Polyadenylation of histone mRNA in *Xenopus* oocytes and embryos

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Oogenesis of amphibians is an atypical situation in which histone mRNA is polyadenylated. The poly(A) tract on H4 mRNA has been examined by S1 nuclease analysis. Throughout oogenesis the poly(A) tract is very short, and nonexistent on some mRNA molecules. The poly(A) tract is completely removed during maturation of the oocyte, and is absent in embryos and cultured cells.

Xenopus *development* *H4 Histone mRNA* *Polyadenylation* *Depolyadenylation*

1. INTRODUCTION

Histone mRNAs are one of the few, perhaps only, classes of mRNA that belong exclusively to the poly(A)$^-$ class of messenger in the somatic cells of animals [1–3]. There are two exceptions to this statement. First, a number of replication-independent or cell-specific histone mRNAs are polyadenylated [4–6]. Second, roughly half of the stored histone mRNA of amphibian oocytes is polyadenylated, as defined by binding to oligo (dT)-cellulose [7–10], as well as ability to be cloned in a typical cDNA protocol, using oligo(dT) priming [11–13]. We have re-investigated this second situation.

Previous data on the polyadenylation of ovary histone mRNA are rather imprecise since they derive solely from translation of RNA that binds or passes through oligo(dT) cellulose. Thus we do not know the length of the poly(A) tract on molecules that bind, nor if there are wholly unpolyadenylated molecules. We have now used S1 nuclease analysis to characterise the poly(A) tract on the histone mRNA. This has allowed us to define its length and to substantiate the previous suggestion that it is lost when the oocyte becomes an egg or embryo.

2. METHODS

RNA was made from oocytes, eggs and embryos as described previously [14]. Oocytes of different sizes were dissected and separated by hand. The RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ fractions on oligo dT-cellulose (Collaborative Research) by conventional procedures. The RNA was cycled five times through the column in 0.5 M LiCl, 1 mM EDTA, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), at room temperature to give the poly(A)$^-$ fraction. The poly(A)$^+$ RNA was eluted in 1 mM EDTA, 0.1% SDS, 10 mM Tris-HCl (pH 7.5) at room temperature.

The probe used in S1 analysis was a *Tth*III 1/*Bam*H1 fragment of pcXbH4W1 [12], see section 3 and fig.1. The whole cloned DNA, which was in pAT153, was digested with *Tth*III 1, extracted with phenol/chloroform and ethanol precipitated. The 3′-ends of the two strands were labelled with [^{32}P]dGTP, using T4 polynucleotide kinase. All enzyme reactions were according to the supplier's instructions. The DNA was phenol/chloroform extracted and ethanol precipitated, then the fragment desired was strand-separated on a standard 8% acrylamide, 7 M urea sequencing gel [15].

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S1-nuclease analysis was performed essentially according to Berk and Sharp [16]. Usually 10–20 μg of RNA were hybridized to the probe in 10 μl 0.4 M NaCl, 10 mM Pipes (pH 6.4), for 3 h. This was rapidly transferred to S1 digestion buffer (0.28 M Nacl, 4.5 mM $ZnSO_4$, 50 mM CH_3COONa, pH 4.6) and 150 units S1 nuclease (Sigma) added. Incubations were for 40 min at 19°C. The reaction was stopped by adding 3 μl of 0.5 M EDTA. The sample was phenol/chloroform extracted, ethanol precipitated and analyzed on a standard 8% acrylamide sequencing gel [15]. The gel was exposed to Fuji RX film, with or without an intensifying screen, at −70°C.

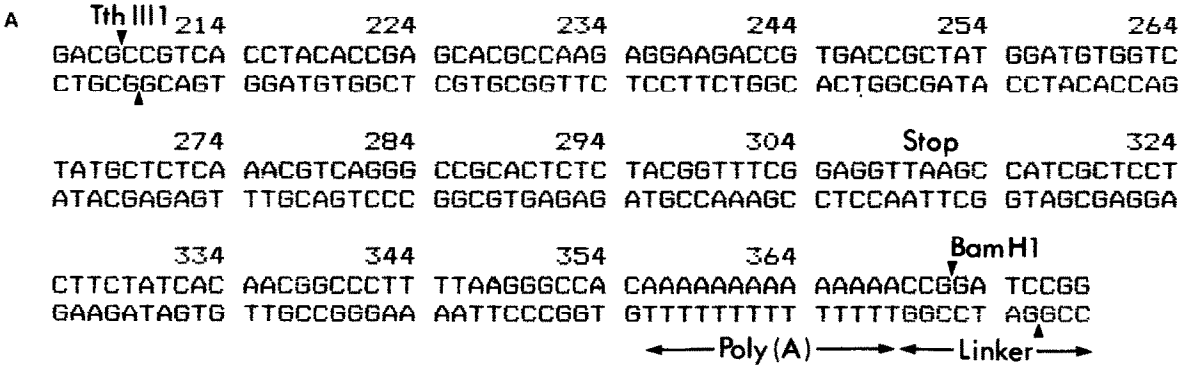
Primer extension was performed with a primer from an *X. laevis* cDNA clone (pcX1H4W1) exactly as described previously [14].

3. RESULTS

The probe used is shown in fig.1A. It is derived from a *Xenopus borealis* ovary cDNA clone encoding histone H4 [12]. The double-stranded cDNA was ligated into pAT 153 via *Bam*H1 linkers, but the linker at the 5′-end of the coding sequence was lost during the cloning. There is a single *Tth*III 1 site within the coding region, and this restriction site is absent from the vector. Thus the fragment shown was cut directly from the rest of the clone by *Bam*H1 digestion, after labelling of the *Tth*III 1 site by addition of [^{32}P]dGTP with T4 polymerase. The labelled non-coding strand was isolated on a sequencing gel. This probe contained the 3′-portion of the coding region, the entire 3′ trailer, 14 residues of poly(A) tract and a terminal CCG sequence derived from the Bam linker.

Fig.1B shows the tuning of the S1 nuclease reaction to reveal poly(A) tracts on ovary H4 mRNA. Above 20°C S1 removes the poly(A) region; at or below 20°C it is protected. Our experiments therefore utilized digestion at either 15 or 19°C.

The poly(A) tract of ovary H4 mRNA is revealed by the experiments shown in fig.1,2,3. Although the experiment was in principle limited by the fact that the probe had a tract of only 14 dTMP residues, this does not seem to have been a serious



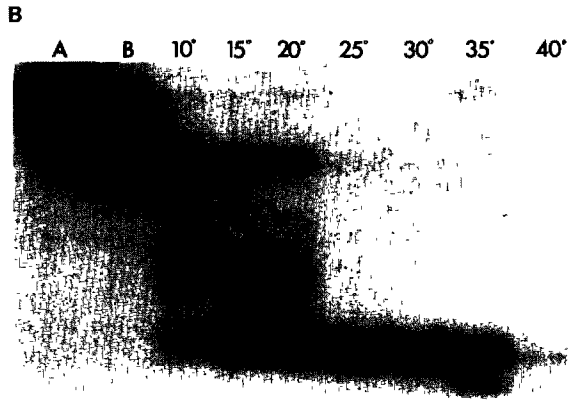


Fig.1. (A) Sequence of the H4 mRNA probe used in these experiments. It is from a *X. borealis* ovary cDNA clone, pcXbH4W1 [12]. The upper sequence is the coding strand; the number of residues from the start of the coding strand is marked, as is the stop codon. The lower sequence yielded the probe, which was labelled by adding a single ^{32}P-GMP residue at the *Tth*III 1 site. (B) Temperature optimization of the S1 nuclease reaction. The labelled probe was hybridized at 65°C to 6 μg of *X. borealis* ovary RNA and the S1 reactions conducted at the temperatures shown. The product was analyzed on an 8% acrylamide sequencing gel and autoradiographed. A and B are the lower and upper strands in fig.1A, respectively. The S1 products are identified in fig.2.

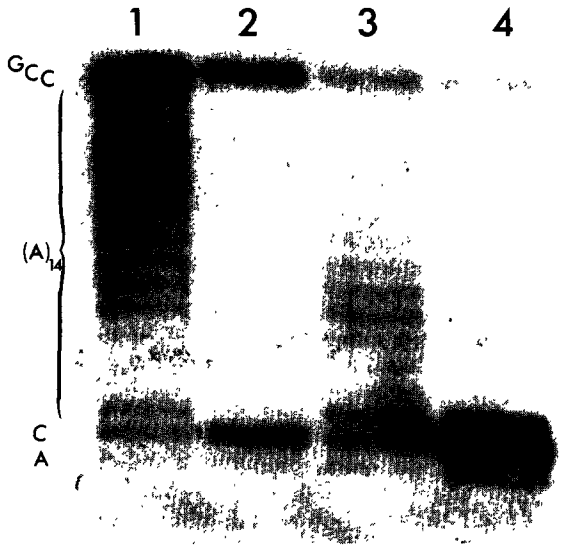


Fig.2. S1 nuclease analysis of *X. borealis* ovary (lanes 1,3) and unfertilized egg (lanes 2,4) total RNA. Hybridizations of 5 μg of total RNA were overnight at 50°C (lanes 1,2) and 70°C (lanes 2,4). The sequence of the protecting mRNA is shown on the left. Other details in fig.1B. Similar patterns were seen when *X. laevis* RNA was used. Protection of the top band (by -CCG) was produced by contamination with the complementary DNA strand of the probe.

Fig.3. S1 nuclease analysis of fractionated *X. borealis* ovary RNA and RNA from different stages of oogenesis. Lane A, protection by total ovary RNA. Lanes B,C, this RNA fractionated into poly(A)$^+$ and poly(A)$^-$ RNA, respectively. A greater equivalent amount of the poly(A)$^+$ RNA was used. Lane D, 12 μg total RNA from full-grown stage VI oocytes [32]. Lane E, 12 μg total RNA from stage IV half-grown oocytes. Lane F, 12 μg total RNA from stage I and II previtellogenic oocytes. Lane G, *E. coli* tRNA control. Lane H, unreacted probe. The position of size markers (bp) is shown on the right. Other details in fig.1B,2.

problem, since there was very little full length protection of the complete poly(dT) tract. There is a band above the poly(A) tract, which is even present after control hybridisation to *E. coli* tRNA. This was caused by trace contamination of the probe with the coding strand. CCG was thus present above the poly(A), but underwent compression to give a single band.

Thus the length of the poly(A) tract is very short, although most H4 mRNA molecules seem to have several A residues. It could be argued that this apparent short length is actually the result of S1 nuclease cuts within a longer region protected by poly(A): There are two arguments against this point of view. (i) The temperature optimisation (fig.1B) indicates that an end-point to the digestion is reached at 20°C; if this were not so one would expect to see longer tracts at lower temperatures. (ii) When ovary RNA is fractionated into poly(A)$^+$ and poly(A)$^-$ fractions, the former is seen to have a longer mean poly(A) tract than the latter or than total RNA, though it is still predominantly less than 14 residues long (fig.3).

The H4 mRNA of the ovary is made up of a number of gene products, differing in sequence, particularly in *X. laevis* [14]. One might therefore ask if ovary H4 mRNA which binds to oligo(dT)-cellulose, and therefore has a longer average poly(A) tract is different in sequence from that which does not bind. S1 analyses of the type presented in fig.3 show no difference, even when the S1 digestion is at a higher temperature. This indicates that the two fractions do not differ at the 3′-end. A more discriminating way to distinguish H4 mRNAs is to use primer extension. Sequencing primer extension products show a range of different mRNA leader sequences, particularly in *X. laevis* [14]. Fig.4 shows the primer extension products of *X. borealis* and *X. laevis* H4 mRNA from the poly(A)$^+$ and poly(A)$^-$ fractions. There are no differences, again indicating that the poly(A) tracts have comparable heterogeneity on all of the abundant kinds of H4 mRNA.

Ruderman et al. [10] reported that the histone mRNA of the egg and embryo did not bind to oligo(dT) cellulose. The molecular basis of this observation is shown in fig.2. In the egg the poly(A) tract vanishes completely; although one A residue remains, this is the conventional end of a histone mRNA [17]. The strength of the signal from an equivalent amount of oocyte and egg RNA is similar, indicating that poly(A)$^+$ oocyte H4 mRNAs are depolyadenylated, rather than that

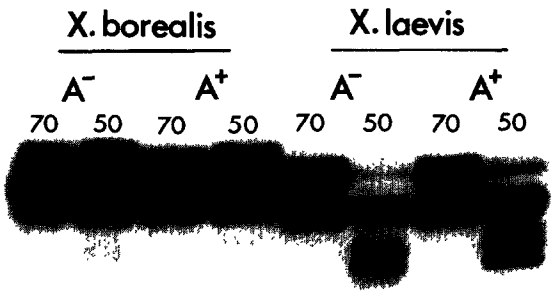


Fig.4. Species of H4 mRNA in the poly(A)$^+$ and poly(A)$^-$ fractions. The 5′-ends of H4 mRNA were analyzed by primer extension exactly as described in [14]. The RNA that bound or passed through oligo(dT)-cellulose was hybridized to the 5′-labelled primer at 50 or 70°C. The hybrid was extended with reverse transcriptase and the product analyzed on a sequencing gel. Only the extended bands are shown. Sequencing shows that in *X. laevis* the different bands represent different sequences, and different mRNAs are revealed at 50 and 70°C [14].

the entire polyadenylated mRNA fraction is degraded in toto.

We have also examined embryo and cultured cell H4 mRNA for poly(A) tracts, but no trace of them was found.

Lastly, we have asked if the size distribution of poly(A) changes during oogenesis. Fig.3 shows that it does not do so.

4. DISCUSSION

The results described above show that ovary histone H4 mRNA is truly polyadenylated, but that the poly(A) tract is very short. A minor proportion of molecules seem to have no poly(A) tract. The H4 and H3 cDNA clones isolated from ovary to date have short poly(A) tracts, but some are longer than the 0–12 suggested here as the predominant size distribution in the ovary [12]. Naturally this is because the clones cannot have a poly(A) tract shorter than the length of the oligo (dT) used to prime reverse transcriptase in the cDNA synthesis.

The fact that histone mRNAs are polyadenylated at all deserves some comment. Typically mRNAs are polyadenylated in a processing reaction relying on an AAUAAA sequence near the 3′-ends so generated [18]. Histone mRNAs, including those polyadenylated in the ovary, lack this sequence. This indicates that there may be something unique about polyadenylation reactions in the oocyte. However, one must be cautious about this conclusion because H5 mRNA is post-transcriptionally polyadenylated in the avian erythroblast, even though it lacks the AAUAAA sequence [4]. H3.3 mRNA is also polyadenylated in these cells, but this may involve a transcribed poly(A) tract [6].

It has been argued that the poly(A) tract of histone mRNA in the ovary may be involved in stabilizing the molecule for long term storage through oogenesis [19]. This can only partly be true, since many molecules lack a poly(A) tract. In addition, it is known that poly(A) stabilises globin mRNA injected into an oocyte, but not when it is less than 20 residues long [20].

Sea urchin and *Xenopus* histone mRNA molecules made on clones injected into oocytes do not bind to oligo(dT)-cellulose, and by this criterion they are not polyadenylated ([21]; unpublished). In this respect they differ from the endogenous transcripts. One explanation could be that endogenous polyadenylated histone mRNA is made only early in oogenesis and poly(A)$^-$ histone mRNA is made later, i.e., at the time cloned DNAs were injected. Comparison of RNA from early and late oocytes does not fit with this idea (fig.3). Thus, either the transcripts from cloned genes are processed differently from those made on chromosomal genes, or the endogenous histone mRNAs are made exclusively early in oogenesis, and the ability to polyadenylate histone mRNA ceases later. This is quite possible, since there is no increase in overall histone mRNA content from previtellogenic stages onwards [22]. However, the lampbrush chromosomes of newts [23,24] transcribe histone genes at stages after the histone mRNA pool is maximal, and *Xenopus* is probably similar. Thus, if this explanation is correct it would be necessary to pro-

pose that histone gene transcripts on lampbrush chromosomes never appear as mature mRNA. It should be mentioned that transcripts made on SV40 DNA molecules injected into the oocyte are polyadenylated, so injected genes do not saturate the oocytes polyadenylation machinery, nor is polyadenylation of mRNA totally absent from these cells [25]. It would be useful to know the size of poly(A) tracts on non-histone mRNAs in the oocyte, but there is no information on this point. Although the size of the poly(A) tract in ovary poly(A)$^+$ RNA has been measured [26,27], it is now known that most of the poly(A)$^+$ RNA of the oocyte is not mRNA [28].

The depolyadenylation of histone mRNA in the egg is dramatic, but its reason is not understood. It has been argued [29] that poly(A)$^+$ mRNA is translated more efficiently by slime moulds than is poly(A)$^-$ RNA. It is true that conversion of the oocyte to the egg produces a dramatic mobilization of histone mRNA [19], but actin mRNA shows reduced binding to oligo(dT)-cellulose at this stage, while being translated less efficiently [30]. Rosenthal et al. [31] have found a complex link between polyadenylation and translatability in molluscan oocytes. On the whole they found a correlation between increased polyadenylation and increased translation, that is the opposite to the situation in slime moulds and for *Xenopus* histone mRNA. A role for changes in polyadenylation state in animals has thus still to be established. Other factors may be important, like the greater diffusibility of poly(A)$^-$ RNA (Drummond and Colman, personal communication).

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