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Tripartite Sequences within and 3′ to the Sea Urchin H2A Histone Gene Display Properties Associated with a Transcriptional Termination Process

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We have defined a DNA sequence that behaves as an RNA polymerase II termination signal by using the human HeLa cell transient expression system. Surprisingly, this sequence is tripartite, including part of the coding region of the sea urchin H2A histone gene together with two separate sequences in the 3′ flanking region of the gene. We demonstrate that this signal functions both in its normal gene environment and also when placed within the human α-globin gene. However, we have failed to detect a discrete 3′ terminus. Rather, our data indicate the presence of an extremely heterogeneous series of nonpolyadenylated RNAs. These heterogeneous nonpolyadenylated RNAs are stable when transcribed from the intact histone gene but are highly unstable within the human α-globin gene. This provides evidence for the role of poly(A) in the stability of mRNA.

Initiation of transcription in eucaryotic RNA polymerase II (*polII*) genes is a highly regulated and in some instances well-defined process (see reference 28 for a review). In contrast, termination of transcription in *polII* genes is ill defined and may not be a regulated process. The principal cause of this uncertainty is the rapid posttranscriptional processing of primary RNA transcripts to mature mRNA (see reference 40 for a review). The fact that introns are spliced out of the *polII* gene transcript is well documented. Similarly, the generation of mRNA 3′ termini for both polyadenylated [poly(A)$^+$] mRNA and nonpolyadenylated [poly(A)$^-$] histone mRNA by a processing event has been recently established (see references 5 and 44 for reviews).

The best evidence for processing of mRNA 3′ ends comes from studies on histone mRNA. Thus, the existence of a histone mRNA 3′ processing activity in *Xenopus laevis* oocytes and *Drosophila melanogaster* tissue culture cell extracts has been clearly demonstrated (4, 25, 43). Furthermore, it has been shown that this activity is associated with a small nuclear RNA U7 (12, 52) in a similar way to the involvement of the small nuclear RNA U1 in intronic splicing (24, 27, 42, 47). These results indicate that transcriptional termination is likely to occur separately from 3′-end processing in histone genes. Evidence of a similar nature in poly(A)$^+$ mRNA genes is now becoming available. Moore and Sharp (37) have recently shown that synthetic RNAs extending beyond an adenovirus poly(A) site can be efficiently and accurately cleaved and polyadenylated by using in vitro cell extracts.

Further evidence for transcriptional termination separate from mRNA 3′-end formation comes from hybridization analysis of pulse-labeled RNA transcripts. Using these techniques, Nevins and Darnell (38) and Fraser et al. (11) deduced that the major late gene transcripts of adenovirus extend beyond the various late mRNA poly(A) sites to near the end of the viral genome. Similarly, transcription extends beyond the adenovirus early region 2 and 4 poly(A) sites and the simian virus 40 (SV40) late poly(A) site (10, 39). These viral transcription experiments were performed on pulse-labeled whole cells. However, similar analysis of chromosomal gene transcription is very difficult due to the lack of radioactivity incorporated into gene-specific pulse-labeled RNAs. To overcome this problem, the more efficient labeling of nascent RNA transcripts in isolated nuclei, the so-called nuclear runoff analysis, has been used as an alternative method (17). With this procedure, hybridization data indicate that transcription proceeds past the poly(A) sites of a number of different genes: the mouse β major globin gene (7, 9, 23), the rabbit β globin gene (48), the chicken and mouse α globin genes (51, 53), the mouse immunoglobulin μ and δ constant region genes (33), the chicken ovalbumin gene (26), and the mouse α amylase gene (18). In two cases the termination processes defined on these methods have been verified in vivo. First, with the mouse β major globin gene, Citron et al. (7) and Falck-Pedersen et al. (9) have demonstrated the same in vitro termination position by using pulse-labeled whole cells. Second, Hagenbuchle et al. (18) have shown the presence of steady-state nuclear poly(A)$^-$ RNA that corresponds to the in vitro termination process in the mouse α amylase gene.

We describe here experiments that indicate the efficient operation of transcriptional termination signals for the sea urchin H2A histone gene. It has been demonstrated by Bendig and Hentschel (1) that the sea urchin H2A histone gene, when transfected into human HeLa cells, will initiate transcription but does not generate authentic mRNA 3′ termini, presumably due to the absence of sufficiently homologous processing activities. We therefore investigated where these H2A transcripts extend to in the 3′ flanking sequence of the gene in HeLa cells. In the H2A gene construct used in the experiments, an SV40 poly(A) site is placed downstream of H2A 3′ flanking sequence. Some transcripts extend to this site and are polyadenylated, but most transcripts end before this position, generating a heterogeneous set of poly(A)$^-$ 3′ ends. We then placed this region of the H2A gene into exon 3 of the human α-globin gene. Similar heterogeneous poly(A)$^-$ transcripts were indicated with this construct but only in the nuclear compartment. These results on steady-state mRNA analysis suggest that the H2A histone gene possesses efficient transcriptional

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termination signals that operate in both a heterologous cellular and a heterologous genetic environment. To further characterize these signals, we finally investigated which H2A gene sequences are required for this putative termination process. Surprisingly, we demonstrate the absolute requirement for sequences within the H2A gene coding sequence together with two separate sequences in the 3′ flanking region for this process.

MATERIALS AND METHODS

DNA construction. (i) H2ASVpBR328. A partial *Taq*I fragment containing the sea urchin H2A gene and 3′ flanking sequence was previously subcloned into the *Cla*I site of pBR322 (a gift from M. L. Birnstiel). The H2A insert was excised from this pBR322 subclone by *Eco*RI and *Hin*dIII digestion, flush ended with DNA polymerase I, and ligated into flush-ended *Bam*HI-*Sal*I double-digested SVpBR328 by standard procedures (31).

(ii) αH2AB/PpSVod. The *TaqI* fragment containing the 3′ portion of the sea urchin H2A gene together with its 3′ flanking sequence was flush ended and ligated into either flush-ended *Bst*EII-cut α1pSVod (35) – α/H2AB or *Pvu*II-cut α1pSVod – α/H2P. Both *Bst*EII and *Pvu*II cut α1pSVod at one site. In each case, the same transcriptional orientation of H2A insert to α1 gene was selected. For α/H2AB the 5′ end of the H2A insert reformed the *Bst*EII site. This site was used for an RNA mapping probe (see Fig. 5).

(iii) α/βI2pSVod. An 800-base-pair (bp) *Hin*fI fragment containing most of the human β globin gene intron 2 but not the donor or acceptor sites was flush ended and ligated into flush-ended *Bst*EII-cut α1pSVod.

(iv) ΔS1 deletion series of H2A insert in α/H2ABpSVod. α/H2ABpSVod was linearized with S1 nuclease, which preferentially cleaves an S1-sensitive site in the H2A gene 3′ flanking region (22). Thus, 10 μg of α/H2ABpSVod was digested with 200 U of S1 in 0.25 M NaCl–0.03 M sodium acetate (pH 4.6)–2 mM zinc acetate for 30 min at 30°C. The linear fragment was purified and subjected to limited Bal31 exonuclease digestion under standard conditions (31). The digested DNA was filled in with DNA polymerase I and ligated to reform a circular deleted plasmid. The different deletion mutants obtained were mapped by DNA sequence analysis (34) to establish the precise extent of each deletion.

(v) Δ5′ deletion series of H2A insert in α/H2ABΔ3pSVod. α/H2ABΔ3pSVod was linearized with *Bst*EII and treated with Bal31 as for iv above. The digested DNA was filled in with DNA polymerase I and religated in the presence of *Bgl*II linkers to reform a circular deleted plasmid. Different size deletions into the 5′ side of the H2A gene insert were identified by accurate restriction enzyme site mapping. Five of these deletion mutants were recloned into αIpSVod. Thus, ΔI-ΔV were made by cutting the deleted plasmids with *Bgl*II (filled in with DNA polymerase) and *Pst*I. The 3′ H2A α globin DNA fragment obtained was then ligated into *Bst*EII- (filled in with DNA polymerase) and *Pst*I-cut αIpSVod (see Fig. 3B). Clones ΔI-ΔV delete 30, 60, 70, 110, and 140 nucleotides, respectively from the H2A *Xho*I site (*TaqI* site) in a 5′-to-3′ direction.

(vi) Δ3′ deletion series of H2A insert in α/H2AB. The 3′ H2A *Taq*I fragment (see Fig. 1A) was cut with *Hin*fI or partially with *Ssp*I or *Eco*RV, and four fragments deleting increasing amounts of H2A 3′ flanking sequence were ligated in *Bst*EII-cut α1pSVod; generating ΔH, ΔS, ΔR1, and ΔR2.

(vii) Reconstructing the H2A termination signal. Different DNA fragments from the H2A 3′ *Taq* fragment were inserted into the polylinker sequence of an SP6 promoter plasmid, pSP62 PL (36). Once suitable combinations were obtained, they were excised from the polylinker and inserted into the *Bst*EII site of α1pSVod. For all R1-R6 constructs an A box fragment *Sfa*NI-*Mbo*I (the *Mbo*I site is positioned against the 3′ side of the A-rich sequence in the deletion clone Δ3) was ligated into the polylinker *Sma*I site. Then an H2A gene fragment (*Bst*EII-*Pvu*I or *Bst*EII-*Ssp*I) was ligated into the polylinker *Hin*dIII site 5′ to the A box fragment. These two C box-A box constructs were then excised from the polylinker (5′ *Nru*I site in H2A gene to 3′ *Eco*RI polylinker site) and ligated into the α1pSVod *Bst*EII site. These clones are R1 and R3. R2 and R4 have an additional 3′ flanking region fragment (partial *Eco*RV-*Taq*) added 3′ to the A box by using a unique *Sac*I site derived from the polylinker. Similarly, R5 and R6 have limit *Ssp*I-*Taq* and *Ssp*I-*Hin*f 3′ flanking region fragments added to the same *Sac*I site.

Transcription analysis. (i) Transient expression in HeLa cells. Transfections were carried out as described previously (35, 45). In outline, 20 μg of plasmid DNA was calcium phosphate precipitated and added to subconfluent petri dishes (90 cm) of HeLa cells. After 10 to 16 h, the medium was changed and the cells were allowed to grow for another 48 h. The HeLa cells were harvested and lysed in Nonidet P-40 detergent buffer, and the cytoplasmic and nuclear fractions were separated by centrifugation through a sucrose cushion. Following incubation with proteinase K, cytoplasmic RNA was purified by phenol-chloroform extraction and ethanol precipitation. Poly(A) selection was carried out by standard procedures with an oligo(dT)-cellulose column. Essentially a total RNA sample was run through the column several times in high salt, and the bound poly(A)$^+$ fraction was eluted in water. The whole poly(A)$^-$ and poly(A)$^+$ fractions were concentrated and analyzed by S1 or exonuclease VII. The ratio of poly(A)$^+$ to poly(A)$^-$ could therefore be estimated. See Maniatis et al. (31) for all these procedures.

(ii) RNA mapping: S1 nuclease. Probe DNAs were in each case double-stranded restriction fragments filled in with the appropriate [α-^{32}P]deoxynucleotide triphosphate with Klenow DNA polymerase (31). These probes (~20 cpm; specific activity, 3,000 Ci/mmol) were annealed to HeLa cell nuclear or cytoplasmic RNAs (about 20 μg) in 30 μl of 80% formamide–0.04 M PIPES (pH 6.8)–0.4 M NaCl–0.1 mM EDTA by denaturation at 80°C for 10 min and then at 53°C overnight. Ice-cold S1 buffer (0.3 ml; 0.25 M NaCl, 0.03 M sodium acetate, pH 4.6, 2 mM $ZnSO_4$, 50 μg of denatured sonicated carrier DNA per ml) plus S1 (3,000 U) was quickly added to each hybridization mixture and incubated for 1 h at 30°C. S1 reactions were ethanol precipitated and fractionated on denaturing 7 M urea–polyacrylamide gels.

(iii) RNA mapping: exonuclease VII. Hybridizations with exonuclease VII were carried out as for the S1 experiments. Exonuclease VII buffer (0.5 ml; 30 mM KCl, 10 mM Tris, pH 7.8, and 10 mM EDTA) with 4 U of exonuclease VII (Bethesda Research Laboratories) per ml was added to hybridization mixtures and incubated at 37°C for 2 h. The reactions were then ethanol precipitated and fractionated as for S1 experiments.

(iv) RNA mapping: primer extension. The DNA primer for α globin mRNA 5′ end analysis was a single-stranded *Hin*fI-*Hae*III fragment as indicated in Fig. 4B. This DNA was obtained by filling in the double-stranded DNA with [α-^{32}P]dATP and fractionating on a denaturing 7 M urea–12% polyacrylamide gel. DNA primer (20 cpm; specific activity, 3,000 Ci/mmol) and RNA were annealed in 10 μl of 10 mM

PIPES (pH 6.4)–0.4 M NaCl at 80°C for 10 min and at 63°C overnight. Reverse transcriptase buffer (50 μl; 50 mM Tris, pH 8.2, 10 mM dithiothreitol, 6 mM $MgCl_2$, 0.5 mM dATP, dCTP, dTTP, and dGTP) plus reverse transcriptase (5 U) was added to hybridization mixtures and incubated at 42°C for 1 h. RNase (2 μg) was added to the incubation mixtures and left a further 15 min at 42°C. The reaction mixture was phenol extracted, ethanol precipitated, and fractionated by electrophoresis on 7 M urea–polyacrylamide gels.

RESULTS

Transient expression of the intact sea urchin H2A gene in HeLa cells. The sea urchin H2A histone gene is part of a tandemly repeated five-gene histone unit and is flanked on its 5′ side by the H3 gene and on its 3′ side by the H1 gene (21) (Fig. 1A). A partial *Taq*I restriction fragment (1.4 kilobases

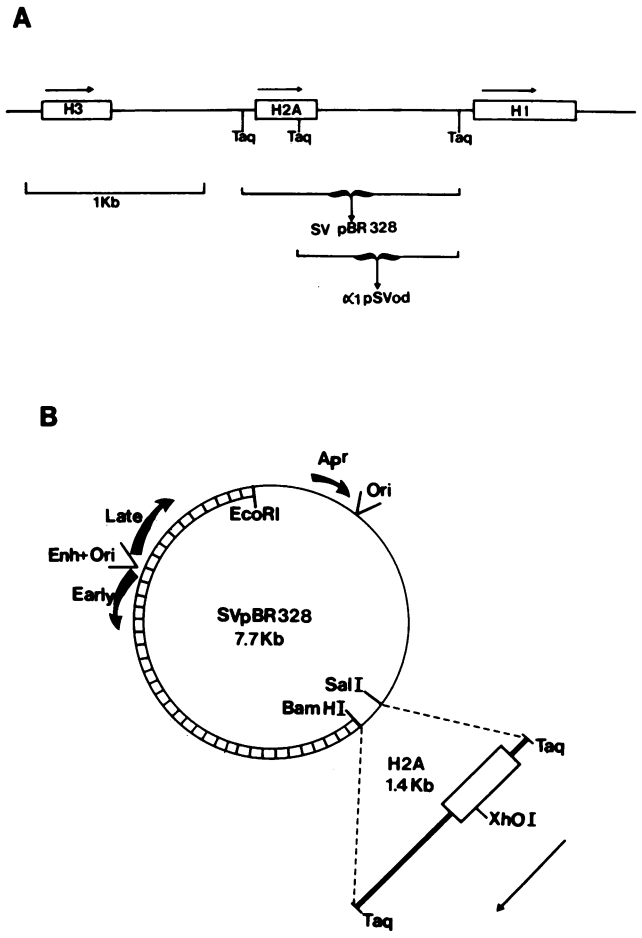


FIG. 1. (A) Line diagram showing the position of the sea urchin H2A histone gene within the repeated histone gene cluster. Line denotes flanking region sequence, and boxes indicate gene sequences. Direction of gene transcription is denoted by arrows. The H2A gene DNA fragments subcloned into SVpBR328 and α1pSVod are indicated by brackets. (B) Line diagram of the H2A SVpBR328 plasmid. Hatched area is SV40 sequence. Thin line is pBR328 sequence. Thick line is H2A gene flanking sequence. Box is H2A gene. The direction of transcription of the SV40 early and late promoters and pBR328 ampicillin resistance gene (Ap^r) are indicated (thick arrows), as is the transcriptional orientation of the H2A insert (thin arrow). Enh, Enhancer; Ori, origin of replication. The restriction sites at the junctions of SV40, pBR328, and H2 are indicated.

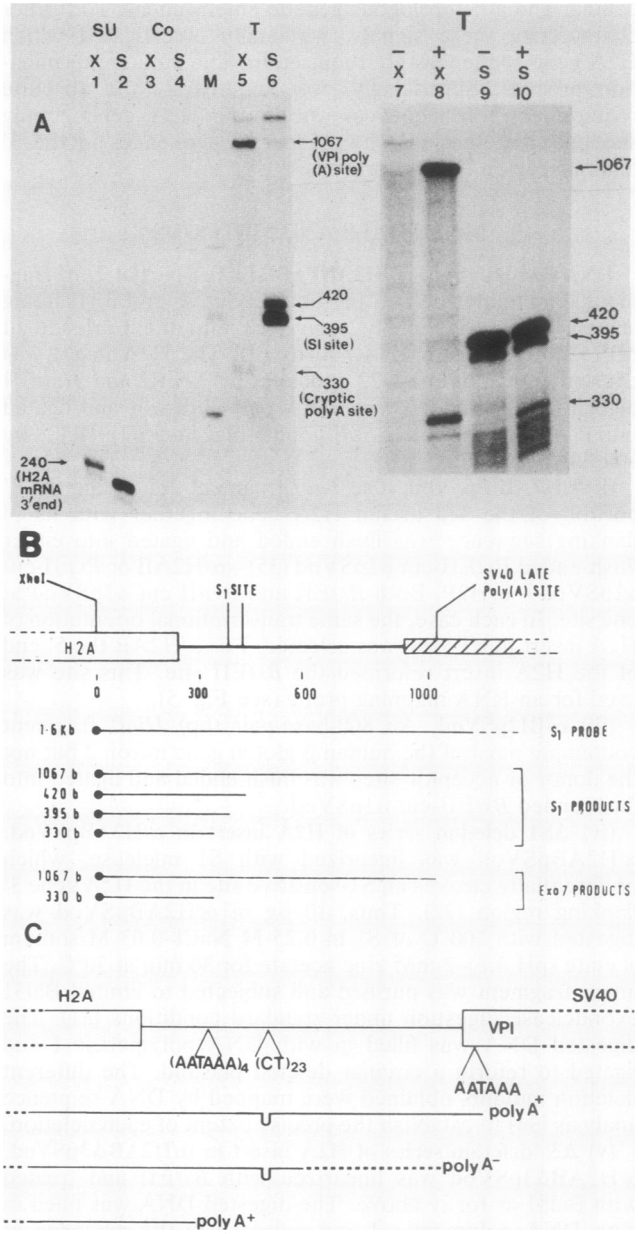


FIG. 2. (A) RNA mapping data on H2A SVpBR328-transfected HeLa cell RNA versus sea urchin RNA. Lanes X are ExoVII digests, and lanes S are S1 nuclease digests of the *Xho*I 3′-end-labelled H2A DNA probe (a *Pst*I-*Xho*I, 1.6-kb double-stranded DNA fragment) hybridized to sea urchin (SU) RNA, yeast control (Co) RNA, and H2A SVpBR328 transfection (T) RNA + and −, $Poly(A)^+$ and $poly(A)^-$ RNA, respectively. Lane M, Size markers (indicated in bases). (B) Line diagram indicating the size and position of the probes and signals obtained in panel A. The open box denotes H2A gene sequences. b, Bases. (C) Line diagram of the three H2A RNA species identified in the RNA mapping experiments on H2A SVpBR328 transfected HeLa cells shown in panel A. Each RNA 3′ terminus is positioned under the gene construct map which shows the relative positions of the H2A gene and SV40 sequences (boxed) and H2A gene flanking sequences (line) together with the positions of the cryptic poly(A) site, S1 site, and VP1 poly(A) site. The heterogeneous $poly(A)^-$ RNA is indicated by a dotted line. A U in the line denotes a mismatch at the S1 site.

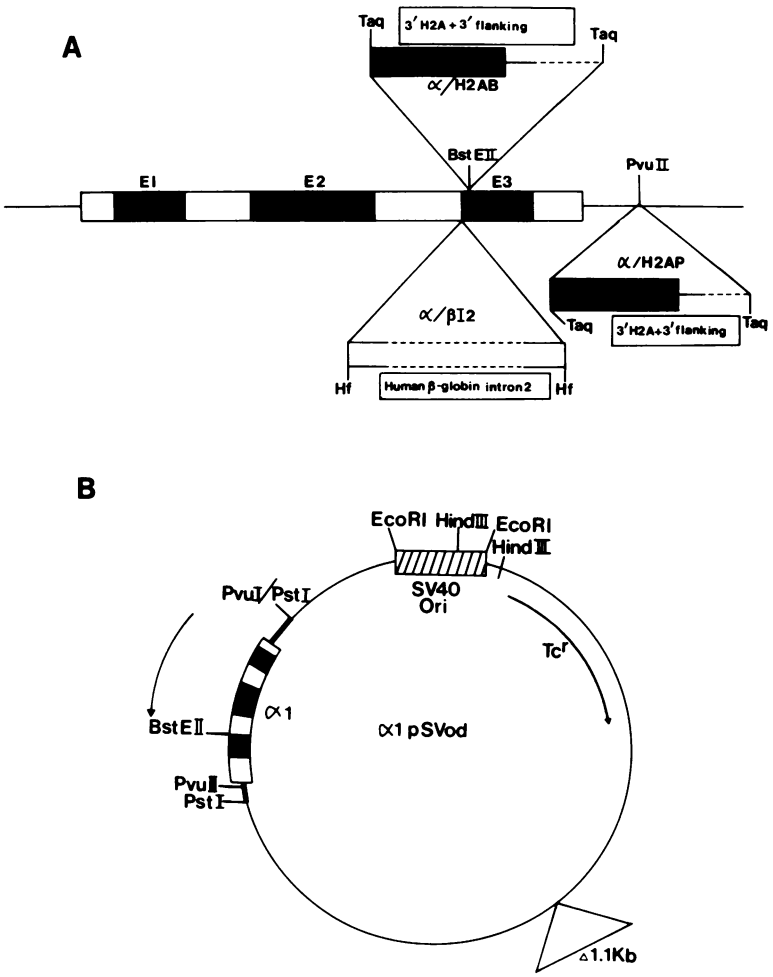


FIG. 3. (A) Line diagram showing the position of the H2A gene 3′ fragment inserted into the human α1 globin gene at the third exon *Bst*EII site (α/H2AB) and 3′ flanking region *Pvu*II site (α/H2AP). The human β globin intron fragment inserted at the α1 globin gene *Bst*EII site is indicated (α/βI2). Filled-in boxes are exons. Open boxes are introns and α globin gene noncoding sequence. Dotted line indicates that the DNA fragment length is longer than drawn. Restriction sites used in construction are shown. (B) Line diagram showing the position of the human α1 globin gene in the pSVod plasmid. Hatched box is SV40 origin (Ori) sequence. Filled-in boxes are α globin gene exons. Open boxes are α globin gene introns and noncoding sequence. Thick line is α globin gene flanking sequence. Thin line is pBR322 sequence. Δ indicates deletion of 1.1 kb from pBR322, a sequence that inhibits replication in tissue culture cells (35). Arrows indicate direction of α globin gene and tetracycline resistance (Tc^r) gene transcription. Restriction sites used in construction are indicated.

[kb]) containing the intact histone H2A gene, including about 200 bp of 5′ flanking sequence and nearly all of the 3′ flanking sequence (14, 15), was subcloned into the transient expression vector SVpBR328 (16). As indicated in Fig. 1B, the clone contains a large portion of SV40, including the origin of replication, the transcriptional enhancer sequence, and the T antigen gene, all necessary for high levels of histone gene expression (1). Following transient expression of this plasmid in HeLa cells by standard procedures (35), cytoplasmic RNA was purified and the 3′ ends of the H2A mRNA were mapped by using both exonuclease VII (exo VII) and S1 nuclease with a DNA probe 3′-end labeled at an *Xho*I site in the H2A coding sequence and extending through all the H2A 3′ flanking sequence into the SV40 vector sequences (Fig. 1B).

Figure 2A shows the RNA mapping data obtained, and Fig. 2B diagrams the positions of the probe and signals obtained with respect to the H2A gene construct map. Identical hybridizations were set up for the exoVII and S1 reactions so that the different signals obtained with the two enzymes are directly comparable for a particular RNA sample. As indicated, total sea urchin RNA gave signals at the authentic H2A mRNA 3′ end with both exoVII (lane 1) and S1 (lane 2). We reproducibly observed that exoVII bands were slightly larger than equivalent S1 bands, presumably reflecting the different specificities of the two nucleases. However, with the transient expression RNA, different patterns were obtained for exoVII and S1. Neither enzyme gave a band at the H2A mRNA 3′ end position. These data indicate that correct histone mRNA 3′-end processing does not occur in the HeLa cell transient expression system. This result is in agreement with Bendig and Hentschel (1) and is probably due to the absence of homologous small nuclear ribonucleoproteins (RNPs) required for sea urchin H2A gene processing (12). Instead, exoVII digestion (lane 5) gave two bands corresponding in position to a cryptic poly(A) site in the H2A 3′-flanking sequence (10 bp 3′ to an A-rich sequence containing four AATAAA sequences in tandem) and to the SV40 VP1 poly(A) site (Fig. 2B). With S1 nuclease (lane 6), in addition to these two bands, a much stronger doublet band

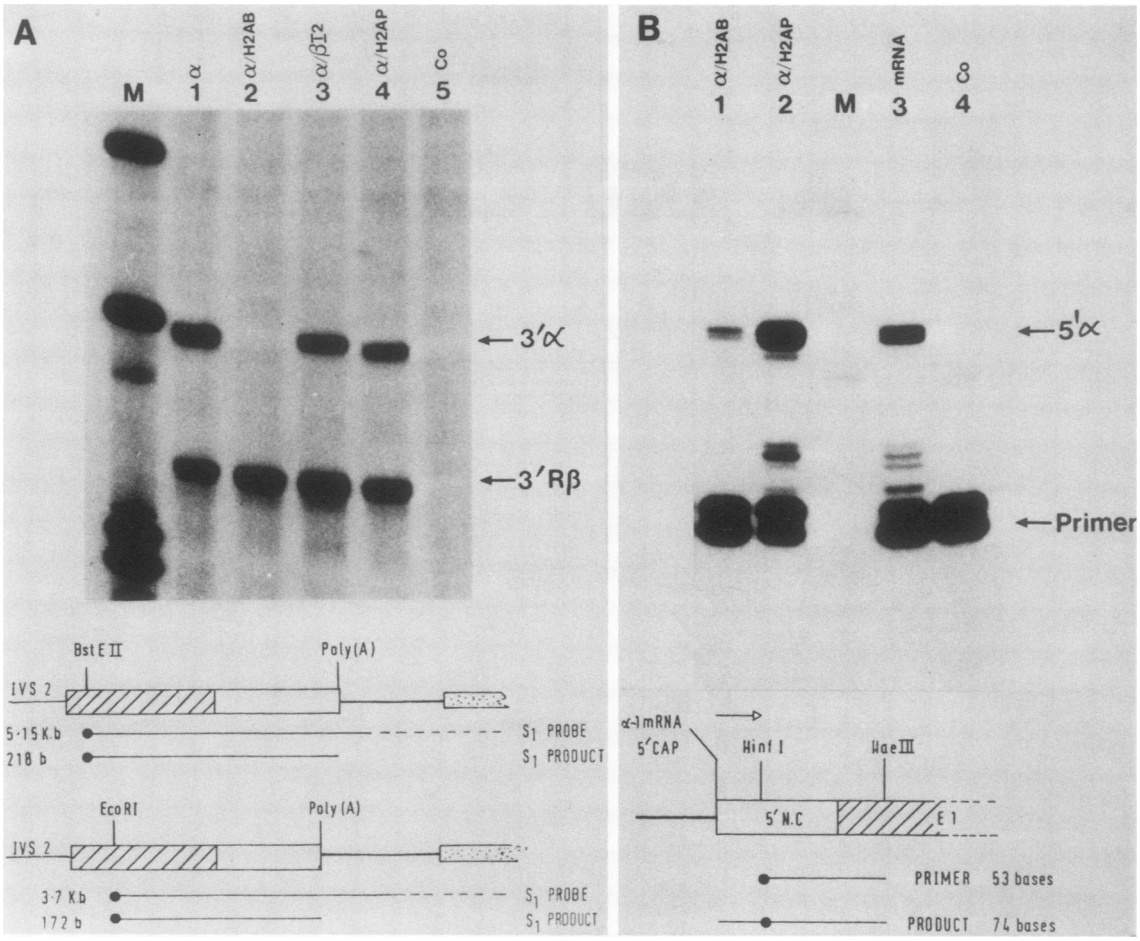


FIG. 4. (A) 3′ end S1 analysis of α1 globin gene, α/H2AB, α/βI2, and α/H2APpSVod transfected HeLa cell RNAs (lanes 1 through 4, respectively) and control yeast RNA (lane 5). The details of the 3′ α1 globin gene probe and control 3′ rabbit β globin gene probe are shown in the diagram below. Both are double-stranded and 3′-end labeled. IVS, Intervening sequence; b, base. The positions of the 3′ α globin (3′*d*) signal and 3′ rabbit β globin signal (3′Rβ) are indicated. In the diagram, the hatched box is exon 3 and the open box is the 3′ noncoding region, the line is 3′ flanking sequence, and the dotted box is vector sequence. The position and size of the S1 probe and product are indicated for both α globin and rabbit β globin. The position of the ^{32}P label is shown (●). (B) 5′ end primer extension analysis of α/H2AB and α/H2AP RNAs (lanes 1 and 2) versus globin mRNA and yeast RNA controls (lanes 3 and 4). 5′α, 5′ alpha globin. Diagram below indicates position of the single-stranded α globin gene primer and product with respect to the α globin gene map. N.C., Noncoding sequence; E1, exon 1; arrow, direction of transcription.

was obtained of intermediate size that corresponds to a CT repeat sequence in the H2A 3′ flanking region. Hentschel (22) has demonstrated that this CT repeat sequence is an S1-sensitive site in linear double-stranded DNA. Furthermore, Pulleyblank et al. (46) have demonstrated that CT repeat sequences form protonated structures that disrupt normal base pairing. We demonstrate here that this same phenomenon occurs with an RNA-DNA duplex to give an S1-sensitive site band not detectable by the single-strand-specific 3′ exoVII.

An interesting feature of this S1 site band is that it is about five times stronger than the only detectable longer RNA in the exoVII digest from which it might be presumed to derive, the VP1 poly(A) site. Any substantial loss of signal between the exoVII and S1 digestions is internally controlled for by the cryptic poly(A) site band in lanes 5 and 6. In fact, this band is somewhat fainter in lane 6 than in lane 5, which would argue for an even greater discrepancy in yield between the VP1 poly(A) site and S1 bands. This discrepancy in yield is further emphasized by the poly(A) selection data shown in Fig. 2A (lanes 7 through 10). As before, the yield of the S1 site band (combination of A^- and A^+, lanes 9 and 10) was about five times stronger than the exoVII VP1 poly(A) site band (lane 8). Also as expected, the two poly(A) site bands are found only in the poly(A)$^+$ fraction (lane 8). In contrast, over 50% of the S1 site signal is poly(A)$^-$ and therefore only a fraction can derive from the VP1 poly(A) site band. Indeed this value is likely to be underrepresented and is presumably near the 5:1 ratio since there is an A-rich sequence close to the S1 site [that includes the cryptic poly(A) site]. Some of the poly(A)$^-$ fraction may therefore be bound by the oligo(dT) column.

Taken together, the high yield and poly(A)$^-$ properties of the S1 site band argue that there is a heterogeneous and therefore undetected series of poly(A)$^-$ 3′ termini between the S1 site and VP1 poly(A) site. We suggest that these transcripts are associated with primary termination events in the H2A 3′ flanking sequences. It is however possible that these heterogeneous ends are generated by rapid exonuclease activity (see Discussion). Figure 2C summarizes these

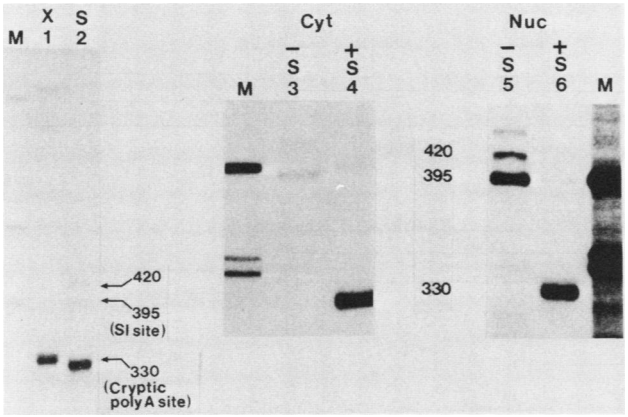


FIG. 5. RNA mapping data on α/H2ABpSVod-transfected HeLa cell cytoplasmic and nuclear RNAs with the 3′-end-labeled *Bst*EII-cut plasmid as the probe. Lanes 1 and 2, exoVII (X) and S1 (S) analysis of α/H2AB cytoplasmic RNA. Lanes 3 and 4, poly(A) selection data on cytoplasmic (Cyt) α/H2AB RNA; lanes 5 and 6, poly (A) selection data on nuclear (Nuc) α/H2AB RNA. The sizes of the bands (in bases) obtained correspond to those obtained with the equivalent 3′ H2A *Xho*I probe described in the legend to Fig. 2. Lanes M, size markers. + and −, poly(A)$^+$ and poly(A)$^-$, respectively.

arguments. As indicated, we predict that there are three types of 3′ termini for the histone gene transcripts. The predominant RNA has heterogeneous poly(A)$^-$ 3′ ends, while the two minor RNAs are poly(A)$^+$, corresponding to the cryptic or VP1 poly(A) sites. Each type of RNA is drawn under the gene map. Both the VP1 poly(A) site RNA and the putative terminated RNA will contribute to the S1 band, as shown by a U in the lines depicting these RNAs.

Transient expression of a hybrid human α1 globin gene-H2A gene construct. To further investigate the possible transcriptional termination process associated with the sea urchin H2A histone gene 3′ flanking sequence, we constructed a hybrid human α1 globin gene into which was inserted (at the beginning of the third exon) the 3′ half of the H2A gene together with its 3′ flanking sequence. Figure 3A shows a diagram of this α globin gene construct, called α/H2AB, together with two control constructs, α/H2AP and α/βI2. The first of these control constructs, α/H2AP, has the H2A insert 3′ to the α globin poly(A) site and was designed to control the possibility that the H2A insert might simply inhibit the α globin gene promoter. The second, α/βI2, has a DNA insert about the same size as the H2A insert (~800 bp) containing the human β globin gene intron 2 but lacking the terminal splice sites, inserted at the same position as the H2A fragment in α/H2AB. This construct was designed to control the possibility that an 800-bp insert in the third exon of the gene might destabilize its mRNA. Each of these α globin gene constructs, together with the intact α1 globin gene, was placed in the transient expression vector pSVod, a plasmid that contains pBR322 sequences together with the SV40 origin of replication (Fig. 3B) (35). Following transient expression in HeLa cells, both cytoplasmic and nuclear RNAs were purified and subjected to RNA mapping procedures.

Initially, the 3′ and 5′ ends of α1 globin mRNA were analyzed. As shown in Fig. 4A, with a *Bst*EII 3′-end-labeled DNA probe derived from the intact α1pSVod (Fig. 3B), α globin mRNA 3′ ends were tested for by using S1 nuclease in the three constructs shown in Fig. 3A. To control for possible variations in transfection efficiency and differential losses in the S1 mapping procedure, the rabbit β globin gene in the SVpBR328 vector (16) was cotransfected with the α globin gene constructs, and a rabbit β globin 3′ probe was mixed with the 3′ α globin probe. Rabbit β globin mRNA 3′-end S1 bands were evident in the cytoplasmic RNA from each transfection. However, although α globin mRNA 3′-end bands were present in nearly equivalent amounts in α1pSVod, α/H2AP, and α/βI2 (lanes 1, 3, and 4), no α globin 3′ ends were detectable in αH2AB (lane 2). These results indicate that no detectable transcripts read through the H2A insert to the α globin gene poly(A) site. Furthermore, this result is not due to promoter inhibition (α/H2AP) or destabilization of α globin mRNA due to expansion of the mRNA size (α/βI2). These results therefore suggested that the putative termination sequence in the H2A gene insert is functional when placed within the α globin gene.

Figure 4B shows 5′-end analysis of the α globin mRNA produced with these α globin gene constructs. The alternative RNA mapping procedure primer extension was used in this experiment. Comparing the levels of correct α globin mRNA 5′ ends between α/H2AB and α/H2AP (lanes 1 and 2), it is evident that although both produced correct 5′ ends, the α/H2AB construct produced 10-fold less than the α/H2AP control. These results imply that the H2A insert results in less stable mRNA production.

To analyze where the 3′ termini of these reduced levels of α globin mRNA map, we probed the α/H2AB cytoplasmic RNA with *Bst*EII-cut α/H2ABpSVod 3′-end-labeled probe. *Bst*EII cuts this plasmid once on the 5′ side of the H2A insert. This position exactly corresponds to the *Xho*I site in the intact H2A gene. The S1 and exoVII products obtained are therefore equivalent to those described in Fig. 2B. We found (Fig. 5) that with both exoVII (lane 1) and S1 nuclease (lane 2) digestion, over 90% of the RNA 3′ ends correspond to the cryptic poly(A) site found to be used at low levels in the intact H2A gene (see Fig. 2A). Only very low signals were obtained at the S1-sensitive-site band position.

Figure 5 also shows a comparison of cytoplasmic and nuclear RNAs obtained from α/H2ABpSVod transfections. Interestingly, although cytoplasmic RNA shows the presence of the poly(A)$^+$ cryptic poly(A) site band and very little poly(A)$^-$ S1 site band (lanes 3 and 4), nuclear RNA revealed a different pattern. Lanes 5 and 6 show that the level of poly(A)$^-$ signal at the S1 site in α/H2AB nuclear RNA (lane 5) was as strong as the cryptic poly(A) site poly(A)$^+$ band (lane 6). Therefore, although this S1 band was only detectable at very low levels in the cytoplasmic fraction, it appears to accumulate in the nuclear compartment.

We interpret the data presented in Fig. 4 and 5 as follows. The histone H2A insert in the human α globin gene prevents transcription reading through to the α globin gene poly(A) site. Rather, transcription terminates beyond the S1-sensitive-site position in the H2A 3′ flanking sequence. Although these poly(A)$^-$ transcripts are detectable as stable cytoplasmic RNAs in the intact histone gene, they are only detectable in the nuclear fraction in the hybrid globin histone gene. Presumably this is because poly(A)$^-$ globin mRNA is highly unstable. However, low levels (10%) of α globin 5′ ends are detectable in the cytoplasm of the α/H2AB transfections due to the utilization of the cryptic poly(A) site. This poly(A) site must be inefficient; if it were as efficient as the α globin gene poly(A) site, normal levels of α globin mRNA would be detectable.

For the rest of this paper we shall refer to the H2A

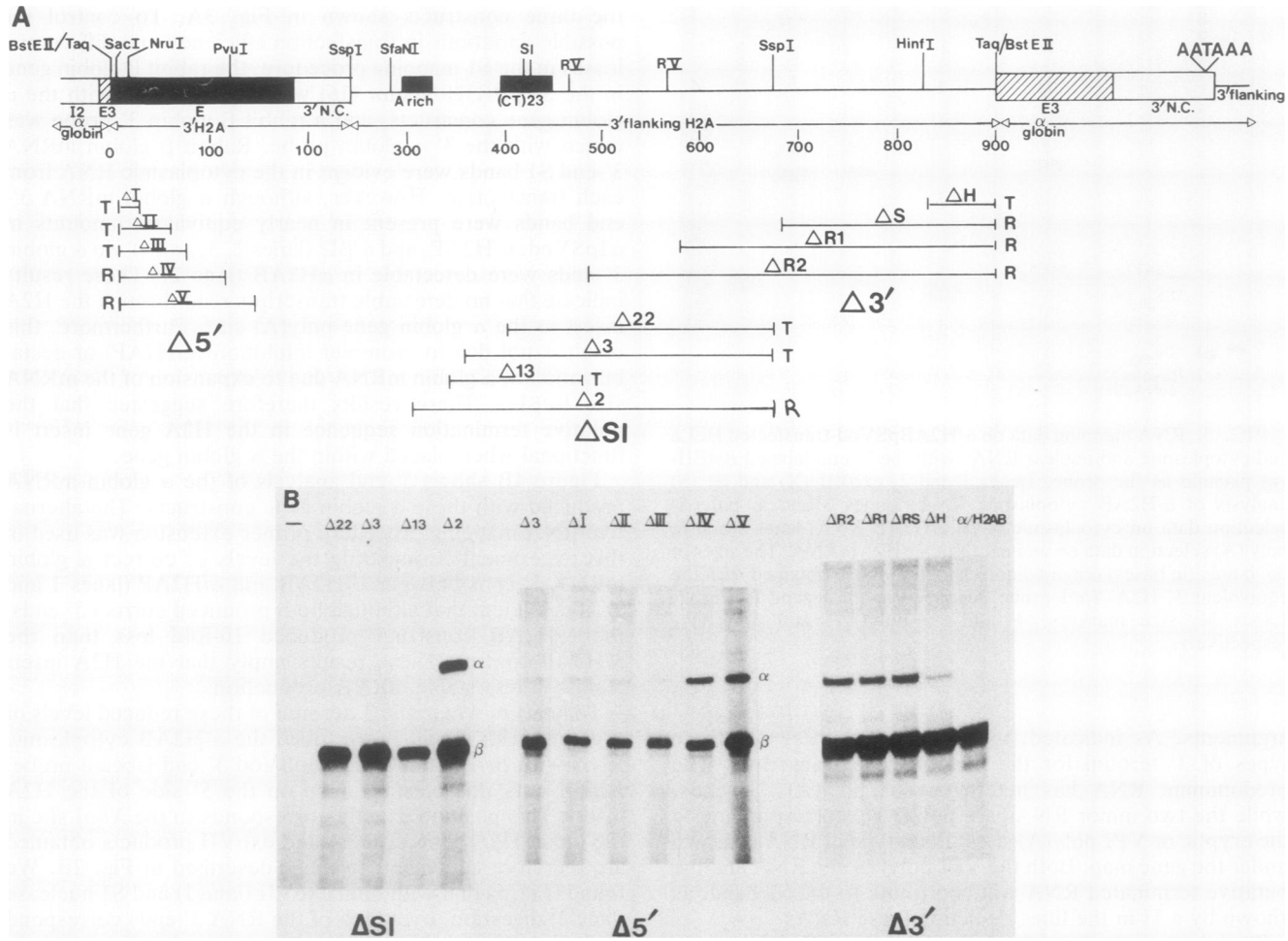
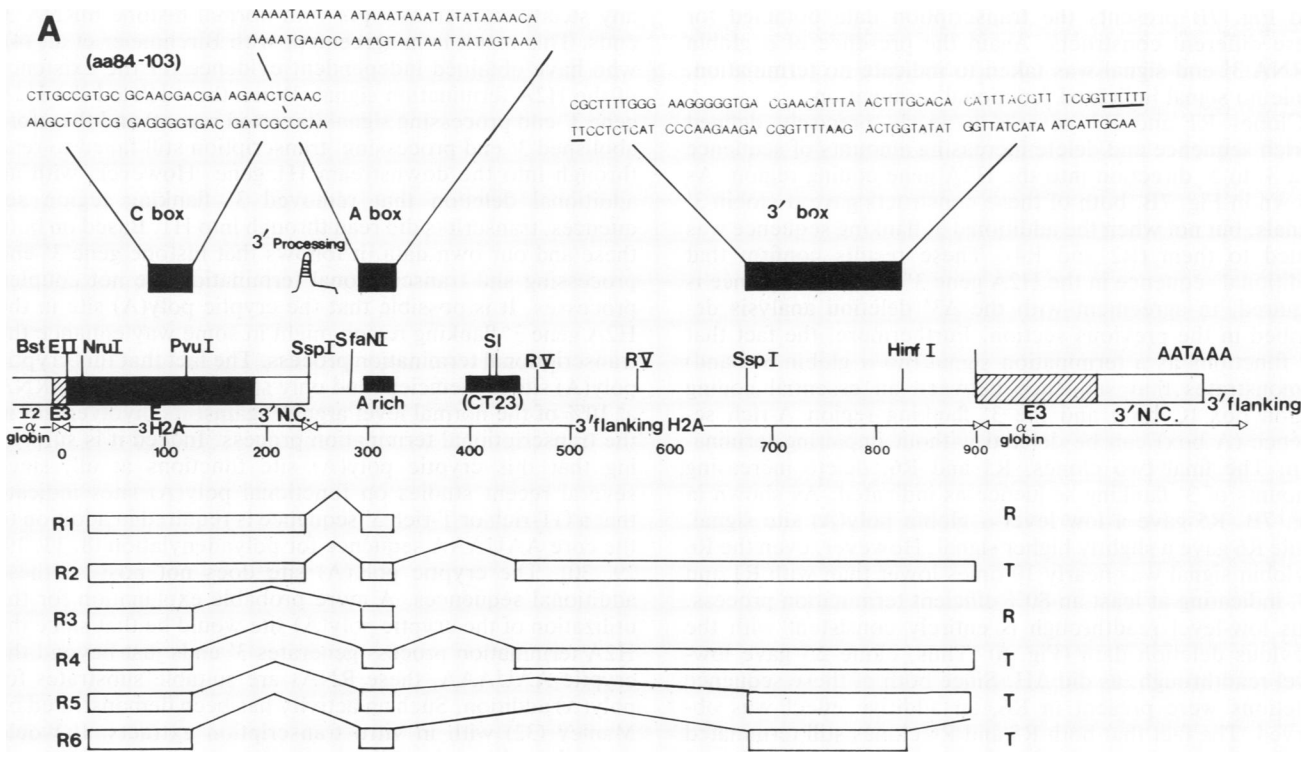


FIG. 6. Restriction enzyme map of H2A gene 3' *Taq* fragment inserted into the α1pSVod *Bst*EII site (α/H2AB) showing the positions of the different deletion clones for the Δ5', ΔS1, and Δ3' series. Deleted sequence denoted by ⊢——⊣. Name of each clone is above each deletion line. T and R denote whether deletion clone terminates or reads through. On map: I, intron, E, exon; N.C., noncoding. Numbers show distances (in bases). (B) 3' and S1 analysis of the deletion mutants shown in panel A. The positions of the 3' α globin signal and 3' rabbit β globin control signal are indicated. See Fig. 4A for details of probes used and signals obtained. Although the ratios of α to β signal are comparable for each separate deletion series, they are not comparable between different deletion series or with the similar analyses shown in Fig. 4 and 7B.

sequence as a transcriptional terminator, although this identification must be qualified as described in the Discussion.

Deletion analysis of the histone H2A termination sequence in the α globin gene construct. Evidence presented in the previous two sections suggests that transcriptional termination occurs in the 3' flanking region of the sea urchin H2A histone gene. We wished to investigate this phenomenon in greater detail by defining the DNA sequences required for this termination process. As a convenient assay we elected to study mutants carrying deletions in the H2A sequence placed within the α globin gene (α/H2AB). The presence or absence of transcripts reading through to the α globin gene poly(A) site could therefore be used as a simple assay for the loss or retention of the termination signal. The rabbit beta globin SVpBR328 cotransfection control signal provides a simple built-in measure of transfection efficiency. As described above, there is a strong S1-sensitive site in the H2A histone gene 3' flanking region DNA (22). At first it seemed plausible to us that such a structural feature in the 3' flanking region might very well be associated with a transcriptional termination effect. We therefore investigated the effect of deleting this S1-sensitive sequence. Thus, the α/H2ABpSVod plasmid was linearized with S1 at the S1-sensitive site, treated with Bal31 exonuclease, and religated. Each deletion mutant was mapped by DNA sequencing, and the extent of the different deletions is indicated in Fig 6A (the ΔS1 series). Clone Δ13 more or less precisely deletes the S1-sensitive sequence. Indeed, the Δ13 plasmid loses its S1 sensitivity at this position (M. R. Johnson, Ph.D. thesis, Oxford University, U.K., 1984). Clones Δ22, Δ3, and Δ2 delete rather more sequence. Interestingly, the Δ3 and Δ2 deletions have nearly the same 3' endpoints but differ at their 5' ends by the A-rich sequence. As shown in Fig. 6B, Δ22, Δ3, and Δ13 gave no readthrough signal to the α globin poly(A) site, while Δ2 did. These results therefore rule out an involvement of the S1-sensitive site in the termination process but strongly implicate the A-rich sequence in this process (compare Δ2 and Δ3).

To further define critical sequences for the termination process we made a series of deletion mutants from both the 5' end (Δ5') and the 3' end (Δ3') of the H2A *Taq* fragment. With Bal31 exonuclease, we deleted increasing amounts of



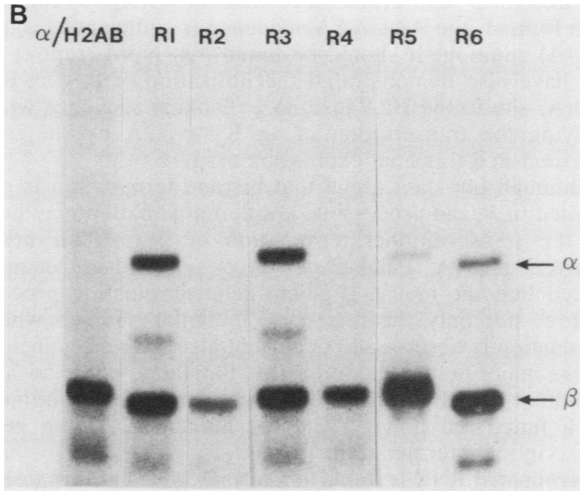


FIG. 7. (A) Restriction map of H2A termination signal. Positions and sequences of the C box, A box, and 3′ box are shown. Distances are indicated (in bases). The reconstruction clones are depicted below the map. Boxes are sequences present, and lines denote sequences deleted. R, Readthrough; T, terminate; aa, amino acid; N.C., noncoding. (B) 3′ and S1 analysis of reconstruction clones R1 through R6 as described for Fig. 6B.

sequence in a 5′ to 3′ direction from the *Bst*EII site of the α/H2AB deletion clone Δ3. This particular 3′ deletion clone was selected for 5′ deletion analysis since it contains the largest deletion of the ΔS1 series that still possesses an intact termination process. To maintain the normal α globin gene sequence on the 5′ side of the *Bst*EII site, each deletion mutant was recloned into the α globin gene *Bst*EII site by using a *Bgl*II linker ligated into each Bal31 deletion endpoint (see Materials and Methods). Clones ΔI through ΔV were thus obtained and precisely mapped as shown in Fig. 6A. Figure 6B shows the transcriptional analysis of these five clones and reveals that while ΔI, ΔII, and ΔIII gave no α globin poly(A) site band, ΔIV and ΔV did. Therefore, the 5′ boundary for the H2A termination signal is surprisingly located within the H2A gene coding sequence between the ΔIII and ΔIV deletion endpoints. For the Δ3′ series we were able to make use of available restriction sites to obtain a series of deletions as indicated in Fig. 6A (see Materials and Methods). Figure 6B reveals that the first deletion mutant (ΔH) gave low levels of α globin poly(A) site signal, while each of the other three larger deletions gave full amounts of α globin poly(A) site signal. Therefore, a 3′ boundary is defined as being close to the *Hin*fI restriction site. Taken together, these data indicate that the sequences required for the putative transcriptional termination process are at least bipartite. One part has a 5′ end in the H2A coding sequence and 3′ end to the A-rich sequence. The other part has a 5′ end 3′ to the ΔS1 Δ3 deletion mutant and a 3′ end near the *Hin*fI site. The 300 bp of sequence deleted in the Δ3 clone represents a nonessential middle section of the H2A terminator sequence.

Reconstruction of the termination sequence reveals a tripartite structure. We wished to confirm and extend the results of deletion analysis described in the previous section by reconstructing the termination signal. In particular we hoped to ascertain whether the whole H2A sequence from the coding sequence through to the 3′ flanking region A-rich sequence was required or whether a middle portion of this sequence was nonessential. Various constructs were produced first by cloning several different DNA fragments into a plasmid polylinker sequence and then by excising the whole construct and placing it in the α globin gene *Bst*EII site used before. Figure 7A shows the various constructs produced,

and Fig. 7B presents the transcription data obtained for these different constructs. Again the presence of α globin mRNA 3′ end signal was taken to indicate no termination, while no signal indicated functional termination

Clones R1 and R3 possess a nearly precisely defined A-rich sequence and delete increasing amounts of sequence in a 3′ to 5′ direction into the H2A gene coding region. As shown in Fig. 7B, both of these constructs gave α globin 3′ signals, but not when the additional 3′ flanking sequence was added to them (R2 and R4). These results confirm that additional sequence in the H2A gene 3′ flanking sequence is required, in agreement with the Δ3′ deletion analysis described in the previous section. Furthermore, the fact that R4 functions as a termination signal (no α globin 3′ band) demonstrates that sequence between an essential coding region box (C box) and the 3′ flanking region A-rich sequence (A box) can be deleted without impairing termination. The final two clones, R5 and R6, delete increasing amounts of 3′ flanking sequence as indicated. As shown in Fig. 7B, R5 gave a low level α globin poly(A) site signal, while R6 gave a slightly higher signal. However, even the R6 α globin signal was nearly 10 times lower than with R1 and R3, indicating at least an 80% efficient termination process. This low-level readthrough is entirely consistent with the previous deletion data (Fig. 6). Thus, clone Δ3 gave low-level readthrough, as did ΔH. Since both of these sequence deletions were present in R6, an additive effect was observed. The fact that both R5 and R6 clones still terminated confirms the previous deletion analysis data, which indicated that a second 3′ flanking region signal (3′ box) was separate from the A box. Together these data demonstrate a tripartite structure for the H2A gene termination signal. The fact that sequence between the C, A, and 3′ boxes could be deleted without significantly affecting the process indicates that the spacing of these three boxes in the H2A gene is unimportant to this process. The precise limits of this tripartite structure are drawn in Fig. 7A together with the sequences of the three essential boxes.

DISCUSSION

We present evidence for a complex RNA polymerase II termination signal in the sea urchin H2A histone gene with the HeLa cell transient expression system. Since all of our data are based on steady-state RNA analysis, we cannot formally distinguish transcriptional termination from RNA instability. However, we did detect RNA species with heterogeneous poly(A)$^-$ 3′ termini from HeLa cells transfected with the intact H2A gene construct (Fig. 2) and in the nuclear fraction of HeLa cells transfected with the hybrid α/H2AB gene construct (Fig. 5). These RNA species are plausible candidates for primary termination events. Furthermore, we consider the possibility that the H2A gene possesses specific sequences that destabilize RNA transcripts unlikely. We have shown that three separate parts of the H2A histone gene are required in combination and that no effect is observed when any one of the three parts is deleted. It is therefore hard to imagine how such sequence specificity could be associated with an artifactual RNA destabilization process. However, we are currently attempting a nuclear runoff analysis on transfected H2A genes to confirm our steady-state RNA data.

Transcriptional termination occurs independently of normal mRNA 3′ end processing. We have demonstrated that the histone termination signals function independently of histone mRNA 3′ end processing, since we did not detect any steady-state transcripts with normal histone mRNA 3′ ends. This result is in agreement with Birchmeier et al. (4), who have obtained independent evidence for the existence of the H2A termination signal. When they deleted the H2A gene 3′ end processing signal (inverted repeat) and therefore abolished 3′ end processing, transcription still failed to read through into the downstream H1 gene. However, with an additional deletion that removed 3′ flanking region sequences, transcripts did read through into H1. Based on both these and our own data, it follows that histone gene 3′ end processing and transcriptional termination are not coupled processes. It is possible that the cryptic poly(A) site in the H2A gene 3′ flanking region might in some way enhance the transcriptional termination process. The fact that this cryptic poly(A) site is inefficient and only stabilizes α globin mRNA at 10% of the normal level argues against its involvement in the transcriptional termination process. Indeed it is surprising that this cryptic poly(A) site functions at all, since several recent studies on functional poly(A) sites indicate that a GT-rich or T-rich 3′ sequence is required in addition to the core AAUAAA sequence for polyadenylation (8, 13, 19, 29, 30). The cryptic poly(A) site does not possess these additional sequences. A more probable explanation for the utilization of the cryptic poly(A) site would be that since the H2A termination process generates 3′ ends just beyond the cryptic AAUAAA, these RNAs are suitable substrates for poly(A) addition. Such an activity has been demonstrated by Manley (32) with in vitro transcription extracts. It would seem that although additional sequences to AAUAAA are required for 3′ end processing, once an mRNA 3′ end has been formed, the AAUAAA sequence is sufficient to signal poly(A) addition. It should be noted that Nordstrom et al. (41) have also demonstrated the utilization of this cryptic poly(A) site in the H2A histone 3′ flanking sequence when studying the transcription of an SV40-H2A hybrid gene construct in a transient expression assay.

Although our data argue that histone termination is not coupled to 3′ end processing, a different situation may hold for the transcriptional termination of genes that make poly(A)$^+$ mRNA. Falck-Pedersen et al. (9) have demonstrated that the mouse β globin gene termination process requires not only the region of 3′ flanking region where termination is observed to occur but also sequences including the major poly(A) addition site. Similarly, Whitelaw and Proudfoot (EMBO J., in press) demonstrate the requirement for a functional poly(A) site for human α2 globin gene transcriptional termination.

Terminated RNA is stable in a histone gene but unstable in a globin gene. Transcription of the intact H2A histone gene in HeLa cells that lack 3′-end-processing activity generates a heterogeneous series of poly(A)$^-$ 3′ termini that account for about 80% of all H2A transcripts. Since these transcripts are present in steady-state populations of cytoplasmic RNA , we cannot directly prove that they represent primary termination events. Indeed it is possible that they derive from the exonucleolytic 3′ end degradation of a primary termination product. However, both Citron et al. (7), studying the mouse β major globin gene termination process, and Hagenbuchle et al. (18), studying the mouse α amalyase gene termination process, suggest that transcriptional termination results in heterogeneous 3′ termini.

The different stabilities of these heterogeneous 3′ end transcripts in histone and globin genes may relate to their poly(A)$^-$ nature. In the intact histone gene these transcripts appear to be relatively stable and are transported into the cytoplasm. These observations demonstrate that 3′ end

processing of histone mRNA is not absolutely required for mRNA stability and transport. Indeed, the processing function may simply operate to "tidy up" the heterogeneous 3' ends into one discrete mRNA species. This may be required for correct packaging of the mRNA into an RNA protein complex (mRNP). In contrast, these heterogeneous poly(A)$^-$ transcripts are highly unstable when synthesized from the α globin gene promoter in the α/H2A gene constructs. They were only detectable in the nucleus since the low level of stable cytoplasmic RNA was poly(A)$^+$, utilizing a poly(A) signal fortuitously present within the A-rich region, a cryptic poly(A) site. These results demonstrate that poly(A)$^-$ globin mRNA is a highly unstable species and therefore provide strong evidence for the role of poly(A) in nonhistone mRNA stability. There must be specific features of histone mRNA that stabilize mRNA without poly(A) tails. Since both the heterogeneous 3' extended transcripts observed in these studies and mature histone mRNA are apparently stable without poly(A) tails, the presence of a hairpin structure at the end of the processed mRNA is unlikely to be the principal feature conferring RNA stability. Indeed, this hairpin structure is present within the unstable transcripts of the hybrid α globin H2A gene. It will be interesting to investigate which features of histone mRNA stabilize poly(A)$^-$ RNA species.

A tripartite termination signal. We have demonstrated that the H2A termination signal is surprisingly complex, containing one part in the coding sequence (C box) and two parts in the 3' flanking sequence (A box and 3' box). It is interesting to compare this signal with the sequences required for H2A histone mRNA 3' end processing. Birchmeier et al. (2, 3), using the *Xenopus laevis* oocyte microinjection transcription system, have clearly demonstrated the requirement for both an inverted repeat sequence at the 3' end of the H2A mRNA and an immediately adjacent 3' flanking region sequence, CAAGAAGA. Both of these sequences are conserved among different histone genes. Interestingly, this 3' processing signal is positioned between the C box and A box regions of the termination signal so that these two distinct transcriptional functions do not overlap.

It is interesting to speculate on the possible roles of the three essential parts of the H2A termination signal. Preliminary computer searches for sequences homologous to the C box in other histone genes do not reveal significant sequence homologies. However, the C box has not yet been precisely defined, so that such sequence comparisons are as yet premature. It seems possible that the A box may not be a sequence-specific requirement but may rather reflect an AT richness requirement for the termination process. Finally, the 3' box is still relatively ill defined. However, it should be noted that within this sequence several oligo(T) sequences are present, especially a T_8 sequence (Fig. 7B). Such sequences have been implicated in both procaryotic termination (49) and eucaryotic RNA polymerase III termination (6).

While this manuscript was in preparation, Sato et al. (50) described a T-rich sequence 3' to the human gastrin gene that when placed 5' to an SV40 poly(A) site, prevents transcripts reading through it to form stable poly(A)$^+$ mRNA that utilizes the SV40 poly(A) site. These and additional data are interpreted as strong evidence that this T-rich sequence is a termination signal. That other sequences in addition to this T-rich sequence are also required was not discounted by these studies. The obvious parallel between those experiments and the ones described here is that in both studies an unusually long, exclusively AT sequence is implicated in the termination process. The fact that a short AT sequence, TTTTATA, is implicated in *Saccharomyces cerevisiae* RNA polymerase II termination (20) may also relate to these observations. Possibly, AT-rich sequences may be general features of eucaryotic RNA polymerase II transcriptional termination.

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