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Sites of transcription initiation *in vivo* on *Xenopus laevis* ribosomal DNA

(40S rRNA precursor/primary transcript/oocyte nucleoli/RNA sequencing/capping enzymes)

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ABSTRACT **We report the results of a novel method for locating sites of transcription initiation using a complex of capping enzymes from vaccinia virions that catalyze the reaction pppG + *S*-adenosylmethionine + (p)ppXpYpZp ..... →^{7m}GpppXpYpZp ..... [Ensinger, M. J., Martin, S. A., Paoletti, E. and Moss, B. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2525–2529]. This enzyme complex will cap di- or triphosphate termini but will not cap monophosphate or hydroxyl termini. *Xenopus laevis* 40S precursor rRNA from oocytes is capped by these enzymes, and we conclude that it has 5′-polyphosphate termini. Therefore, 40S RNA must represent the primary transcript of amplified *X. laevis* ribosomal DNA. The majority of 40S molecules with polyphosphate termini begin with the sequence (p)ppAAG. There is evidence, however, that the 5′ terminus may be heterogeneous. The majority of all detectable initiation events were localized close to the region coding for the 5′ end of the 40S RNA. No initiation sites were detected in the nontranscribed spacer, but an apparent initiation site in the middle of the transcribed region was also observed.**

When cells of *Xenopus laevis* are labeled with RNA precursors, the first distinct transcription product that arises from the rDNAs is a 2.7×10^6 dalton molecule, the so-called 40S rRNA precursor. (1). Through a series of known processing steps, this precursor then gives rise to the 18S and 28S rRNAs found in cytoplasmic ribosomes (2). Electron micrographs of active rDNA show a series of transcribed 40S gene regions alternating tandemly with spacer regions that are not transcribed (3). The assumption has been that transcription initiates close to the 5′ end of each 40S gene.

In this paper, we report the results of a novel method for locating transcription initiation sites. The method involves use of a complex of capping enzymes from vaccinia virus that catalyze the reaction pppG + *S*-adenosylmethionine + (p)ppXpYpZp ..... →^{7m}GpppXpYpZp ..... (4). This enzyme complex will cap 5′ termini bearing a di- or triphosphate but will not cap monophosphate or hydroxyl termini. Moss (7) established that the capping and methylating enzymes of vaccinia virus could be used to label, identify, and sequence the primary polyphosphate-containing ends of heterologous RNA molecules and pointed out its wide applicability to viral, prokaryotic, and eukaryotic systems. On the assumption that 5′ di- or triphosphate termini mark the site of RNA chain initiation, we have used this reaction to cap *Xenopus* nucleolar RNA radioactively and to locate sites of initiation on the rDNA. The large majority of all detectable initiation events were found to occur close to the 5′ end of the 40S precursor molecule.

METHODS

Isolation of Nucleolar RNA from Oocytes. Amplified nucleoli were isolated from immature oocytes of *X. laevis* as described (5). Such nucleolar preparations contain no DNA other than rDNA. The nucleoli were dissolved in sodium dodecyl sulfate ($NaDodSO_4$) and their RNA was fractionated on 15–30% sucrose/$NaDodSO_4$ gradients as described by Penman *et al.* (6). Pooled fractions from the gradient were extracted with phenol, ethanol precipitated, and used as substrate for capping.

Capping with Vaccinia Enzymes. Partially purified capping enzymes from vaccinia virions were a generous gift from B. Moss. The preparation had been purified through the first DEAE column step and contained at least three different activities: a RNA guanylyltransferase, a RNA (guanine-7-)-methyltransferase, and a RNA (nucleoside-2′-)-methyltransferase (7). Capping reactions were usually performed as described by Moss (7) in a volume of 100 μl containing 10–20 μg of RNA, 50 mM Tris·HCl (pH 7.6), 1 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 2 mM GTP, 65 pmol of *S*-adenosyl[*methyl*-^3H]methionine (65 Ci/mmol, New England Nuclear), and 5 μl of enzyme extract. After 90 min at 37°, reactions were terminated by adding $NaDodSO_4$ to 0.5%, 100 μg of *Escherichia coli* transfer RNA carrier was added, and the mixture was extracted with phenol. The phenol extract was passed through a column of Sephadex G-25 (1.5 × 30 cm, equilibrated with 0.1 M NaCl/0.01 M Tris·HCl, pH 8 0.5% $NaDodSO_4$) and the void volume containing the capped RNA was collected and ethanol precipitated.

Alternatively, in some experiments, RNA was capped by using [α-^{32}P]GTP (200–300 Ci/mmol, 50–100 μCi/100 μl of reaction mixture) as the radioactive donor, leaving out the unlabeled GTP and substituting 0.2 mM unlabeled *S*-adenosylmethionine for the ^{3}H-labeled compound. The enzyme preparation used in these experiments also contains a terminal transferase activity that appears to add nucleotides to the 3′ end of RNA molecules (B. Moss, personal communication; our own observations). This transferase does not interfere with [^{3}H]-methyl-labeling but, when [α-^{32}P]GTP is used, it can cause up to 50% of the label to be in noncap structures. Addition of 0.16 mM ATP suppressed this unwanted side reaction and resulted in over 90% of the ^{32}P label appearing in caps.

RNase Digestions and DEAE Column Chromatography. ^{3}H-Capped RNA was digested with either nuclease P_1 (Calbiochem, 5 μg in 50 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA for 1 hr at 37°), RNase T_1 (Calbiochem, 50 units in 50 μl of 10 mM Tris·HCl, pH 8, for 1 hr at 37°), RNase T_2 (10 units in 50 μl of 10 mM Na acetate, pH 4.5/0.1 mM EDTA for 1 hr at 37°), or RNase A (Sigma, 10 μg in 50 μl of 10 mM Tris·HCl, pH 8, for 1 hr at 37°). Labeled digests were mixed with unlabeled marker oligonucleotides (2 mg of *E. coli* transfer RNA digested to completion with RNase A), applied to a column of DEAE-Sephadex (0.6 × 25 cm in 7 M urea/0.05 M Tris·HCl, pH 8), and eluted with a 300-ml gradient from 0.1 to 0.5 M NaCl in

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

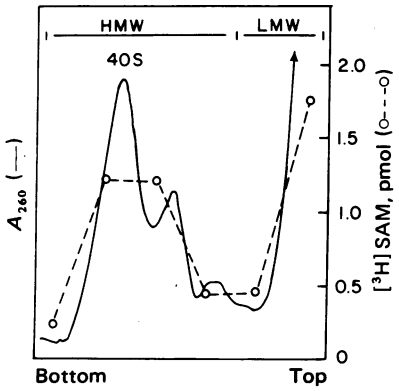

FIG. 1. Sucrose gradient analysis of RNA bearing di- or triphosphate termini from nucleoli of *X. laevis* oocytes. Pooled fractions from the gradient were then tested for their relative ability to accept 5′ terminal caps with *S*-adenosyl[^{3}H]methionine ([^{3}H]SAM) as the label donor. HMW, high molecular weight; LMW, low molecular weight.

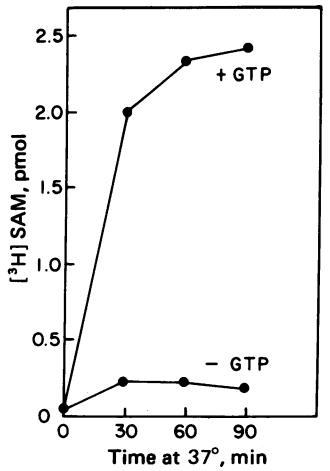

FIG. 2. GTP dependence of *S*-adenosyl[^{3}H]methionine incorporation into caps. Reactions were in 100-μl volumes containing 8.5 μg of 40S rRNA in the presence or absence of 2 mM GTP. Aliquots were taken at intervals to determine incorporation of [^{3}H]methyl into acid-precipitable material.

7 M urea/0.05 M Tris·HCl, pH 8. Fractions of about 4.5 ml were mixed with 15 ml of Aquasol (New England Nuclear) and assayed for radioactivity in a scintillation counter.

The specificities of the above nucleases are: nuclease P_1, cleaves RNA chains at all four bases leaving 5′ phosphates; RNase T_1, cleaves after G leaving a 3′ phosphate; RNase T_2, cleaves at all four bases leaving 3′ phosphates; RNase A, cleaves after C or U leaving 3′ phosphates.

Thin-Layer Chromatography of Cap Structures. Capped RNA was digested with nuclease P_1 and chromatographed in two dimensions on thin-layer cellulose sheets (Eastman 6065 Chromagram). The first dimension solvent was isobutyric acid/29% NH_4OH/H_2O 66:0.5:33 (vol/vol). After overnight drying, the second dimension was saturated $(NH_4)_2SO_4$/1 M Na acetate/isopropanol, 80:18:2 (vol/vol). Unlabeled markers of the type $^{7m}GpppX^m$ were purchased from P-L Laboratories.

Gel Electrophoresis of rDNA Restriction Fragments. Chimeric plasmid pXlr11 (15 μg) was digested with *Eco*RI. Plasmid pXlr13 (30 μg) was doubly digested with *Bam*HI and *Eco*RI. Together these plasmids yielded fragments that spanned the entire rDNA repeating unit [see Fig. 4 for map positions of the fragments; the plasmids and restriction digestion conditions are more fully described elsewhere (8, 9)]. The pXlr11 and pXlr13 digests were combined and electrophoresed on 1% agarose as described (10). The fragments were then transferred to a nitrocellulose filter (11) and hybridized with ^{3}H-capped nucleolar RNA.

An *Eco*RI digest of pXlr11 (30 μg) was strand separated by applying it in alkali to a neutral 1% agarose gel (12). After electrophoresis, the DNA was transferred to a nitrocellulose filter by the procedure of Southern (11) except that the denaturing step was omitted.

Hybridization of ^{3}H-Capped RNA to rDNA Fragments. ^{3}H-Capped RNA was added to rDNA on filters in 1 ml of 0.6 M NaCl/0.2 M Tris·HCl, pH 8/20 mM EDTA/50% formamide and incubated overnight at 40°. Hybrids were trimmed with RNase A (50 μg/ml in 0.3 M NaCl/0.1 M Tris·HCl, pH 8/10 mM EDTA) at room temperature for 15 min before the filters were rinsed, dried, and assayed for radioactivity in a toluene-based scintillation fluor.

Hybrids were recovered for further analysis by rinsing the filter four times with $CHCl_3$ to remove fluor. Filters were then incubated for 10 min in 1 ml of 0.25 mM $MgCl_2$/0.5 mM Tris, pH 8, containing 50 μg of RNase-free DNase and then heated at 100° for 10 min. The liquid was cooled to 37° and to it was added 250 μl of RNase A (1 mg/ml), 50 μl of DNase (1 mg/ml), and 2 mg of *E. coli* transfer RNA as carrier. Digestion at 37° was continued for another hour, at which time the filters were removed and the remaining liquid was made 7 M in urea and 0.05 M in Tris·HCl (pH 8) and applied to a DEAE-Sephadex column for analysis of the ^{3}H-labeled RNase A oligonucleotides. Overall recovery of hybridized radioactivity was about 60%.

RESULTS

Capping of Nucleolar RNA. The major RNA species isolated from the nucleoli of immature oocytes was the 40S rRNA precursor (Fig. 1), with a second component at 30 S, the size of one of the more stable processing intermediates. Little, if any mature 28S and 18S RNA was observed. The high UV absorbance at the top of the gradient was mostly due to Metrizamide from the preceding density gradient step used to purify the nucleoli. Pooled fractions from the gradient were extracted with phenol, precipitated with ethanol, and tested for their ability to serve as substrate for the vaccinia capping enzymes. With *S*-adenosyl[^{3}H]methionine as the radioactive donor, capping acceptor activity was found in a broad peak from 30 S to 40 S and also in the low molecular weight fraction.

Evidence that the Label Is in Cap Structures. Fig. 2 shows the time course of capping of RNA taken from the heavy side of the 40S peak. The incorporation of [^{3}H]methyl label was completely dependent upon GTP, as expected from the known mechanism of these enzymes (13). The GTP dependence is good evidence that the methylation is in cap structures and not at internal sites along the RNA chain. The reaction reached a plateau at 0.44 mol of [^{3}H]methyl groups incorporated per mol of 40S rRNA. Assuming that each cap contains two methyl groups (results shown in Fig. 3 demonstrate that this assumption is correct), this means that 22% of the 40S termini were able to accept caps. The level of incorporation was high enough to make it likely that the 40S itself was being capped rather than some minor contaminant. The less-than-stoichiometric amount of capping is not unexpected in view of the apparent lability of polyphosphate termini (14, 15).

An independent method for checking that the label is really in cap structures is to digest the labeled RNA with nuclease P_1. This nuclease leaves 5′-phosphoryl termini and is not blocked by methylation of the 2′-hydroxyl on the adjacent ribose. However, it cannot cleave the 5′ → 5′ pyrophosphate linkage

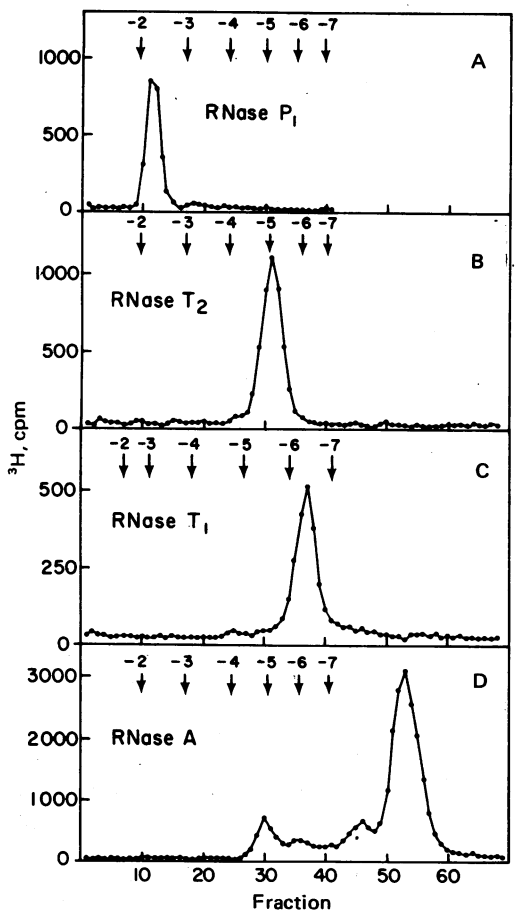

FIG. 3. Digestion of capped high molecular weight (HMW) nucleolar RNA with various RNases. The HMW region of the gradient shown in Fig. 1 was pooled and capped with *S*-adenosyl[^{3}H]methionine as the label. The capped RNA was then digested with various RNases and the resulting oligonucleotides were fractionated on columns of DEAE-sephadex. (*A*) RNase P_1; (*B*) RNase T_2; (*C*) RNase T_1; and (*D*) RNase A. The elution position of unlabeled marker oligonucleotides of known charge is indicated for each column profile.

within the cap itself and, thus, from capped structures should generate a labeled fragment with −2.5 charges (16). If the methylation were occurring at internal sites along the RNA chain, P_1 should generate fragments with −2 or fewer charges. Fig. 3*A* shows that P_1 nuclease liberates a single fragment of about −2.5 charges.

We also checked to see if the capped 40S is methylated on the 2′-hydroxyl of the terminal ribose because Ensinger *et al.* (4) found this position to be methylated with the substrates they were using. If the 2′-hydroxyl is methylated, digestion with RNase T_2 (which leaves 3′-phosphoryl termini and is blocked by methylation of the 2′-hydroxyl on the adjacent ribose) should yield a single fragment with a charge of −5.5. Fig. 3*B* shows that a fragment of −5.5 charges was obtained.

We conclude from these results that all of the [^{3}H]methyl label is incorporated into structures of the dimethylated or cap 1 (16) variety and is not incorporated into internal sites along the RNA chain.

Is Capping by Vaccinia Enzymes Specific for Polyphosphate Termini? Only molecules that are known to terminate in 5′ di- or triphosphates have been observed to accept caps. For example, Martin and Moss (13) showed that $ppA(pA)_n$ will cap whereas $pA(pA)_n$ will not. We have found that processed molecules such as *E. coli* transfer RNA and *Xenopus* 18S and 28S rRNA will not accept caps but *Xenopus* 5S rRNA will cap (data not shown).

Table 1. Capping of nucleolar RNA with *S*-adenosyl[^{3}H]-methionine and [β,γ-^{32}P]GTP*

Radioactive group	pmol incorporated
^{3}H methyl	0.63
^{32}P (β or γ)	0.003

* Reaction in 100 μl contained 23 μg of unfractionated nucleolar RNA, 50 mM Tris·HCl (pH 7.6), 1 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 65 pmol of *S*-adenosyl[^{3}H]methionine, (7700 counts/pmol), 2.6 nmol of [β,γ^{32}P]GTP (3.68×10^5 counts/pmol), and 5 μl of enzyme. Incubation was for 90 min at 37°.

In an alternate approach to the specificity question, total nucleolar RNA was capped with *S*-adenosyl[^{3}H]methionine and [β,γ-^{32}P]GTP as radioactive donors in the same reaction. With the enzymes from vaccinia, Martin and Moss (13) have shown that only the α phosphate from GTP is incorporated into the cap, and incorporation of the G residue is obligatory before the methyl groups can be added. Incorporation of only the α phosphate agrees with the enzymes' specificity for polyphosphate termini. Enzymes that do cap monophosphate termini (17) have been shown to incorporate both the β and γ phosphates from GTP. For every mol of termini labeled with methyl groups, at most only 0.01 mol of termini could have been labeled with the β or γ phosphates (Table 1). We feel confident, therefore, that the capping enzymes we used were highly specific for di- or triphosphate termini.

Partial Sequencing of Capped RNA. High molecular weight nucleolar RNA (30S–40S) was pooled, capped with a [^{3}H]-methyl donor, and digested with RNase A; the oligonucleotides were separated according to charge (Fig. 3*D*). As we will show in the next section, the two oligonucleotides that had more than −7 charges also were recovered from capped RNA hybridized to DNA containing the 5′ end of the 40S sequence. The oligonucleotides with less than −7 charges apparently did not result from rDNA transcription and have not been further analyzed. The largest RNase A fragment was recovered and redigested with RNase T_1. This yielded a single fragment with −6.5 charges (data not shown). RNase T_1 digestion of total capped 30S–40S RNA also yielded a single fragment of −6.5 charges (Fig. 3*C*). This number of charges is consistent with the structure being ^{7m}GpppXpApGp. The second nucleotide was deduced to be A because RNase T_2 cleaves at this point (Fig. 3*B*) but T_1 and A do not. The X nucleotide was determined by capping 40S RNA with [α-^{32}P]GTP, digesting the capped RNA with nuclease P_1, and chromatographing the digest in the two-dimensional system capable of separating all four possible caps of the type ^{7m}GpppX^m (X is A, G. U, or C). About 70% of the labeled material cochromatographed with authentic ^{7m}GpppA^m, 19% with ^{7m}GpppG^m, and 11% with 5′-GMP (data not shown). We assume that the 5′-GMP came from residual incorporation of label at the 3′ end of the RNA that was not suppressed by addition of unlabeled ATP. We conclude that the majority of cappable 40S molecules begin with 5′-(p)-ppAAG. At present we do not know if the molecules initiating with G represent a subset of 40S molecules or if they are due to contamination by some other RNA.

Hybridization of Capped RNA to rDNA. To determine which of the RNase A oligonucleotides in Fig. 3*D* were associated with rDNA transcription, capped nucleolar RNA was hybridized to cloned DNA fragments that spanned the entire repeating unit of rDNA. DNA from the chimeric plasmid pXlr11 was digested with *Eco*RI endonuclease and combined

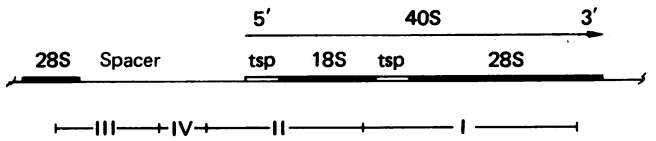

FIG. 4. Map of one repeating unit of *X. laevis* rDNA. This map shows the location of fragments I, II, III, and IV used for the hybridization experiment shown in Fig. 5. Fragments were derived from clones Xlr11 and Xlr13 by using *Bam*HI and *Eco*RI (see ref. 8 for more detailed restriction maps).

with DNA from pXlr13 that had been doubly digested with *Bam*HI and *Eco*RI.

Fig. 4 shows the location on the rDNA map of each of the fragments used in this study. The combined fragments were electrophoresed on 1% agarose and transferred onto a strip of nitrocellulose filter. One such filter strip was hybridized with capped 30S–40S nucleolar RNA; another strip was hybridized with capped low molecular weight RNA. The filters were then treated with RNase A to remove nonhybridized RNA, dried, cut into pieces, and assayed for radioactivity. The result for the

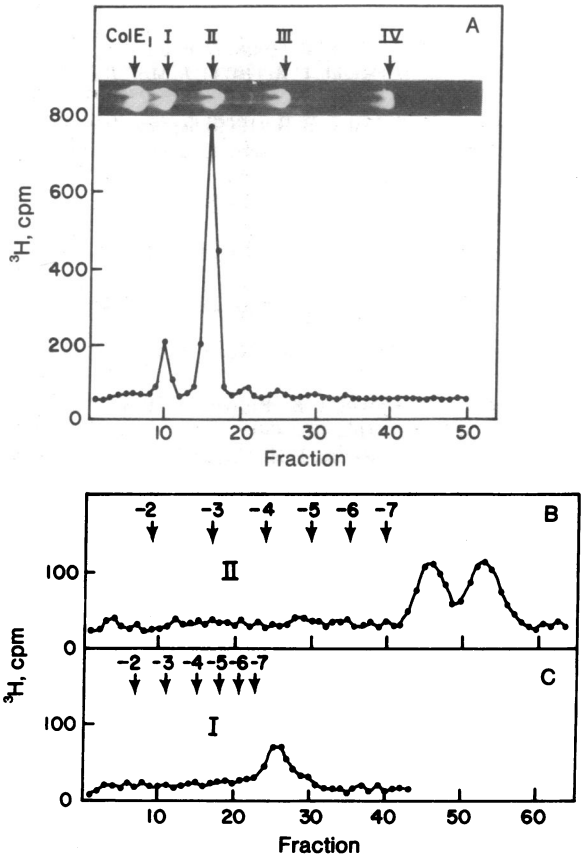

FIG. 5. Hybridization of capped nucleolar RNA to rDNA restriction fragments. ^{3}H-Capped nucleolar RNA (HMW fraction from Fig. 3) was hybridized to restriction fragments immobilized on a nitrocellulose filter (50,000 cpm of ^{3}H-capped RNA in 1 ml of 0.6 M NaCl/0.2 M Tris, pH 8/50% formamide at 40° overnight). The map location of the fragments is shown in Fig. 4. After trimming hybrid with RNase A, the filter was cut into pieces and assayed for radioactivity. Hybrid RNA was then removed from the filter pieces and digested with RNase A, and the labeled oligonucleotides were fractionated on DEAE-Sephadex as described in Fig. 3. (*A*) Photograph of the agarose gel used to separate the rDNA fragments; underneath is represented the amount of RNase-resistant radioactivity hybridizing to each fragment. (*B*) DEAE chromatography of RNase A oligonucleotides recovered from hybrid to fragment II. (*C*) Oligonucleotides recovered from hybrid to fragment I.

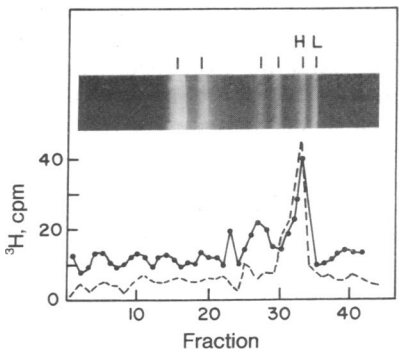

FIG. 6. Hybridization of capped nucleolar RNA to separated strands of fragment I. pXlr11 was digested with *Eco*RI, and the fragments were strand separated on an agarose gel. After transfer to a nitrocellulose filter they were hybridized with *S*-adenosyl[^{3}H]-methionine-capped total nucleolar RNA (42,000 cpm of ^{3}H-capped RNA in 1 ml of 0.6 M NaCl/0.2 M Tris, pH 8/50% formamide at 40° overnight). A parallel filter was hybridized with 40S [^{32}P]rRNA to locate the coding (H) strand of the rDNA fragment. (●—●), ^{3}H-Capped nucleolar RNA; (- - - -), 40S [^{32}P]rRNA.

30S–40S RNA is shown in Fig. 5*A*. A closely similar result was obtained with the low molecular weight RNA and is not shown.

About 85% of the hybridization occurred with fragment II which spanned the 5′ end of the 40S precursor. There was no hybridization to the fragment spanning the 3′ end (fragment III), to the center of the nontranscribed spacer (fragment IV), or to the ColE1 vehicle. The RNA bound to fragment II was recovered and digested with RNase A, and the oligonucleotides were run on a DEAE column (Fig. 5*B*). Two peaks were observed, corresponding in charge to the two largest peaks obtained from RNase A digestion of unhybridized 30S–40S rRNA (Fig. 3*D*). The two peaks recovered from the hybrid (Fig. 5*B*) were of about equal size whereas before hybridization one was 5 times larger than the other. This change in ratio probably reflects the fact that RNA was saturating in the hybridization reaction. These results strongly support the conclusion that the majority of capped structures are actually on the 5′ ends of 40S molecules. This leads to the further conclusion that the majority of all detectable initiation events on rDNA occur close to or at the 5′ end of the 40S RNA. The data also show that the 5′ end of the 40S RNA may be heterogeneous at the position of the first RNase A cleavage.

A somewhat surprising result, also shown in Fig. 5*A* is that 15% of the hybridization is associated with fragment I, a fragment from the middle of the transcribed gene. RNase A digestion of RNA from this hybrid yielded a single peak of greater than −7 charges (Fig. 5*C*). The significance of this apparent initiation event is unknown at present. To confirm that the hybridization was in fact occurring with fragment I and not with some partial digestion product that contained fragment I, capped RNA was hybridized to pXlr11 (the plasmid that contains only fragment I plus ColE1) that had been *Eco*RI digested and strand separated on an agarose gel. Fig. 6 shows that the hybridization occurred to the coding (H) strand of the rDNA insert.

DISCUSSION

The size of the primary transcription product is still unknown for most eukaryotic genes that have been studied. Only in the case of the 5S RNA from ribosomes has it been possible to demonstrate a triphosphate terminus on the 5′ end of the RNA (18, 19) and therefore to pinpoint the site of chain initiation.

In all other cases, the approach has been to isolate the largest RNA that can be detected after brief labeling and assume that it is the primary transcript. Experience with prokaryotes (20) has shown, however, that processing may occur so rapidly for some genes that under normal circumstances the primary transcript never appears intact and the earliest discernable products may already be processed intermediates.

The approach described in this report provides a novel and powerful method for locating the 5′ ends of primary transcripts that is completely independent of size or the kinetics of labeling. Its only requirements are that (*i*) the capping enzymes be absolutely specific for di- or triphosphate termini and (*ii*) such termini are only present as the result of chain initiation and not as the result of secondary rephosphorylation. Concerning the first point, Martin and Moss (13) have shown that on homoribopolymers the vaccinia enzymes ignore monophosphate termini. We have found that the 5′-monophosphates on 18S and 28S rRNA are not capped, nor are the processed ends of *E. coli* tRNA. Approaching the same question from another route, we have shown that neither β nor γ phosphate of GTP is incorporated into capped structures in nucleolar RNA. Therefore, we feel confident that the guanylation reaction is absolutely specific for di- or triphosphate termini. The second requirement, that di- or triphosphate termini arise only from chain initiation and not by rephosphorylation, is more difficult to establish. U. Schibler and R. Perry (personal communication) have found that, in L-cell heterogeneous nuclear RNA, polyphosphate termini are only seen adjacent to adenosine or guanine. This suggests that, even in whole nuclear RNA, rephosphorylation may not interfere with this method. It is highly unlikely that rephosphorylation is responsible for the results we have described here with nucleolar RNA. For that to be the case, one would have to argue that the kinase is also sequence-specific because only certain termini are capped and others are not.

Slack and Loening (14) have searched for triphosphate termini on 40S rRNA from *Xenopus* cultured cells and reported they could only detect 5′-monophosphate termini with guanine as the terminal base. This monophosphate terminus could in theory result from cleavage of a larger precursor or it could represent the true transcription initiation site with two of its phosphates removed.

Another line of evidence concerning possible RNA chain initiatior sites on rDNA comes from the observation by Scheer *et al.* (21) that structures resembling RNA polymerase and small transcription complexes are occasionally seen in electron micrographs of the nontranscribed spacer. Rungger and Crippa (22) have made the related observation that treatment of oocytes with high concentrations of 5-fluorouridine results in the appearance of large amounts of transcription in the spacer region. Both results could be due to polymerase initiation in the spacer region. In view of our own data, however, it seems more likely that their results are due to a failure of proper termination at the 3′ end of the 40S coupled with a variable degree of processing of the aberrant transcript. We obviously cannot rule out the possibility that a low level of initiation does occur in the spacer with these 5′ termini being extra sensitive to degradation and thus not detected. But we can say that the majority of the detectable initiation events occur close to the 5′ end of the 40S sequence and we are therefore justified in concentrating on this region to find the promoter and other possible control sequences. It seems likely that there is nothing within the spacer itself that is inimicable to transcription; rather, it is the presence upstream of a termination site that normally results in the absence of transcription.

There is evidence that 40S processing in oocytes is slower than in cultured cells (23). This fact coupled with the much greater sensitivity of the capping method provides a plausible explanation of why we have been able to detect polyphosphate termini where Slack and Loening (14) could not. Partial sequencing of the capped 40S rRNA shows that the majority of molecules begin with the sequence 5′ AAG .... There is evidence for heterogeneity, however, at the location of the first RNase A cleavage. This heterogeneity could be due to hetrogeneity in the sequence of different copies of the gene. Alternatively, it is possible that the polymerase can start in more than one place. Sequencing of the various RNA oligonucleotides and of the DNA to which they hybridize will be required to clarify this question.

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