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3′ editing of mRNAs: Sequence requirements and involvement of a 60-nucleotide RNA in maturation of histone mRNA precursors

(histone genes/RNA processing/palindrome/small nuclear ribonucleoprotein/surrogate genetics)

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ABSTRACT ***In vitro*-synthesized transcripts of the sea urchin histone H2A gene with 3′ extensions are efficiently and rapidly processed to H2A mRNA with faithful 3′ ends in *Xenopus laevis* oocyte nuclei. Processing requires the presence of a histone-specific dyad symmetry element and of H2A-proximal spacer sequences in the precursor RNA. In DNA injection experiments with a processing-deficient H2A mutant, the transcription products appear to terminate heterogeneously in the first 100–200 base pairs of the post-H2A spacer. Processing of synthetic H3 RNA precursors requires the prior injection of a 60-nucleotide RNA from sea urchin embryos that seems to be a component of a small nuclear ribonucleoprotein.**

RNA molecules acquire their 3′ ends either by transcription termination or by processing of a high molecular weight precursor RNA. A transcription termination event is usually responsible for the 3′ end formation of prokaryotic mRNAs. In contrast, in all higher eukaryotes the genes coding for polyadenylylated mRNAs that have been analyzed to date are transcribed well beyond the 3′ ends of the mature mRNAs. Therefore, the 3′ termini of mature mRNAs are commonly thought to arise by an intramolecular cleavage followed by the sequential addition of adenosines (1). Part of the signal for the generation of 3′ ends of those transcripts is the sequence A-A-U-A-A-A commonly found at a relatively fixed distance upstream of the mature 3′ end (2, 3).

Histone genes, unlike other genes transcribed by RNA polymerase II, are usually not polyadenylylated nor do they contain the canonical A-A-U-A-A-A sequence near the 3′ end, although exceptions to these rules are known. In general, histone genes end in a conserved 23-nucleotide-long sequence that includes a 16-base-pair (bp) hyphenated inverted repeat that, once transcribed into RNA, would be expected to form a stable RNA hairpin structure (4, 5).

Previous investigations concerning the possible existence of histone transcripts with spacer extensions or of polycistronic histone precursors have led to confusing and contradictory results (6, 7). Therefore, it remained an open question whether or not the 3′ ends of histone mRNAs are generated directly by transcription termination (8). We reasoned that a post-transcriptional processing event should be independent of transcription and should, by this criterion, be distinguishable from a primary termination event. Because we knew from previous experiments that frog oocytes are able to form correct histone H2A mRNA 3′ ends when confronted with sea urchin H2A genes by DNA injection (9, 10) we synthesized sea urchin H2A gene transcripts containing 3′ spacer extensions by *Escherichia coli* RNA polymerase *in vitro* and injected these transcripts into the nuclei of *Xenopus* oocytes. In this way, we have identified an RNA processing mechanism as most likely being responsible for the formation of histone mRNA 3′ termini and have determined the RNA sequences responsible for this process. We also present circumstantial evidence that the precursors synthesized in DNA injection experiments terminate heterogeneously within the H2A gene-proximal spacer sequences. Finally, we show that the processing of histone H3 transcripts requires a previously identified 60-nucleotide RNA (11), which seems to act as a component of a small nuclear ribonucleoprotein (RNP).

MATERIALS AND METHODS

Preparation of Templates for *in Vitro* Transcription. Various restriction fragments from the H2A and H3 genes of the h22 repeat unit (see figures) were introduced by means of *Eco*RI linkers into plasmid pPLc2819 (12) within the polylinker sequence located 120 bp downstream from the transcription initiation site of the bacteriophage λ p_L promoter. Ligation mixtures were used to transform λ wild-type lysogen K514 (a derivative of *E. coli* C600, a generous gift of E. Remaut and W. Fiers). CsCl gradient-purified DNAs from the desired recombinants were cleaved downstream of the histone DNA insert with *Sal* I, before being used as templates in run-off transcriptions.

***In Vitro* Transcription of pPLc2819 Recombinants.** *In vitro* transcription with *E. coli* RNA polymerase was performed essentially as described (13) except that the RNA was primed with an excess of presynthesized cap structure m^7GpppA (14) purchased from P-L Biochemicals. This modification ensures at least partial capping of the synthesized transcripts and should therefore greatly increase their stability in *Xenopus* oocyte nuclear injections (15). Incubation was for 30 min at 37°C; reactions were stopped by adding 5 μg of DNase I (RNase-free, prepared and kindly provided by W. Schaffner) and 10 μg of *E. coli* tRNA; and the mixture was further incubated for 10 min at room temperature. The desired transcripts were isolated on preparative 1.5% low-melting agarose gels.

***Xenopus laevis* Oocyte Injection and Analysis of the RNA Product.** Nuclear injection of vector-free circles from mutant h22 DNAs and the subsequent analysis of the newly synthesized RNAs were performed as described (9, 16). RNA synthesized *in vitro* was injected in a volume of 25 nl per oocyte (equivalent to 300–2000 cpm). For the experiment with H3 precursor, the cytoplasm of oocytes had previously been injected with 30 nl of sea urchin 60-nucleotide RNA (11). RNA was isolated and analyzed on 6% polyacrylamide/7 M urea gels or by S1 nuclease mapping (17, 18).

RESULTS

***In Vitro* Synthesis of Histone "Precursor" RNA.** The H2A gene of the cloned histone repeat unit h22 from the sea ur-

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Abbreviations: bp, base pair(s); RNP, ribonucleoprotein.

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chin *Psammechinus miliaris* was used for the synthesis of H2A gene transcripts containing 3′ spacer extensions. Fragments containing the 3′ portion of the wild-type or mutant H2A genes and various lengths of spacer DNAs (19) were subcloned in the plasmid vector pPLc2819, which contains the strong p_L promoter of bacteriophage λ (12). ^{32}P-labeled run-off transcripts, starting at the p_L promoter and traversing the entire histone DNA insert (Fig. 1), were synthesized *in vitro* by using *E. coli* RNA polymerase. To ensure capping of the artificial precursor, and hence its stability in oocyte nuclear injection experiments (15), the transcription reaction was run in the presence of a high excess of m^{7}GpppA, a cap analogue, which is efficiently incorporated as a first nucleotide under the conditions used (14). The RNA molecules of desired lengths were purified by preparative gel electrophoresis and injected into *Xenopus* oocyte nuclei. Injected oocytes were collected after different incubation times and the RNA was isolated and analyzed by gel electrophoresis and autogradiography or by S1 nuclease mapping experiments.

H2A RNAs Synthesized *in Vitro* with 3′ Extensions Are Processed to Yield Genuine mRNA 3′ Ends. We had previously injected wild-type and mutant H2A genes into the frog oocyte nucleus and knew from these experiments that both the terminal inverted DNA repeat and spacer sequences are required for the faithful generation of 3′ termini of H2A mRNA (6). Therefore, the constructions used initially for the *in vitro* synthesis of input RNA contained the 3′ half of the H2A gene, including the terminal palindrome, as well as 230 nucleotides of the adjacent spacer. Moreover, the first 120 and the last 50 nucleotides of the synthetic RNA corresponded to the p_L promoter region and to the poly-linker sequence (12) into which sea urchin histone gene sequences had been inserted (see Fig. 1).

After injection of the run-off RNA of about 600 nucleotides into nuclei of centrifuged *Xenopus* oocytes, the input RNA was seen to disappear gradually, while a new 350-nucleotide RNA accumulated (Fig. 1). The time course suggests a precursor–product relationship between these two RNA species and a precursor half-life of the order of 30 min. When the same input RNA was injected into the cytoplasmic compartment of *Xenopus* oocytes, no small RNA was generated even after a prolonged incubation of 8 hr (Fig. 1).

The length of the 350-nucleotide RNA species corresponds to an RNA molecule starting at the p_L promoter and extending approximately to the position of the 3′ termini of authentic H2A mRNA. To analyze the RNA more accurately, we performed an S1 nuclease mapping experiment with a DNA probe 3′-end-labeled at the *Xho* I site of the H2A gene (Fig. 1). This probe is colinear with the injected input RNA for 360 nucleotides (Fig. 2). The unprocessed input RNA protects the DNA over the full length of the 360 nucleotides, as expected (Fig. 2). After incubation of the synthetic RNA in the oocyte and S1 nuclease mapping of the RNA, a predominant S1-resistant DNA fragment of 240 nucleotides is found that comigrates precisely with the DNA fragment protected by authentic sea urchin H2A mRNA. These S1 mapping experiments confirm the results obtained by analytical gel electrophoresis (Fig. 1) and reveal accurate and rapid processing of the input RNA.

Terminal Stem and Loop Structure of H2A mRNA and Adjacent Spacer Sequences Are Required for Rapid RNA Processing. Next, we investigated whether the terminal RNA hairpin loop was required for RNA processing. A fragment from H2A mutant 122 (19) was subcloned in plasmid pPLc2819 and transcribed *in vitro*. This mutant DNA contains two base changes in the terminal inverted DNA repeat that would be expected to prevent a folding of the RNA into

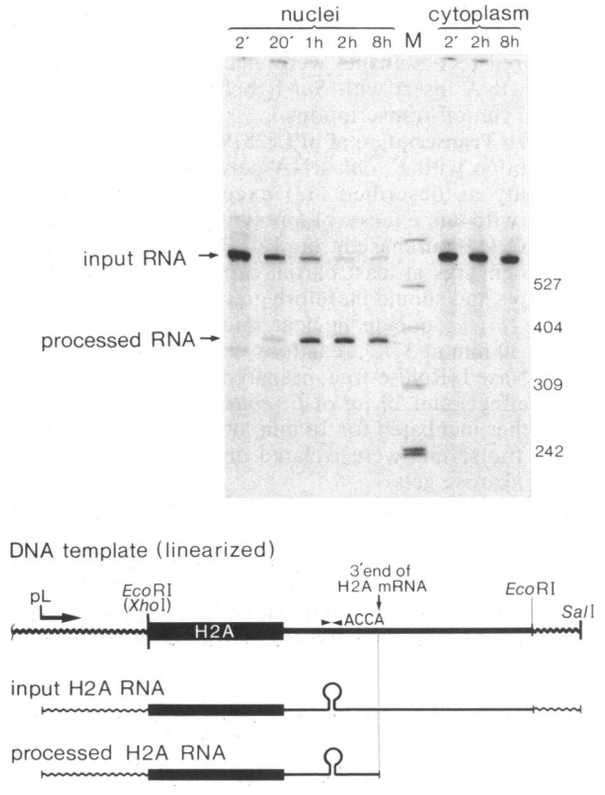

FIG. 1. Maturation of a synthetic sea urchin H2A transcript in the frog oocyte nucleus. Autoradiogram of a denaturing gel of synthetic ^{32}P-labeled H2A RNA incubated in *Xenopus* oocytes for the indicated times (′, min; h, hr). Lengths in nucleotides of markers (lane M) are indicated on the right. The same conventions are used in subsequent figures. The structures of the DNA template and of the input or matured H2A transcripts are depicted at the bottom. Plasmid sequences are indicated by wavy lines, and the transcription start point at the p_L promoter, by an arrow.

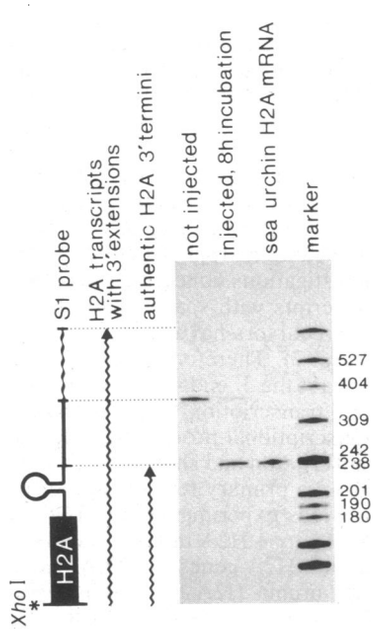

FIG. 2. Comparison of processed H2A precursors with authentic histone mRNA by S1 nuclease mapping. The 3′ ends of the *in vitro* transcripts shown in Fig. 1 were mapped with S1 nuclease, either before or after incubation in frog oocyte nuclei, using sea urchin blastula RNA as a reference (marker). The 3′-end-labeled DNA probe was homologous to the synthetic RNA for only 360 nucleotides. This allowed distinction between the renatured DNA probe and the fragment protected by the input RNA.

a hairpin structure. Mutant 122 fails to generate faithful H2A mRNA 3′ ends in DNA injection experiments (19).

When the synthetic RNA of mutant 122 is injected into the oocyte nucleus, no RNA maturation occurs, whereas in parallel experiments input RNA with a wild-type sequence is processed efficiently to yield correct 3′ ends (Fig. 3). This demonstrates that the palindrome acts in the oocyte as a signal for the maturation of histone mRNA transcripts.

Since we had observed an absolute requirement for H2A proximal spacer sequences for the generation of 3′ termini in DNA injection experiments (19), we tested whether the same sequences were also necessary for RNA processing. A series of DNA fragments generated by BAL31 resection, extending from the *Xho* I site (Fig. 4) to different positions in the spacer (for construction see ref. 19) were subcloned in pPLc2819. The various subclones were transcribed and maturation of the RNA transcripts was studied in the *Xenopus* oocyte nucleus. The shortest of these transcripts (w+4) contains only four nucleotides of the H2A spacer. These transcripts are not processed when injected into the oocyte nucleus (Fig. 4). The next class of transcripts (w+24) contains an additional 20 nucleotides of the H2A spacer. These RNA molecules are processed with low efficiency. RNA molecules with additional spacer transcripts attached to them are processed efficiently. Nearly stoichiometric conversion of the RNA input to processed molecules is seen when a minimum of 200 nucleotides of the spacer DNA is present in the RNA transcripts (Figs. 1 and 4). This also indicates that the synthetic transcripts are stable in oocyte nuclear injections, presumably because of the presence of a 5′ cap structure.

Transcription Termination in the H2A Spacer Is Heterogeneous. The above experiments demonstrate that it is possible to prevent RNA processing either by interference with the terminal RNA hairpin structure or by deletion of spacer sequences. DNA injection of mutant H2A genes from which the terminal inverted repeat is deleted should therefore reveal if and where transcription termination occurs downstream of the processing site, because such genes would give rise to unprocessed RNAs only. With this in mind, we constructed a series of H2A spacer resection mutants by using a mutant h22 DNA clone (ΔG, or del +35/+47 in ref. 6) in which the inverted DNA repeat of the H2A gene was deleted (for details see ref. 6 and Fig. 5). The end points of the resected spacers were determined by restriction analysis. The 3′ spacer termini were then fused by means of *Eco*RI DNA linkers to a truncated 3′ segment of the H1 gene. In this way, the terminal portion of the H1 coding sequence and its palindrome and spacer sequences were present 3′ to the resected H2A transcription unit in an otherwise intact h22 DNA.

First, we determined that the transcripts obtained from all DNA constructions were stable in the oocyte. The ratio between the 5′ ends of H2B and H2A mRNA was determined by S1 nuclease mapping. This ratio did not change (data not shown), hence there was no evidence for instability of the transcripts as a consequence of DNA manipulation. We then studied the level of readthrough products in the various 3′ deletion mutants. As a base line control we used the H2A mutant del +47/+406(H1) (for details see ref. 6 and Fig. 5), which lacks all spacer sequences and therefore does not contain the presumptive H2A terminator. RNA transcripts of this H2A gene are also defective in RNA processing, as can be deduced from Fig. 4. Indeed, readthrough of the RNA polymerase into the H1 gene is clearly in evidence (Fig. 5), giving rise to H2A/H1 fusion RNA molecules in the relative amounts anticipated from the promoter strength of the H2A and the H2B genes. The ratio of fusion RNA to H2B mRNA is about the same for a member of the mentioned Bal31 resection series that contains 25 bp of spacer sequences but no inverted DNA repeat (mutant ΔG+25). However, the amount of labeled fusion RNA is reduced to a low level when 130 nucleotides of the spacer are included in mutant ΔG+130, while 230 bp of the spacer virtually abolish all

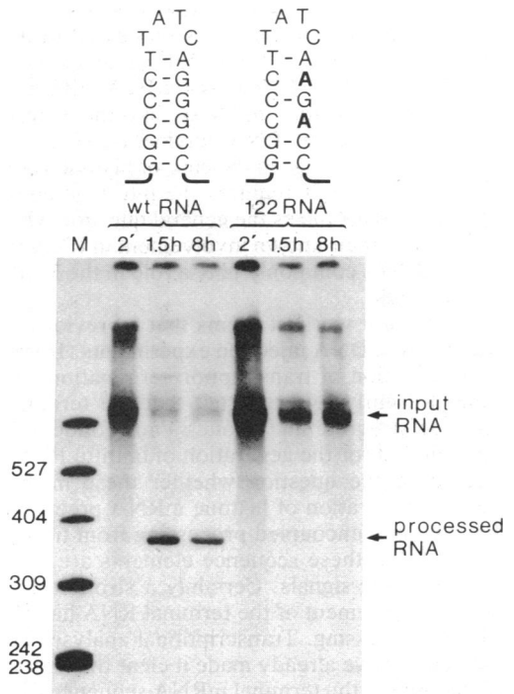

FIG. 3. Lack of maturation of an H2A transcript with point mutations in the palindromic sequence. The structures of the terminal palindromes from H2A wild type (wt) and mutant 122 are shown at the top of the figure. The DNA templates used for the *in vitro* synthesis of the transcripts were analogous to the one depicted in Fig. 1 but contained all the H2A spacer sequences up to position +700.

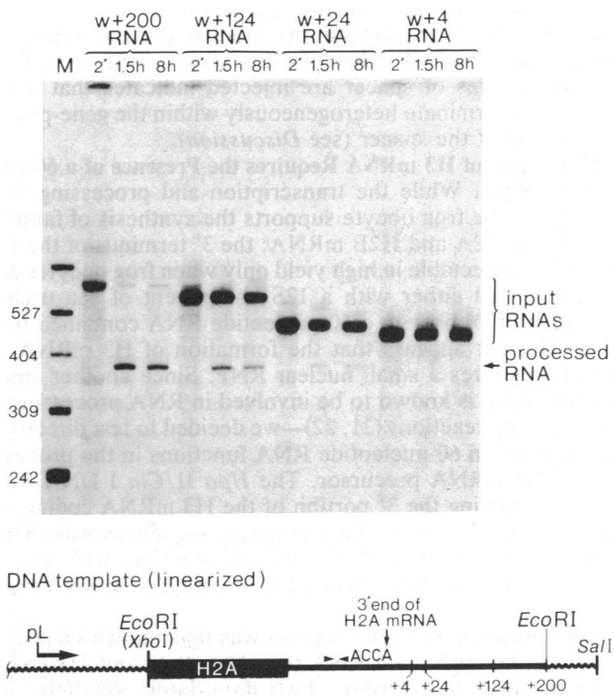

FIG. 4. Maturation of *in vitro*-synthesized H2A RNAs with different amounts of 3′ spacer extensions. The DNA template for w+200 RNA is depicted at the bottom. w+4, w+24, and w+124 RNAs were transcribed from analogous constructions and contained 4, 24, and 124 nucleotides of spacer sequences, respectively, which were fused to the same vector sequences as in w+200.

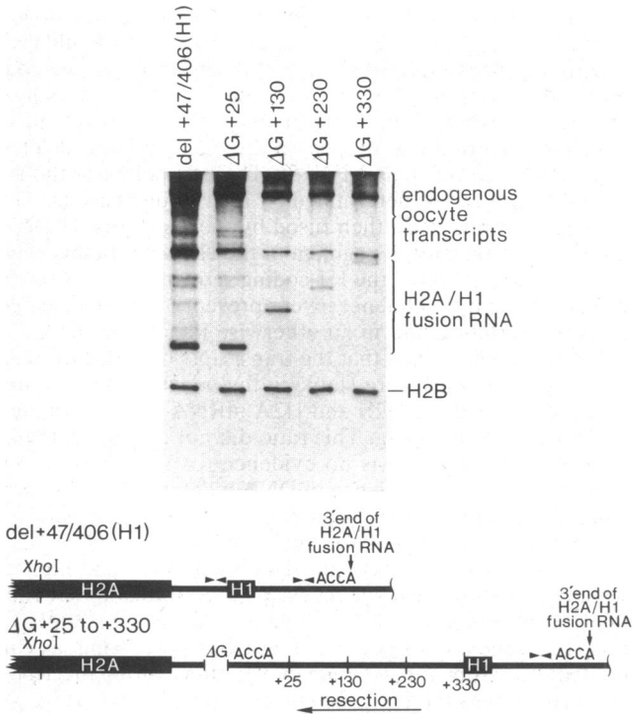

FIG. 5. Transcription termination in the H2A spacer. Autoradiogram of a partially denaturing gel of RNA extracted from oocytes after injection of mutant h22 DNA together with [α-^{32}P]GTP. The relevant structure of mutant del +47/406(H1) and of the G resection series are shown at the bottom. The end points of the spacer deletions are directly fused to the end of the H1 coding sequences, thus allowing the detection of readthrough transcripts as RNA molecules ending at the 3′ terminus of the H1 gene. The 3′ ends of the H2A/H1 fusion RNAs are indicated.

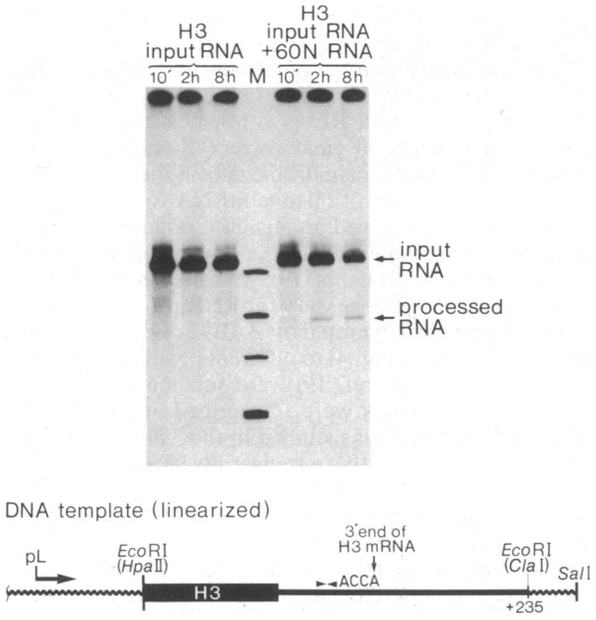

FIG. 6. Maturation of H3 transcripts in the presence of a 60-nucleotide (60N) RNA. Autoradiogram of a denaturing gel of synthetic H3 RNA incubated in *Xenopus* oocytes injected 2 days before either with buffer alone or with buffer containing 60-nucleotide RNA from sea urchin embryos (11). The structure of the template used for the synthesis of the H3 precursor is shown at the bottom.

readthrough transcription (Fig. 5). The absence of specific additional RNA bands and the gradual disappearance of readthrough products when constructions containing 130 to 230 nucleotides of spacer are injected indicates that most transcripts terminate heterogeneously within the gene-proximal 200 bp of the spacer (see *Discussion*).

Processing of H3 mRNA Requires the Presence of a 60-Nucleotide RNA. While the transcription and processing machinery of the frog oocyte supports the synthesis of faithful sea urchin H2A and H2B mRNA, the 3′ terminus of the H3 mRNA is detectable in high yield only when frog oocytes are supplemented either with a 12S component of sea urchin chromatin (20) or with a 60-nucleotide RNA contained in it (11). Thus, it appears that the formation of H3 mRNA 3′ termini requires a small nuclear RNP. Since another small nuclear RNP is known to be involved in RNA processing—i.e., splicing reactions (21, 22)—we decided to test directly if the sea urchin 60-nucleotide RNA functions in the processing of H3 mRNA precursor. The *Hpa* II/*Cla* I DNA fragment containing the 3′ portion of the H3 mRNA coding sequence and 235 bp of adjacent spacer sequences was therefore placed into pPLc2819, and H3 mRNAs with spacer extensions were synthesized *in vitro* with *E. coli* RNA polymerase.

One group of *Xenopus* oocytes was injected with a preparation of 60-nucleotide RNA (see *Materials and Methods*), another with buffer only. Two days later, synthetic H3 mRNA transcripts were injected into the oocyte nuclei and the fate of the input RNA was followed by analytical gel electrophoresis and autoradiography. As seen in Fig. 6, only oocytes previously injected with the 60-nucleotide RNA are able to process H3 RNA, while mock-injected oocytes are not. The yield of processed H3 mRNA is conspicuously lower than in the case of the H2A RNA experiment. Nevertheless, these results suggest that the 60-nucleotide RNA may normally be involved in the maturation of the H3 mRNA precursors in the sea urchin embryos.

DISCUSSION

We have demonstrated that synthetic sea urchin H2A mRNA with 3′ spacer extensions is processed in *Xenopus* oocyte nuclei to produce 3′ ends that are indistinguishable from authentic 3′ termini of sea urchin H2A mRNA. Therefore, the 3′ ends of histone mRNAs, like the 3′ termini of other genes transcribed by RNA polymerase II, can be generated by RNA processing. In the case of histone H3 mRNA the generation of 3′ ends requires the intervention of a 60-nucleotide RNA. This raises the general question whether or not, in addition to their known involvement in RNA splicing, small nuclear RNPs commonly play a role in the 3′ editing of mRNA precursors.

From the present work, it seems that a previous analysis of H2A mutants in DNA injection experiments (16) reflected the combined action of transcription termination and RNA processing. Recently, we reported that the terminal RNA stem and loop structure and about 80 nucleotides of spacer DNA are required for the generation of faithful H2A mRNA ends and raised the question whether these might be involved in the maturation of histone mRNA precursors (19). Now that we have uncoupled processing from transcription we can assert that these sequence elements are, or at least contain, processing signals. Certainly a strong case can be made for an involvement of the terminal RNA hairpin structure in RNA processing. Transcriptional analyses of hybrid DNA molecules have already made it clear that H2A 3′ ends are obtained only if the terminal mRNA sequence can form a stem and loop structure (19). This is in accord with our present findings that point mutations in the stem that destabilize the RNA hairpin prevent the maturation reaction.

The role of the spacer sequences is less well characterized except that they also appear to be required for RNA processing. Where they are absent, no processing can be observed

and RNA containing only 24 spacer nucleotides is matured very inefficiently. This finding contrasts with our previous conclusion that 24 bp of the spacer DNA in conjunction with the terminal palindrome supports the formation of mRNA 3′ ends at 50% of wild-type level. Indeed, DNA injection experiments suggest that important RNA processing signals do no occur 3′ to spacer nucleotide +80 (19).

At present, these discrepancies are not fully understood, but there are two, possibly important, differences between the RNA and the DNA injection experiments. In the RNA injection experiments about 2×10^9 RNA molecules are delivered into the nuclear compartment at once, whereas the synthesis of a similar number of RNA molecules from injected DNA requires at least 24 hr. Therefore, RNA injection may tax the processing machinery of the nucleus to a much larger extent than does RNA synthesis *in situ* and may reveal rate-limiting steps of a different nature than those seen in DNA injection experiments. Second, the synthetic precursor RNAs all terminate in highly palindromic poly-linker sequences, whereas in DNA injection experiments the spacer sequences abutt onto H1 protein coding sequences. For instance, these palindromic linker transcripts might impair processing, unless they are placed at a considerable distance from the processing signals. Both these aspects may also explain the relative inefficiency of H3 mRNA processing when the oocyte is complemented with the 60-nucleotide RNA.

Our studies suggest that, in the absence of processing the formation of hybrid H2A/H1 transcripts is suppressed when 200 bp of post-H2A spacer DNA intervenes between these two genes. This is consistent with the view that, at least in mutants lacking the terminal palindrome, the RNA polymerase terminates, presumably heterogeneously, within a short stretch of spacer DNA of 100–200 nucleotides. Termination rather than a secondary site of RNA processing is suggested because we have not as yet found any evidence for such a processing site in oocyte RNA injection experiments (unpublished results). It remains to be seen to what extent transcription termination and RNA processing are temporally or functionally coupled in the sea urchin.

In summary, all our surrogate genetics experiments on the mode of sea urchin histone gene transcription taken together suggest that, in the *Xenopus* oocyte test system, transcription termination occurs heterogeneously within the first 100–200 nucleotides of the spacer, 3′ to the terminal inverted DNA repeat of sea urchin histone genes. This implies that short histone mRNA precursors are made and then processed rapidly. Processing requires an intact palindrome structure at the RNA level. A contribution of the spacer sequences, in particular of the canonical C-A-A-G-A-A-A-G-A sequence (4) to RNA processing seems likely but has not as yet been conclusively proven. The functional involvement of a 60-nucleotide RNA isolated from sea urchin embryonic chromatin in the maturation of artificial histone mRNA precursors strongly suggests that an identical, or at least closely similar, sequence of events occurs during the biogenesis of histone mRNAs in the sea urchin embryo.

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