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Transcription termination factor La is also an initiation factor for RNA polymerase III

(autoimmune antigen/ribonucleoprotein/transcription complexes)

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ABSTRACT **La RNA-binding protein is a transcription termination factor that facilitates recycling of template and RNA polymerase (pol) III. Transcription complexes preassembled on immobilized templates were depleted of pol III after a single round of RNA synthesis in the presence of heparin and sarkosyl. The isolated complexes could then be complemented with highly purified pol III and/or recombinant La to test if La is required for transcription reinitiation. VA1, 7SL, and B1 transcription complexes cannot be transcribed by supplemental pol III in single or multiple-round transcription assays unless La is also provided. La mediates concentration-dependent activation of pol III initiation and thereby controls the use of preassembled stable transcription complexes. The initiation factor activity of La augments its termination factor activity to produce a novel mechanism of activated reinitiation. A model in which La serves pol III upon transcription initiation and again at termination is discussed.**

Genes that encode tRNA and VA1 RNAs can be transcribed *in vitro* from nuclear extracts that have been chromatographically separated into three basic fractions: transcription factor (TF) IIIB, TFIIIC, and RNA polymerase III (pol III; recently reviewed in ref. 1). Once transcription complexes have been assembled on class III genes, they remain stable to challenge by dilution, high salt, polyanion, equivalent promoter, or low concentrations of the detergent sarkosyl (2–6). *In vivo*, small RNAs accumulate to copy numbers several orders of magnitude higher than their template copy numbers, suggesting that reinitiation by pol III is the means by which the great majority of these transcripts are synthesized. Moreover, efficient small RNA production requires that transcription termination as well as initiation be accurate and efficient.

Evidence that pol III terminators play a basic role in transcription has been accumulating. Templates that lack a consensus terminator do not assemble transcription complexes or function efficiently *in vivo* (7, 8). A TFIIIC component binds to the termination signals of some class III genes (9, 10). Mammalian TFIIIC can be separated into TFIIIC2, the B box-binding component, and TFIIIC1, a factor that extends the TFIIIC2 footprint 5′ to the start site of transcription and 3′ to the terminator of the VA1 gene (10–12). While mammalian TFIIIB has been reconstituted from recombinant proteins, some of the components of the TFIIIC fraction remain to be identified (1). Moreover, highly purified TFIIIB, TFIIIC1, TFIIIC2, and pol III are insufficient to reconstitute transcription because an as yet unidentified factor present in crude TFIIIB and TFIIIC fractions is also required (see Discussion in ref. 1).

La protein is an autoimmune antigen that is transiently associated with the precursors for tRNAs, 5S rRNA, and other transcripts synthesized by pol III (13, 14). La binds to these RNAs via their common 3′ terminal motif UUU_{OH} which corresponds to the oligo(dT) termination signal for pol III (15, 16). The specificity for this motif reflects La's role as a transcription termination factor that mediates nascent transcript release and facilitates recycling of pol III onto stable preinitiation complexes (17–19).

Transcription of immobilized templates provides a system to study termination and reinitiation (19). Washing of native pol III-containing complexes has two effects on RNA synthesis: reduction in the rate to basal levels and reduction in termination efficiency. Termination efficiency is itself monitored by two criteria: efficiency of nascent transcript release and percentage of polymerases that read through the termination signal. Reductions in each of these activities are reversed by La (19). La's ability to increase the overall rate of transcription was previously thought to be due to a "singular molecular event" (18) at termination by simply facilitating dissociation of pol III and template. According to this model, pol III and template would be passively recycled (17, 18). However, the high efficiency with which a limiting amount of pol III was recycled in the presence of La (19) suggested that, contrary to this passive model, La might actively direct pol III to reinitiate transcription. In the present report, the hypothesis that La is required for reinitiation by pol III is tested. The experimental system relies on the remarkable stability of immobilized transcription complexes; this allows them to be washed and depleted of loosely associated factors as well as factors such as pol III that dissociate as a result of transcription (4).

MATERIALS AND METHODS

Preparation of pol III-depleted initiation complexes was as follows. First, 0.6 μg of each biotinylated plasmid-derived DNA (enough for four reactions), immobilized on 60 μl of agarose-streptavidin as described previously, was incubated with 200 μl of transcription buffer (TB) containing 20 μl of nuclear extract and 0.1 mM ATP for 30 min at 30°C (19). Second, the supernatant was replaced with 80 μl of TB containing nonradioactive NTPs (0.5 mM each), heparin at 0.1 mg/ml, and 0.05% sarkosyl, and the reaction mixture was then incubated for an additional 6 min at 30°C. Third, the immobilized complexes were washed once with 250 μl of TB containing heparin at 0.1 mg/ml and 0.05% sarkosyl and twice with 500 μl of TB lacking NTPs. Finally, the complexes were aliquoted, and the residual liquid was aspirated.

Standard transcription assay was performed as follows. La and/or pol III was added to the pol III-depleted complexes, followed by 25 μl of synthesis mix (TB containing 0.5 mM ATP, UTP, and CTP; 0.03 mM GTP with 0.75 μCi (1 Ci = 37 GBq) of [α-^{32}P]GTP, and RNasin). Reactions were stopped after 30 min at 30°C by addition of 150 μl of 2% SDS, 10 mM EDTA, 50 mM Tris·HCl, and tRNA at 100 μg/ml, and total

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Abbreviations: TF, transcription factor; pol III, RNA polymerase III; TB, transcription buffer.

RNA was purified. Transcripts were visualized by autoradiography after 8 M urea/6% PAGE. Multiple-round assay (see Fig. 3) was performed as follows. After addition of La (500 pmol) and pol III (120 units) to a batch of 110 μl of drained complexes, 174 μl of synthesis mix was added, and the mix was incubated at 30°C. At various times thereafter, aliquots were removed, reactions were stopped, and RNA was prepared. Single-round (re)initiation assay was performed as follows. After addition of La and/or pol III, TB containing CTP, [α-^{32}P]GTP, and ATP was added to the complexes and allowed to incubate for 1 min to arrest pol III (the first U occurs at position 7 of VA1 RNA). A mixture containing UTP and sarkosyl was then added to bring the sarkosyl concentration to 0.05% (6) and incubated for the times indicated. Reactions were stopped, and RNA was prepared.

Recombinant La (gift of D. Kenan, Duke University Medical Center) was expressed in *Escherichia coli* from human La cDNA and purified by conventional chromatography to ≈98% homogeneity as evidenced by PAGE (refs. 20 and 21; data not shown). Pol III (gift of A. Wolffe; ref. 22) was purified from *Xenopus laevis* ovary by the method of Cozzarelli (23). TFIIIA was purified from 7S particles or provided by D. Lee and A. Wolffe. Recombinant U1A protein (ref. 24; gift of D. Kenan) was purified from the same *E. coli* strain as was La. *E. coli* single strand binding protein (cloned) was from Pharmacia. T4 gene 32 protein and soy bean trypsin inhibitor were from Boehringer Mannheim.

RESULTS

Preassembled transcription complexes formed on an immobilized VA1 RNA gene were first depleted of pol III initiation activity by incubation with unlabeled NTPs in the presence of heparin at 100 μg/ml and 0.05% sarkosyl. On the basis of work by others, it was expected that under these conditions, pol III would be stripped from the immobilized template after one round of transcription and removed by the heparin and sarkosyl wash, leaving complexes that could be reinitiated upon introduction of pol III and any other necessary components (6, 9). These complexes were then equilibrated with transcription buffer and equal amounts were aliquoted to different reaction vessels and incubated with [α-^{32}P]NTPs. As expected, the complexes directed no VA1 RNA synthesis (Fig. 1*A*, lane 1). More significantly, no VA1 RNA synthesis could be detected after the addition of supplemental purified pol III (lane 2), indicating that none of the complexes was competent for transcription during the 30-min incubation period. The addition of pol III and recombinant La led to a high level of VA1 RNA synthesis (lane 3), demonstrating both the integrity of these transcription complexes as well as the positive effect of La. The synergistic effect of La and pol III in this assay is underscored by the experiment shown in Fig. 1*A*, lane 4, which reflected low reproducible VA1 RNA synthesis in response to the addition of La without pol III supplementation. La protein was purified from recombinant bacteria and was nearly homogeneous. The possibility that the La preparation contained pol III-like activity is unlikely since transcription becomes saturated at low levels of La while remaining responsive to supplemental pol III at the same high concentrations of La (below). Therefore, VA1 RNA synthesis in response to La alone (Fig. 1*A*, lane 4) was almost certainly due to activation of a small amount of residual latent pol III that remained associated with the immobilized DNA. On the basis of previous work it seems likely that some of the residual pol III was arrested at the VA1 terminator (19) in a latent form that could be rescued by La.

Because a 200- or 220-nt transcript that might result from pol III reading through the VA1 termination signal (see Fig. 1*A*) was not observed, and because total RNA represented released and unreleased transcripts (19), inefficient termination could not account for the negative results obtained in the absence of La. The possibility that nascent transcripts were differentially stabilized by La could also be excluded since purified VA1 [^{32}P]RNA remained intact in transcription reactions whether or not La was added (data not shown).

Although latent residual pol III could be activated by La (Fig. 1*A*, compare lanes 1 and 4), the nature of the association between the latent pol III and the immobilized DNA was not determined. Latent residual pol III could not be significantly reduced by incubation with supplemental La with NTPs before the NTP/heparin/sarkosyl and subsequent washes (data not shown). More importantly however, this pretreatment with La

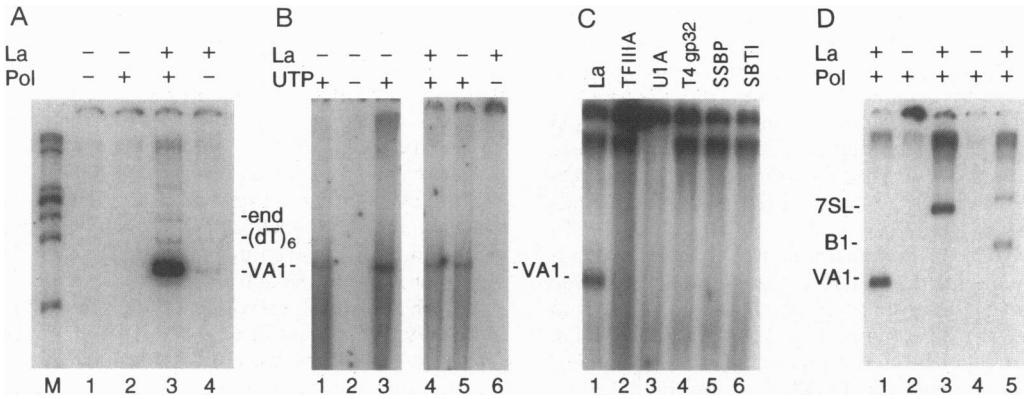

FIG. 1. La is required for transcription by pol III. (*A*) After isolation of pol III-depleted VA1 complexes, equal amounts were aliquoted to different tubes to which purified recombinant La (85 pmol) and/or pol III (30 units) were added as indicated. Reactions were started and RNA was assayed after a 30-min incubation. Termination occurs at +160 to produce VA1 RNA, while read-through to a $(dT)_6$ tract yields a 200-nt RNA, and further read-through to the end of the linear plasmid yields a ≈220-nt RNA. Lane M contains *Hae* III/ΦX174 markers; VA1 migrates between the 118-nt and 194-nt markers. (*B*) *De novo* single round transcription was monitored from complexes assembled in extract that contained endogenous La and pol III. After preincubation, complexes were aliquotted and a mix of heparin, sarkosyl, CTP, ATP, and [α-^{32}P]GTP was added with (lane 1) or without (lane 2) UTP. For lane 3, CTP, ATP and [α-^{32}P]GTP were preincubated with the complexes before addition of heparin and sarkosyl, and UTP was added last. A separate experiment is represented in lanes 4–6. After preincubation, pol III was advanced in the presence of ATP, [α-^{32}P]GTP, and CTP and stalled for lack of UTP. La was added to reactions in lanes 4 and 6 and UTP was then added (lanes 4 and 5) or withheld (lane 6) together with sarkosyl and heparin. RNA was purified after a ≈3-min incubation. (*C*) La was compared to equivalent amounts of control proteins TFIIIA, U1A, SSBP (single strand binding protein), T4 gene 32 protein, and soybean trypsin inhibitor (SBTI), each in the presence of supplemental pol III. (*D*) VA1 (lane 1), 7SL (lanes 2 and 3), and B1 (lanes 4 and 5) transcription complexes were analyzed as in *A*, all in the presence of supplemental pol III, with or without La (odd- and even-numbered lanes, respectively).

plus NTPs did not convert the complexes to initiation-competent complexes upon subsequent supplementation with pol III alone (data not shown). This suggests that La does not stably activate the transcription complexes or simply reposition the residual pol III for initiation but rather that La and pol III must be present simultaneously for RNA synthesis to occur. It can be concluded that La is required for reinitiation under conditions used here. In addition, La activates pol III that is otherwise inactive for reinitiation regardless of whether the source of pol III is endogenous (complex-associated) or exogenous (soluble).

For the experiments represented in Fig. 1*A*, the presumed RNA product of the first round of transcription went undetected since it was synthesized with nonradioactive NTPs. Therefore, the experiments shown in Fig. 1*B* monitored *de novo* transcription from native preassembled complexes. After assembly of preinitiation complexes in nuclear extract, a mixture of heparin, sarkosyl, and [α-^{32}P]NTPs including (Fig. 1*B*, lane 1) or lacking (lane 2) UTP was added, and RNA was purified after a 3-min incubation. This assay differs from the single-round assay used by others in which pol III is elongated and stalled before addition of heparin (5), although prestalling is not required for limiting pol III to a single round (6). The reaction in lane 1 was therefore compared to the more conventional single round transcription reaction in lane 3, which was deprived of UTP before addition of heparin and sarkosyl. Conditions used for prestalling (lane 3) led to somewhat more VA1 transcript than in the case of adding heparin, sarkosyl, and all four NTPs simultaneously (lane 1), presumably because pol III could be stripped from a preinitiation complex more readily than from a stalled elongation complex. This result suggests that for some of the templates, pol III was removed from the preinitiation complex before the first round of RNA synthesis. Transcription from stalled complexes generated by *de novo* initiation was further examined in lanes 4–6 of Fig. 1*B*. After prestalling, heparin and sarkosyl were added with (Fig. 1*B*, lanes 4 and 5) or without (lane 6) UTP, and a single round of transcription was monitored. La (lane 4) or buffer alone (lane 5) was added before UTP addition. Supplemental La was not required for prestalled pol III to synthesize full-length RNA under these conditions (lanes 4 and 5). Note that these experiments do not address whether La is required for *de novo* assembly of the preinitiation complex since assembly occurred in nuclear extract which contains La. Collectively, the experiments in Fig. 1*B* revealed that in this system: (*i*) engaged pol III is stable as it is in other systems that use heparin or sarkosyl, (*ii*) engaged pol III can synthesize full-length RNA when provided with all four NTPs but not when UTP is withheld as expected, and (*iii*) for some of the complexes, the first round of transcription indeed occurred in the presence of heparin, sarkosyl, and NTPs, while some appeared to be depleted of pol III before *de novo* RNA synthesis (Fig. 1*B*, lanes 1–3). From these data it could be concluded that the NTP/heparin/sarkosyl-treated complexes used in Fig. 1*A* and below did appear to be appropriate substrates for (re)initiation.

Reinitiation cofactor activity was specific to La since other purified proteins including recombinant as well as native RNA- and DNA-binding proteins did not exhibit this activity (Fig. 1*C*). The pol III cofactor activity of La was also apparent using pol III-depleted initiation complexes formed on human 7SL and rodent B1 genes (Fig. 1*D*).

La-mediated transcription from isolated complexes was readily responsive to increasing amounts of soluble pol III (Fig. 2*A*). La also exhibited concentration-dependent activation of these complexes as shown in Fig. 2*B*. While the response to pol III was linear over the concentrations tested, a sharp activation curve was reproducibly observed below 1 μM La. The data indicate that La controls the use of stable transcription complexes by pol III.

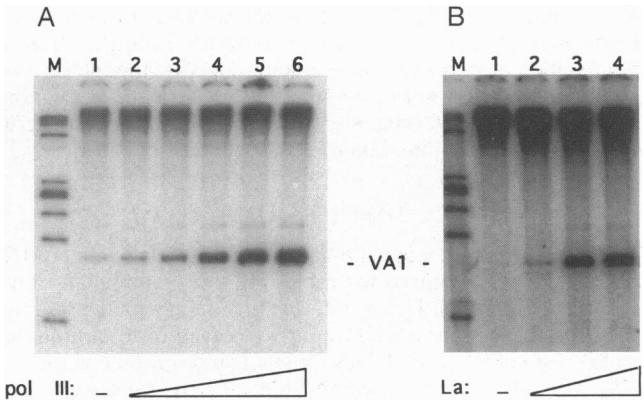

FIG. 2. Concentration-dependent response of a constant amount of pol III-depleted VA1 complexes to pol III and La. (*A*) Varying amounts of pol III were added as indicated: lane 1, none; lane 2, 1.5 units; lane 3, 5 units; lane 4, 15 units; lane 5, 30 units; and lane 6, 45 units. As expected, lane 1 revealed detectable VA1 transcription in the absence of supplemental pol III (see text). (*B*) Varying amounts of La were added as indicated: lane 1, no addition; lane 2, 6 pmol; lane 3, 21 pmol; and lane 4, 28 pmol. Twenty-five microliters of synthesis mix including recombinant 85 pmol La (*A*) or 30 units pol III (*B*) was added to each tube, incubated at 30°C for 30 min, and RNA was analyzed. *A* and *B* represent separate experiments.

To determine if La indeed does stimulate multiple reinitiations using this system, the kinetics of its effects were investigated using conditions that could monitor single and multiple- round transcriptions simultaneously (Fig. 3). For the multiple- round part of this experiment, pol III-depleted initiation complexes were incubated with La, pol III, and [α-^{32}P]NTPs, and aliquots were removed for analysis at various times thereafter as indicated at the top of lanes 1–4. The results revealed a time-dependent accumulation of VA1 RNA consistent with multiple round transcription. As an index of transcription efficiency relative to the multiple round synthesis, and to control for inhibition of reinitiation, single round assays after 2 min- and 45 min-incubation under conditions which limit transcription to a single cycle were performed simultaneously with the multiple-round conditions, either in the presence or absence of La (Fig. 3, lanes 5–8). In these reactions, the complexes supported transcription in the presence of La at levels consistent with a single round of VA1 RNA synthesis (lanes 5 and 7), while no VA1 RNA synthesis was detected in the absence of La (lanes 6 and 8). Importantly, under these conditions which limit pol III to a single round of

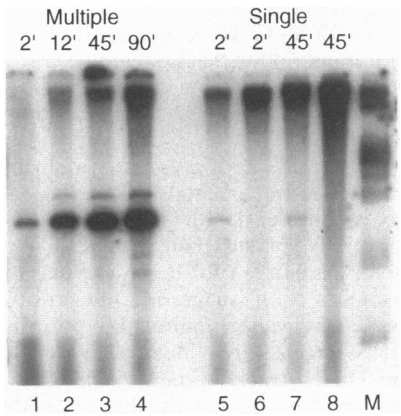

FIG. 3. La mediates single and multiple initiations from isolated complexes. Lanes 1–4, time course of VA1 transcription in the presence of La; lanes 5–8, single round transcription in the presence (lanes 5 and 7) and absence (lanes 6 and 8) of La after 2-min (lanes 5 and 6) and 45-min (lanes 7 and 8) incubations. All reactions contained supplemental pol III.

transcription, VA1 RNA synthesis did not increase with time (compare lanes 5 and 7). In addition to documenting the efficacy of inhibition of reinitiation, this finding supports the interpretation that La's effect in this system is not to affect the elongation rate of engaged yet otherwise sluggish pol III but rather, it appears to mediate initiation of RNA synthesis by pol III.

DISCUSSION

The result reported here is that La was identified as a pol III cofactor that is required for reinitiation of mammalian transcription complexes. This work is in agreement with the finding by others that (yeast) pol III requires a cofactor for reinitiation that is distinct from pol III and stable transcription complexes (25). In addition, La activates latent pol III, presumably by facilitating pol III release from the termination complex (19) and perhaps by rescuing pol III from arrest sites beyond the terminator and directing it to the initiation complex. The cumulative data indicate that La functions as a pol III initiation factor and a termination factor. These activities would appear to coordinate termination and reinitiation and together produce activated reinitiation.

The method of transcription complex preparation used here was designed to provide templates with which to monitor the reuse of stable transcription complexes. High salt washing before the first round of synthesis was also tested. However, unlike the NTP/heparin/sarkosyl treatment, high salt alone could not completely remove pol III initiation activity from the complexes (data not shown). Although the composition of the complexes that survived heparin/sarkosyl/NTP fractionation was not determined, they were competent to direct initiation by pol III if La was present. By comparison to the effects of heparin on the yeast pol III system, it is expected that these complexes minimally contain TFIIIB (9).

Comparison of La and the yeast transcription factor TFIIIE as reported by Dieci *et al.* is noteworthy. The active component of TFIIIE is a small proteinaceous factor that is required for *de novo* initiation as well as reinitiation by pol III but not for formation of stable transcription complexes (25). Although a requirement for a factor similar to TFIIIE had not been reported for other systems that use fractionated yeast extracts (26), Dieci *et al.* (25) found that TFIIIE activity was present in all of the conventionally prepared fractions of yeast class III transcription machinery. Likewise, La cofractionates with mammalian TFIIIC (27). The possibility that La may also be present in partially purified preparations of pol III and/or TFIIIB may explain why a factor requirement for reinitiation has not been obvious in previous use of vertebrate fractions (4, 6). Reportedly, TFIIIB constituted from recombinant components together with TFIIIC1, TFIIIC2, and pol III requires an additional factor for transcription that is present in crude TFIIIB and TFIIIC fractions (see Discussion in ref. 1). On the basis of available data, it should be suspected that La constitutes this as yet unidentified factor.

The yeast polypeptide known as La homologous protein 1 (lhp1), which is found associated with nascent pol III transcripts *in vivo*, is nonessential for yeast growth (28). However, lhp1 is substantially smaller than vertebrate La and its sequence homology is limited to the N-terminal RNA-binding domain of vertebrate La, suggesting the possibility that an additional protein may be required for pol III reinitiation cofactor activity in yeast. The identity of the essential component of the TFIIIE fraction and its relationship to La and lhp1, if any, may be revealed by further biochemical and genetic analysis.

Previous studies attributed all of the stimulatory effects of La to a single mechanistic event at termination (17). By demonstrating that La is required for initiation of transcription from complexes that had previously undergone RNA synthesis and depletion of pol III, the present data extend our understanding of the role that La plays in transcription. The data reported here also help reconcile the dramatic reduction in transcription observed after depletion of La from nuclear extracts (17, 18). The use of nuclear extract that contains La for preinitiation complex assembly precludes the conclusion that La is required for *de novo* initiation by pol III, although this seems likely; in any case, La is indeed required for each reinitiation. According to the definition of activation as "an increase in the efficiency of transcription of a particular gene within a defined period of time" (29), La unequivocally qualifies as an activator as well as a general transcription factor.

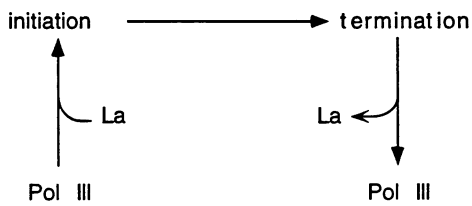

FIG. 4. Model of La action.

Given the available data, we have created a model of La action (Fig. 4). According to this model, at least one molecule of La is required for each molecule of RNA synthesized; its first demonstrable effect is just before initiation by pol III. La assists in terminating the cycle and the transcript exits the complex bound to La (17–19). In this model, La acts like a chaperone for pol III, assisting its entrance into and exit from the template upon dissociation from the transcription complex at termination (4, 30). Although evidence that indicates that La associates with pol III complexes already exists (27), distinguishing interaction at initiation versus termination sites must await higher resolution experiments using reconstitution from highly purified components—i.e., TFIIIC2, TFIIIC1, TFIIIB, La, and pol III. Furthermore, it must be noted that proof of distinct activities of La at termination and reinitiation must await analyses of appropriate mutants of La in the appropriate assays.

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