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Processing and nucleo-cytoplasmic transport of histone gene transcripts

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ABSTRACT

Precursors of Xenopus and sea urchin histone mRNAs were synthesized in vitro with the SP6 transcription system, and their maturation and nucleo-cytoplasmic transport was studied by frog oocyte injection. 3' processing is most rapid for homologous histone messenger sequences and does not require either genuine 5' or specific 3' ends of the precursor, but capping of the 5' terminus strongly influences the efficiency of 3' processing. No generation of 5' histone mRNA ends can be detected when precursors containing 5' spacer sequence extensions are injected into the oocyte nucleus. This finding may have some implications for the question whether histone gene transcription could be polycistronic. Using a novel oocyte technique, we have separated nuclei from cytoplasm and have studied the time course of exit of the processed RNA from the oocyte nucleus into the cytoplasm. The results suggest that RNA maturation and nucleo-cytoplasmic transport are not temporally coupled processes.

INTRODUCTION

From the investigation of the biogenesis of a series of mRNAs (1-6) it has emerged that in higher eukaryotes, as a rule, 3' termini of mRNAs, including those of histone mRNAs (7,8) are generated by RNA processing rather than transcription termination. By contrast, there is good evidence that the 5' termini of mRNAs are produced during transcription initiation rather than post-transcriptional cleavage (9-11) and that the RNA precursors are capped post-transcriptionally (reviewed in ref.12). In many instances pre-mRNAs are edited by excising the intron sequences (reviewed in ref.13). Recent investigations suggest that in these latter steps small nuclear RNPs play a major role (14-18). For most mRNAs other than histone mRNAs (19,20) polyadenylation follows 3' processing (21,22).

In this paper we have studied several features of RNA 3'

processing of in vitro synthesized SP6/histone mRNA precursors from sea urchin and Xenopus using frog oocyte injection techniques. We have found that the homologous Xenopus H4 mRNA accumulates rapidly from in vitro synthesized precursors in the nucleus and then exits with a short lag into the cytoplasm. The exact sequences at the 5' and the 3' termini of the histone mRNA precursors are of no importance, but capping of the SP6/mRNA precursors not only imparts a degree of stability to the transcripts inside the nucleus (see also ref. 7,8,23), it also influences the efficiency of 3' processing. Production of genuine histone mRNA 5' ends by means of a specific endonucleolytic cut within the precursor could not be observed in any of our experiments.

MATERIALS AND METHODS

Enzymes and substrates:

Restriction enzymes, S_1 nuclease, calf intestine phosphatase, DNA polymerase (Klenow), were purchased from Boehringer Mannheim, SP6 polymerase was from New England Nuclear. ^{m7}G(5')ppp(5')G, GMP-PNP and rNTPs were purchased from P-L Biochemical Inc. ^{32}P-dCTP (3000Ci/mmol) and ^{32}P GTP (400Ci/mmol) were from Amersham. RNAsin was purchased from Promega Biotec.

Preparation of templates for in vitro transcription:

Various restriction fragments (see Figures) containing portions or the entirety of H2A, H2B and H3 genes of the sea urchin h22 repeat unit, or the H4 gene of Xenopus laevis were introduced into plasmid pSP62 (8) within the polylinker sequence. DNAs from desired recombinants were cleaved downstream of the histone DNA insert before being used as templates in run-off transcriptions.

Standard transcription reactions (20µl) containined 1µg linearized DNA template, 20 U RNAsin; 0.5mM of each CTP, ATP, UTP; 3µM GTP, 165µM cap analog; 3 U SP6 polymerase, 10-15µCi ^{32}P GTP. The ratio of cap analog to GTP of about 1:50 ensures capping. Reaction mixtures were incubated at 37°C for 1 hr followed by addition of RNAse-free DNAse, kindly provided by W. Schaffner, and subsequent incubation for 10 min at room temperature. After phenol-chloroform extraction and ethanol

precipitation ^{32}P RNA was run on a 4% polyacrylamide gel, containing 7M urea, and the desired RNA molecule was isolated.

Analysis of processed RNA:

In vitro synthesized RNA was dissolved in injection buffer (40mM NaCl; 25mM Tris, pH 7.5) and a volume of 20-30nl per oocyte containing 5-10x10^3 cpm or approx. 50-100 pg ^{32}P labelled RNA was injected (7). After incubation of the oocytes RNA was isolated from oocytes and analyzed on 6 or 8% polyacrylamide gels containing 7M urea or by S_1 mapping. For procedural details of oocyte injection and RNA analysis consult ref. 7, 23 and papers cited therein.

RESULTS

In vitro synthesis of capped histone mRNA containing 5' and 3' sequence extensions.

Histone genes of the sea urchin Psammechinus or the frog Xenopus laevis, together with the corresponding 5' and 3' flanking sequences, were excised and reclaimed from the respective recombinant DNAs pBR h22 (24) and pBR Xlhi-1 (25) and inserted into the polylinker sequence of pSP62 (23; kindly provided by D.A. Melton), as described in Methods and Legends to Figures. Superhelical DNA was prepared and cleaved with an appropriate restriction enzyme within either the polylinker segment or 3' to it in pSP62 DNA. RNA was synthesized with SP6 RNA polymerase in an in vitro transcription system (23) which contained ^{32}P-GTP as labelled RNA precursor and which was supplemented, following the protocol of Fiers' laboratory (26), with $m^7G(5')ppp(5')G$ (see also Methods). In this way, transcripts of defined lengths were obtained which did not require further treatment with guanylyl-transferase (27) and appeared to be predominantly capped on the basis of their relative stability in frog oocyte injection experiments.

3', but not 5' termini of histone mRNAs are generated from mRNA precursor molecules.

The recombinant DNAs used in previous studies of histone mRNA maturation (7,8) contained only the 3' portion of histone genes. We wanted to investigate the question whether, using appropriate constructions, any evidence could be obtained that

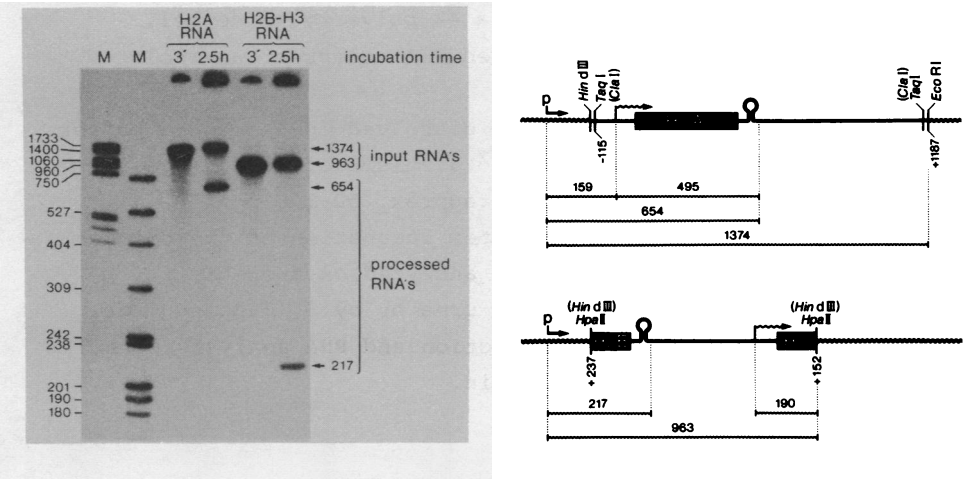

Fig. 1. Processing of in vitro synthesized sea urchin H2A and H2B-H3 RNA precursors in the frog oocyte nucleus. Autoradiogram of a 6% denaturing gel. Oocytes were incubated for the times indicated and RNA was isolated. Lengths in nucleotides of markers (M), of input and processed RNAs are shown on the left and right of the panel. The structures of the DNA templates are depicted at the bottom of the graph. Plasmid sequences are denoted by wavy lines and the transcription start-point by an arrow.

5' processing of histone mRNA precursors occurred. Transcripts from a novel H2A recombinant DNA were prepared which contained, in addition to the 42 nucleotides transcribed from the bacterial pSP62 DNA a 117 base spacer extension at the 5' terminus and 370 base spacer extension at the 3' end of the Psammechinus H2A gene (see Fig. 1). The lengths of RNA molecules anticipated from 3' processing only or from combined 5' and 3' processing events are given at the bottom of Fig. 1.

After injection of the transcripts into the nucleus it was found that in the case of the H2A pre-mRNA an RNA species of 654 nucleotides was generated in about 30-50% molar yield (Fig.1) with a time course similar to that reported earlier (7). The processed RNA can be shown to yield faithful 3' termini by S1 mapping (Fig.2). Note, that an RNA with a length of a genuine H2A mRNA is not seen on the autoradiograms. This is because either cleavage at the 5' messenger terminus does not occur in the oocyte or because such 5' termini are not

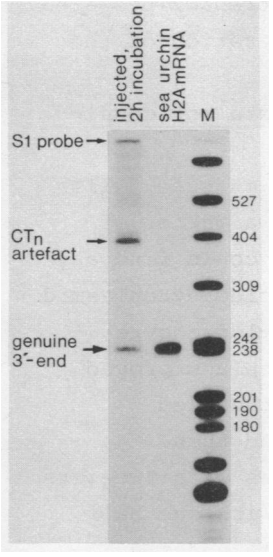

Fig. 2. Comparison of processed H2A precursors with authentic histone mRNA by S1 nuclease mapping.
The 3' ends of the matured transcripts shown in Fig.1 were mapped with S1 nuclease using sea urchin blastula RNA as marker. In vitro synthesized H2A precursor is seen to yield genuine 3' termini. DNA fragments of larger size arise by a slippage mechanism in the run of CTs present downstream of the H2A gene (38) in both spacer transcripts and template DNA.

capped and the mRNAs are therefore unstable.

However, if 5' cleavage does occur the 159 base 5' fragment of the SP6/H2A mRNA molecule so generated (Fig.1) would have a cap and should be seen on the autoradiograph. This prediction was tested experimentally. The cap sequences of the H2A gene contain a HindII restriction site (28,24) and can be used to produce a capped run-off transcript from the SP6/H2A constructions in vitro. Such a short spacer transcript, when injected into the oocyte nucleus, was found to have a half life of about 30 to 40 minutes (results not shown). Therefore, similar molecules should have become visible, at least during the initial stages of H2A mRNA maturation, if indeed rapid and efficient 5' processing of the H2A mRNA were an important feature of the oocyte processing machinery, but this was not the case.

Similarly, a construction in which the 3' and the 5' termini of the H2B and the H3 gene, respectively, and the intervening spacers are transcribed into RNA, yield only a short matured RNA species of 217 nucleotides length (Fig.1) which initiates at the SP6 promoter and terminates at the 3' terminal palindrome of the H2B gene, as verified by S1 mapping

experiments (results not shown). Spacer and H3 transcripts are not preserved, nor is there any evidence of generation of the 5' ends to H3 mRNA.

3' maturation and nucleo-cytoplasmic transport are not coupled processes.

It has long been known (29) that in tissue culture cells histone mRNAs exit from the cell nucleus within a matter of minutes. We therefore wanted to know whether we could find any evidence for a coupling of 3' processing and nucleo-cytoplasmic transport of histone mRNA. For this, a novel boiled oocyte technique for the separation of nuclei from cytoplasm proved very useful. Oocytes injected with in vitro synthesized precursor RNAs were heated to 100°C for 3 min and then immediately chilled on ice. Squeezing the oocytes with tweezers fragmented the cells, usually into two halves, with one half displaying a blue-ish nucleus clearly discernable under a binocular microscope. The nucleus was poked with a needle to dislodge it from the cytoplasm and transferred to an Eppendorf tube where the RNA was extracted by SDS-protease K treatment. The cytoplasmic and the nuclear histone mRNA molecules remained intact throughout the boiling step as well as during storage on ice for at least four hours. This procedure greatly facilitated time course studies at short time intervals.

In preliminary experiments the nucleo-cytoplasmic transport of the matured sea urchin H2A mRNAs of Fig.1 was studied. After 20 minutes of incubation cytoplasmic pools of H2A mRNAs became detectable and after 40 minutes significant amounts of 3' matured RNA were found in the cytoplasmic compartment. Because the maturation of sea urchin H2A mRNA in frog oocytes is relatively slow, the kinetics of RNA processing and RNA migration nearly coincided.

However, the kinetics of maturation and passage through the nuclear membrane are distinctly different from one another in the case of homologous Xenopus H4 mRNA precursors. Processing of the in vitro synthesized RNA precursor containing both 5' and 3' spacer extensions (see Legend of Fig.3) was rapid and after a 3 minute incubation period processed RNA molecules are already in evidence within the nucleus (Fig.3). After 20 minutes the nuclear pool of processed RNA molecules

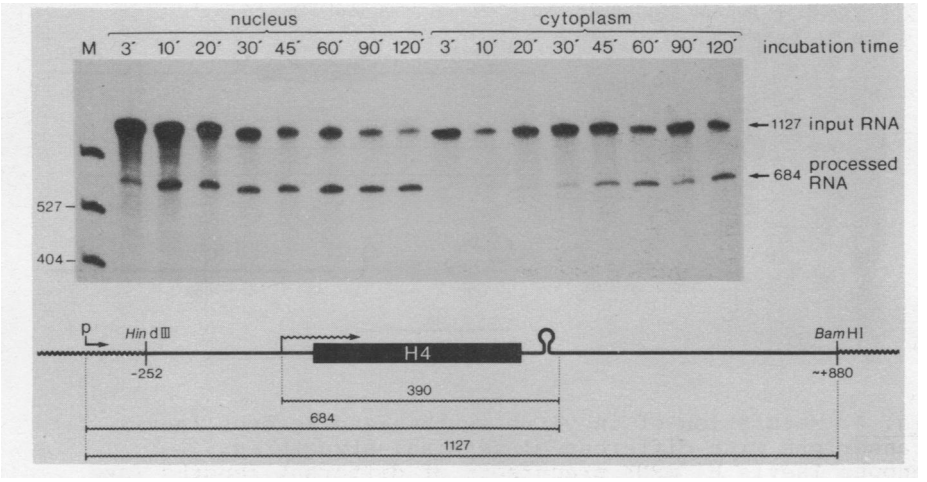

Fig. 3. Nucleo-cytoplasmic transport of a processed Xenopus laevis H4 mRNA.
After injection of ^{32}P-labelled H3 mRNA precursors in the Xenopus laevis oocyte, nucleus and cytoplasm were separated after the indicated times of incubation. RNA was isolated and analyzed by 6% denaturing polyacrylamide gel electrophoresis. Lengths of input and processed RNAs as well as of (denatured) DNA markers are indicated on the right and left of the panel, respectively. The graph at the bottom gives the DNA template used in the experiment. + and - indicate nucleotide distances from the 5' and 3' end of the mRNA coding sequences, respectively.

appeared constant, whereas the amount of precursor molecules decreased rapidly. The cytoplasmic compartment contained a small and variable concentration of precursor molecules throughout the incubation period studied, which presumably resulted from some inaccuracy in our injection procedure and should not be taken as evidence for nuclear cytoplasmic transport of precursor molecules. Processed RNA molecules became apparent in the cytoplasm after a lag of about 20-30 minutes and from then on could be found in increasing concentration. They could only have arisen from processing of the precursor in the nucleus because precursor injected into the cytoplasm is not matured (7). The results show the classical precursor product relationship of the RNAs in the different cell compartments. They suggest that processing is not coupled to histone mRNA transport although RNA migration may depend on prior maturation.

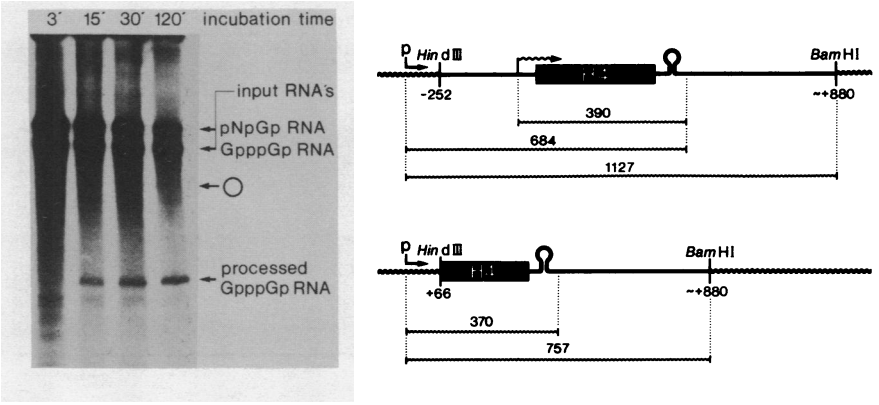

Fig. 4. Maturation of in vitro synthesized Xenopus laevis transcripts with different 5' terminal structures. Xenopus laevis H4 mRNA precursors of different lengths (see lower part of figure) were synthesized in the presence of m7 GpppG or pNpGMP. GpppGpRNA and pNpCp RNAs were combined and injected into the Xenopus oocyte nuclei and incubated for the times indicated. After isolation of the RNA, the RNA was run on denaturing gels. Arrows indicate positions of the input and processed m7 GpppG RNA and input pNpGMP RNA. Symbol o indicates the position of a potential pNpGMP RNA maturation product whose identity has not as yet been verified.

The efficiency of 3' processing of the Xenopus H4 pre-mRNA requires a capped 5' end.

Capping of globin mRNA-precursors significantly affects the specificity and extent of the in vitro splicing reaction. In the absence of a cap only 30-40% of the β-globin-RNA is accurately matured (30). Similarly, the in vitro splicing of the adeno major late mRNA is dependent on the 5' modification of the pre-mRNA (P. Sharp and W. Keller, personal communications). Since there is a clear involvement of the cap structure in RNA splicing, we decided to see whether it was also a prerequisite for 3' processing.

Uncapped SP6/H4 mRNA precursors, when injected into the frog oocyte nucleus, do not yield any detectable amounts of 3' processed RNA molecules (our unpublished observation) but uncapped precursors decay rapidly in the oocyte nucleus and so the detection of equally unstable processed RNA would be more difficult. However, another set of experiments indicates that capping may be a prerequisite for 3' end maturation.

H4 pre-mRNA SP6 transcripts which contain at their 5' end

a non-hydrolysable GMP-pNp have been found to be as stable as capped transcripts in the frog oocytes. RNA transcripts carrying such terminal groups cannot be capped (31) during incubation in the oocyte nucleus since their 5' ends contain a guanosine with a non-hydrolyzable, and hence non-reactive, β-γ bond which is the known site of attack for the capping reaction (27). A situation therefore prevails which should allow us to determine the relative contribution of a modified 5' terminus to 3' processing.

When two Xenopus H4 histone gene transcripts of different length (see Legend to Fig.4), one capped, the other donated with a GMP-pNp terminus, are co-injected into the oocyte nucleus, the capped transcripts yield 3' processed RNA of the anticipated length (Fig.4), whereas cleavage of the GMP-pNp transcripts is severely inhibited. Some cleavage product is present (designated by the symbol o in Fig.4) but in such low amounts that its identity as 3' maturation product could not be verified.

DISCUSSION

Oocyte injection experiments have demonstrated that the 3' termini of histone mRNAs can arise by RNA processing (7,8). Such 3' maturation of in vitro synthesized precursors has been shown to occur for transcripts of a variety of sea urchin histone genes (7; this paper), the H2B gene of the chicken (8), the H4 gene of Xenopus laevis (this paper) and the mouse (D. Schümperli, unpublished results), in fact for all histone pre-mRNA tested so far, with the sole exception of the H3 gene transcripts of the sea urchin Psammechinus miliaris. In this latter case, processing requires the complementation of the frog oocyte with a 60 nucleotide U_7 RNA species as an adaptor molecule (32,7,39). Not surprisingly, the in vitro synthesized homologous H4 pre-mRNA is processed in the frog oocyte considerably faster than the heterologous sea urchin histone pre-mRNAs.

Temporal aspects of RNA processing and nucleocytoplasmic transport.

In tissue culture cells, histone mRNAs exit very rapidly from the nucleus (29). Similarly, the homologous SP6/H4

transcripts with genuine 3' ends migrate from the oocyte nucleus with a time lag of only 20 to 30 minutes despite the fact that the volume of an oocyte nucleus is 10^6 times larger than that of a somatic nucleus. RNA processing of the homologous precursor RNAs is faster still (Fig.3), so that RNA processing and RNA migration appear to rely on quite separate mechanisms. DeLeon et al. (36) have suggested that in the sea urchin egg the newly synthesized mRNAs (most likely identical to those under study in this paper) accumulate inside the nucleus and are released into the cytoplasm only after the nuclear membrane disintegrates during mitosis. The heterologous frog oocyte system obviously does not reflect such a developmental programming and appears to be more permissive than the sea urchin egg nucleus.

5' versus 3' RNA processing.

Histone genes are transcribed monocistronically in most tissues (33,34) but during the lampbrush chromosome stages of the immature oocytes of amphibians the quintet of histone genes is transcribed polycistronically (34,41, and J. Gall, personal communication). Similar transcripts have also been reported to occur in sea urchin oocytes (G. Spinelli, personal communication). In the immature amphibian oocyte each of the five promoters is known to give rise to long precursor molecules which can be analyzed by in situ hybridization techniques (41 and J. Gall, personal communication). In accordance with present dogma, namely that 5' ends of mRNAs are produced by transcription initiation, whereas 3' ends can be generated by processing, we detect no generation of genuine 5' ends to histone mRNAs from in vitro synthesized precursors when these are injected into nearly mature oocytes. This raises the interesting question as to whether or not during the lampbrush chromosome stages of early oocytes a special 5' processing and capping system exists or whether, alternatively, histone mRNA production in the immature oocyte is exceptional in that the "first" capped mRNA stretch, located closest to the 5'-end of the polycistronic transcript, is the sole precursor to the cytoplasmic monocistronic mRNA, whereas all other mRNA sequences remain uncapped and subsequently decay (J. Gall,

personal communication). This would be an inefficient mode of operation, but perhaps efficient enough for the highly repeated histone genes. But cases are known where a polycistronic transcription mechanism is apparently not operative i.e. in the HeLa cell (33) or the early sea urchin embryo (34; but see ref.40 for a dissenting view). Furthermore, our oocyte injection experiments have revealed polydisperse transcription termination sites within the spacer following the H2A histone sea urchin gene (36)and these might be real and functional in the early sea urchin embryo.

Parameters determining the efficiency of 3' processing.

3' processing of histone mRNA is directed by the small nuclear RNA U_7 (32,39) and by specific RNA sequences within the mRNA precursor molecule, most importantly the terminal mRNA palindrome (7,37) and the CAAGAAAGA spacer sequence (our unpublished results). A recent sequence analysis of U_7-cDNA clones reveals striking base complementarities between the U_7 RNA and the conserved terminal sequences of the putative mRNA precursor (39).

As seen in the experiments reported above the 5' sequence of the histone mRNA itself is of no demonstrable importance for the generation of 3' ends (or nucleo-cytoplasmic transport) as it can be replaced with impunity by any other sequence such as E.coli (37) or SP62 RNA sequences (this paper). Similarly, there is no exacting sequence requirement for the 3' terminus of the precursor, provided the precursor contains at least 50 nucleotides 3' to the conserved CAAGAAAGA sequence (37; our unpublished results).

While the exact nature of the 5' sequence of the precursor is of no import, our results do suggest that a 5' modification of the transcripts can affect the efficiency with which the 3' ends are generated. This is reminiscent of the finding that the presence of a cap structure enhances the faithful splicing of intron-containing mRNAs (30; P. Sharp, personal communication). Our preliminary results, that processing can be inhibited, at least at early times of incubation, with cap analogues also argues for an involvement of a 5' cap structure (unpublished results). More clear cut are the experiments in this paper with

transcripts containing the unhydrolyzable GMP-pNp 5' terminus. These cannot be capped and are at the same time poor precursors for the 3' processing reaction. A very helpful and unexpected feature of the precursor with a pNp modified 5' terminus was its relative high stability within the oocyte nucleus. Quite clearly, these observations call for an investigation in an in vitro processing system.

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