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The RNA binding protein La influences both the accuracy and the efficiency of RNA polymerase III transcription *in vitro*

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The autoantigen La binds the U-rich 3′ ends of all nascent RNA polymerase III transcripts. Here, we demonstrate that this abundant nuclear phosphoprotein not only binds these RNAs but appears to be required for their synthesis. HeLa cell extracts immunochemically depleted of La by either patient or mouse monoclonal antibodies lose >99% of their transcription activity on class III genes. The few transcripts synthesized in the absence of La have fewer uridylate residues at their 3′ ends than those made in its presence. Reconstitution of La-depleted extracts with biochemically purified HeLa La protein stimulates transcription levels and completely restores transcript length. A model coupling transcription levels to the action of La at the RNA polymerase III termination signal is presented.

Key words: autoantibodies/La protein/RNA polymerase III/transcription factor/transcription termination

Introduction

Highly conserved cellular components are the targets of antibodies produced by patients with autoimmune disorders such as systemic lupus erythematosus and Sjögren's syndrome (Provost, 1979). These autoantibodies have proven to be potent probes for elucidating both the structure and function of a number of important nucleic acid–protein complexes in mammalian cells. Sera from patients exhibiting the anti-La serotype (also called anti-SSB or anti-Ha; Alspaugh and Maddison, 1979; Provost, 1979; Lerner *et al.*, 1981b) recognize a 50-kd phosphoprotein from human cells, denoted the La antigen (Francoeur and Mathews, 1982; Habets *et al.*, 1983; Pizer *et al.*, 1983; Steitz *et al.*, 1983; Hoch and Billings, 1984; Stefano, 1984). Cross-reactive material is present in all mammalian species examined (summarized in Chan *et al.*, 1986), amphibians (E.Gottlieb and J.A.Steitz, unpublished observations; Asselbergs, A.M.Francoeur and M.B.Mathews, unpublished observations) and protozoa (Francoeur *et al.*, 1985), indicating that La has been highly conserved through evolution. Mammalian cells contain $\sim 2 \times 10^7$ copies of this protein, localized predominantly in the nucleus (Alspaugh and Tan, 1975; Alspaugh *et al.*, 1976; Akizuki *et al.*, 1977; Hendrick *et al.*, 1981; Salden *et al.*, 1982; Smith *et al.*, 1985; Bachmann *et al.*, 1986a).

La's hallmark has been its association with all newly synthesized RNA polymerase III transcripts in mammalian cells. Cellular RNAs precipitable by anti-La antibodies include 5S rRNA and tRNA precursors (Rinke and Steitz, 1982), newly synthesized 7S-L (Chambers *et al.*, 1983) and 7-2 RNA (Hashimoto and Steitz, 1983; Reddy *et al.*, 1983b), murine 4.5S RNA (Hendrick *et al.*, 1981), transcripts of Alu sequences (Shen and Maniatis, 1982) and the Ro small cytoplasmic RNAs (Hendrick *et al.*, 1981). Four abundant small RNAs specified by viral genomes are also La associated: the adenovirus VAI and VAII RNAs (Rosa *et al.*, 1981; Lerner *et al.*, 1981b) and EBER1 and EBER2 encoded by Epstein-Barr virus (Lerner *et al.*, 1981a).

The RNA sequences required for interaction with the La protein have been analyzed in several ways. Mixing isolated RNAs with cell extracts containing the La protein has revealed that the 3′ terminal fragments of both VAI RNA (Francoeur and Mathews, 1982) and 4.5S RNA (Reddy *et al.*, 1983a) are specifically bound. La has been UV crosslinked to the terminal uridylate tail of VAI RNA both *in vivo* and *in vitro*, indicating that it is found in close proximity to this conserved feature of class III transcripts (van Eekelen *et al.*, 1982; Mathews and Francoeur, 1984). Finally, Stefano (1984) studied the binding of biochemically purified La protein to 3′-extended tRNA substrates, while Mathews and Francoeur (1984) examined the fingerprints of *in vitro* synthesized VAI RNA contained in anti-La immunoprecipitates and supernatants. Both concluded that three or more uridylate residues followed by a hydroxyl group at the 3′ terminus is the minimal sequence conferring optimal La recognition. This sequence corresponds to the RNA polymerase III transcription termination signal (Bogenhagen and Brown, 1981).

Proposals for La protein function rest on several prior observations. First, La associates with RNA polymerase III products immediately after their synthesis *in vivo* (Rinke and Steitz, 1982); *in vitro*, the kinetics of transcription and of incorporation into anti-La precipitable RNPs are similar (Francoeur and Mathews, 1982). Second, La localization in the cell nucleus is consistent with a primary involvement early in the life of class III transcripts (Hendrick *et al.*, 1981). Hence, it has been suggested that La participates in the synthesis, maturation or nuclear export of this class of RNA molecules (Rinke and Steitz, 1982). The elegant demonstration that TFIIIA, a *Xenopus* 5S RNA-specific transcription factor, binds both to the 5S gene (Engelke *et al.*, 1980; Sakonju *et al.*, 1981), effecting transcription and to its RNA product (Pelham and Brown, 1980; Honda and Roeder, 1980) makes the possibility that the La protein is also a RNA polymerase III transcription factor most intriguing.

Here, we have directly tested the hypothesis that the La antigen participates in the synthesis of RNA polymerase III products. Removal of the protein from HeLa cell extracts

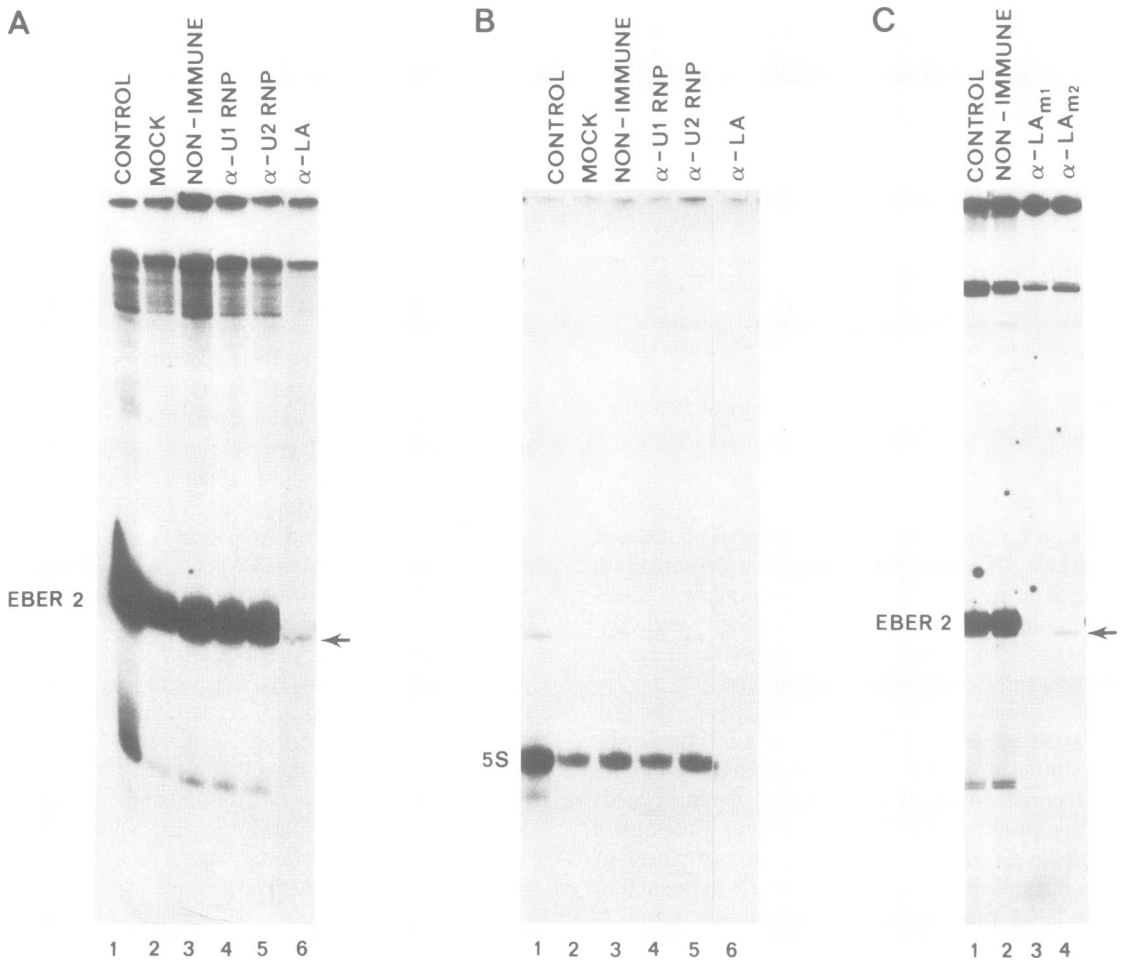

Fig. 1. La immunodepletion results in a drastic diminution of transcription activity. HeLa extracts were incubated with purified antibodies followed by protein A (see Materials and methods). The resulting depleted supernatants were tested for their ability to transcribe viral (EBER2; **panels A,C**) or cellular (5S rRNA; **panel B**) genes. Radiolabeled RNA transcription products were fractionated on 5% (**panels A,B**) or 6% (**panel C**) polyacrylamide gels. **Panels A,B**: Immunodepletion with patient autoantibodies as follows: **lanes 1** and **2**, buffer; **lane 3**, non-immune human serum; **lane 4**, anti-(U1)RNP serum; **lane 5**, anti-(U2)RNP serum; **lane 6**, anti-La serum. **Panel C**: Immunodepletion with mouse monoclonal antibodies as follows: **lane 1**, buffer; **lane 2**, non-immune mouse monoclonal antibodies; **lanes 3** and **4**, two independently derived anti-La mouse monoclonal antibodies, m_1 (SW1) and m_2 (SW5) (Smith, 1985). Lanes A2–6, B2–6 and C2–4 all were exposed to protein A positive *Staphylococcus* cells. Arrows to the right of panels A and C denote the few RNA molecules exhibiting increased gel mobility synthesized in the absence of La. These can easily be seen in lanes A6 and C4 as well as in lane C3 following longer exposure. The RNA species migrating between EBER2 RNA and the dye front (panels A and C) is an EBER2 breakdown product.

using anti-La antibodies alters both the efficiency and nature of transcription. Analyses of the few transcripts made in depleted extracts further suggest that La may be involved in the termination of RNA polymerase III transcription.

Results

Depletion of La protein results in transcription inhibition

A transcription factor is defined as a macromolecular component whose presence during transcription is required to produce normal levels of normal products. To assess whether the La protein is such a *trans*-acting factor for RNA polymerase III, we employed HeLa cell extracts (Weil *et al.*, 1979) that accurately and efficiently synthesize high levels of RNA transcripts when programmed with exogenous viral or cellular class III genes. Immunoblots reveal that these extracts contain large amounts of La protein, and RNAs made in such systems are quantitatively precipitated by anti-La antibodies (Francoeur and Mathews, 1982; Rinke and Steitz, 1982; E.Gottlieb and J.A.Steitz, unpublished observations), mimicking the *in vivo* situation.

We depleted active extracts of the La protein by incubation with purified IgG fractions from anti-La patient sera or from mouse monoclonal ascites fluid followed by addition of fixed protein A-bearing *Staphylococcus aureus* cells (Boehringer). The resulting antigen–antibody–*Staphylococcus* cell conjugates were spun out of the mixture and the depleted supernatants tested for their ability to transcribe various cloned class III genes.

Figure 1A shows the ^{32}P-labeled EBER2 transcripts synthesized in extracts subjected to this depletion protocol. The products displayed in lanes 1 and 2 were transcribed in an untreated extract or an extract exposed to protein A-bearing cells respectively. While the addition of the fixed *Staphylococcus* cells results in some decrease in transcriptional activity, a significant portion of the extract's synthetic capability remains, allowing the effects of different antibodies to be assessed. Exposure to non-immune serum (lane 3) or to lupus sera directed against other abundant

nuclear components [anti-(U1)RNP, lane 4; anti-(U2)RNP lane 5] does not affect transcription. In contrast, exposure to anti-La antibodies results in >99% diminution of transcription ability (lane 6).

Similar striking results are observed when La-depleted extracts are programmed with class III genes of cellular origin. Figure 1B, lanes 1 and 2, show the transcription of a 5S rRNA gene in an untreated extract verses one exposed to fixed *Staphylococcus* cells respectively. Depletion of the La protein again results in a dramatic decrease in RNA synthesis (lane 6). Transcription of a human $tRNA^{Met}$ gene (Santos and Zasloff, 1981) is likewise crippled; we observe neither primary tRNA transcripts nor any processed products following La depletion (data not shown).

We have observed comparable diminution of transcription ability with five out of five high titer patient anti-La sera, each selected for its relative monospecificity using three assays: indirect immunofluorescence, Western immunoblots and immunoprecipitation of *in vivo* ^{32}P-labeled RNAs (see Materials and methods). Inhibition appears specific to the anti-La antibodies rather than some commonly occurring cospecificity since depletion using either of two independently derived mouse anti-La monoclonal antibodies (Smith *et al.*, 1985) likewise results in transcription inhibition (Figure 1C, lanes 3,4). Pre-boiled anti-La antibodies of either patient or mouse monoclonal origin have no inhibitory effect (data not shown).

The observed loss of transcription ability coincides with nearly complete removal of the La protein from the extract as analyzed by immunoblotting (Figure 2). Samples subjected to our depletion protocol with anti-La sera contained little detectable 50-kd La antigen (lane 4) while exposure to protein A-bearing *Staphylococcus* cells alone (lane 2) or with normal human serum (lane 3) did not alter La levels. Reconstruction experiments revealed that extracts displaying >99% diminution of transcription activity contained <2% of the La protein originally present (see lane 5 for comparison).

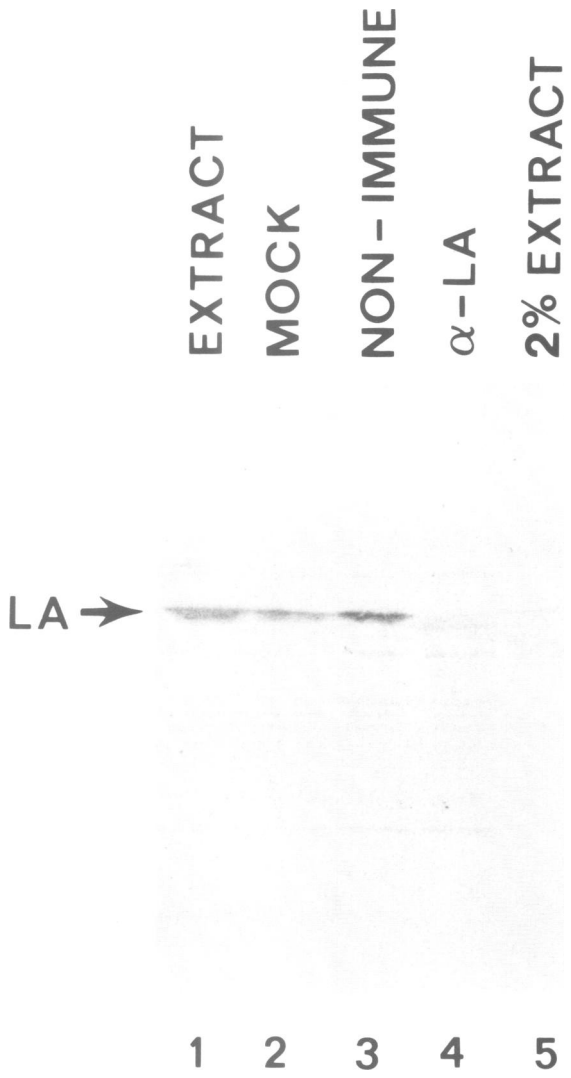

Fig. 2. Anti-La Western blot of immunodepleted transcription extracts. Extracts were immunodepleted (see Materials and methods) and a fraction of each was assayed for transcription ability as in Figure 1. Aliquots of the remainder of each depleted extract were separated on a 10% Laemmli gel and the proteins were electrophoretically blotted onto nitrocellulose. The blot was sequentially probed with anti-La antibodies and alkaline phosphatase coupled anti-human IgG antibodies prior to development. Extracts in **lanes 2–4** were exposed to protein A-bearing cells and to the sera indicated, while the extract in **lane 1** was untreated. Samples containing 2–100% of the amount of extract loaded in lane 1 were processed in parallel; **lane 5** shows the 2% sample. The minor bands in lanes 1–4 are non-specific since they also appear when the blot is probed with non-immune serum.

La depletion also alters transcript mobility

The above results indicated that the La antigen either is itself or is strongly associated with a factor required for normal levels of RNA polymerase III transcription. A second unanticipated effect of La removal is an alteration in mobility of the few transcripts synthesized in the absence of La. These RNAs reproducibly migrate more rapidly on our gels than those made in the presence of La. For example, the EBER2 product synthesized in an extract depleted with either anti-La patient sera (Figure 1A, lane 6) or with anti-La monoclonal antibodies (Figure 1C, lanes 3,4) appears as a discrete band (denoted by arrows to the right of panels A and C) below the middle of the normal broad EBER2 band. Similar mobility shifts are also observed for the few 5S and VA_{200} RNAs synthesized in the absence of La (see below).

Addition of purified La protein restores transcript mobility

To determine whether either the lowered transcription activity or the change in product mobility could be ascribed to the La protein itself, we attempted to reconstitute the La-depleted transcription system with purified La protein. This was important since we knew from studies using [^{35}S]methionine-labeled cell extracts that, in addition to the 50-kd La protein, several less abundant polypeptides appear in anti-La immunoprecipitates generated in low salt buffers similar to those used in transcription extracts (not shown).

We first established that mixing a normal (La-containing) extract wth a La-depleted extract prior to transcription totally restores normal transcription ability, including the normal average mobility of the RNA products (Figure 3, compare lanes 3 and 4). This result argues that both transcription inhibition and the alteration in product mobility are the result of depletion of some essential component(s) rather than the addition or creation of an inhibitor during the depletion protocol.

La protein was purified from HeLa cells using a biochemical procedure devoid of immunoaffinity columns

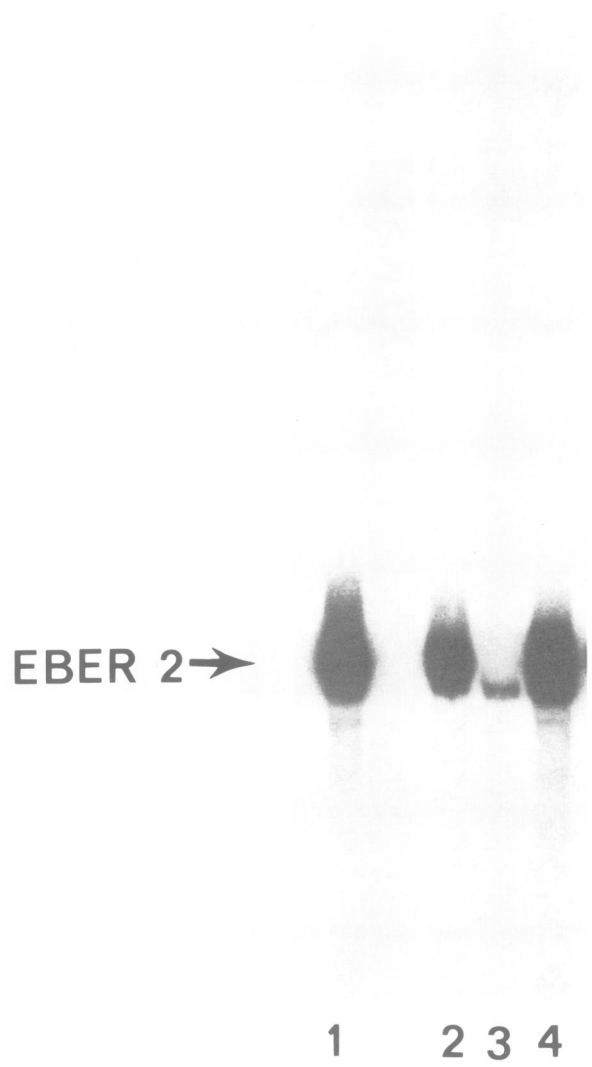

Fig. 3. Mixing non-immune depleted and anti-La depleted extracts restores normal transcription ability. Immunodepleted extracts were supplemented with either 5 μl of transcription buffer (**lanes 1–3**) or 5 μl of S100 dialyzed into transcription buffer (**lane 4**), and assayed for their ability to transcribe the EBER2 gene. Resulting radiolabeled EBER2 transcripts were fractionated on a 6% polyacrylamide gel. Extracts were untreated (**lane 1**), non-immune depleted (**lane 2**) or anti-La depleted (**lanes 3** and **4**).

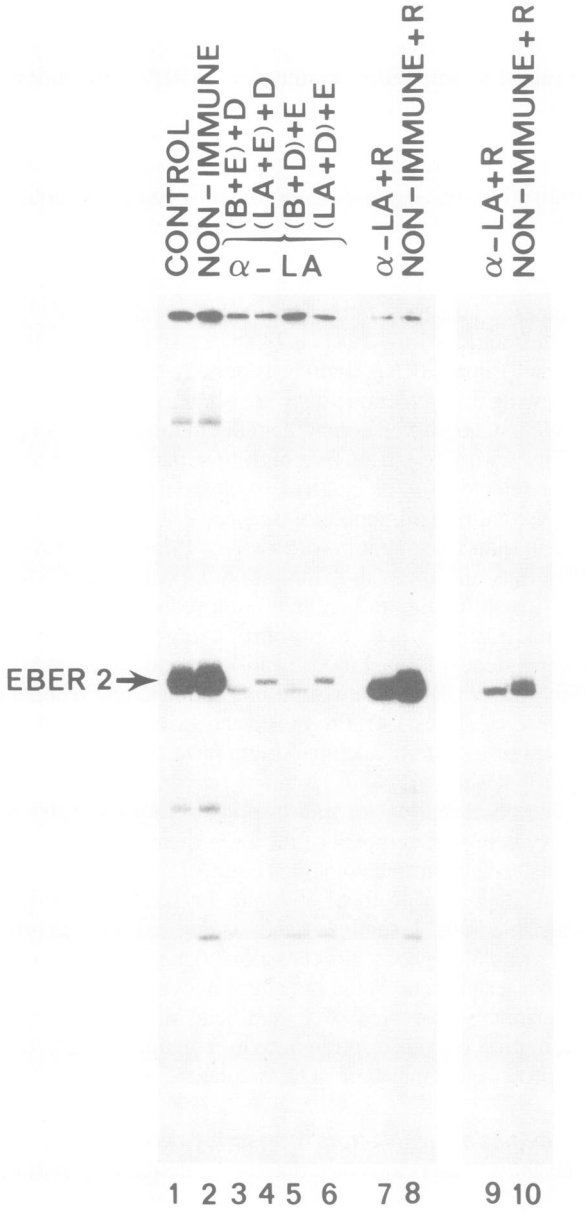

Fig. 4. Reconstitution of biochemically purified La protein stimulates transcription slightly and quantitatively restores transcript length. Extracts were untreated (**lane 1**) or immunodepleted with non-immune (**lanes 2** and **8**) or anti-La (**lanes 3–7**) antibodies and exposed to protein A-bearing *Staphylococcus* cells (**lanes 2–8**). Then extracts were preincubated for 15 min at 30°C with transcription buffer (**lanes 1–3**) or biochemically purified La protein in transcription buffer (**lane 4**) before DNA addition; alternatively, DNA was preincubated with buffer (**lane 5**) or purified La protein (**lane 6**) before extract addition. Reactions were supplemented with ribonucleotides to initiate transcription, incubated for an additional 2 h at 30°C, and the resulting radiolabeled EBER2 transcripts were electrophoretically separated on a 5% polyacrylamide gel. For **lanes 7** and **8**, ^{32}P-labeled EBER2 RNA synthesized in a reaction similar to that in lane 1 was gel purified and incubated in other aliquots of the extracts used in lanes 3 and 2, respectively, during the transcription period of the experiment. **Lanes 9** and **10** are lighter exposures of lanes 7 and 8, respectively, to show the mobility of RNAs. Abbreviations: B, transcription buffer; E, extract pretreated as indicated; D, DNA template; R, presynthesized radiolabeled EBER2 RNA. Preincubated components are denoted by parentheses.

(Stefano, 1984), significantly decreasing the possibility of contamination by cross-reacting antigens. Briefly, the protein was fractionated through four steps as a heterogeneous collection of ribonucleoprotein particles, dissociated from bound RNA during anionic affinity column chromatography, concentrated and dialyzed into transcription buffer. Highly purified material from the final poly(U)–Sepharose column contained a single prominent 50-kd polypeptide (see the silver-stained gel profile of this material in Figure 2C, lane f, in Stefano, 1984, and Materials and methods), which is identified by immunoblotting as the La antigen.

Purified La protein was added to a La-depleted extract programmed with the EBER2 gene, and the resulting ^{32}P-labeled RNA transcripts were resolved on a denaturing polyacrylamide gel (Figure 4). Reconstitution produces two effects (Figure 4, compare lanes 4 and 6 with lanes 3 and 5). First, a slight but reproducible stimulation of transcription is detected: in this experiment, a 3-fold increase resulted from the addition of 10% of the amount of La protein present in the extract before depletion. Second, transcript mobility is quantitatively restored to that of transcripts made in the

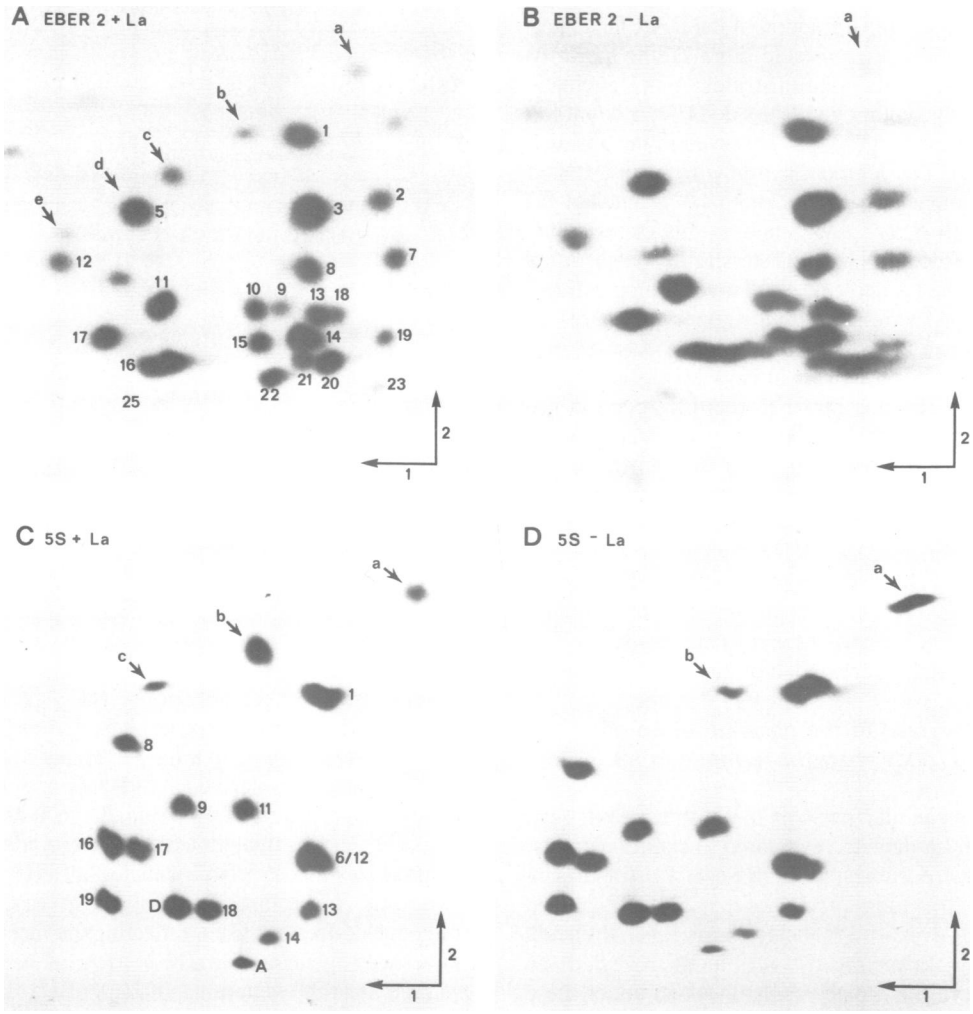

Fig. 5. RNase T1 fingerprints of EBER2 and 5S transcripts synthesized in the presence and absence of La. EBER2 RNAs (**panels A** and **B**) and 5S RNAs (**panels C** and **D**) were synthesized in the presence (**A** and **C**) or absence (**B** and **D**) of La. Gel-purified products were subjected to RNase T1 digestion and separation by electrophoresis on cellulose acetate (horizontal dimension) followed by homochromatography on PEI plates (vertical dimension). Identical fingerprints are obtained following depletion with patient and mouse monoclonal anti-La antibodies. The identity of each spot was confirmed by pancreatic RNase digestion and electrophoresis on DEAE paper. 3′ terminal spots (denoted by arrows) of EBER2 are: (**a**) $CUAU_{OH}$, (**b**) $CUA(U)_{2OH}$, (**c**) $CUA(U)_{3OH}$, (**d**) $CUA(U)_{4OH}$, (**e**) $CUA(U)_{5OH}$. The presence of 3′ end spot d in the major UG spot (5) is revealed by secondary analysis. The remaining spots are numbered according to Rosa *et al.* (1981) and have intensities which differ from those in *in vivo* fingerprints since this RNA was labeled with only [α-^{32}P]GTP and [α-^{32}P]UTP. In **A** and **B**, spot 25 is under-represented due to poor transfer efficiency and the 5′ end has been run off; in **B**, spot 22 has moved to comigrate with 20 and 21. The 3′ end spots of 5S (arrows) are: (**a**) $C(U)_{2OH}$; (**b**) $C(U)_{3OH}$; (**c**) $C(U)_{4OH}$. Remaining spots are numbered according to Brown and Gurdon (1977); only a subset of spots are present since these samples were labeled with [α-^{32}P]UTP. Spot **D** is AUAUCG, which is specific to the *Xenopus borealis* somatic 5S rRNA.

control extract. These two effects occur irrespective of the order of addition of the transcription components following depletion: similar results are obtained when the La protein is incubated with the La-depleted extract prior to DNA addition (lane 4) or when the La protein is incubated with the DNA template prior to extract addition (lane 6). Reconstitution using purified La RNPs (obtained after salt elution from the DEAE–Sepharose column step in the isolation procedure; Stefano, 1984) gave qualitatively similar results (data not shown). We conclude that a modest stimulation of transcription and a complete restoration of transcript mobility can be ascribed to La protein itself.

In other experiments (not shown), we asked whether a greater fraction of transcription activity could be restored by adding larger amounts of purified La protein. We repeatedly observed increasing levels of transcription following the addition of increasing amounts of La protein to aliquots of a given La-depleted extract. Moreover, addition of purified La antigen to 100% the amount found in an untreated extract resulted in the same level of transcription as was obtained by mixing the La-depleted extract with 30% as much dialyzed untreated transcription extract. The restoration of transcription activity plateaued at the 30% level and was insensitive to further increases of La protein. Possible reasons for our inability to restore transcription levels completely are considered in the Discussion.

One possible explanation for the results seen with La-depleted extracts is that newly synthesized transcripts become degraded by endogenous nucleases when La protein is not available to bind and protect their 3′ ends. To investigate this possibility, radiolabeled EBER2 was synthesized *in vitro*, gel purified and incubated under transcription conditions in

the same non-immune or anti-La treated extracts as used in Figure 4, lanes 2–6. While a fraction of the RNA incubated in the La-depleted extract is unstable (lane 7), the majority (>50%) survives. This demonstrates that nuclease degradation of newly synthesized RNA does not account for the low levels of transcript (~1%) observed in the absence of La (lane 3 and 5). The gel mobility of the recovered RNAs in such an experiment is variable, often being unchanged upon incubation. In other cases, such as this experiment, the exogenously added RNAs migrate slightly faster after incubation in both the La-depleted and La-containing extracts (see lanes 9 and 10, which represent lighter exposures of lanes 7 and 8). Since the RNAs incubated in parallel in a La-depleted and a control extract always comigrate, these observations are in sharp contrast to the difference in gel mobility invariably seen when RNAs are synthesized *de novo* in the same pair of extracts. Furthermore, when presynthesized RNAs become shortened upon incubation in transcription extracts, fingerprint analyses reveal that they are even more 3′ truncated than RNAs made in the absence of La (see below). These findings argue that differential nuclease trimming is not responsible for the second distinguishing characteristic of transcripts synthesized in La-depleted extracts, their increased mobility.

Transcripts synthesized in the absence of La are shorter at their 3′ ends than those made in its presence

To establish the molecular basis of the mobility shift seen in transcripts from La-depleted compared to control extracts, RNA products were subjected to RNase T1 fingerprint analyses (Figure 5). Fingerprints of [α-^{32}P]GTP-labeled EBER2 (in which every oligonucleotide is labeled except for those arising from the 3′ terminus) revealed no differences between the molecules synthesized under these two conditions: each spot, including the 5′ pppAG oligonucleotide, appeared in the expected molar ratio (not shown). In contrast, T1 fingerprints of [α-^{32}P]UTP and [α-^{32}P]GTP doubly labeled EBER2 made in the presence of La contained five additional spots (Figure 5A, a–e), four of which are missing from the fingerprint of EBER2 synthesized in the La-depleted extract (Figure 5B). Secondary analyses confirmed that these five oligonucleotides are derived from the heterogeneous EBER2 3′ terminus. Fingerprints of [α-^{32}P]UTP-labeled EBER2 (not shown) allowed quantitation of these spots: $CUA(U)_{3OH}$ is the most abundant; $CUA(U)_{2OH}$ and $CUA(U)_{4OH}$ appear in lower but approximately equal amounts; and trace levels of both $CUAU_{OH}$ and $CUA(U)_{5OH}$ were detected. The more rapidly migrating EBER2 (Figure 5B) obtained from the La-depleted extract possesses only the shortest of these 3′ end spots, $CUAU_{OH}$, the identity of which was confirmed by analysis of its P1 RNase digestion products. The penultimate oligonucleotide (UCCG), spot 9 of EBER2, is present in stoichiometric amounts in transcripts made either in the presence or absence of La as revealed in fingerprints of RNAs labeled with [α-^{32}P]GTP or doubly labeled with [α-^{32}P]GTP and [α-^{32}P]UTP.

Analyses of 5S rRNA transcripts likewise revealed a difference at the 3′ end. The fingerprint of [α-^{32}P]UTP-labeled 5S rRNA made in the presence of La shows three 3′ end spots (Figure 5C, spots a–c): $C(U)_{2OH}$, $C(U)_{3OH}$ and $C(U)_{4OH}$ respectively. One of these spots, $C(U)_{4OH}$, is absent and a second spot $C(U)_{3OH}$, greatly underrepresented in the fingerprint of the [α-^{32}P]UTP-labeled 5S RNA transcript made in a La-depleted extract (Figure 5D).

Table I. Fewer uridylate residues in the absence of La

RNA	3′ Ends +La	3′ Ends −La
EBER 2 RNA	(a) $CUA(U)_{OH}$	(a) $CUA(U)_{OH}$
	(b) $CUA(U)_2OH$	—
	(c) $CUA(U)_3OH$ major	—
	(d) $CUA(U)_4OH$	—
	(e) $CUA(U)_5OH$ trace	—
5S rRNA	(a) $C(U)_2OH$	(a) $C(U)_2OH$ major
	(b) $C(U)_3OH$ major	(b) $C(U)_3OH$ trace
	(c) $C(U)_4OH$	—
VA 200 RNA	(a) $C(U)_3OH$	(a) $C(U)_3OH$ major
	(b) $C(U)_4OH$ major	(b) $C(U)_4OH$ trace
	(c) $C(U)_5OH$	—

A compilation of 3′ end T1 oligonucleotides detected on transcripts synthesized in the presence (column 2) and absence (column 3) of La. Information is from T1 fingerprints of transcripts derived from three class III genes (column 1) as displayed in Figures 5 and 6. Absence of a detectable oligonucleotide is denoted by a dash. In most cases, multiple 3′ oligonucleotides are listed reflecting the fact that class III RNAs exhibit 3′ heterogeneity *in vitro* as well as *in vivo*.

For transcripts derived from both the EBER2 and 5S genes, fingerprints of RNA made in untreated and non-immune serum treated extracts were identical. Table I summarizes the 3′ T1 oligonucleotides derived from these two sets of transcripts. In most cases, several T1 oligonucleotides are listed reflecting the fact that these RNAs have heterogeneous termini *in vitro* as well as *in vivo*.

To extend the generality of La's effect on the 3′ ends of class III gene products, we examined an additional transcript, VA_{200}. This RNA is synthesized from the same internal promoter region as VAI RNA, which terminates 88–90 nucleotides 3′ to its internal control region within a stretch of four U residues beyond the base of an RNA hairpin. VA_{200} RNA is produced when transcription reads through this site and ends within a second stretch of six U residues ~40 nucleotides downstream. Read-through products are usually synthesized at ~5% the level of VAI RNA, but we have employed a mutant gene in which 100% of the transcripts extend to the second terminator. (The normal terminator in this gene has been deleted by B.Thimmappaya; see Materials and methods). The VA_{200} transcript therefore differs from most RNA polymerase III products in that its 3′ end is not adjacent to a stable stem structure formed by base pairing of sequences at the 5′ and 3′ termini of the RNA molecule.

Fingerprint analyses of VA_{200} RNA synthesized in the presence and absence of La (Figure 6) gave findings consistent with those reported above. In VA_{200}, three 3′ termini are normally observed (Figure 6A): a major spot $C(U)_{4OH}$ (spot b) and two less abundant oligonucleotides $C(U)_{3OH}$ and $C(U)_{5OH}$ (spots a and c respectively). In a La-depleted extract (Figure 6B), most of the RNA (which is made at about the 5% level compared to RNA synthesized in a non-immune depleted extract) terminates with $C(U)_{3OH}$; $C(U)_{4OH}$ is a minor end; and RNA ending at $C(U)_{5OH}$ is not detected. In addition, the penultimate

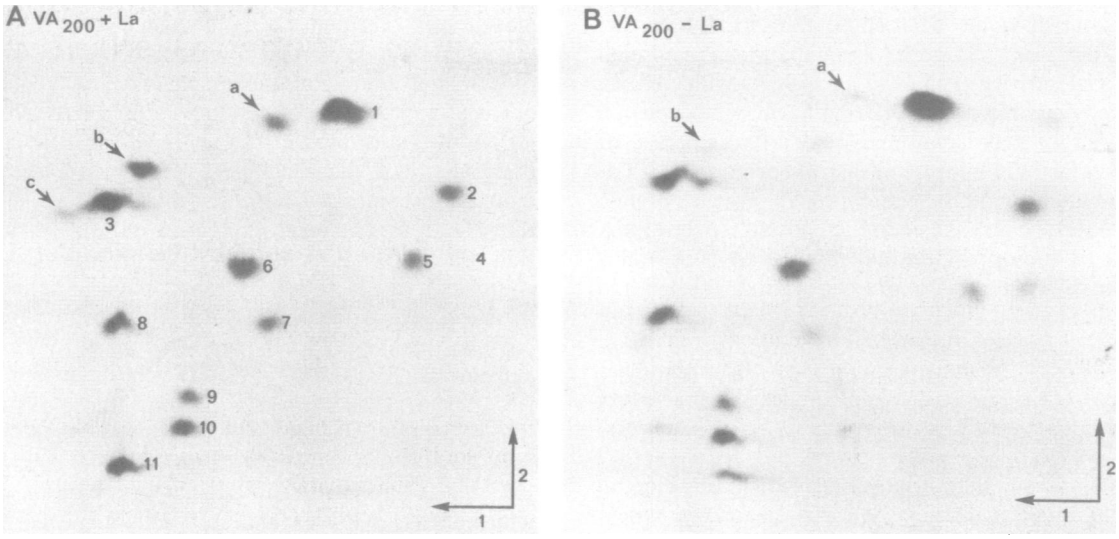

Fig. 6. RNase T1 fingerprints of VA_{200} transcripts synthesized in the presence and absence of La. [α-^{32}P]UTP-labeled VA_{200} RNA synthesized in the presence (**A**) and absence (**B**) of La were subjected to T1 fingerprint analysis as in Figure 5. Oligonucleotide identities were confirmed by pancreatic secondary analysis and comparison with both fingerprint and secondary analyses of VAI RNA and a different VA_{200} mutant. They agree with the predictions of Akusjärvi *et al.* (1980) and Ohe and Weissman (1971). Arrows denote 3′ oligonucleotides: (**a**) $C(U)_{3OH}$; (**b**) $C(U)_{4OH}$; (**c**) $C(U)_{5OH}$. Remaining spots are: (**1**) G; (**2**) CG; (**3**) UG; (**4**) CCG [which is present in variable yields in fingerprints of VAI made by us and others (Mathews and Francoeur, 1984) as well as in fingerprints of VA_{200} and another VA_{200} mutant]; (**5**) ACG; (**6**) CUG; (**7**) CUAG; (**8**) UUCG + UCUG; (**9**) U_2AC_2G; (**10**) UAUCAUG + AUC_2AUG; (**11**) AUA_3U_2CG (which we find moves variably in the first dimension). Spots **6** and **7** are derived from the 3′ extension between the VAI and VA_{200} terminators.

labeled spot (7, CUAG) is diminished. In both extracts, spot 6 (CUG), which is diagnostic of the RNA extension between the VAI and VA_{200} terminators, is present in significant yield. Hence, the majority of VA_{200} molecules synthesized in the absence of La are a few nucleotides shorter than normal (similar to the difference observed with other transcripts), rather than being truncated to the RNA hairpin.

Discussion

We have shown that the autoantigen La, initially identified as an RNA binding protein, also possesses properties characteristic of an RNA polymerase III transcription factor. Following exposure to either patient or monoclonal anti-La antibodies, competent HeLa transcription extracts lose most of their ability to transcribe exogenous viral (EBER2) and cellular (5S rRNA, tRNA) class III genes. In contrast, treatment with control antibodies [anti-(U1)RNP, anti-(U2)RNP, non-immune] has no apparent effect. The few remaining RNA transcripts produced in the absence of La are shorter than those made in its presence, having fewer uridylate residues at their 3′ ends. Reconstitution of the La-depleted extracts with biochemically purified La protein results in partial stimulation of the transcription level and quantitative restoration of transcript length.

Using antibody-treated extracts, two groups of investigators (Francoeur and Mathews, 1982; Gottesfeld *et al.*, 1984) previously addressed the hypothesis that the La protein might be a polymerase III transcription factor and came to a negative conclusion. Our study differs from theirs in several important respects. First, we have directly assessed the amount of La remaining in depleted extracts and have shown that the La protein has been effectively removed (Figure 2). Using covalently coupled antibody columns similar to those of Francoeur and Mathews, we were also unable to detect reproducible transcription inhibition; however, in these experiments, the amount of La protein removed was variable and we found 20% of the endogenous La protein to be inaccessible to depletion (data not shown). Second, we have obtained transcription inhibition using two mouse monoclonal antibodies as well as five high titer patient anti-La antibodies, arguing strongly that inhibition does not result from a commonly occurring second specificity. Third, we have used biochemically rather than immunochemically purified La protein to reconstitute depleted extracts. This allows us to avoid the use of harsh denaturants required to recover antigens from antibody columns and markedly decreases the probability that we are adding back a minor constituent removed during the depletion protocol. Finally, we have shown that aberrant products foreshortened at their 3′ ends are made in La-depleted extracts. These RNAs behave as though they are components of stalled transcription complexes (Gottlieb and Steitz, 1989).

We were able to restore only a fraction of the original transcription activity by the addition of purified La protein to La-depleted extracts: in Figure 4, readdition of 10% the amount of protein normally contained in the extract resulted in only a 3-fold stimulation of transcription. While addition of increasing amounts of other preparations of purified La resulted in further increases in the level of transcription up to 30% (data not shown), we were never able to restore the vast majority of activity. There are several possible explanations for this result. First, the La protein present in our purified preparations may not be the most transcriptionally competent form. La is known to exist in several discrete phosphorylated forms (Pizer *et al.*, 1983; Francoeur *et al.*, 1984), which could contribute differentially to transcription; increased phosphorylation *in vitro* appears to decrease La's RNA binding ability (Bachmann *et al.*, 1986b; Pfeifle *et al.*, 1987). Second, a required cofactor may have

been separated from the La protein during purification. Recall that TFIIIA, the 5S-specific transcription factor, requires zinc for 5S gene association and optimal transcriptional activity (Hanas *et al.*, 1983; Miller *et al.*, 1985). Finally and most likely, during La-immunodepletion of our extract we may have removed other polymerase III transcription factors by virtue of their association with La. These could interact with La either through protein−protein or protein−RNA contacts (since La binds all nascent RNA polymerase III transcripts). Several observations support this explanation: (i) La protein is present in crude TFIIIB and C preparations and a small fraction continues to copurify with TFIIIC through multiple chromatographic steps (Gottlieb and Steitz, 1987; P.Martin, E.Gottlieb, R.Roeder and J.A.Steitz, unpublished observations). (ii) A DNA template containing a class III gene can be selectively immunoprecipitated by anti-La antibodies (Gottlieb and Steitz, 1987) despite the fact that La has not been observed to bind DNA directly (Stefano, 1984; Gottesfeld *et al.*, 1984; Gottlieb and Steitz, 1987). (iii) Previously La has been suggested to associate with a 64- to 68-kd protein implicated in the transcription process (Gottesfeld *et al.*, 1984).

The synthesis of 3′ shortened transcripts in La-depleted extracts was not anticipated, yet these products seem central to deciphering La function. It is striking that the sequence at the 3′ terminus of RNA polymerase III transcripts is dependent upon the La protein for its appearance as well as being the documented RNA binding site for La. We have considered three molecular explanations for the generation of these 3′ shortened transcripts. First, since La binds RNA polymerase III transcripts at their 3′ termini, preferring 3−4 uridylate residues followed by a hydroxyl group, nucleases present in our *in vitro* system could degrade newly synthesized full-length transcripts in the absence of La packaging and protection. Control experiments involving presynthesized EBER2 (Figure 4) argue against this as a sole explanation for all our results. However, degradation cannot be ruled out completely since RNA synthesized *in situ* could have different requirements for stability. In addition, we have examined the effects of incubating VA_{200} RNA in extracts containing or lacking La; in La-depleted extracts this RNA becomes shortened to the base of the RNA hairpin (data not shown) ~40 nts upstream from the 3′ uridylate stretch, arguing that the several nucleotide shorter VA_{200} synthesized in the absence of La (see Figure 6) is not produced by the same mechanism. Second, La could be a poly(U) polymerase. Tests of this possibility show that biochemically purified La protein fails to polymerize [α-^{32}P]UTP or add [α-^{32}P]UTP to pre-existing RNA molecules and that our La-depleted extracts retain poly(U) polymerase activity (E.Gottlieb, unpublished observations; J.E.Stefano, unpublished observations). Further, the number of terminal uridylate residues in each transcript analyzed does not exceed the number encoded by its corresponding gene. Finally and most intriguing, La could be a transcription termination factor. We favor this possibility because both effects of La depletion—the drastic decrease in transcription ability and the synthesis of 3′ foreshortened transcripts—could be ascribed to a single molecular event occurring at the termination signal.

The RNA polymerase III termination signal, defined by Bogenhagen and Brown (1981) as T_n (where $n \geq 4$), corresponds precisely to the region that is affected in transcripts synthesized in La-deficient extracts. In addition, the U-rich 3′ terminus is the documented binding site for the La protein in normal polymerase III transcripts. We propose that during transcription, an initial round of synthesis proceeds until RNA polymerase III pauses at the termination signal. If La is present, the polymerase transcribes the uridylate stretch, the resulting RNA is released, and the DNA template becomes available for a second and subsequent rounds of reuse. In the absence of La, the polymerase would stall prior to synthesizing the 3′ U-rich stretch, unable to complete a single round of transcription. The incomplete transcript would remain bound to the frozen transcription complex blocking further synthetic activity. This model explains why La is required for efficient (multiple rounds of) RNA synthesis as well as for the appearance of full-length transcripts. It is also compatible with the data of Cozzarelli *et al.* (1983), who concluded that RNA polymerase III can recognize the transcription termination consensus sequence, which we feel may constitute an RNA polymerase pause site (see Gottlieb and Steitz, 1989). Although the results presented here do not rule out the possibility that another protein is responsible for the effects we observe, such a molecule would have to be both removed in our immunodepletion by virtue of its association with the La protein and present in purified La (which has been isolated first as an RNA−protein complex and then as a protein). Further data presented in the accompanying manuscript make it likely that these effects are due directly to La.

Two well-characterized prokaryotic transcription termination factors affect the exact positioning of the 3′ ends of nascent transcripts. Rho is a 46-kd protein implicated in termination and transcript release by *Escherichia coli* RNA polymerase. It interacts with RNA polymerase, auxiliary *E.coli* transcription factors and the resulting nascent transcript (reviewed in von Hippel *et al.*, 1984, and Platt, 1986; Chen *et al.*, 1986). Rosenberg *et al.* (1975) found that the addition of purified rho protein resulted in a 10-fold increase in the level of the λ4S transcript synthesized by purified RNA polymerase *in vitro*. Enhancement of synthetic activity was coupled to an alteration in transcript length: 70% of the λ4S RNAs made in the presence of rho were longer, having an extra uridylate residue at their 3′ ends. Recently, a second termination factor, tau, has been identified (Briat and Chamberlin, 1984; Briat *et al.*, 1987) in whose presence early T7 RNA transcripts terminate four nts upstream of those made in its absence. The shortened RNAs, which have ends corresponding to *in vivo* termini, are generated by transcription termination rather then post-transcriptional processing or nuclease degradation. Analogous to our observations, the partially purified protein was capable of restoring the *in vivo* length but only a small fraction of the synthetic ability to extracts deficient in tau.

Finally, La's ability to bind all newly synthesized class III transcripts sets it apart from the previously described general class III transcription factors and has several implications. La may mediate its effects, in part, by transcript release, binding nascent transcripts while still template associated. If so, it likely acts stoichiometrically, explaining why La is such a major component of mammalian cells (2×10^7 molecules/cell). Stoichiometric action is further consistent with the kinetics of transcript synthesis and La association reported by Francoeur and Mathews (1982) and suggests that packaging into La RNPs may be mechanistically

coupled to transcription. This would ensure that the 3′ ends of all nascent transcripts become La associated and thereby protected from nuclease attack. Further, La is ideally positioned to regulate the balance of all newly synthesized polymerase III transcripts in the eukaryotic cell, potentially participating in a feedback loop through association with both transcription complexes and the resulting RNA products.

Materials and methods

Cells, antibodies and enzymes

HeLa cells were grown 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. Cells were maintained at 37°C in a 5% CO_2 environment at a density of ~7 × 10^5 cells/ml.

Sera from patients with autoimmune disorders were kindly supplied by J.Hardin (Yale University), T.Mimori (Keio University), G.McCarty (Georgetown University) and P.Schur (Brigham and Women's Hospital, Boston). Each serum was selected for its high titer and relative monospecificity following three independent assays: immunofluorescence, Western immunoblots (Towbin *et al.*, 1979; Yen and Webster, 1981; Mimori *et al.*, 1984) and immunoprecipitation of *in vivo* [^{32}P]orthophosphate-labeled RNAs from a HeLa whole-cell extract (Hendrick *et al.*, 1981; Matter *et al.*, 1982). Control sera were obtained from healthy laboratory personnel. IgG fractions were derived from each crude serum preparation by three successive ammonium sulfate precipitations, redissolved in 150 mM NaCl, 40 mM Tris pH 7.5, dialyzed into 17.5 mM sodium phosphate pH 6.3, adjusted to 40 mM Tris pH 7.5, 150 mM NaCl, and stored at −20°C. Preparations employed for transcription inhibition were dialyzed into 1× transcription buffer (70 mM KCl, 5 mM $MgCl_2$, 15 mM Hepes pH 7.9, 0.5 mM DTT) and contained ~17 mg/ml protein. Anti-La monoclonal antibodies were the kind gift of D.Williams and P.Venables (Smith *et al.*, 1985). They were grown in mouse ascites and IgGs prepared by isolation on protein A−Sepharose columns (Pharmacia). Purified IgGs were dialyzed against 150 mM NaCl, 40 mM Tris pH 7.5 and stored at −20°C. Each preparation was confirmed to contain anti-La antibodies by the three assays noted above. Mouse IgG controls were derived from the ascites fluid of sibling mice (BALB/c) injected with the parent myeloma cells (NS-1). Protein A−Sepharose purified ascites preparations contained ~0.5 mg/ml protein.

Proteinase K was obtained from Beckman. T1, P1 and pancreatic RNase were from Calbiochem. Protein A-bearing *Staphylococcus aureus* cells were purchased from Boehringer Mannheim.

RNA analysis

Radiolabeled RNAs were fractionated under denaturing conditions on 5%, 6% or 10% polyacrylamide gels containing 7 M urea. Analytical gels were dried and autoradiographed at −70°C. For further analysis, RNA species were extracted from gel slices by the crush-and-soak method (Maxam and Gilbert, 1980), phenol extracted and ethanol precipitated. T1 RNase digests were fingerprinted on cellulose acetate in pH 3.5 buffer (Barrell, 1971), followed by thin-layer homochromatography on PEI 300 plates (Brinkmann, see Lerner and Steitz, 1979). Secondary analyses of eluted oligonucleotides were performed by pancreatic RNase digestion and electrophoretic separation of the products on DEAE paper (Barrell, 1971).

Extracts and transcription reactions

Soluble transcription (S100) extracts were prepared from ~4 × 10^9 log phase HeLa cells (Weil *et al.*, 1979), aliquoted, frozen in liquid nitrogen and stored at −70°C. A standard 25-μl transcription reaction contained 10 μl S100; 70 mM KCl; 5 mM $MgCl_2$; 15 mM Hepes pH 7.9; 0.5 mM DTT; 0.5 mM each ATP, CTP, and UTP; 0.025 mM GTP; 20 μCi [α-^{32}P]GTP (410 Ci/mmole; Amersham), and 10−40 μg/ml of DNA template. DNA templates were all supercoiled derivatives of pBR322. Each extract was optimized for DNA and $MgCl_2$ concentrations. Transcription proceeded at 30°C for 2 h followed by treatment with proteinase K (Beckman) and ethanol precipitation. Preparative reactions varied from 100 to 300 μl and were performed in the presence of 800 μCi/ml of 410 Ci/mmol (Amersham) [α-^{32}P]GTP, [α-^{32}P]UTP, or both.

Antibody depletion

Depletions were performed at 0°C in transcription buffer (70 mM KCl, 15 mM Hepes pH 7.9, 5 mM $MgCl_2$, 0.5 mM DTT). Basically 20 μl of transcription extract was incubated for 10 min with 5 μl of an IgG preparation followed by a 90-min incubation in the presence of 12.5 μl of a 20% protein A-bearing *Staphylococcus aureus* cell preparation. The optimal amount of antibody required to deplete La from each extract was determined by titration. Many sources of protein A were tested; the reagent purchased from Boehringer Mannheim as a lyophile gave the most reproducible results. Prior to use, it was washed several times with ice-cold transcription complementation buffer (5.8 mM Hepes pH 7.9, 58.4 mM KCl, 12.4 mM $MgCl_2$, 0.5 mM DTT) and resuspended in the same buffer. Following centrifugation to remove the antigen−antibody−protein A complexes, the depleted extract was supplemented with DNA template, salts, cold and [α-^{32}P]-labeled ribonucleotides as above and assayed for transcription. Our extracts lack polymerase II activity, making it impossible to assess the effect of La immunodepletion on the synthesis of class II gene products. Furthermore, attempts to immunodeplete an extract exhibiting both polymerase II and III activity were unsuccessful as indicated by Western blots, ELISAs and the retention of polymerase III transcription activity.

Immunoblots

Aliquots of transcription extract were boiled in Laemmli sample buffer and separated on 10% SDS−polyacrylamide gels (Laemmli, 1970). They were subsequently electrophoretically transferred to nitrocellulose (BA85 0.45 μm; Schleicher and Schuell) for 2 h at 225 mA in 4°C in 25 mM Tris, 192 mM glycine, 25% methanol, 0.1% SDS (Towbin *et al.*, 1979; Mimori *et al.*, 1984).

Blots were processed according to the procedure of Yen and Webster (1981). Antigen−antibody complexes were detected with [^{125}I]protein A (Amersham) followed by autoradiography. Alternatively, complexes were decorated using an alkaline phosphatase coupled anti-human IgG antibody (Jackson Immuno Research Laboratories) and visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma; procedure of S.Edelstein, Yale University, unpublished data).

Protein purification

La protein was isolated from 6−9 l of exponentially growing HeLa cells according to the procedure of Stefano (1984). Protein employed for the reconstitution was taken through the heparin−agarose or poly(U)−Sepharose column steps and dialyzed against 15 mM Hepes pH 7.9, 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 20% glycerol. Purification was monitored by ELISA (Thoen *et al.*, 1980) using patient anti-La sera and horseradish peroxidase conjugated protein A (Boehringer Mannheim Biochemicals). Antigenicity was monitored at 492 nm on a Titertek Multiskan. Following this procedure, protein was determined to be functional by two criteria: it retained antigenicity and it selectively bound appropriate RNA substrates. Determination of the amount of purified La added in each reconstitution was achieved by comparative ELISAs and Western immunoblots of the purified protein preparation and the transcription extract. This measures the total amount of antigenic protein, not necessarily the amount of transcriptionally functional protein.

Plasmids

EBER2 RNA was transcribed from pEBV EcoRIJ (Rosa *et al.*, 1981) or pJJJ2 (Jat and Arrand, 1982). The former contains the 3 kb EcoRIJ fragment of EBV DNA cloned into the *Eco*RI site of pBR325 while the latter is a subfragment of ~500 bp cloned into pAT153 between the *Eco*RI and *Bam*HI sites. pxbs1 (kindly provided by R.Roeder, Rockefeller University) encodes the *Xenopus borealis* somatic 5S gene cloned into pBR322 (Peterson *et al.*, 1980). VA_{200} RNA was transcribed from an *Xba*I−*Bal*I fragment of adenovirus 2 cloned between the *Pvu*II and *Eco*RI sites of pBR322. This construction which has a 6-bp deletion (T_4G_2) encompassing positions +157 to +162 of the VAI RNA gene (where T_4 is the normal VAI terminator), was generously supplied by B.Thimmappaya (Northwestern University).

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