Formation of the 3' End of Sea Urchin U1 Small Nuclear RNA Occurs Independently of the Conserved 3' Box and on Transcripts Initiated from a Histone Promoter†

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The formation of the 3' end of vertebrate small nuclear RNAs (snRNAs) requires that transcription initiate from an snRNA promoter. There is a loosely conserved required box 5 to 20 nucleotides (nt) 3' of the gene. The sea urchin snRNA genes contain promoter elements different from those of the vertebrate snRNAs. They also contain a characteristic 3' 15-nt sequence which is conserved among different sea urchin snRNA genes. We used microinjection of sea urchin U1 snRNA genes into sea urchin zygotes to define the sequence requirements for U1 snRNA 3'-end formation. Surprisingly, the conserved 3' box is not required for efficient 3'-end formation in vivo. Deletion analysis reveals that the 6 nt immediately 3' of the U1 snRNA are involved in 3'-end formation. Substitution analysis revealed that either these 6 nt 3' of the U1 RNA or the conserved 3' box could direct 3'-end formation. Transcripts initiated from a histone H4 promoter formed U1 3' ends about 50% as efficiently as transcripts initiated from the U1 promoter, even when the U1 end was placed in tandem with a histone 3'-processing signal, suggesting that transcription from an snRNA promoter is not necessary for formation of the 3' end of sea urchin U1 snRNA.

The small nuclear RNA (snRNA) genes transcribed by RNA polymerase II contain distinct promoter elements from the genes encoding mRNAs (24). Initiation of transcription is determined by a proximal sequence element located at about -55. Formation of the snRNA 3' end requires that transcription initiate from an snRNA promoter (14, 23), and 3'-end formation is mediated by a conserved sequence element present 15 to 20 nucleotides (nt) downstream of the end of the snRNA (1, 14). Transcripts which initiate from promoters of genes encoding mRNAs, including herpesvirus tk, simian virus 40, globin (14, 23) and histone mRNAs (25), continue downstream of the end of the snRNA and do not form mature snRNAs. The formation of the 3' ends of vertebrate snRNAs is thought to occur by transcription termination (13, 22). The 3' box downstream of the vertebrate snRNA genes varies significantly between different snRNA genes, indicating that there is not a strict sequence requirement for snRNA 3'-end formation (1).

In contrast, the 3' ends of the other transcripts synthesized by RNA polymerase II, i.e., the polyadenylated mRNAs and the histone mRNAs, are formed by RNA processing reactions (2). Each of these reactions requires a bipartite signal, and cleavage occurs between the two elements. In the polyadenylated mRNAs, 3'-end formation is catalyzed by a complex of proteins and apparently does not require an RNA component (35), while the formation of histone 3' ends requires the U7 small nuclear ribonucleoprotein as well as several other components (10, 20, 21, 33). The

U7 small nuclear ribonucleoprotein recognizes the downstream conserved purine-rich sequence by base pairing (3, 30).

The mechanism of 3'-end formation of snRNAs in invertebrate systems has not been studied. The 3' ends of yeast snRNAs are formed properly on transcripts initiated from promoters of genes encoding mRNAs (11). In the sea urchin, there is an AG-rich sequence 3' of the snRNA genes encoding U1, U2, and U7 snRNAs (4, 8, 27). This sequence is similar to the sequence 3' of the histone genes, which interacts with U7 snRNA. The structure of the sea urchin U1 snRNA promoter is similar to those of the vertebrate snRNA promoters. There are two essential sequences, the proximal sequence element, located at about -55, and a distal sequence element located at about -300 (34). The sea urchin snRNA genes are not transcribed in *Xenopus* oocytes (30), indicating that the promoter elements interact with different factors, although the 3' end of sea urchin U7 snRNA was formed properly when the U7 snRNA was transcribed by using a *Xenopus* U2 snRNA promoter (30).

Here, we examine the sequence requirements for formation of the 3' end of U1 RNA in sea urchin embryos. We find that there is a bipartite signal, either part of which suffices to direct 3'-end formation. We also find that, unlike in vertebrate systems, there is efficient formation of U1 3' ends on transcripts which are initiated by using a sea urchin histone gene promoter.

MATERIALS AND METHODS

Construction of 3' deletion mutants. All of the constructs were made by using the LvU1.2 maxigene, which has been previously described (29, 34). Briefly, deletion mutants +122, +66, and +34 were constructed by taking advantage of convenient restriction enzyme sites located in the 3' noncoding region of the gene. All other 3' deletion mutants were generated by digesting clone +34 with *Pst*I, which was

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originally part of the polylinker of pUC18. The linearized DNA was treated with *Bal* 31 exonuclease for 30, 60, and 120 s to create the deletions. The DNA was treated with the Klenow fragment of DNA polymerase I to ensure that most of the ends were blunt, and the plasmids were religated by using T4 DNA ligase. The clone 3'ApaI, which has the six bases immediately 3' of the U1 gene changed, was generated by the method of Kunkel (15). The U13' Δ clone was constructed by digesting the 3'ApaI clone with *Apa*I and inserting the fragment containing the promoter and coding region of the U1 gene into the *Apa*I site of the plasmid pGEM7zf(+). The 3' flanking region of the U1 gene in this clone consists only of polylinker sequences in the vector.

Construction of U1-histone hybrid genes. Hybrid genes were constructed from the *Lytechinus variegatus* LvU1.2 maxigene, the *Strongylocentrotus purpuratus* α -histone H2a gene (31), and the *S. purpuratus* α -histone H4 gene (32). The 3' end of the H2a histone gene was an *Hind*III-*Eco*RI fragment extending from codon 94 to 30 nt 3' of the end of the mRNA. This fragment was attached to the *Bgl*II site at nt 140 of the U1 RNA or to the *Nde*I site 122 nt 3' of the end of the U1 RNA to construct the clones U1B/H2a and U1N/H2a, respectively.

The EH4/U1 construct was generated by cloning the α -histone H4 promoter, the *Xba*I-*Hind*III fragment from p+19EH4 (32) (a generous gift from E. Weinberg), into the *Eag*I site 19 nt 5' of the start of the U1 RNA. This 436-nt histone promoter fragment contains all of the *cis*-acting DNA elements required for transcription of the α -histone H4 gene (32) in addition to 19 bases of the 5' nontranslated region of the H4 gene. Two additional hybrid clones, EH4/U1B/H2a and EH4/U1N/H2a, were constructed by fusing the 3' ends of U1B/H2a and U1N/H2a by using the *Nco*I restriction site in the U1 maxigene.

Microinjection of DNA into sea urchin zygotes. Microinjection of template DNAs was performed by the method of McMahon and coworkers (17) as modified by Colin and coworkers (6). In some experiments, the test gene was coinjected with a control gene, the LvU2_H gene (28). The internal control gene and all test genes were linearized at the 3' end at least 500 nt 3' of the end of the RNA, and the concentration of each was adjusted to 50 μ g/ml. Test gene and internal control DNAs were then mixed in equal volumes so that the overall DNA concentration remained constant. Approximately 10 to 20 μ l of DNA solution was injected into either *S. purpuratus* or *Lytechinus pictus* zygotes, and 100 to 200 embryos were injected with each DNA sample. The embryos were incubated at 15°C and were harvested shortly (20 h) after hatching. The embryos were collected and total cellular RNA was isolated as previously described (28).

Analysis of RNA transcripts. The transcripts from the injected gene were analyzed by using a riboprobe assay, essentially as described previously (27, 29). The riboprobe assay used for the U1_M genes is shown in Fig. 1C, and the assays for the U1-histone hybrid genes are described in each figure legend. When the LvU2_H gene was used as an internal control gene, the transcripts from the test gene and internal control gene were assayed by using two riboprobes in the RNase protection assay. The template for transcribing the U1 riboprobe was the U1_M gene linearized at the *Eag*I site at nt -19. The LvU2_H gene was analyzed by using a riboprobe from the LvU2_M gene as described previously (28). This probe protects a 158-nt fragment of the 177-nt transcript from the LvU2_H gene, allowing better separation from the U1 maxigene transcript (28). Equal amounts of the two

probes were mixed together and the RNase protection assay was carried out as described above. The samples were analyzed by electrophoresis on 8% polyacrylamide gels in 7 M urea, and the protected fragments were detected by autoradiography.

RESULTS

Expression of 3' truncated deletion clones. To assay the expression of the sea urchin U1 snRNA gene in a homologous system, a maxigene was constructed by inserting a 19-nt oligonucleotide into the coding region at nt 112 of the U1 gene. In order to assess the importance of 3'-end sequences to U1 3'-end formation, we first generated a series of 3' truncated genes from the U1 maxigene. We also changed the 6 nt directly flanking the 3' end of the U1 RNA. Figure 1A shows the genes with altered 3' ends which were used in these experiments. Figure 1B shows a second set of constructs in which hybrid genes combining the sea urchin U1 gene and the α -histone H2a 3' end and/or the α -histone H4 promoter were constructed. These genes were used to assess the role of the promoter in 3'-end formation and to assess the possible competition between the different 3'-end signals.

The transcripts from the U1_M maxigene and the endogenous U1 snRNA can be mapped as discrete protected fragments by using the U1_M maxigene as a riboprobe (Fig. 1C). The LvU1_M gene was injected together with an LvU2_H gene as an internal control in some experiments. When the LvU2_H gene was used as an internal control, transcripts from both the U1_M gene and the LvU2_H gene were analyzed simultaneously by using riboprobes derived from the LvU1_M and LvU2_M maxigenes. The LvU2_M maxigene has been described previously (28). The LvU2_M riboprobe protected a 158-nt fragment of the transcript from the LvU2_H gene (Fig. 1C), which was readily distinguished from the 180-nt fragment protected by the LvU1_M riboprobe. The assay is diagrammed in Fig. 1C.

The plasmids were linearized by using a restriction site either in the polylinker or in the plasmid at least 500 nt from the 3' end of the gene and were injected into sea urchin zygotes. The embryos were harvested after they hatched (at 18 h), which is the time of maximal expression of the U1 genes (26). Initial experiments showed that genes containing 122, 64, or 34 nt of 3' flanking sequence (including the CAAGAAGAA box) were all expressed at the same level as the intact gene (data not shown).

Figure 2 shows the results of the microinjection assay of an additional set of deletions extending closer to the 3' end of the gene. The genes were injected either without (Fig. 2A) or with the LvU2_H gene as an internal control gene. All of these genes had either part (+24) or all (+16 and +7) of the purine-rich element deleted (Fig. 1A). In two genes (-1 and -7) the deletion extended either 1 or 7 nt into the U1 RNA. Close examination of the flanking vector sequences did not reveal any sequences that resembled the U1 3' snRNA signal and therefore should not functionally replace it. The U1 maxigenes containing 7, 16, or 24 nt 3' of the gene were all expressed (Fig. 2A, lanes 5 through 7), yielding transcripts identical to those from the U1 maxigene (Fig. 2A, lane 3). Note that any transcripts longer than the U1 RNA would have protected fragments 7, 16, or 24 nt larger than the U1 RNA because the probe used was derived from the wild-type U1 maxigene. Since no longer transcripts were observed, it appears that 3'-end formation was efficient. The absolute level of expression of an injected gene varies from batch to

batch of embryos. To allow quantitative comparison between different genes, another hybrid gene with the U2 5' half and U1 3' half, the LvU2_H gene, was coinjected with the U1 genes as an internal standard. The transcripts from the two genes were assayed simultaneously. The transcripts from the LvU2_H gene protected two fragments when the gene was injected into *S. purpuratus* embryos (Fig. 2B). Comparison of the intensity of the transcripts from the test and control genes shows that constructs with the purine-rich sequence completely deleted are still expressed well, to at least 30% of the level of the wild-type gene (Fig. 2B, lanes 4 through 7). No transcripts extending past the U1 3' end were detected.

In contrast, when the deletion included the last nucleotide of U1 RNA or the last seven nt of U1 RNA, expression of the U1_M gene was greatly reduced (Fig. 2A, lane 4, and 2B, lanes 2 and 3). Note that the riboprobe used would protect all transcripts from these genes as a single fragment, regardless of the 3' ends of these transcripts. The protected fragment produced from the U1(-7) gene is 7 nt smaller than that protected by the wild-type U1 transcripts. The way these genes were constructed precluded synthesis of a riboprobe from these genes, so it was not possible to map the 3' ends of these transcripts. The gene lacking only the last nucleotide of the U1 RNA contains all the secondary structure elements of U1 RNA and hence would be expected to give stable transcripts. There was a great reduction in the amount of transcripts detected from both of these genes (<10% of the amount detected from the wild-type gene), and the

FIG. 1. Genes with altered U1 3' ends. (A) The *L. variegatus* U1 maxigene (28) is diagrammed. The essential promoter elements, i.e., the distal sequence element (DSE) at -300 and the proximal sequence element (PSE) at -50 to -70, are indicated (34). The conserved CAGA box sequence located 3' of the gene is indicated. The solid box in the coding region indicates the position of the inserted oligonucleotide within the coding region. The construction of the maxigene (maxi) has been described previously (28). Below the maxigene, the structures of the 3' deletion mutants are given. The mutants -7 and -1 are missing the last 7 nt and the last nucleotide of the U1 coding region, while the mutants +7, +16, and +24 contain 7, 16, and 24 nt of U1 3' flanking sequence, respectively. The sequence of the CAGA box is given at the bottom, and the nucleotides of the CAGA box present in each gene are shown above the +16 and +24 deletion mutants. (B) The site-specific mutants and the U1-histone hybrid genes are diagrammed. The α -histone H4 gene promoter was attached 5' of the U1 gene in the EH4/U1, EH4/U1N/H2a, and EH4/U1B/H2a genes. The α -histone H2a 3' end was attached at the *Bgl*III site in the U1 gene or at the *Nde*I site 3' of the gene. The U13'Apal gene has an *Apal* site substituted for the 6 nt 3' of the gene, and the U13' Δ gene has all the 3' flanking sequences deleted. (C) The riboprobe assay used to measure the expression of the U1_M gene (maxi) and the endogenous (endo) U1 snRNA is diagrammed. The LvU2_H gene was coinjected with the U1_M genes as an internal control in most experiments. In experiments in which internal control genes were injected, the two riboprobes were mixed and the RNA samples were analyzed in a single reaction. The riboprobe assay used to detect the transcripts from the LvU2_M gene utilized a U2 maxigene riboprobe (28) that protects a 158-nt fragment from the transcripts of the LvU2_H gene, which was readily distinguishable from the 180-nt transcript protected by the U1_M transcripts and from the fragments protected by the endogenous U1 and U2 snRNAs.

results below (see Fig. 4) suggest that these transcripts probably end heterogeneously 3' of the gene.

These results suggest that as few as 7 nt 3' to the U1 RNA are sufficient to specify proper 3'-end formation but that removal of the last nucleotide of the U1 sequence and all the 3' flanking sequences greatly reduces, and possibly abolishes, 3'-end formation.

The 6 nt directly 3' of the U1 RNA are not required for 3'-end formation. The results above suggested that the 6 nt

FIG. 2. Expression of 3' deletion mutants. (A) The $U1_M$ gene deletions were injected into *S. purpuratus* zygotes (lanes 3 through 7). RNA was prepared from embryos at the blastula stage and was analyzed by using the $U1_M$ riboprobe. The gene used is indicated below each lane. RNA from 100 embryos was assayed in each lane. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), RNA from control embryos; lane 3 (wt), wild-type. The protected fragments are as follows: $U1_M$, protection of transcripts from the $U1_M$ gene; E, protection of the endogenous U1 snRNA. (B) The $U1_M$ gene deletions were injected together with the $U2_H$ gene into *S. purpuratus* zygotes, and RNA was prepared from embryos harvested at the blastula stage (lanes 2 through 8). The gene used is indicated below each lane. The RNAs were analyzed by using a mixture of the $U1_M$ and $U2_M$ riboprobes as described for Fig. 1C. Lane 1, pUC18 digested with *Hpa*II; lane 8 (t), analysis of tRNA. The band migrating at 190 in lane 2 [$U13'(-7)$] may be from shortened transcripts as a result of the probes' divergence with the transcripts from this clone. The longer protected fragments are probably due to protection of the injected DNA by the riboprobe. The protected fragments are as follows: $U1_M$, protection of transcripts from the $U1_M$ gene; $U2_H$, protection of transcripts from the $U2_H$ gene; E, protection of the endogenous U1 and U2 snRNAs. The minor transcript at ca. 170 nt (relative to the DNA marker) is also derived from the $U2_H$ gene.

directly 3' of the U1 RNA might play an important role in 3'-end formation. To examine this possibility, two additional genes were constructed. An *Apa*I restriction site (GGGCC) was substituted for the 6 nt directly 3' of the U1 gene to yield the gene $U13'Apa$ I. This construct contains an intact purine-rich element. Taking advantage of the *Apa*I site introduced in construction of the $U13'Apa$ I gene, an additional gene containing only the U1 coding sequences ($U13'\Delta$), including the last nucleotide of the RNA lacking in the $U13'(-1)$ gene, was constructed. The transcript from the $U13'\Delta$ gene would contain all of the nucleotides in U1 snRNA; therefore, if the 3' end is formed properly, the transcript should be stable.

Figure 3 shows the results from an RNase protection assay of the $U13'Apa$ I mutant coinjected with the $LvU2_H$ hybrid gene into *S. purpuratus* embryos. In this experiment, $U13'Apa$ I transcripts were mapped by using the heterologous $U1_M$ riboprobe, and the internal control $LvU2_H$ transcripts were mapped with the $LvU2_M$ riboprobe, which yields a 158-nt protected fragment. The heterologous riboprobe will map all the transcripts from the $U13'Apa$ I gene as

FIG. 3. The nucleotides directly 3' of the U1 RNA are not required for 3'-end formation. (A) The $U1_M$ (lane 3) or the $U13'Apa$ I (lanes 4 and 5) gene was injected into *S. purpuratus* zygotes, and the embryos were harvested at the blastula stage. The $LvU2_H$ gene was coinjected in lanes 3 through 5. The transcripts were mapped with the $U1_M$ and $U2_M$ probes. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), RNA from uninjected control embryos. The protected fragments are as follows: $U1_M$, protection of transcripts from the $U1_M$ gene; $U2_H$, protection of transcripts from the $U2_H$ gene; E, protection of the endogenous U1 and U2 snRNA; p, fragments derived from the probe. The minor transcript at ca. 180 nt is also from the $U2_H$ gene. (B) The $U1_M$ gene (lane 3) and the $U13'Apa$ I gene (lane 4) were injected into *L. pictus* embryos together with the $LvU2_H$ gene, and the embryos were harvested at the blastula stage. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), RNA from uninjected control embryos, lane 5 (t), analysis of yeast tRNA. The transcripts were mapped with riboprobes from the $U13'Apa$ I gene and the $U2_M$ gene. The protected fragments are as follows: $U1_M$, protection of transcripts from the $U1_M$ gene or $U13'Apa$ I gene; $U2_H$, protection of transcripts from the $U2_H$ gene; e, fragment protected by an endogenous transcript present in *L. pictus* embryos; E, protection of the endogenous U1 and U2 snRNAs.

a single fragment corresponding to the size of the transcript from the intact U1 maxigene, since the probe diverges from the transcript precisely at the end of the U1 RNA sequence. The results from two independent injections of the $U13'Apa$ I gene with the internal control gene are shown in Fig. 3A, lanes 4 and 5. The $U13'Apa$ I gene was expressed at a level similar to that of the wild-type U1 gene.

To accurately map the 3' ends of the transcript from the $U13'Apa$ I gene, RNAs from embryos injected with the wild-type gene or the $U13'Apa$ I gene together with the $LvU2_H$ gene were mapped by using a riboprobe made from the $U13'Apa$ I gene. This probe will map all the transcripts from the wild-type gene as a single fragment the size of the U1 maxigene transcript, regardless of their 3' ends. The transcripts from the $U13'Apa$ I gene will be mapped accurately. The results clearly show that the proper U1 3' end is formed efficiently on transcripts from the $U13'Apa$ I gene (Fig. 3B, lane 4) and that the expression of the $U13'Apa$ I gene is similar to the expression of the U1 maxigene (Fig. 3B, lane 3). Thus, simply altering the 6 nt at the 3' end of the

FIG. 4. Expression of the U13'Δ gene. (A) The U1_M gene or the U13'Δ gene was injected into *L. pictus* embryos together with the LvU2_H gene. The transcripts were mapped by using the U1_M riboprobe mixed with the LvU2_M riboprobe. The U1_M probe will map any transcripts from the U13'Δ gene as the same size as the U1_M transcript. Lane 1 (Con), RNA from uninjected control embryos; lane 5 (t), analysis of yeast tRNA. The protected fragments are as follows: U1_M, protection of transcripts from the U1_M gene; U2_H, protection of transcripts from the U2_H gene; p, fragment derived from the probe (the top probe band is from the LvU2_H probe; the bottom probe band is from the U1_M probe); E, protection of the endogenous U1 and U2 snRNAs. (B) The U13'Δ gene was injected into *L. pictus* embryos (lanes 4 and 5), and the embryos were harvested at the blastula stage. The transcripts were mapped with the U13'Δ riboprobe. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), RNA from uninjected control embryos. The position of the transcript from the U1_M gene is shown. The sequence of the U1Δ gene 3' of the end of the U1 RNA is given at the bottom. The sequence 3' of the wild-type U1 gene is given in Fig. 7. The protected fragments are as follows: U1_M, protection of transcripts from the U1_M gene; U2_H, protection of transcripts from the U2_H gene. e, endogenous transcript from the endogenous *L. pictus* gene; E, protection of the endogenous U1 snRNA.

sea urchin U1 gene has no effect on the efficiency or accuracy of 3'-end formation.

Sequences 3' of the U1 RNA are required for 3'-end formation. The two 3' deletion clones whose expression was severely diminished, the U13'(-7) and U13'(-1) genes, lacked any downstream sequences. The failure to find significant amounts of transcripts from these genes probably means that any transcripts formed were unstable. Because of the way the gene was constructed, we were not able to map the 3' ends of the transcripts. To test whether any downstream sequences were required for U1 3'-end formation, the U13'Δ gene was constructed. This gene contains all the nucleotides of the U1 RNA but differs completely from the wild-type gene 3' of the end of U1 RNA. The sequence of the 3' region is shown in Fig. 4. The U13'Δ gene was injected together with the LvU2_H gene and assayed with either the

heterologous U1_M riboprobe (Fig. 4A) or the homologous U13'Δ riboprobe (Fig. 4B) together with the LvU2_M riboprobe. Only a small amount of transcripts (<10% of that of the wild-type gene) from the U13'Δ gene were detected with the heterologous U1_M riboprobe (Fig. 4A, lanes 3 and 4), and no transcripts were detected with the homologous probe even though expression of the wild-type U1 gene was readily detected. Since the U13'Δ gene was transcribed and because the coinjected internal control gene was expressed, these results indicate that the transcripts from the U13'Δ gene probably end heterogeneously and hence were unstable. The level of transcripts from the U13'Δ gene was similar to that for the U13'(-1) gene with the heterologous probe (compare Fig. 4A, lanes 3 and 4, with Fig. 2B, lane 3). Note that this result rules out the possibility that the formation of the proper U1 3' ends in the U13'(+7) and U13'(+16) clones described above was due to a cryptic 3' signal from the plasmid, since the sequence in this region does not resemble that of the purine-rich element. Thus, formation of the U1 3' end requires some 3' flanking sequences. These results suggest that either the 6 nt immediately 3' of the U1 gene or the purine-rich signal is required for proper 3'-end formation. Moreover, these results demonstrate that the sequence requirements for U1 3'-end formation differ between sea urchin and vertebrate snRNAs.

The U1 3' end can form on transcripts which are initiated from a histone promoter. A striking characteristic of 3'-end formation of vertebrate snRNA genes is that formation of the 3' end depends on initiation from an snRNA promoter. Transcripts which are initiated at mRNA promoters read through the U1 3' signal (14, 23). The mechanism of coupling the promoter to 3'-end formation is not well understood.

We investigated whether there was a similar dependence of 3'-end formation on the promoter in sea urchins by constructing chimeric genes containing histone promoters and U1 3' ends. The *S. purpuratus* α-histone H4 promoter and α-histone H2a 3' end were fused to the U1 gene to generate the chimeric genes shown in Fig. 1B. The results presented below indicate that the sea urchin U1 3' end can form efficiently on transcripts initiated from a histone promoter. In the first chimeric gene, the U1 promoter was replaced by the α-histone H4 promoter. This promoter contains all of the *cis*-acting DNA elements required for transcription in addition to 19 nt of the 5' untranslated region of the histone H4 gene. The histone promoter was fused to the *Eag*I site 19 nt 5' of the start site of the U1 gene (see Materials and Methods). This clone contains all of the U1-coding-region and 3' flanking region sequences. The gene was injected into sea urchin zygotes, and the transcripts from each gene were mapped by using the appropriate riboprobes.

When transcripts from the EH4/U1_M gene were mapped with the U1_M riboprobe, a protected fragment 19 nt longer than the transcript from the U1_M gene was expected. The U1_M riboprobe is identical to the 19 nt 5' of the U1 start site present in these transcripts (Fig. 5A, cf. lane 3 with lanes 4 and 5). The internal control LvU2_H gene was coinjected with the test genes in this experiment, and the expression of the EH4/U1 gene was about 10 to 20% of that of the U1_M gene. This difference in expression could be due to the different promoters used, different stabilities of the transcripts, or inefficient 3'-end formation.

The transcripts of the EH4/U1 chimeric gene were also mapped by using the homologous EH4/U1_M riboprobe (Fig. 5B). The EH4/U1_M probe will map the transcripts which are initiated at the histone promoter and ending at the U1 end as

FIG. 5. Expression of the EH4/U1 gene. (A) The U1_M gene (lane 3) and the EH4/U1_M gene (lanes 4 and 5) were injected together with the LvU2_H gene into *S. purpuratus* zygotes, and the embryos were collected at the blastula stage. The transcripts were analyzed by using the U1_M and the U2_M riboprobes as described in the legend to Fig. 1C. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), analysis of RNA from uninjected control embryos; lane 6 (t), analysis of yeast tRNA. The protected fragments are as follows: EH4/U1, transcripts from the EH4/U1_M gene; U2_H, transcripts from the U2_H gene; E, transcripts from the endogenous U1 and U2 snRNA genes. The riboprobe assay is diagrammed at the bottom. (B) The U1_M gene (lane 3) or the EH4/U1 gene (lane 4) was injected into *L. pictus* embryos, and the embryos were harvested at the blastula stage. The transcripts were mapped with the EH4/U1 riboprobe. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), analysis of RNA from uninjected embryos. The protected fragments are as follows: EH4/U1, protection of transcripts ending at the U1 end from the EH4/U1 gene (note the difference in size between fragments protected by the EH4/U1 transcripts in panels A and B); U1_M, protection of transcripts from the U1_M gene; E, protection of the endogenous U1 snRNA.

a 218-nt protected fragment. The U1_M gene and the EH4/U1 gene were injected in parallel. If the transcripts were initiated from the histone promoter, then the protected fragment would be 19 nt longer than those protected by the U1_M probe and 38 nt longer than the transcripts from the U1_M gene. The expected 218-nt fragment was observed (Fig. 5B, lane 4). The embryos injected with the U1 maxigene were also analyzed with the EH4/U1 probe which protects the entire U1_M transcript (Fig. 5B, lane 3).

These results show that the transcripts from the EH4/U1 gene are initiated from the histone promoter and have U1 3' ends. The efficiency of U1 3'-end formation of these transcripts cannot be measured with the probes used in these experiments. These riboprobes will not detect any read-through transcription products which do not have defined 3' ends. Transcripts which end heterogeneously would give a broad spectrum of protected fragments which would not be detected as specific bands. The read-through transcripts can be detected with a probe which diverges from the gene at a site 3' of the end of the U1 gene (see below [Fig. 6B]).

Transcripts initiated from the histone promoter form U1 3' ends efficiently. To compare the relative efficiencies of U1 3'-end formation on transcripts initiated from the histone promoter and the U1 promoter, additional chimeric genes were analyzed. The EH4/U1B/H2a and the EH4/U1N/H2a

genes contain the histone promoter, the U1 coding region, and the H2a 3' end attached either at the *Bgl*II site 20 nt from the end of the U1 RNA or at a site 122 nt 3' of the gene. The U1N/H2a gene has the histone 3' end placed 3' of the intact U1 maxigene and differs from the EH4/U1N/H2a gene only in the promoter. These genes are also shown in Fig. 1B. In the EH4/U1N/H2a and the U1N/H2a genes, there are two different 3'-end signals present, a U1 signal followed by a histone signal. It is likely that any transcripts which read through the U1 signal will be processed at the histone 3' signal.

These three genes were injected into sea urchin zygotes, and the transcripts were analyzed by using RNase protection assays. The EH4/U1B/H2a gene was injected in parallel with the U1 maxigene (Fig. 6A). Transcripts from the EH4/U1B/H2a gene which end at the histone 3' end were readily detected (Fig. 6A, lane 4). This gene was expressed at a lower level than the U1 maxigene (Fig. 6A, lane 3), probably because the histone promoter is weaker than the U1 promoter, although the transcripts with histone 3' ends could be less stable than the U1_M transcript. Note that the probe used in this experiment is a continuously labeled probe, so there are more radioactive nucleotides incorporated into the probe fragment protecting the EH4/U1B/H2a transcripts than in those protecting the U1_M transcripts. The EH4/U1B/H2a

FIG. 6. Expression of histone-U1 chimeric genes. (A) The EH4/U1B/H2a gene was injected into the same batch of *L. pictus* embryos as in Fig. 5B, which were harvested at the blastula stage. The transcripts were mapped with the U1_M gene (lane 3) or the EH4/U1B/H2a gene (lane 4), and the RNase-resistant fragments were analyzed by gel electrophoresis. Lane 1 (M), pUC18 digested with *Hpa*II; lane 2 (Con), analysis of uninjected control embryos. The protected fragments are as follows: EH4/U1B/H2a, protection by transcripts ending at the histone end; U1_M, protection by transcripts from the U1_M gene; E, protection by the endogenous U1 snRNA. The riboprobe assays are diagrammed at the bottom. (B) *L. pictus* zygotes were injected with the EH4/U1 gene (lane 3) or the EH4/U1N/H2a gene (lane 4) and harvested at the blastula stage. The transcripts were mapped with a riboprobe from the EH4/U1N/H2a gene and were analyzed by gel electrophoresis. Lane 1, pUC18 digested with *Hpa*II; lane 2 (Con), analysis of RNA from uninjected control embryos; lane 5 (t), analysis of yeast tRNA. The protected fragments are as follows: EH4/UN/H2a, protection by transcripts ending at the histone 3' end; RT (read through), protection by transcripts which extend past the U1_M 3' end from the EH4/U1 gene; U1_M, protection by transcripts ending at the U1 3' end; e, protection by endogenous transcripts from the *L. pictus* embryos; E, protection by endogenous U1 snRNA. The riboprobe assay is diagrammed at the bottom. (C) *L. pictus* zygotes were injected with the EH4/U1 gene (lane 3), the EH4/U1N/H2a gene (lane 4), or the U1N/H2a gene (lane 5), and the transcripts were mapped by using a riboprobe derived from the EH4/U1N/H2a gene. Lane 1, pUC18 digested with *Hpa*II; lane 2 (Con), analysis of RNA from uninjected control embryos; lane 6 (t), of yeast tRNA. The protected fragments are as follows: EH4/UN/H2a, protection by transcripts ending at the histone 3' end; U1_M, protection by transcripts ending at the U1_M 3' end; e, protection by endogenous transcripts in the *L. pictus* embryos; E, protection by endogenous U1 snRNA. The riboprobe assay is diagrammed at the bottom.

gene is expressed at a level similar to that of the EH4/U1 gene, on the basis of the expression of both of these genes relative to the expression of the U1 maxigene (Fig. 5 and 6C and data not shown).

The EH4/U1 and EH4/U1N/H2a genes were injected in parallel into *L. pictus* embryos, and the transcripts from these genes were mapped with the EH4/U1N/H2a riboprobe (Fig. 6B). This probe will map the transcripts ending at the U1 end from both genes. Any longer transcripts from the EH4/U1 gene which extend farther than 122 nt from the end of the U1 RNA will be detected as a single fragment 340 nt in length (labeled "RT" in Fig. 6B) by using this heterologous probe. These read-through transcripts would not be detected with a homologous probe. Longer transcripts from the EH4/U1N/H2a gene should be processed at the H2a 3' end and are likely to be more stable than the transcripts which end heterogeneously. The results clearly show that both genes produce similar levels of transcripts which end at the U1 3' end (Fig. 6B, lanes 3 and 4). A small amount of read-through transcripts that map as the predicted size (Fig. 6B, lane 3) were detected from the EH4/U1 gene. A larger proportion of transcripts from the EH4/U1N/H2a gene extended past the U1 end to the histone 3' end. The intensities of the two protected fragments are similar. However, since the probe is uniformly labeled, there are twice as many transcripts ending at the U1 3' end than at the histone 3' end. About 70% of the transcripts had U1 3' ends.

Results from an independent experiment with the same genes injected in parallel with the U1N/H2a gene are shown in Fig. 6C. The transcripts from these genes were all mapped with the EH4/U1N/H2a probe. This probe will map the U1N/H2a transcripts accurately (with protected fragments of 180 nt from transcripts ending at the U1 end, or 445 nt from transcripts ending at the histone end) since it contains all of the sequence of the U1 maxigene. The EH4/U1 gene and the EH4/U1N/H2a gene produced similar amounts of transcripts (Fig. 6C, lanes 3 and 4). About 70% of the transcripts from the EH4/U1N/H2a gene contained a U1 3' end, and the other 30% had a histone 3' end. All the transcripts detected from the U1N/H2a gene ended at the U1 3' end. Given the intensity of the band ending at the U1 3' end and the relative lengths of the two transcripts we would readily have detected as few as 5% of the transcripts ending at the histone 3' end. These results demonstrate that formation of the U1 3' end on transcripts which initiate at a histone promoter occurs efficiently in sea urchins.

DISCUSSION

Sequence requirements for U1 snRNA 3'-end formation. The 3'-end signal located 15 to 20 nt downstream of all vertebrate snRNA genes examined is required for proper 3'-end formation (1, 5, 12). In vertebrates, snRNA 3'-end formation is thought to occur cotranscriptionally because transcripts that contain this sequence are not processed to the proper length (7) after microinjection in *Xenopus* oocytes. In addition, correct 3'-end formation depends on transcription being initiated from an snRNA promoter. Transcripts which are initiated from mRNA promoters read through the U1 3'-end signal and end heterogeneously downstream of the snRNA 3' end or at polyadenylation sites that have been placed downstream of the gene (14, 23, 25).

Here, we have examined the sequence and promoter requirements for 3'-end formation of the sea urchin U1 snRNA. These results suggest that the 3' end of the sea urchin U1 snRNA is formed by a bipartite signal, involving

Lv U1.2: ACAATTCAACTAATGCAAAGAAAGAAAA
 Lv U1.1: ACAATTCAACTAATGCAAAGAAAGAAAA
 Sp U1: ACAAAACGAATGTAAGAAAAAGTAAATA
 Ap U1: TCAATTCAACCCAAAGAAAGACATACTC
 Lv U2E: CTCAAGGGTTAATAAAATACCAAAGAAA
 Lv U2L: CTCAAGGGGTAACAATAAACCAAAGAAA
 Sp U2E: CTCAGGGTTAAATATACCAAAGAAAGA
 Pm U7: AATCAAACCAAAGACAGATCTTTACCTTC

FIG. 7. Comparison of the 3' flanking regions of sea urchin U1 snRNA genes. The sequences 3' of the snRNA genes from different sea urchin species are compared. The underlined sequences indicate the conserved purine-rich 3' signal. The sequences of the U1 genes are from references 36 and 37, those of the U2 genes are from reference 27, and those of the U7 genes are from reference 8. The sequence of the *A. punctulata* (Ap) U1 snRNA is from reference 25a. Lv, *L. variegatus*; Sp, *S. purpuratus*; Pm, *Psammechinus miliaris*.

the nucleotides directly 3' to the U1 gene and the conserved purine-rich sequence 15 to 20 nt 3' of the gene. Accurate and efficient (to at least 50% of the level from the intact gene) formation of U1 3' ends can occur (in the absence of any competing 3'-end signals) if either of these sequences is removed (Fig. 2) and with only 7 nt of 3' flanking sequence present. However, if all of the 3' flanking sequence is removed, then U1 3' ends are not formed (Fig. 4). The sequences at the 3' ends of different sea urchin U1 snRNA genes are compared in Fig. 7. The 4 nt directly 3' of the U1 RNA are conserved between the *L. variegatus* and *S. purpuratus* U1 snRNA genes, and there is extensive similarity with the much more distantly related sea urchin, *Arbacia punctulata*. Similarly, the sequences directly 3' of the three different sea urchin U2 snRNA genes show extensive sequence similarity with each other (Fig. 7). In both the U1 and U2 genes, the sequence similarity starts immediately after the end of the snRNA. There is no similarity between the U1 and U2 snRNA genes in this region. Sequences directly 3' of the U2 snRNA as well as the purine-rich sequence are necessary for efficient 3'-end formation (15a). Thus, the finding of an extensive 3'-sequence requirement may be general for sea urchin snRNA 3'-end formation.

It is very unlikely that the 3' ends observed in these experiments were formed by exonuclease digestion following random termination. Two genes with all the 3' flanking sequences removed, U13'(-1) and U13'Δ, did not produce transcripts with U1 3' ends, even though they contain the complete U1 snRNA sequence. Inspection of the vector sequences 3' of the U1 gene in these constructs did not reveal any similarities to the purine-rich 3' signal, indicating that there are no cryptic sequences that might guide 3'-end formation. If the U1 3' end on the mutant genes was being formed by recognition of cryptic 3' signals followed by exonuclease trimming to the 3' end of the U1 gene, then this should have occurred just as efficiently on the transcripts from the U13'(-1) and U13'Δ genes.

Promoter requirements for U1 snRNA 3'-end formation. We tested the ability of a histone promoter to direct formation of sea urchin snRNA 3' ends by constructing chimeric genes containing a histone promoter with a U1 3' end. In addition, genes with both a U1 3' end and a histone 3' end placed in tandem were tested. Histone 3' ends are formed by an RNA-processing reaction (9) and thus should be formed relatively efficiently on any transcripts which include the histone 3'-end signal. Histone 3' ends are formed efficiently

on transcripts which initiate at U1 promoters in both mammals (25) and sea urchins (Fig. 6C).

Transcripts which are initiated at the sea urchin α -histone H4 promoter formed substantial amounts of U1 3' ends. Since the transcription rate of the genes with the different promoters and the stability of the transcripts may differ, it is difficult to compare the absolute amount of transcripts formed from the endogenous U1 genes with the amount formed from the histone-U1 chimeric genes. Several observations suggest that the U1 3' end is being formed relatively efficiently on transcripts initiated from a histone promoter. The U1 promoter is likely to be at least as strong as, if not stronger than, the histone promoter. About 10 to 20% as many transcripts accumulate from U1 genes driven by the histone promoter as from U1 genes driven by the U1 promoter. Similar amounts of transcripts accumulated from the EH4/U1B/H2a and the EH4/U1 genes (compare Fig. 5 and 6), which differ only in their 3' ends. These results suggest that U1 3'-end formation on transcripts initiated from a histone promoter is as efficient as histone 3'-end formation.

The suggestion that the U1 3' end is formed efficiently on transcripts initiating from a histone promoter is further supported by the observation that when the U1 3'-end signal is followed by a histone 3'-end signal, the U1 3' end is still formed. In the EH4/U1N/H2a gene, two 3' signals were placed in tandem. We assume that any transcripts which reach the histone 3' end are processed. Transcripts which end at the U1 3' end must then be formed prior to the transcription of the histone 3' signal (as a result of either termination or an RNA processing event). It is possible, although unlikely, that U1 3' ends can form by shortening transcripts which first form histone 3' ends, assuming that the U1 3' ends are formed by an RNA processing reaction. If the U1 3' ends are formed at a reduced rate, then the predominant transcripts should end at the histone 3' end. We observed essentially the same number of transcripts ending at the U1 end, regardless of whether there was a histone 3' end placed downstream (Fig. 6B, lanes 3 and 4). About 30% of the transcripts ended at the histone 3' end, indicating that the U1 3'-end formation on transcripts from a histone promoter was not completely efficient. Since we do not know the relative stabilities of the two transcripts we cannot make a definitive conclusion about the absolute efficiency of 3' end formation. However, since the same amounts of transcripts from the EH4/U1 and EH4/U1N/H2a genes form U1 3' ends, we can rule out the possibility that the U1 3' ends form slowly and nonspecifically after transcription of more than 250 nt 3' of the end of the U1 gene. Rather, the U1 ends must have formed rapidly, but not with 100% efficiency.

In contrast, transcripts which initiated from a U1 promoter formed U1 ends very efficiently. The same two 3' signals were put in competition on a transcript we initiated from a U1 promoter, and only transcripts with U1 ends were detected. As few as 5% of transcripts with histone 3' ends would have been readily detected. Thus, while transcription from a U1 promoter is not absolutely required for proper U1 3'-end formation, U1 3' ends are formed more efficiently on transcripts initiated from a U1 promoter than from a histone promoter.

These results differ from those obtained with the mammalian snRNA genes, for which initiation of transcription from an snRNA promoter is reported to be an obligatory requirement for proper 3'-end formation (14, 23). Some of these experiments utilized genes with essentially no U1 coding

sequence, so that any effects of U1 RNA secondary structure would not have been detected. However transcripts initiating from a mouse histone promoter containing U1 3'-end signals (including the secondary structure elements in the 3' half of U1 RNA) did not form U1 3' ends (25). Results similar to those reported here have been obtained in studies of snRNA biosynthesis in plants (6a); the formation of plant snRNA 3' ends is independent of snRNA promoters and is not dependent on a conserved 3' sequence. This result suggests that the strict coupling of U1 snRNA 3'-end formation to the promoter may be restricted to vertebrate snRNA genes.

These experiments do not address the mechanism of snRNA 3'-end formation: transcription termination versus processing. It is very possible that the U1 3' end is formed cotranscriptionally in sea urchins, as it is in vertebrates. The fact that there was no decrease in the amount of U1 3' ends formed when the histone 3' end was placed downstream of the U1 3' signal is consistent with the formation of the U1 3'-end being cotranscriptional. U1 3'-end formation occurs very rapidly, prior to the polymerase transcribing the additional 300 nt to the histone 3' signal. The 3' end of the sea urchin U1 snRNA is formed accurately in isolated nuclei (18) and in an *in vitro* transcription system (19). Although these same *in vitro* systems process synthetic prehistone mRNAs they do not "process" synthetic pre-U1 RNAs, even though 3' ends are formed accurately on transcripts synthesized under identical conditions (34a). In contrast, 3' ends are not formed efficiently on mouse U1 snRNA transcripts initiated in isolated nuclei (16). Thus, 3'-end formation of snRNAs is likely to be cotranscriptional in both vertebrates and sea urchins, although the promoter dependence is much stronger in vertebrates than in sea urchins.

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