions as described in the text. RNA from each fraction was analyzed by S1 nuclease mapping using the 3' end of the H3-1 gene as a probe as described in Fig. 3C and the text. The protected fragments are H3-1 mRNA (282 nt), H3 coding region (231 nt), and the extended transcript (530 nt) as denoted by the arrow. RNA from an equal number of cells was used in each assay. The nuclei were prepared by a gentle cell lysis procedure that results in contamination by cytoplasm, accounting for the relatively large amounts of H3 mRNA in this fraction. Lanes: 1, nuclear RNA; 2, cytoplasmic RNA. (B) Total RNA from transfectant cell line 22A was fractionated by chromatography on oligo(dT)-cellulose as described in the text. RNAs in the bound and unbound fractions were analyzed by S1 nuclease mapping as described in the legend to panel A. RNA from an equivalent number of cells was used in each assay. Lanes: 1 and 2, poly(A)⁻ RNA; 3, poly(A)⁺ RNA. (C) The bound and unbound RNA fractions from oligo(dT)-cellulose were analyzed by S1 nuclease mapping using the 5' end of the H3-2 gene as a probe (Fig. 3C). The protected fragments are H3-2 mRNA (195 nt) and H3 coding region (174 nt). Lanes: 1, mouse myeloma RNA; 2, poly(A)⁻ RNA; 3, poly(A)⁺ RNA.

and then stimulated to reenter the cell division cycle by refeeding serum and (ii) when growing cells are treated with DNA synthesis inhibitors.

Cultures of transfected cells were maintained in growth medium containing 0.5% FBS for 10 days. Upon addition of medium containing 10% FBS, DNA synthesis began after about 8 h and reached a maximum at about 20 h (Fig. 6B). The levels of H3 mRNAs present at various times after serum stimulation were analyzed by an S1 nuclease assay, using the 3' end of the H3-1 gene as a probe. As shown in Fig. 3, more than 90% of the transcripts with H3-1 3' ends in these transfected cells are derived from the chimeric gene, and they protect a 282-nt DNA fragment. Most other H3 mRNAs produced from endogenous genes have different 3' ends that give rise to a 231-nt protected DNA fragment. Low levels of the chimeric mRNA as well as the other H3 mRNAs were detected in serum-deprived cells (Fig. 6A, lane 1). The

FIG. 6. Analysis of H3 histone mRNA levels in transfected cells synchronized by serum deprivation. (A) Cell line LH3-(2,1)-24A was synchronized by serum starvation as described in the text. Total RNA was isolated at the indicated times after addition of growth medium containing 10% FBS. The RNA was hybridized with *Sall*-digested H3-1 DNA labeled at the 3' terminus (Fig. 3C), and protected fragments were fractionated on denaturing acrylamide gels. The protected fragments are H3-1 mRNA (282 nt), H3 coding region (231 nt), and the extended transcript (530 nt) as denoted by the arrow. Lanes: 1, 0 h; 2, 4 h; 3, 8 h; 4, 12 h; 5, 20 h; 6, 22 h. (B) The rate of DNA synthesis following addition of growth medium was measured in parallel cultures as described in the text. The data represent an average of duplicate determinations of [³H]thymidine incorporated during pulse labeling for 30 min.

amounts of both types of H3 mRNAs increased steadily following serum stimulation, reaching a maximum at 20 to 22 h. The ratio of the two types of mRNA remained constant during the recovery from serum starvation. The results indicate that chimeric mRNA levels are regulated in parallel with endogenous H3 mRNAs during the serum-stimulated transition to a new cell cycle. However, the levels of the longer chimeric transcript, reflected in the 530-nt protected DNA fragment, changed with different kinetics. This transcript increased to a smaller extent 4 h after serum stimulation, and it did not increase further during the period when the rate of DNA synthesis increased.

Treatment of growing cells with several different types of DNA synthesis inhibitors leads to a rapid decline in the levels of histone mRNAs (5, 6). We analyzed chimeric mRNA levels in transfected cells during treatment with two inhibitors: FUDR, which blocks thymidylate synthetase, and aphidicolin, which inhibits DNA polymerase. Figure 7A shows the results of treating transfected cells with 2 μ M FUDR for various lengths of times. The levels of chimeric mRNA and endogenous H3 mRNAs declined to about the same extent and with similar kinetics during the 90-min treatment. The effect of FUDR is readily reversible by the addition of excess thymidine. Upon addition of 10 μ M thymidine to FUDR-inhibited cultures, the levels of both the chimeric mRNA and the other H3 mRNAs rose rapidly to pretreatment levels (Fig. 7A). Similar results were obtained

after treatment of several transfectant cell lines with aphidicolin. The levels of both chimeric mRNA and endogenous H3 mRNAs were markedly reduced after 60 min of aphidicolin treatment (Fig. 7B). In other experiments we found that the kinetics of decline for both types of mRNA were similar (data not shown). However, as observed during serum stimulation, changes in the amounts of the longer poly(A)⁺ chimeric transcript did not exactly parallel that of the other mRNAs. In FUDR-treated cultures this transcript declined shortly after the addition of the inhibitor, but it was present at nearly pretreatment levels after longer treatments (Fig. 7A). This difference in the behavior of the extended transcript as compared to the histone mRNAs was especially apparent in cultures treated for 60 min with aphidicolin (Fig. 7B).

DISCUSSION

DNA-mediated gene transfer is now a widely used technique for studying the role of specific DNA sequences in gene expression. The approach allows one to modify DNA sequences and to determine the effect of the modifications on expression *in vivo*. The histone genes provide excellent material for such studies because they are expressed in all growing cells and their expression is regulated periodically during the cell cycle (1, 3, 6, 18). Although gene transfer is usually carried out by introducing a gene from one species into cells of another species, we were able to study expression of a mouse histone gene transferred into mouse cells. This was accomplished by joining two H3 histone genes that differ in their 5' and 3' untranslated regions at a common restriction enzyme site in the coding region. The resulting chimeric gene contains functional promoter sequences from the H3-2 gene and sequences required for formation of the mRNA 3' terminus from the H3-1 gene. The chimeric gene encodes a normal histone protein of the H3.1 subtype.

After its introduction into mouse fibroblasts, the chimeric gene was expressed in a large number of independently isolated cell lines. In some lines nearly 50% of the H3 mRNA was derived from the chimeric gene, even though the H3-2 gene that comprised the 5' end of the chimeric gene normally encodes only about 5% of the H3 mRNA present in the parental L cells. The large amount of chimeric mRNA produced in the transfected cells was reflected in the histone protein composition of their chromatin. The H3.1 subtype constitutes about 25% of the total H3 protein in L cells, whereas it represented the majority of H3 histones in these transfectants. Presumably, the large amount of chimeric mRNA produced in these cells is due to the presence of many (20 to 200) copies of the transfected DNA. However, the level of expression per gene copy is probably less than that of the endogenous H3-2 gene which is present as a single copy per haploid genome.

An important conclusion from the present work is that expression of the transfected gene is regulated in parallel with that of endogenous histone genes. The levels of chimeric mRNA and endogenous H3 mRNAs were measured during stimulation of DNA synthesis in cells synchronized by serum starvation. Both types of mRNA were increased to about the same extent and with similar kinetics (Fig. 6). Recent work has shown that the increase in histone mRNAs in serum-stimulated fibroblasts is due to both an increase in the level of transcription and a posttranscriptional mechanism, possibly mRNA stabilization (3). Each step contributes about equally to the increase in histone mRNA levels. We were not able to measure directly the transcription rate from endogenous and chimeric genes because they contain

FIG. 7. H3 histone RNA levels in transfected cells treated with inhibitors of DNA synthesis. (A) Cell line LH3-(2,1)-21B was treated with 2 μ M FUDR as described in the text. Total RNA was isolated at the indicated times and analyzed by S1 nuclease assay with the 3' end of the H3-1 gene as a probe as described in the legend to Fig. 3C. Lanes: 1, untreated; 2 to 5, treated with FUDR for 10, 30, 60, and 90 min, respectively; 6 and 7, treated with FUDR for 60 min followed by incubation in growth medium containing 10 μ M thymidine for 15 and 90 min, respectively. (B) Cultures of transfected cells were treated with aphidicolin as described in the text. Total RNA was isolated, and H3 mRNA was analyzed by S1 nuclease assay with the 3' end of the H3-1 gene as a probe as described in the legend to Fig. 3C. Lanes: 1, tk⁺ transfectant which does not contain the chimeric histone gene, untreated; 2, transfectant cell line LH3-(2,1)-21B, untreated; 3, line 21B treated with aphidicolin (5 μ g/ml) for 60 min; 4, transfectant cell line LH3-(2,1)-24A, untreated; 5, line 24A treated with aphidicolin (5 μ g/ml) for 60 min.

very similar DNA sequences. However, the large increase (greater than 20-fold) in chimeric mRNA levels following serum stimulation strongly suggests that transcription of the transfected genes was regulated. This conclusion is strengthened by the observation that both mRNAs were decreased proportionally in serum-starved cells. Since the chimeric gene construct contains about 1 kb 5' and 0.35 kb 3' to the gene, the signals needed for transcriptional control must lie within or near the gene. Further manipulation of the chimeric gene should allow us to define the sequences required for transcriptional regulation.

Control of chimeric mRNA levels also was observed in cells treated with two different DNA synthesis inhibitors (Fig. 7). Chimeric mRNA levels were found to decline to about the same extent and with similar kinetics to that of the bulk H3 mRNAs, and the effect was reversible in FUDR-treated cells by the addition of excess thymidine. Since under these conditions histone mRNA levels appear to be controlled primarily by changes in mRNA turnover (6, 13), the metabolism of the chimeric mRNA must closely parallel that of the endogenous H3 mRNAs. This is not unexpected since the chimeric mRNA shares certain unique structural features with endogenous histone mRNAs. These features include the lack of a poly(A) tail and the presence of a 3'-terminal stem-loop structure. However, the rapid turnover of histone mRNAs in cells inhibited for DNA synthesis may not be due simply to the absence of poly(A) because the mRNA for the replacement histone H3.3 also lacks this feature and it is not subject to rapid turnover on inhibition of DNA synthesis. It is more likely that the sequences determining histone mRNA metabolism are located within the mRNA itself, possibly in the 5' or 3' noncoding regions. It should now be possible to identify these regions by studying the expression of other types of chimeric histone genes after their transfer to cultured cells.

An unexpected finding was the detection of a discrete longer transcript present in all transfected cell lines expressing the chimeric gene. This transcript is initiated at the normal start site in the H3-2 gene, but it extends about 250 nt past the normal mRNA 3' terminus. Preliminary sequence information indicates the presence of a consensus hexanucleotide polyadenylation signal in this region, and we found that the extended transcript is indeed polyadenylated and accumulates in the cytoplasm of the transfected cells. Recent reports indicate that the 3' ends of histone mRNAs from sea urchin, *Drosophila melanogaster*, and chickens can be formed by endonucleolytic cleavage of longer transcripts (11, 19, 24). Although we have not detected extended transcripts from endogenous mouse histone genes, the presence of the longer transcript in transfected cells suggests that such a mechanism may be used to generate histone mRNA 3' termini in mouse cells as well. Presumably, the longer transcript results from a failure to cleave some of the chimeric transcripts at the normal 3' site, followed by polyadenylation when the RNA polymerase passes the polyadenylation signal in the 3' flanking DNA.

The production of the longer transcript in transfected cells appears to be intrinsic to the introduction of the H3-(2,1) chimeric gene into these cells. We have not been able to detect such a transcript in other cultured mouse cells, including the parental L fibroblasts, even when very large amounts of RNA have been assayed by S1 nuclease mapping. This suggests two possible explanations for the accumulation of the extended transcript. Perhaps the high level of transcription of the chimeric genes results in overloading of the processing system, allowing some transcripts to escape

normal 3'-end formation. Alternatively, the chimeric nature of the H3-(2,1) transcript may result in a reduced affinity for the processing machinery. In either case, once the RNA polymerase reaches the polyadenylation signal, the extended transcript may be cleaved at that site and polyadenylated.

Even though such transcripts may not normally be produced in mouse fibroblasts, possibly there are circumstances in which histone mRNAs are polyadenylated in mouse cells. Histone mRNAs are polyadenylated during maternal mRNA formation in amphibian oocytes, whereas they are not polyadenylated in somatic cells (21). Differences in the state of polyadenylation of the histone mRNAs might play a role in mRNA turnover. In this context it is interesting that the longer, poly(A)⁺ transcripts accumulate in the cytoplasm of the transfected cells. Moreover, their metabolism is different from the poly(A)⁻ chimeric mRNA. These observations have prompted us to ask whether other histone genes have polyadenylation signals near their 3' ends and whether mouse histone mRNAs are polyadenylated at some point during development.

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