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Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III

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We have tested the hypothesis that the mammalian La protein, which appears to be required for accurate and efficient RNA polymerase III transcription, is a transcription termination factor. Our data suggest that 3′ foreshortened transcripts generated in La's absence are components of a novel transcription intermediate containing a paused polymerase. These transcripts are produced by fractionated transcription complexes, are synthesized with kinetics different from full-length transcripts, and are chasable to completion from the stalled transcription complexes. Together, these findings argue that termination by RNA polymerase III requires auxilliary factor(s) and implicate La as such a factor. Since La appears to facilitate transcript completion and release and also binds the resulting RNA product, it may be a regulator of RNA polymerase III transcription.
Key words: La protein/RNA polymerase III/transcription factor/transcription regulation/transcription termination

Introduction

RNA polymerase III is responsible for the synthesis of most small RNAs in eukaryotic cells [see Geiduschek and Tocchini-Valentini (1988) for a review]. Cellular species include 5S rRNA, all tRNAs, 7S-L, 4.5S and Alu transcripts; VAI, VAIII, EBER1 and EBER2 are specified by class III genes carried on viral genomes. Immediately following synthesis, all RNA polymerase III transcripts are packaged into RNA−protein complexes with the 50-kd La protein (Francoeur and Mathews, 1982; Rinke and Steitz, 1982; Steitz *et al.*, 1983).

Initiation of polymerase III transcription is directed by multipartite sequences within the coding region termed internal control regions (ICR) (Bogenhagen *et al.*, 1980; Sakonju *et al.*, 1980), while its efficiency can be influenced by flanking sequences (comprehensively reviewed in Geiduschek and Tocchini-Valentini, 1988; for apparent exceptions see Carbon *et al.*, 1987; Murphy *et al.*, 1987; Das *et al.*, 1988). Chromatographic fractionation, initially of HeLa and *Xenopus* extracts, revealed that RNA polymerase III alone is incapable of accurate initiation: a minimum of two additional factor fractions (TFIIIB and TFIIIC) are required for transcription of all class III genes (Segall *et al.*, 1980; Shastry *et al.*, 1982). These fractions may contain multiple general factors (Lassar *et al.*, 1983; Bieker *et al.*, 1985) as evidenced by the fact that TFIIIC was recently resolved into two complementing components (Yoshinaga *et al.*, 1987). An additional 5S-specific factor, TFIIIA (Engelke *et al.*, 1980; Honda and Roeder, 1980; Pelham and Brown, 1980) has been purified to homogeneity (Engelke *et al.*, 1980) and its gene cloned (Ginsberg *et al.*, 1984). Prior to initiation, TFIIIA binds to the 5S gene ICR; a TFIIIC component associates with the ICR of both tRNA and VA genes (Engelke *et al.*, 1980; Pelham and Brown, 1980; Sakonju *et al.*, 1981; Klemenz *et al.*, 1982; Lassar *et al.*, 1983; Fuhrman *et al.*, 1984; Stillman and Geiduschek, 1984; Stillman *et al.*, 1984; Camier *et al.*, 1985; Carey *et al.*, 1986; Yoshinaga *et al.*, 1987). Remaining general factors are believed to associate primarily through protein−protein interactions (Lassar *et al.*, 1983; Setzer and Brown, 1985; Carey *et al.*, 1986; Klekamp *et al.*, 1986; Wingender *et al.*, 1986). The resulting transcription complexes remain stable through multiple rounds of transcription (Bogenhagen *et al.*, 1982; Lassar *et al.*, 1983; Schaack *et al.*, 1983).

Considerably less is known about RNA polymerase III termination, the process including cessation of elongation, transcript release and polymerase dissociation. Four or more thymidine residues embedded in a G-C rich environment constitute a transcription termination signal (Bogenhagen and Brown, 1981). Purified *Xenopus* RNA polymerase III has been shown to be capable of recognizing this simple consensus sequence in the absence of auxiliary factors (Cozzarelli *et al.*, 1983) and similar properties have been ascribed to calf thymus polymerase III (Watson *et al.*, 1984). While auxiliary factors facilitating the last two aspects of the process have not yet been identified, several observations suggest that they may exist. First, the simple termination signal and/or its 3′ flanking sequences are protected by components present in transcription factor fractions (Klemenz *et al.*, 1982; Camier *et al.*, 1985; Van Dyke and Roeder, 1987) and some 3′ deletion mutants compete poorly for the transcription apparatus (Schaack *et al.*, 1983; Sharp *et al.*, 1983; Allison and Hall, 1985; Wilson *et al.*, 1985; Wu *et al.*, 1987). Second, seemingly equivalent consensus sequences behave differently in various sequence environments (DeFranco *et al.*, 1982; Koski *et al.*, 1982; Mazabraud *et al.*, 1987) and there are hints that terminators may exhibit tissue-specific expression (Mazabraud *et al.*, 1987).

In the accompanying paper (Gottlieb and Steitz, 1989), we suggested that the mammalian La protein may be an RNA polymerase III transcription termination factor. This 50-kd phosphoprotein (Francoeur and Mathews, 1982; Steitz *et al.*, 1983; Habets *et al.*, 1983; Pizer *et al.*, 1983; Francoeur *et al.*, 1984; Hoch and Billings, 1984) associates with all nascent polymerase III transcripts, selectively binding the 3′ uridylate stretch encoded by the termination signal (Stefano, 1984; Mathews and Francoeur, 1984). Its presence can be detected in active transcription complexes and in both crude and highly purified TFIIIC preparations (Gottlieb and

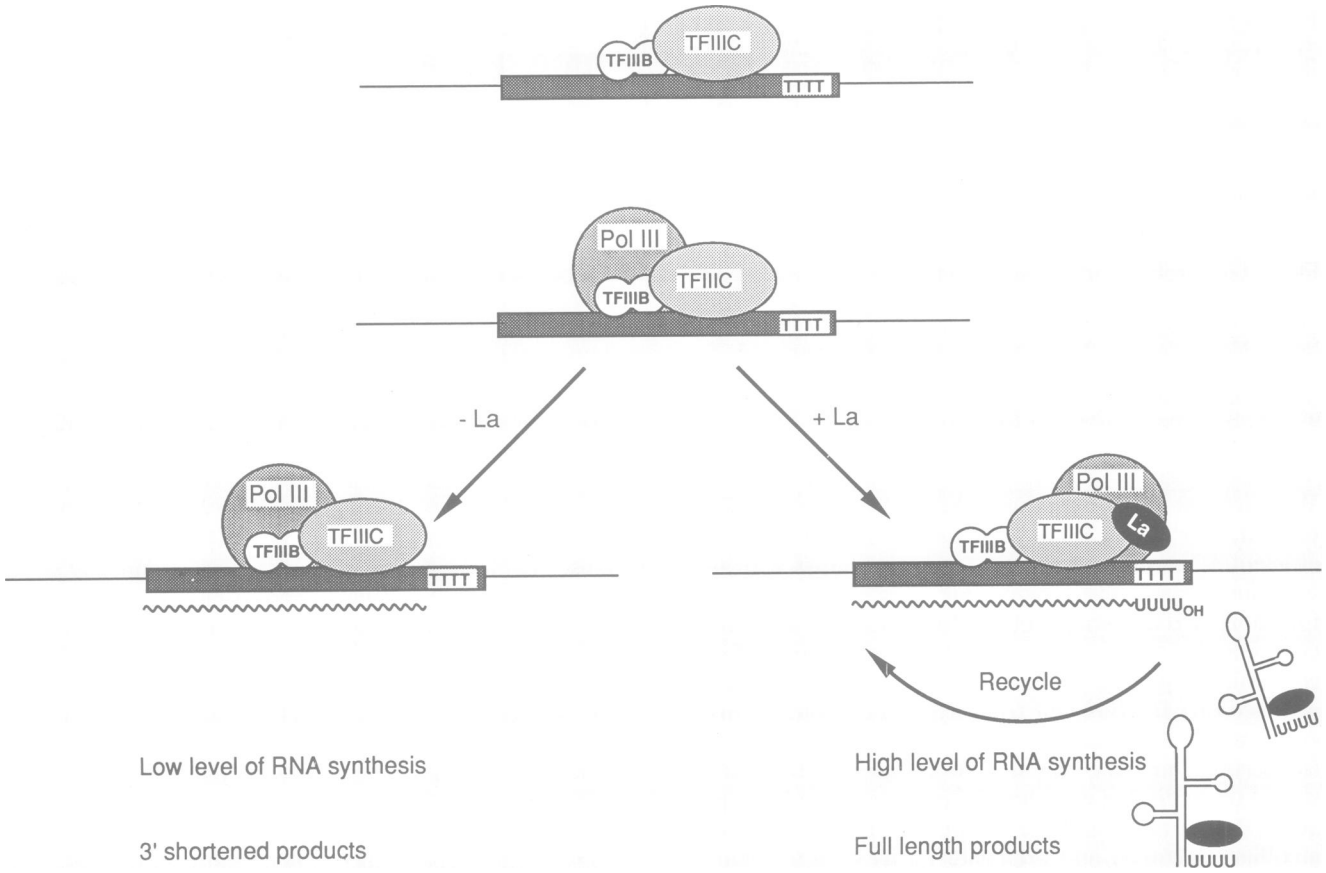

Fig. 1. A model for La action. Schematic representation of a stable transcription complex containing TFIIIB, TFIIIC and a class III gene (top), which can be recognized by RNA polymerase III (middle). (If this were a 5S gene, the additional gene-specific initiation factor, TFIIIA, would directly contact the internal control region.) Transcription in the absence of La yields a stalled transcription complex containing the 3′ foreshortened transcript (bottom left), while the presence of La produces an active transcription complex capable of multiple rounds of re-use (bottom right). Expected transcriptional properties of each of the two complexes are summarized below. Class III genes are depicted as stippled boxes with termination signals (TTTT); nascent transcripts are wavy lines; released transcripts are represented as hairpin structures. Other components include: TFIIIC, a large globular complex assigned various sizes ≥300 kd (Lassar *et al.*, 1983; Ruet *et al.*, 1984; Stillman *et al.*, 1985; Yoshinaga *et al.*, 1987), which footprints over the internal control region and may protect (or associate with something that protects) the terminator; TFIIIB, probably a single molecule of 60 kd or a dimer of two 60-kd subunits (Klekamp and Weil, 1986; P.Martin and R.Roeder, unpublished observations), which does not appear to produce a footprint but stabilizes the complex; RNA polymerase III, a multisubunit enzyme of ~700 kd (see Roeder, 1976); La, a 50-kd phosphoprotein, which can be found associated with TFIIIC, with active transcription complexes, and with the 3′-uridylate sequence of full-length transcripts. La might induce a conformational change in the polymerase (compare complexes on the bottom left and right), effecting transcript completion.

Steitz, 1987). La immunodepletion of HeLa-derived transcription extracts results in a dramatic diminution of transcription ability; and the few transcripts synthesized in the absence of La have fewer uridylate residues at their 3′ ends compared to those synthesized in the presence of La. These phenomena are reversed to different extents by the addition of biochemically purified La protein (Gottlieb and Steitz, 1989).

Here, we present a detailed model for La action at the termination signal and employ a variety of *in vitro* approaches to test this hypothesis. We conclude that auxiliary factors facilitate termination of polymerase III transcription and that the mammalian La protein is a good candidate for such a termination factor. In addition, we present evidence for a novel stalled transcription intermediate containing a paused polymerase molecule and a 3′ foreshortened unreleased transcript.

Results

Model

Figure 1 depicts a model for RNA polymerase III transcription. Initially TFIIIB and TFIIIC associate with a class III gene forming a stable transcription complex (top) (Bogenhagen *et al.*, 1982; Lassar *et al.*, 1983; Schaack *et al.*, 1983). RNA polymerase III recognizes this complex and initiates synthesis (middle). We propose that elongation of the initial RNA chain proceeds until the polymerase halts just prior to the termination signal. In the absence of La, a stalled transcription complex (bottom left) and a low level of 3′ truncated product result. In contrast, when La is present (the normal situation) the polymerase overcomes the pause and transcribes the 3′ uridylate stretch (bottom right). The resulting full-length transcript is released, freeing the complex for multiple rounds of re-use. This model

accommodates all known polymerase III transcription data and ascribes both of La's functional properties—its influence on the overall transcription level and the 3′ foreshortened transcript termini found in its absence (Gottlieb and Steitz, 1989)—to a single molecular event.

To test this model and to assess whether La plays a role in the termination process, we have performed a series of experiments monitoring the behavior of the EBER2 gene (Rosa *et al.*, 1981; Jat and Arrand, 1982). This prototypical class III gene contains an intragenic control region (Rosa *et al.*, 1981) and requires TFIIIB and TFIIIC in conjunction with RNA polymerase III for its transcription (P.Martin, unpublished observations). EBER2 is synthesized at high levels and is quantitatively precipitable by anti-La antibodies *in vivo* and *in vitro* (Rosa *et al.*, 1981; E.Gottlieb and J.A.Steitz, unpublished observations). Moreover, the low level of EBER2 3′ foreshortened product synthesized after La immunodepletion of a HeLa-derived S-100 extract is easily distinguished from full-length EBER2 (Gottlieb and Steitz, 1989). We infer that our conclusions with EBER2 can be extended to other class III genes which behave similarly in La-depleted extracts (Gottlieb and Steitz, 1989).

Transcription complexes differ in the presence and absence of La

Transcription complexes formed on class III genes *in vitro* (Bogenhagen *et al.*, 1982; Lassar *et al.*, 1983; Schaack *et al.*, 1983) are stable through multiple rounds of transcription. They can be isolated on glycerol gradients in a synthetically active form and contain $<1\%$ of the total extract protein (Wingender *et al.*, 1984; Jahn *et al.*, 1987), enabling us to analyze both their catalytic properties and molecular constituents. EBER2 complexes were assembled under transcription conditions in an untreated HeLa derived S-100 (Weil *et al.*, 1979) containing added nucleotides. After glycerol gradient fractionation, each fraction was supplemented with ribonucleotides containing [α-^{32}P]GTP and incubated for 90 min. Since additional DNA template was not added after ultracentrifugation, the resulting products reflect the sedimentation position of active transcription complexes formed prior to fractionation.

Two distinct populations of complexes can be visualized (Figure 2A). One population (denoted α) migrated at $\sim$60S and synthesized full-length EBER2. Surprisingly, a second slower sedimenting fraction (denoted β) produced 3′ foreshortened transcripts, characteristic of polymerase III transcription in the absence of La (Gottlieb and Steitz, 1989). The levels of RNA synthesized by these two populations are likely a reflection of the relative number of active complexes in each population: the ratio of labeled RNA transcripts synthesized by these two populations is constant upon refractionation of a given extract, but varies from extract to extract.

Only a fraction of the total transcription activity survives gradient fractionation (also previously observed by K.H. Seifart, unpublished observations), suggesting loss of one or more essential components. To determine whether the La protein is preferentially retained by complexes in the α region, La protein was located in the gradient by ELISA (Thoen *et al.*, 1980). Unfortunately, La antigenicity peaked at the top and smeared downwards with levels in the central portion of the gradient high enough that any repeaking of

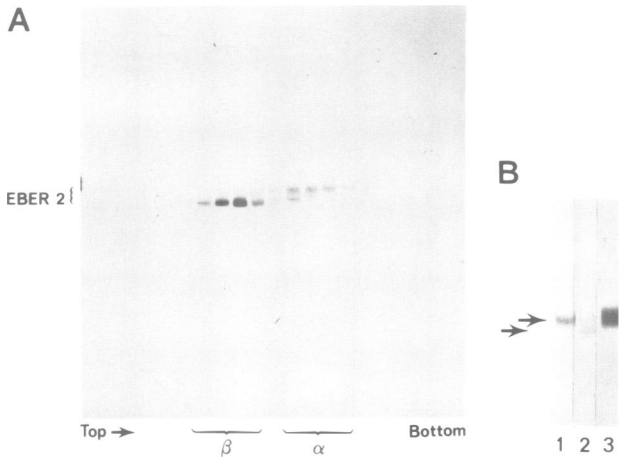

Fig. 2. Gradient fractionation of EBER2 transcription complexes. **Panel A**: EBER2 DNA was incubated in a HeLa-derived S-100 for 30 min, and the resulting transcription complexes were sedimented through a 20–45% glycerol gradient (17 fractions collected). Active transcription complexes were detected by their ability to support the synthesis of radiolabeled EBER2 following the addition of [α-^{32}P]GTP. Products are displayed on a 6% polyacrylamide gel. The direction of sedimentation is indicated, and the two complex populations are denoted α and β. **Panel B**: Transcription complexes were formed and gradient purified as in (A). Radiolabeled products synthesized by complexes in peak α (**lane 1**), peak β (**lane 2**), or by a mixture of the fractions used in lanes 1 and 2 (**lane 3**) after a 90-min incubation at 30°C are displayed on this 6% gel. Lanes were derived from a single exposure of a single gel.

La with either of the two complex populations could not be detected (data not shown). Recall that most of the La in cells exists in the form of RNPs (Stefano, 1984). In an alternative approach, we compared the sedimentation profile of complexes formed in anti-La versus non-immune depleted extracts. Following La immunodepletion, enhanced levels of the slower sedimenting complex population (β) were seen while levels of the more rapidly migrating population (α) were diminished (not shown). This suggests that at least one difference between these two transcription complex populations is a deficiency of La protein in β relative to α.

An alternative explanation for the synthesis of shortened products by the β fractions (Figure 2A) is that a nuclease sediments in this region of the gradient. To exclude this possibility, mixing experiments were performed (Figure 2B). Aliquots of gradient fractions from regions α (lane 1) and β (lane 2) were combined prior to the 90-min transcription reaction and the products analyzed as in Figure 2A. Figure 2B (lane 3) shows a typical result: about a 20-fold stimulation of transcription of full-length product. In other trials, the lengths of the products reflected the length distribution seen in the two component fractions. These results argue that the appearance of shorter products in region β is not due to a nuclease specifically present in these fractions and further suggest that La may recycle between rounds of transcription (see Discussion).

The absence of the La protein affects the kinetics of transcript accumulation

To determine whether transcription complexes containing or lacking La might also exhibit dissimilar transcription kinetics, we subjected a transcription extract (Weil *et al.*, 1979) to depletion with non-immune or anti-La antibodies followed by exposure to a protein A preparation (Gottlieb

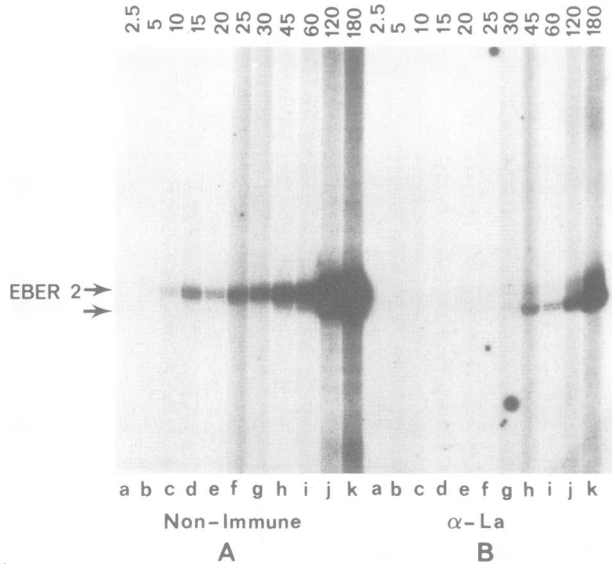

Fig. 3. Time course of EBER2 transcription in the presence and absence of La. Parallel reactions were initiated by adding non-immune (**panel A**) or anti-La (**panel B**) depleted extracts (see Materials and methods) to pre-mixed transcription reactions including the EBER2 template and [α-^{32}P]GTP (0 min). Reactions were incubated at 30°C, with aliquots removed at 11 time points and immediately treated with EDTA, SDS and proteinase K to halt synthesis. Following ethanol precipitation, the radiolabeled products were electrophoresed through a single 6% polyacrylamide gel. Arrows denote the full-length and 3′ foreshortened EBER2 molecules. A small amount of the sample in **lane e**, panel A, was lost upon gel loading.

and Steitz, 1989). To initiate synthesis, the resulting depleted supernatants were added to previously assembled reaction mixtures containing optimal salt concentrations, excess ribonucleotides, [α-^{32}P]GTP and saturating amounts of template. Radiolabeled transcripts appearing at various times were resolved on a denaturing polyacrylamide gel (Figure 3). We sampled several early points (<1 h) anticipating that these would be most likely to reveal differences between the reactions containing and lacking La. Because transcription complexes were not preformed prior to the addition of label, we could examine both the transcriptional lag and later reaction phases previously described for other class III genes, where similar kinetic analyses were performed (Birkenmeier *et al.*, 1978; Schaack *et al.*, 1983; Bieker *et al.*, 1985).

The rate of transcript appearance is strikingly different in the presence (Figure 3A) and absence (Figure 3B) of La protein. In the extract immunodepleted with non-immune serum (Figure 3A), newly synthesized products were detected after 5 min of incubation (lane b). Product accumulation lagged over the first 30 min but then increased steadily with time. All detectable transcripts, including those observed at the earliest time, were full-length. An extract that was not subjected to the immunodepletion protocol gave a similar transcription pattern except that the total incorporated counts were slightly greater (not shown). Therefore, the immunodepletion procedure itself does not alter the overall pattern of transcription. In contrast, in the absence of La (Figure 3B), the first observable transcripts appeared 25 min after initiation of the reaction (lane f) and all detectable EBER transcripts exhibited a rapid gel mobility.

Consistent with the proposed model (Figure 1) is the fact that even at the earliest time of transcript detection in La's absence only 3′ foreshortened molecules appear. Full-length products do not seem to be synthesized and subsequently trimmed, although an exceedingly rapid process cannot be excluded. Additionally, the low transcript levels observed in the absence of La cannot be wholly ascribed to transcript instability; there is no initial burst followed by subsequent decay. Instead transcripts build up late in the reaction (cf. lanes f and k, Figure 3B; see Discussion).

Visualization of a stalled transcription intermediate

The above experiments indicate that transcription complexes with different physical and catalytic properties are formed in the presence or absence of the La protein. Our model (Figure 1) makes the further specific prediction that the 3′ foreshortened transcripts synthesized in La-depleted extracts are a reflection of stalled transcription complexes in which the nascent transcript remains attached to the DNA template blocking further rounds of synthesis. To look for such a transcription intermediate, S-100 extracts were subjected to immunodepletion with non-immune or anti-La serum as above and then supplemented with labeled and unlabeled nucleotides and the EBER2 gene. After a 45-min incubation, reaction mixtures were fractionated on glycerol gradients (Wingender *et al.*, 1984; see Figure 2) and the radiolabeled transcripts in each fraction analyzed on denaturing polyacrylamide gels (Figure 4). This experiment directly monitors the sedimentation behavior of transcripts synthesized before fractionation, whereas in Figure 2 the behavior of preformed transcription complexes was examined. Here, if transcripts migrate into the gradient, they are likely to be associated with large complexes since EBER2 RNPs, consisting of only the 172 $\pm$ 1 nucleotide RNA and the 50-kd La protein, have a sedimentation value of $\sim$8S and would remain at the top of the gradient.

Figure 4, panel B, shows the gradient containing products synthesized in the absence of La. All transcripts are short and their level is low, as expected. While most transcripts remained at the top of the gradient (lanes 1–3), some migrated into the region where competent transcription complexes sediment (lanes 6–14; compare with Figure 2A). Agarose gel analysis of aliquots of each fraction confirmed the presence of the DNA template in this region (data not shown). In contrast, in the gradient containing the labeled products synthesized in the presence of La (panel A), all of the EBER2 transcripts are full-length and appear at the top of the gradient (lanes 1–4). These are presumably La-containing ribonucleoprotein particles since EBERs synthesized in a La-containing extract are quantitatively La precipitable. Despite the fact that more incorporated counts were loaded onto gradient A than gradient B, no full-length EBER2 transcripts are detected cosedimenting with the transcription apparatus (panel A, lanes 6–14). Light exposures of the third fraction from these gradients (panel C, lanes 1, 2) show that the predominant transcription products were full-length EBER2 and 3′ foreshortened EBER2 in the presence and absence of La, respectively. This length difference was confirmed by analyzing small samples of the total transcription reactions fractionated in A and B (data not shown). The second most abundant product (denoted by *) has an EBER2 fingerprint; it could represent a multimer formed during the gradient procedure, since it is absent when these reactions are directly gel fractionated.

It appears that La-immunodepletion has enabled us to

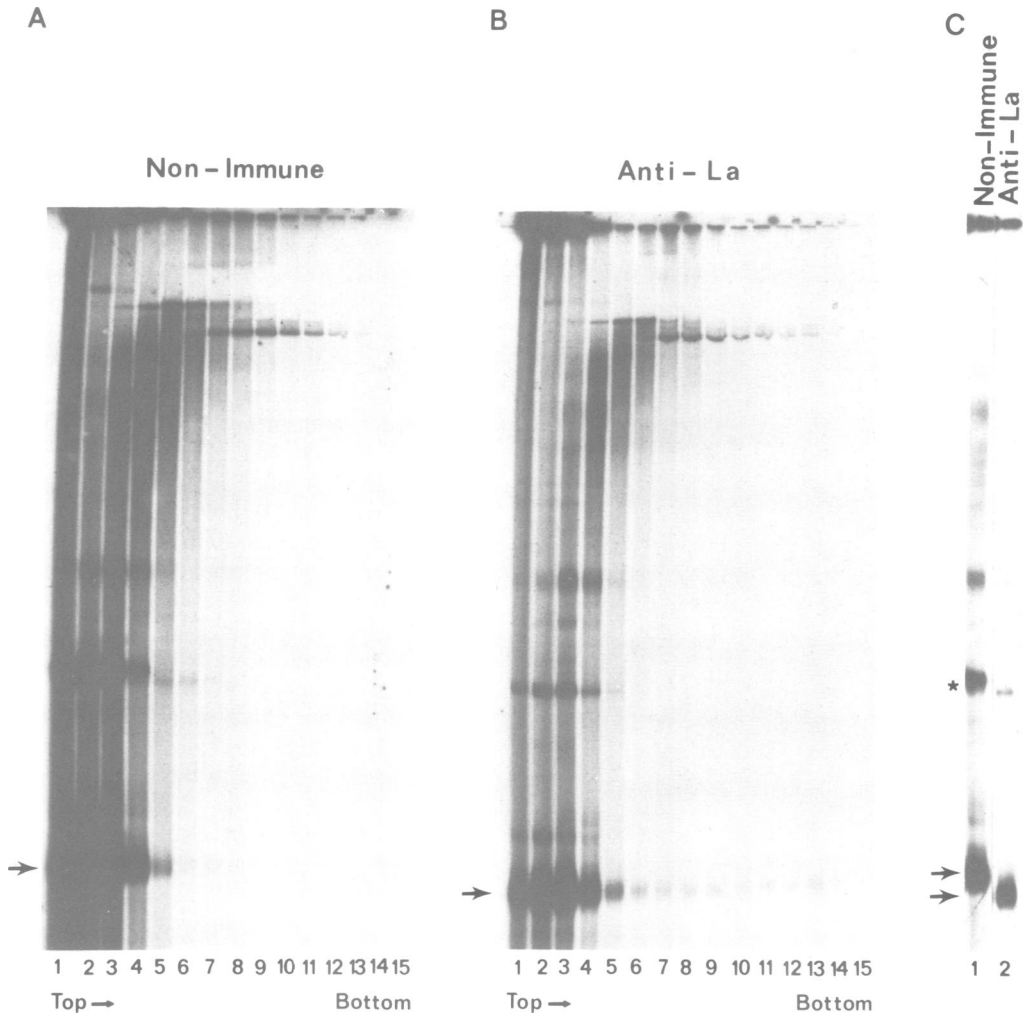

Fig. 4. Visualization of a stalled transcription complex. Immunodepleted extracts (see Materials and methods) were supplemented with [α-^{32}P]GTP and [α-^{32}P]UTP, unlabeled ribonucleotides, salts and the EBER2 template, incubated at 30°C for 45 min, and fractionated on parallel 20–45% glycerol gradients. Each resulting fraction was proteinase K treated, ethanol precipitated and displayed on a 6% polyacrylamide gel. **Panels A** and **B**: Migration behavior of radiolabeled transcription products from non-immune and anti-La depleted extracts respectively. Numbers correspond to gradient fractions and arrows denote the EBER2 transcripts. The direction of sedimentation is indicated (top → bottom). **Panel C, lanes 1** and **2**: Lighter exposures of lanes A3 and B3. The major transcription products include full-length and 3′ foreshortened EBERs respectively. Products denoted by * have EBER2 fingerprints, are not observed when aliquots of each reaction are gel fractionated without prior gradient separation, and are likely to be EBER2 multimers formed during gradient fractionation. Most of the additional radioactivity in the top fractions of panels A and B represents overexposure of minor products. An additional radiolabeled RNA with very low gel mobility appears in the middle of both gradients, but we could never retrieve enough of this material to analyze it.

visualize unreleased polymerase III transcription products associated with stalled transcription complexes. The 3′ truncated RNAs sedimenting at the top of the −La gradient (Figure 4B) could have dissociated from transcription complexes because of stress during gradient fractionation and/or slow but spontaneous release. The latter possibility would also explain the delayed transcript build-up seen at late reaction times in a La-depleted extract (Figure 3B). In the presence of La, transcript release appears to be a rapid, active process coupled to completion; thus, we (Figure 4A) and others (Ackerman *et al.*, 1983; Wingender *et al.*, 1984; Jahn *et al.*, 1987) have been unable to detect this intermediate in complete transcription reactions.

The stalled transcription complex can be rescued

The stalled transcription complexes visualized in Figure 4B could represent either non-productive side products of the synthetic process or *bone fide* transcription intermediates. If the latter were the case, the presynthesized short RNAs might be susceptible to completion (Maderious and Chen-Kiang, 1984; Grayhack *et al.*, 1985). Ideally, we would have liked to add La protein to gradient fractions containing these nascent RNAs and analyzed their lengths. However, the low recovery of transcripts retained in such complexes after fractionation demanded that we use unfractionated transcription extracts for this experiment instead.

Figure 5A indicates that the 3′ foreshortened transcripts generated in a La-immunodepleted extract can be completed by subsequent addition of a source of La protein. Extracts depleted with non-immune (lanes 1,2) or anti-La (lanes 3,4) serum were supplemented with nucleotides, the EBER2 template, and [α-^{32}P]GTP, and incubated for 30 min, long enough for labeled 3′ foreshortened transcripts to appear (see Figure 3B). By selecting this short incubation period we hoped to maximize the percentage of transcripts remaining associated with the stalled transcription complexes (Figure

Fig. 5. Stalled transcription complexes can be chased to completion. Radiolabeled EBER2 transcripts were synthesized for 30 min at 30°C in reactions containing extracts depleted with non-immune (**lanes A 1–2, B 1–2**) or anti-La (**Lanes A 3–4, B 3–5**) antibodies. Samples then received prewarmed transcription buffer (**lanes A1** and **3, B1** and **3**) or prewarmed S-100 dialyzed into transcription buffer (**lanes A2** and **4**; **B2, 4** and **5**) containing either a 200-fold excess of unlabeled nucleotides (**A**) or 300 μg/ml α-amanitin (**B**). Incubation was for 5 min in all cases except **lane B5**, where the chase was extended to a total of 15 min. Resulting radiolabeled products were proteinase K treated and separated on denaturing 6% polyacrylamide gels.

4B). Then, following the simultaneous addition of a 200-fold excess of unlabeled ribonucleotides (to dilute the labeled precursors) and either transcription buffer (lanes 1 and 3) or a La protein dialyzed into transcription buffer (lanes 2 and 4), incubation continued for ≥5 additional minutes. The shift of the presynthesized 3′ foreshortened species (lane 3) to full-length (lane 4) indicates that at least some of the RNA components of stalled complexes can be chased to completion. It is highly unlikely that the full-length molecules detected after the 5-min chase of the stalled transcription complexes result from *de novo* synthesis since this time period is too short for substantial amounts of new molecules to appear (Figure 3A, lane 2) and the few made would have a specific activity 200-fold less than the pre-existing transcripts. [Note that these complexes do not sequester appreciable amounts of nucleotides (Wingender *et al.*, 1984).] Transcript completion exhibits the same α-amanitin sensitivity as synthesis by RNA polymerase III: inclusion of 300 μg/ml α-amanitin (Figure 5B) during the 5-min chase period abolishes the shift in transcript mobility (lane 3 versus 4). Similar results are seen following chase periods of 15 (lane 5) and 60 min (data not shown).

Thus, our results indicate that the discrete 3′ foreshortened RNA synthesized in La depleted extracts is a *bone fide* transcription intermediate, probably the product of a paused polymerase. Transcript completion appears to require active RNA polymerase III in conjunction with the La protein preparation. Although other data (Gottlieb and Steitz, 1987; this manuscript) indicate that La is normally associated with the transcription complex, the chase data suggest that La can functionally interact even after initiation of synthesis. This conclusion does not contradict the results in Figure 2 since the concentration of La in the gradient-fractionated transcription complexes was calculated to be about four orders of magnitude less than in this experiment.

Chelating La with RNA also yields stalled transcription complexes

To ask further whether La itself is a termination factor that facilitates transcript completion and release, we exploited the protein's documented ability to bind the U-rich 3′ end of polymerase III transcripts. A transcription reaction was pre-incubated with excess unlabeled EBER2 (Figure 6D, lane labeled EBER) in the hope that incorporation into ribonucleoprotein particles would render the La protein unavailable to the transcription apparatus. La is the only protein known to bind the EBERs (J.Stefano, unpublished observations; Glickman *et al.*, 1988). Thus, this depletion strategy is more selective than use of anti-La antibodies since La immunodepletion under conditions that preserve transcription activity also removes several less abundant proteins by virtue of their association with La and/or La-bound RNAs. As a control, an equivalent reaction was pre-incubated with an equimolar amount of unlabeled EBER2 from which the U-rich La binding site had been specifically removed. These 3′ΔEBERs, generated by oligonucleotide-directed RNase H cleavage of EBER2 are shown in Figure 6D. After pre-incubation, the reactions were supplemented with the EBER2 gene and both labeled and unlabeled nucleotides, incubated for 45 min and fractionated in parallel on glycerol gradients as in Figure 4.

Figure 6B reveals that removal of La by RNA chelation again results in visualization of stalled transcription intermediates: a fraction of the radiolabeled transcripts synthesized in the EBER2-treated extract migrates into the central region of the glycerol gradient (lanes 4–11). The mobility of full-length EBER2 transcripts generated in a parallel untreated reaction and loaded onto the far right lane of the gel demonstrates that these rapidly sedimenting RNAs are short, as expected for paused transcripts. This effect is

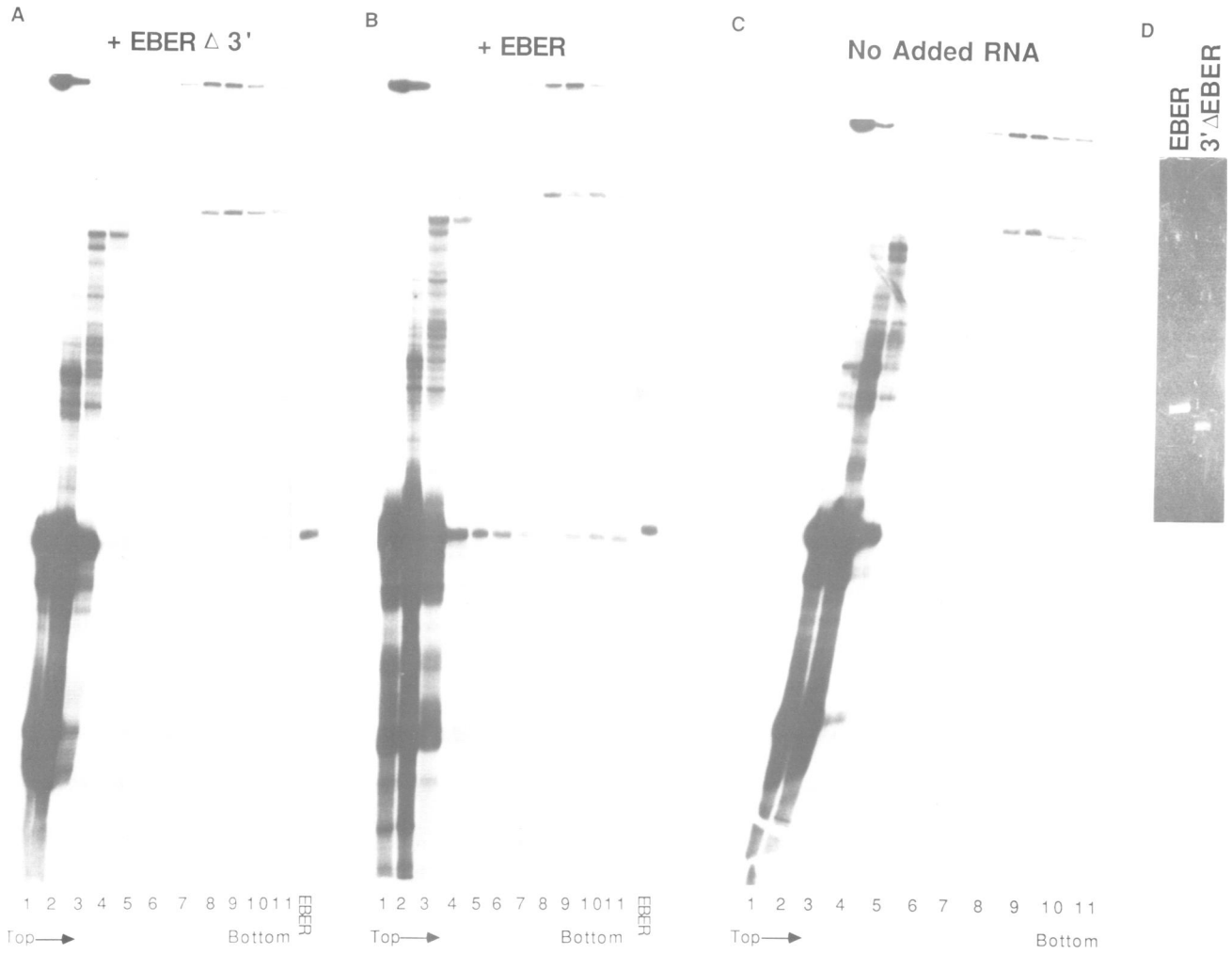

Fig. 6. A stalled transcription complex can be generated by chelation of the La protein with RNA. **Panels A–C**: Gradient profiles showing the migration behavior of radiolabeled EBER2 synthesized in extracts preincubated with excess unlabeled EBER2 from which the La binding site had been removed (3′ΔEBER, **A**), with full-length EBER2 (**B**), or no added RNA (**C**). Equivalent c.p.m. were loaded onto each gradient. The direction of sedimentation in the glycerol gradient (left to right) is indicated (top→bottom); the far right-hand lane of **panels A** and **B** contain full-length EBER2 transcript as a marker. In **panel B**, the EBER2 transcript running into the gradient exhibits the shorter length characteristic of transcription in the absence of La (see Gottlieb and Steitz, 1989), compared to the full-length transcript in **panels A** and **C**. **Panel D**: An ethidium bromide stained gel profile of the unlabeled full-length EBER2 and of EBER2 RNA that has had its La-binding site removed by oligonucleotide-directed RNase H mediated cleavage (3′ΔEBER).

produced by adding EBER2 at an RNA:La protein ratio estimated to be one-tenth that found in the cell and without appreciable diminution of transcription. (In experiments not shown, depression of transcript levels was observed at higher EBER2 concentrations.) In contrast, labeled EBER2 made in the extract pre-incubated with 3′ΔEBERs (Figure 6A) or in an untreated extract (Figure 6C) are full length and do not significantly migrate into the gradient. Simply being incomplete does not cause a transcription product to move into the gradient: while RNAs of other lengths are present in the profiles, only those lacking just their 3′ terminal Us cosediment with transcription complexes (Figure 6B). Moreover, control experiments in which full-length or 3′ foreshortened EBER2 transcripts were mixed with unlabeled transcription reactions prior to fractionation revealed that neither detectably sediments into the gradient (data not shown). Hence, a 3′ foreshortened RNA must have been synthesized in the reaction if it is to migrate with the transcription complex upon fractionation. The simplest interpretation of these results is that the factor whose activity we have been monitoring is the La protein itself.

Discussion

Existence of a transcription termination factor for RNA polymerase III

Two important conclusions emerge from our results: first, termination by RNA polymerase III seems to require auxiliary factor(s) and second, the 50-kd mammalian La protein appears to be such a transcription termination factor. Ultimate confirmation of the latter conclusion will require the addition of biologically active La protein synthesized from a cDNA clone to a transcription system reconstituted from more highly purified components than are now available. As discussed in Gottlieb and Steitz (1989), current protocols for reconstitution of transcription utilize fractions still containing La. Yet, the fact that stalled complexes can be generated by two independent methods

of La depletion (Figures 4 and 6) implicates La itself as the termination factor required for their resolution. If another molecule were responsible, it would have to meet three criteria: (i) very tight association with La, allowing complete immunodepletion by anti-La antibodies (Figure 4); (ii) specific recognition of the U-rich 3′ ends of polymerase III transcripts (Figure 6); and (iii) copurification with La through six steps in which La first fractionates as an RNP and later as a protein (Gottlieb and Steitz, 1989).

Several predictions of our model (Figure 1) are supported by the analyses presented here. Distinct transcription complexes appear to be assembled in the presence and absence of La (Figure 2). A fraction of the 3′ foreshortened transcripts generated in La's absence appear to be retained in stalled transcription complexes (Figure 4–6), providing the best indication that the 3′ foreshortened RNA arises via a transcription event. We infer that this novel transcription intermediate minimally contains TFIIIB, TFIIIC (since both are non-cycling factors required for initiation), RNA polymerase III, the DNA template and the nascent RNA transcript. It would therefore be distinct from previously identified metastable, stable and pre-initiation transcription complexes [see Bieker *et al.* (1985) for distinctions] in at least two regards: it would represent a step immediately preceding termination and it would contain the incomplete transcript. La appears to be an integral component of the normal transcription complex not only because its presence in gradient purified complexes can be inferred (Figure 2) but also because DNA carrying class III genes can be selectively immunoprecipitated from synthetically active extracts with anti-La antibodies (Gottlieb and Steitz, 1987). Figure 5 suggests that La may also be able to interact functionally with the transcription complex even after initiation of synthesis.

The aspect of the simplest form of the model (as presented in Figure 1) that may require revision is the prediction that only one round of synthesis should be detected when transcript completion and release are not La facilitated. In other words, we observe that transcript level and length are not obligatorily coupled: while we do see a low level of transcript in the absence of La, this level does not remain constant over time in Figure 3. This unexpected observation could be explained in at least two ways. Conceivably, transcription complex assembly could be altered by removal of La-associated transcription initiation factors (e.g. TFIIIC) upon La immunodepletion, effecting a decrease in transcript level not directly related to La. This explanation could be viewed as consistent with our reconstitution data (Gottlieb and Steitz, 1989) and with the absence of significantly depressed transcription levels when La is removed by RNA chelation (Figure 6). However, slow transcription complex assembly is not entirely responsible since transcript accumulation in the presence and absence of La (Figure 3) differ in the rate of product accumulation as well as in the duration of the transcriptional lag. Alternatively, in a scenario compatible with the data in Figures 4 and 6, a single round of synthesis may simply not be detectable; later transcript build up in the absence of La may result from slow, spontaneous release of truncated products.

La and transcription termination

Assuming that eukaryotic transcription termination (like initiation) bears some similarity to the comparable prokaryotic process, it should involve at least three distinct events: polymerase pausing, transcript release and polymerase dissociation (Platt and Bear, 1983). With *Escherichia coli* RNA polymerase, pausing is the first step in the termination process; while all pauses do not result in termination, pausing is an obligate prerequisite for termination (von Hippel *et al.*, 1984; Grayhack *et al.*, 1985; Platt, 1986). The order of the remaining two processes is not known.

Detection of stalled transcription complexes in the absence of La argues that La is not required for polymerase pausing. Rather, it seems reasonable that RNA polymerase III, like *E.coli* RNA polymerase (Grayhack *et al.*, 1985; J.Roberts, personal communication), possesses the inherent capacity to pause. Parameters inducing pausing by RNA polymerases are unknown, even for the *E.coli* enzyme. Pausing could result from the formation of a helical stem between complementary sequences present near the 5′ and 3′ ends of most nascent RNA polymerase III transcripts. Alternatively, it may be induced by the structure of the DNA template. It is intriguing that polymerase III termination signals (which include ≥ 4 adenine residues in the coding strand) coincide with DNA sequences that are believed to induce bending (Koo *et al.*, 1986).

Several mechanisms for La protein action after polymerase pausing are compatible with our data. Since La does not appear to bind DNA strongly but is present in RNA polymerase III transcription complexes (Figure 2; Gottlieb and Steitz, 1987) and in TFIIIC preparations (Gottlieb and Steitz, 1987), it most likely interacts with other protein components and could thereby induce conformational changes in the transcription complex causing polymerase to effect transcript completion. This may be reflected in the substantial S-value difference between transcription complexes that synthesize short versus full-length transcripts (Figure 2A), which cannot be accounted for solely by the absence of a 50-kd protein. [Note that the mere presence of nucleotides during complex formation in a HeLa extract significantly increases the mean sedimentation rate (Wingender *et al.*, 1984) and that *E.coli* transcription complexes containing nascent RNAs can exist in alternate conformations (Straney and Crothers, 1985).] Once the transcript's U-rich 3′ terminus has been synthesized, the transcription complex configuration may be incompatible with transcript retention. Alternatively, La may be correctly positioned in the transcription complex to bind the newly synthesized RNA 3′ terminus when it becomes exposed because of dA–rU heteroduplex instability (Martin and Tinoco, 1980). Similar to the *E.coli* termination factor rho, upon binding the nascent transcript (Chen *et al.*, 1986; Brennan *et al.*, 1987), La might melt the remaining DNA–RNA duplex using an ATP-dependent helicase activity (Brennan *et al.*, 1987). This scenario is supported by recent sequencing of a *Xenopus* La cDNA clone, which has revealed a potential NTP binding site (D.Scherly and S.Clarkson, personal communication). Finally, a more indirect role for La in transcript completion and release can also be envisioned. Here, polymerase pausing at the U tract may result from an increased rate of depolymerization versus polymerization by the enzyme (Kassavetis *et al.*, 1986). La could shift the equilibrium by sequestering the longer transcripts, driving the reaction towards polymerization.

Clearly, additional work is required to determine the

details of La's contribution to the termination of RNA synthesis by RNA polymerase III. It should be realized that the possible mechanisms for La action outlined above are not necessarily mutually exclusive. Further, La's participation in the termination process does not eliminate a subsequent role of the protein in 3′ end protection of resulting transcripts; La's unique RNA binding ability may couple RNA polymerase III transcription termination to RNA packaging. Finally, future efforts should also resolve the issue of whether (Bieker *et al.*, 1985; Setzer and Brown, 1985; Carey *et al.*, 1986) or not (Jahn *et al.*, 1987) RNA polymerase III dissociates after each round of synthesis and (if so) whether dissociation occurs concomitant with or following transcript release.

In a detailed study, Cozzarelli *et al.* (1983) previously concluded that *Xenopus* RNA polymerase III alone is capable of transcription termination. These authors analyzed their 5S transcription products by T_1 nuclease mapping, which probably was not sensitive enough to discriminate the 1−2 uridylate residue difference between 5S transcripts produced in the presence and absence of La (Gottlieb and Steitz, 1989). We suggest that the RNAs Cozzarelli *et al.* analyzed may have been components of stalled transcription complexes. Their data would then strongly argue that RNA polymerase III alone is capable of the first step in termination—recognition of and pausing at the termination signal. Other ways of resolving the apparent contradictions between their and our conclusions are: (i) there might exist species-specific differences between *Xenopus* and HeLa RNA polymerase III, although the observations of Watson *et al.* (1984) on calf thymus RNA polymerase III can likewise be explained by pausing at the termination signal; (ii) polymerase III alone might be able to recognize the termination signal and complete transcription on a naked DNA template (their experiments) but require additional factors when confronted with a protein-covered transcription complex (our experiments); or (iii) *Xenopus* RNA polymerase III purified to 90% homogeneity might retain some La protein, even though La is not detectable in purified HeLa RNA polymerase III preparations either by immunochemical criteria (Gottlieb and Steitz, 1987) or by activity (W.K.Hoeffler and E.Gottlieb, unpublished observations).

A regulatory role for La?

Figure 1 suggests that La's role in termination may have a direct affect on initiation by resetting the transcription complex for its documented multiple rounds of re-use. La's presence might therefore amplify the transcriptional output. Interaction between transcription termination and initiation would not be unique to the RNA polymerase III system. RNA polymerase I termination affects initiation at the adjacent rRNA transcription unit (Grummt *et al.*, 1986; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986) and the 3′ end formation signals of U-RNAs (synthesized by RNA polymerase II) overlap the promoter (Hernandez and Weiner, 1986; Neuman de Vegvar *et al.*, 1986; Ciliberto *et al.*, 1986).

Possible feedback regulation of transcription is suggested by the fact that La is both a factor required for transcript completion and binds the resulting products: La could therefore modulate the synthesis of all RNA polymerase III transcripts in the mammalian cell. If a large number of substrate RNAs were present, La would be sequestered in RNPs, unavailable to catalyze further rounds of transcription. Such a scenario is reminiscent of the 5S-specific regulatory loop proposed for TFIIIA in the amphibian oocyte (Pelham and Brown, 1980). La's relative affinity for various transcripts and transcription complexes formed on different class III genes coupled to its phosphorylation state could then be used to fine-tune this regulation.

Materials and methods

Cells, antibodies and enzymes

HeLa and Raji cells were maintained at 37°C in a 5% Co_2 environment at $\sim 7 \times 10^5$ or 5×10^5 cells/ml respectively. Cells were cultured in spinners (HeLa) or in suspension (Raji) in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 60 μg/ml penicillin, 100 μg/ml streptomycin and 300 μg/ml glutamine.

Autoimmune patient sera (kindly provided by J.Hardin and J.Craft, Yale University) were selected for their high titer and relative monospecificity as determined by three independent assays: immunofluorescence, immunoprecipitation of *in vivo* [^{32}P]orthophosphate-labeled RNAs from a HeLa whole cell extract (Hendrick *et al.*, 1981; Matter *et al.*, 1982) and Western immunoblots (Towbin *et al.*, 1979; Yen and Webster, 1981; Mimori *et al.*, 1984). Non-immune sera were donated by healthy laboratory personnel. IgG preparations were obtained by ammonium sulfate precipitation and contained ~17 mg/ml protein. Anti-La monoclonal IgGs and mouse control IgG (the kind gift of D.Williams and P.Venables; Smith *et al.*, 1985) were grown in the ascites of BALB/c mice, protein A−Sepharose selected and were ~0.5 mg/ml protein.

Lyophilized protein A-bearing *Staphylococcus aureus* cells, α-amanitin and RNA markers (MS2, 16S and 23S RNAs) were purchased from Boehringer and Mannheim and proteinase K was obtained from Beckman. RNase H was purchased from Pharmacia.

Extracts, antibody depletions and transcription reactions

Soluble transcription (S-100) extracts were prepared from log phase HeLa cells (Weil *et al.*, 1979), aliquoted, frozen in liquid nitrogen and stored at −70°C. Antibody depletions were performed at 0°C in transcription buffer (70 mM KCl, 5 mM $MgCl_2$, 15 mM Hepes pH 7.9, 0.5 mM DTT) as described (Gottlieb and Steitz, 1989). Prior to transcription, extracts were supplemented with 0.5 mM each ATP, CTP and UTP, 0.025 mM GTP, [α-^{32}P]GTP and template, unless otherwise indicated. Each was optimized for DNA and $MgCl_2$ concentrations. EBER2 gene containing plasmids, pJJJ2 (Jat and Arrand, 1982) or pEBV R1J (Rosa *et al.*, 1981), were supplied as supercoiled templates at 10−40 μg/ml. Transcription proceeded at 30°C for the times indicated. Reactions (typically 25−50 μl) were then supplemented with 20 μg yeast carrier RNA, 0.4% SDS and 20 μg proteinase K, heated at 65°C for 20−25 min, made 0.25 M in ammonium acetate and ethanol precipitated. Radiolabeled RNA products were fractionated on 5%, 6% or 10% polyacrylamide/7 M urea/0.5 × TBE (0.5 M Tris, 0.5 M sodium borate, 2 mM EDTA pH 8.3) gels, which were dried and autoradiographed at −70°C.

Glycerol gradient analyses

Gradient fractionation was performed by a modification of the procedure of Wingender *et al.* (1984). Stable transcription complexes were formed in 150-μl reactions containing 60 μl S-100, 10 μg/ml of EBER2 template, 0.5 mM each ATP, CTP, GTP and UTP, 1.75 mM sodium phosphate (pH 6.3), 15 mM NaCl and the other components of the transcription buffer listed above. After incubation at 30°C for 30 min, reactions were chilled and layered on 4.8-ml 20−45% glycerol gradients (v/v) containing 70 mM KCl, 5 mM $MgCl_2$, 15 mM Hepes pH 7.9, 0.5 mM DTT, 1.5 mM EDTA. Gradients were centrifuged in an SW50.1 rotor at 50K r.p.m. for 2.2 h at 0°C. To assay the transcription ability of each resulting fraction, 200-μl reactions were assembled containing 100 μl of the fraction, 0.5 mM each ATP, CTP and UTP, 0.025 mM GTP, 20 μCi [α-^{32}P]GTP (410 Ci/mmol), 70 mM KCl, 15 mM Hepes pH 7.9, 5 mM $MgCl_2$ and 0.5 mM DTT. Following a 90-min incubation at 30°C and proteinase K treatment, transcripts were ethanol precipitated and resolved on polyacrylamide gels. Mol. wt standards fractionated on a parallel gradient included *E.coli* ribosomal RNA (16S, 23S), MS2 RNA (28S), HeLa ribosomal subunits (40S, 60S) and R17 phage (80S).

Mixing experiments were performed immediately after the collection of

gradient fractions. A volume of 25 μl from a fraction judged to synthesize full-length RNA and from one judged to synthesize 3′ foreshortened RNA were mixed with an equal volume (50 μl) of reaction mixture (1 mM ATP, CTP and UTP, 0.05 mM GTP, 10 μCi [α-^{32}P]GTP, 70 mM KCl, 15 mM Hepes pH 7.9, 5 mM $MgCl_2$, 0.5 mM DTT), incubated at 30°C for 2 h and processed as above. To control for dilution effects, 25 μl of each fraction was also mixed with 25 μl of transcription buffer and the reaction treated as above; no alterations due to dilution were detected. La protein concentration in each fraction was determined by ELISA (Thoen *et al.*, 1980) on 100 μl of each gradient fraction.

In vitro kinetics of transcript accumulation

HeLa S-100 extracts were immunodepleted with non-immune or anti-La antibodies as described (Gottlieb and Steitz, 1989). Parallel transcription reactions (1 ml each) were assembled by adding the depleted supernatant to a pre-compiled mixture of salts, unlabeled ribonucleotides, [α-^{32}P]GTP and the EBER2 template. Incubation proceeded at 30°C over a 3 h period. Aliquots of 50 μl were removed from each reaction at the times indicated, stopped by EDTA and SDS addition, and proteinase K treated. The ethanol precipitated radiolabeled products were gel fractionated.

Visualization of stalled transcription complexes

Transcription reactions of 100 μl containing extract (immunodepleted with non-immune or anti-La serum), salts, ribonucleotides, EBER2 template and 60 μCi each of [α-^{32}P]GTP and [α-^{32}P]UTP were incubated for 45 min at 30°C and diluted 2-fold with transcription buffer. A volume of 150 μl from each reaction was layered on a 4.8-ml, 20–45% glycerol gradient and centrifuged in an SW50.1 rotor at 50K r.p.m. for 2–2.2 h. Gradients were fractionated, diluted 2-fold with transcription buffer and proteinase K treated. Radiolabeled products were ethanol precipitated and displayed on denaturing 6% polyacrylamide gels. To assess the effectiveness of La depletion, the remaining 50 μl of each transcription reaction, which was not gradient fractionated, was proteinase K treated and gel fractionated.

Alternatively, extracts were preincubated on ice for 20 min with 12 μg/ml EBER2 or EBER2 devoid of its La protein binding site (3′ΔEBER). Extracts were then supplemented for transcription and processed as above. EBER2 for this experiment was prepared by anti-La immunoprecipitation from Raji cells (Matter *et al.*, 1982) followed by purification on a 5% polyacrylamide/7 M urea/1 × TBE gel, visualized with ethidium bromide, excised and eluted in 0.5 M sodium acetate, 10 mM EDTA, 10 mM Tris–HCl pH 7.5, 0.1% SDS, PCA extracted, ether washed and ethanol precipitated. Although the vast majority of EBER2 in the cell is complexed with the La protein, the use of anti-La selected RNA ensured that the isolated RNA was intact and capable of binding La; non-immunoselected RNA was, however, successfully employed as well. To generate EBER2 lacking the La binding site, gel-purified EBER2 was mixed in water with a 220-fold molar excess of a 20 mer oligonucleotide (5′-AAAAATAGCGGACAAGC-CGA-3′) complementary to its 3′ end, heated to 80°C for 5 min to facilitate hybridization, equilibrated to 30°C for 15 min, made 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, and incubated with RNase H (20 U/μg RNA) at 30°C. After 60 min, additional RNase H (10 U/μg RNA) was added and incubation continued for 30 min to facilitate quantitative cleavage. The resulting 3′-deleted EBER2 was gel purified, eluted, PCA extracted, ether washed and ethanol precipitated. A shorter oligonucleotide (5′-AAAAATAGC-3′), complementary to the terminal 9 residues of EBER2, was also tried, but only 20% cleavage was obtained at a 500-fold molar excess; presumably the high proportion of A-U base pairs resulted in instability of the hybrid. The concentrations of all RNAs were determined by both UV absorption and intensity of ethidium bromide staining compared to RNA standards. The addition of synthetic polymers (U_5 and A_5) had no effect (either transcriptionally or post-transcriptionally) in our system.

Chase

Non-immune and anti-La immunodepleted extracts were supplemented with the EBER2 template, salts, nucleotides (600 μM each ATP, CTP, UTP, 25 μM GTP) and 400 μCi/ml [α-^{32}P]GTP. Transcription proceeded at 30°C for 30 min in 25 μl reactions, which were then supplemented with either 12.5 μl transcription buffer (70 mM KCl, 5 mM $MgCl_2$, 15 mM Hepes pH 7.9, 0.5 mM DTT) or S-100 dialyzed into transcription buffer. Each mixture (prewarmed to 30°C) contained unlabeled ribonucleotides such that the final 37.5 μl reaction was 600 μM each in ATP, CTP, and UTP and 5 mM in GTP. Some reactions were supplemented with 300 μg/ml α-amanitin instead of excess unlabeled GTP. Incubation proceeded at 30°C for an additional chase period and was then halted by the addition of SDS, EDTA and proteinase K. To assess the level of radiolabeled products synthesized during the chase period, reactions containing all components with the exception of [α-^{32}P]GTP were processed in parallel; after 30 min of transcription, [α-^{32}P]GTP was added with the other components.

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