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La Antigen Recognizes and Binds to the 3′-Oligouridylate Tail of a Small RNA

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The La antigen is a cellular protein which interacts with many RNA species that are products of RNA polymerase III, including the adenovirus virus-associated (VA) RNAs. We demonstrate that the efficiency of antigen binding in vitro is determined by the number of U residues at the RNA 3′ terminus. Forms of VA RNA_I with more than two terminal U residues are fully bound, forms with two U residues are partially bound, and forms with fewer than two U residues are not bound at all. The antigen can be covalently linked to VA RNA by UV irradiation, and the site of cross-linking is shown to contain the 3′ terminus of the RNA. We conclude that the antigen recognizes the U-rich 3′ tail of VA RNA, and presumably that of other polymerase III products, and that it binds at or close to this site.

The La antigen is a cellular protein defined by its reaction with human autoantibodies of the La (also Ha or SS-B) type (see references 15 and 20 for reviews). Anti-La antibodies are characteristic of autoimmune diseases, especially systemic lupus erythematosus, in which they are found in about 10% of patients, and the related condition of primary sicca syndrome, in which they are found in 40% of patients (R. M. Bernstein, C. C. Bunn, G. R. V. Hughes, A. M. Francoeur, and M. B. Mathews, submitted for publication). The La antigen is a phosphoprotein with an apparent molecular weight of 46,000 (5) and is principally nuclear in location.

In common with the antigens recognized by other autoantibodies (11), interest in this protein was stimulated by the discovery that it binds RNA (10). In uninfected cells it associates with several RNA species including precursors of ribosomal 5S RNA and tRNA, mouse 4.5S RNA, and transcripts of Alu sequences (6, 16, 19). In adenovirus-infected cells it binds the virus-associated (VA) RNAs I and II (5, 10, 17). Likewise, in cells infected or transformed with Epstein-Barr virus, it binds the EBER I and II species (9). All of these RNAs are relatively small and are products of RNA polymerase III, sharing 5′-terminal pppG or pppA residues and 3′-terminal oligouridylate runs.

To illuminate the function of La antigen, we investigated its binding to VA RNA_I. By a combination of RNA fingerprinting and protein-RNA cross-linking, we showed that the principal recognition feature and binding site for the antigen comprises the string of uridylate residues at the 3′ end of the RNA molecule.

MATERIALS AND METHODS

Transcription. VA RNA_I of adenovirus 2 was transcribed in vitro from the pBalM plasmid (originally provided by S. Berget) in a HeLa cell extract containing RNA polymerase III activity (25) as described previously (5). VA RNA_{II} was synthesized from the pD* plasmid (5) in the same way. Reactions contained [α-^{32}P]GTP or [α-^{32}P]UTP (New England Nuclear Corp.), as specified for each experiment, and were incubated at 30°C for 4 h.

Cross-linking. Transcription reactions were cooled, placed as drops on a plastic surface resting on ice, and exposed to UV irradiation. The UV source was a germicidal lamp (Atlantic Ultraviolet G15T8) set at a distance of 10 cm from the sample. Its output at this range was 1.1 mW/cm^2 at 254 nm. Routinely, the samples were irradiated for 60 min.

Reconstitution assay. Ribonucleoprotein (RNP) particles containing the La antigen and VA RNA were reconstituted as described previously (5). Transcripts were labeled with UTP for 4 h in a 100-μl reaction containing 50 μl of HeLa cell extract. After phenol extraction, chloroform extraction, and ethanol precipitation, the RNA was incubated with 50 μl of HeLa cell extract–50 μl of buffer C (5)–2.5 mM supplementary $MgCl_2$ for 20 min at 20°C.

Immunoprecipitation. The complex between VA RNA and the La antigen was isolated by reaction with monospecific anti-La antibodies (from patient R serum provided by R. Bernstein) and adsorption of immune complexes to protein A on the surface of *Staphylococcus aureus* cells. Details are given by Francoeur and Mathews (5).

Gel procedures. For analysis in 15 or 20% sodium dodecyl sulfate (SDS)-polyacrylamide gels (8), the washed immunoprecipitate was suspended in gel sample buffer, heated to 100°C, and loaded directly; samples of the supernatant from the immunoprecipitation or of the unfractionated reaction products were treated the same way. For analysis in high-resolution urea-polyacrylamide gels (18), RNA was dissociated from the immunoprecipitate by heating for 1 min at 100°C in the presence of sodium dodecyl sulfate and was isolated by phenol and chloroform extraction, followed by ethanol precipitation.

RNA analysis. RNA and RNP species were eluted from gel pieces by the crush-and-soak method (13), and fingerprints were prepared by two-dimensional separation of RNase T_1 digests (1). The first dimension, shown in our figures as running from left to right, was electrophoresis in urea-pH 3.5 buffer on cellulose acetate paper (Schleicher & Schuell). The second dimension, shown bottom to top, was homochromatography with homomix C on DEAE-cellulose thin-layer plates (Brinkmann Instruments). Spots were identified by redigestion with pancreatic RNase or alkali (1). Structures of 3′-terminal oligonucleotides were deduced from the Cp:Up ratio after alkaline hydrolysis.

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RESULTS

RNAs with longer U-rich tails selected by La antigen. The La antigen, a cellular phosphoprotein of M_r 46,000, interacts with VA RNA synthesized de novo in a HeLa cell extract containing RNA polymerase III activity (5). Fixed proportions of VA RNA_I and VA RNA_{II} bound the antigen and became precipitable with anti-La antibody (Fig. 1). The percent bound differed between the two RNA species but did not alter during the course of the incubation. The unbound material did not become precipitable upon addition of more antigen (data not shown), implying the existence of two populations of VA RNA molecules. Comparison of the La-bound and unbound RNA, separated by immunoprecipitation with anti-La antibody, showed that the bound material migrated more slowly than the unbound fraction in high-resolution polyacrylamide-urea gels (Fig. 2). Mixing the two fractions regenerated the original pattern of gel bands, indicating that the difference in mobility is not artefactual. This finding suggested that the antigen interacts preferentially with longer VA RNA molecules.

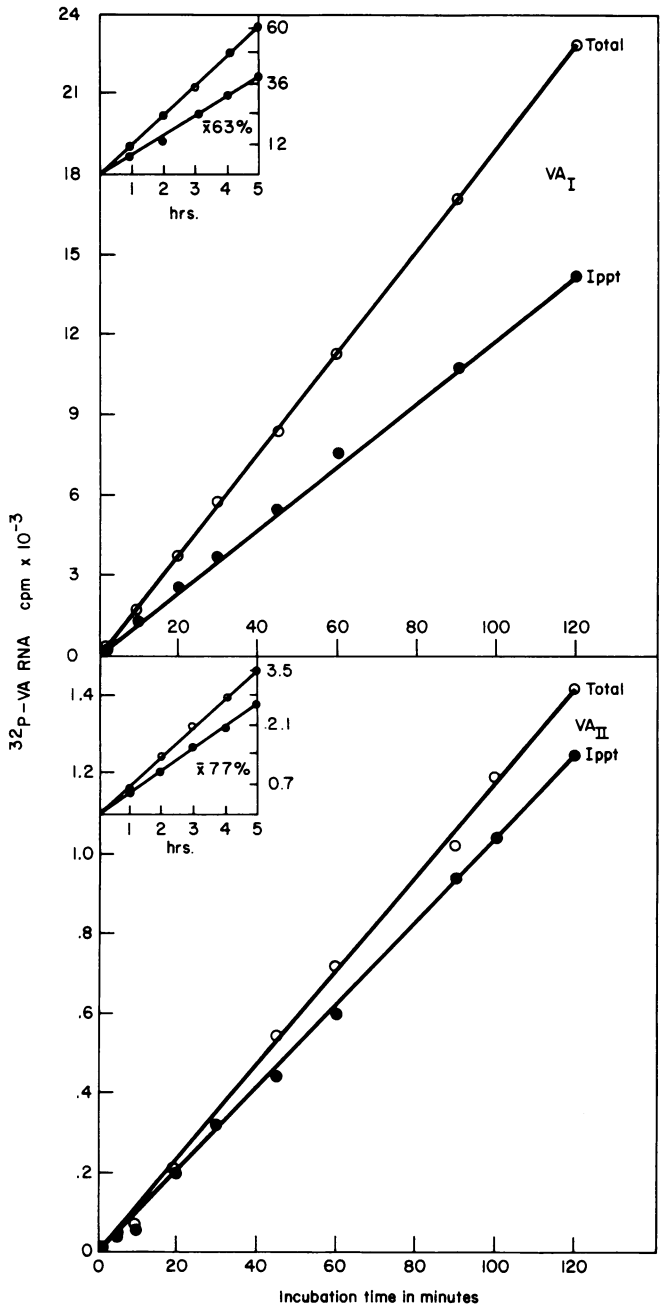

FIG. 1. Kinetics of RNP formation. Portions of transcription reactions synthesizing VA RNA_I (upper panel) or VA RNA_{II} (lower panel) were removed at the times shown. The amounts of VA RNA made (Total, ○) and of VA RNA bound to the La antigen (Ippt, ●) were estimated by gel electrophoresis, followed by counting of the radioactivity in gel slices. The percent precipitability of each RNA, x̄, is shown in the insert panels.

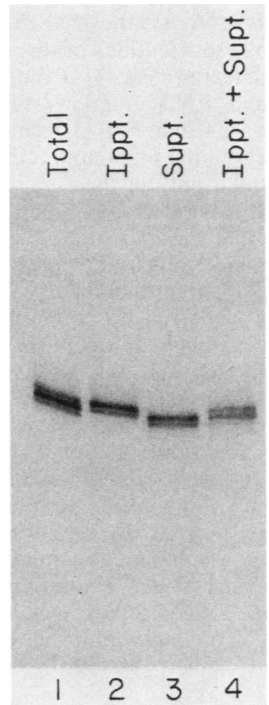

FIG. 2. Size analysis of La-bound and free VA RNA_I. A portion of a transcription reaction was fractionated by immunoprecipitation with anti-La antibody. RNA was isolated and examined by electrophoresis through a urea-polyacrylamide gel. Lanes: 1, unfractionated RNA (Total); 2, La-bound RNA (Ippt.); 3, free RNA (Supt.); 4, a mixture of bound and free RNA.

VA RNA_I is heterogeneous at both its 5′ and 3′ termini (2, 3, 23). Previous work has indicated that the "G-start" form of the RNA was precipitable by anti-La antibody (5), so the 5′ trinucleotide extension pppAGC found on the "A-start" form is not a prerequisite for La antigen binding. Subsequent experiments showed that molecules terminating with 5′ tri-, di-, and monophosphate G residues were all present in the immunoprecipitate and that complete dephosphorylation of the 5′ end by alkaline phosphatase failed to diminish precipitability (unpublished data). Furthermore, digestion of the RNA from its 5′ end by spleen phosphodiesterase gave rise to shorter molecules, many of which were able to combine with the La antigen, whereas binding was obliterated by digestion from the 3′ end with venom phosphodiesterase (data not shown). These results focused attention on the 3′ terminus.

The 3′-terminal oligonucleotide of VA RNA_I released by RNase T_1 digestion has the structure $CUCCU_n$-OH, where n can vary from 1 to 6 but is commonly 2 or 3 (2). In

fingerprints of VA RNA_I synthesized in vitro and labeled with [α-^{32}P]UTP, oligonucleotides of this structure and with n = 1 to 4 were detected (Fig. 3). Comparison of the La-bound and unbound RNA fractions revealed a marked distinction. The n = 4 and n = 3 forms were recovered almost quantitatively in the immunoprecipitate, whereas the n = 1 form was found only in the supernatant. The n = 2 form was present in both RNA fractions and was the predominant form in the supernatant. Taking into account the relative amounts of RNA in the precipitate and supernatant (ca. 1.7:1), it is apparent that the n = 2 form was divided roughly equally between the La-bound and unbound fractions. Very similar results were obtained when the complex between the La antigen and VA RNA was reconstituted from deproteinized RNA in the absence of transcription. The La-bound RNA again moved more slowly in gels than did unbound VA RNA (data not shown), and fingerprints demonstrated that the antigen preferentially interacts with molecules containing longer oligouridylate tracts (Fig. 4).

Covalent complex between the La antigen and VA RNA. Although alternative explanations can readily be devised, the heavy bias toward longer 3′-terminal oligonucleotides suggested that the antigen recognizes the oligouridylate tracts themselves. To show that the protein is closely associated with the RNA 3′ terminus, we needed a means to cross-link the two species. van Eekelen et al. (22) found that VA RNA became covalently linked to a protein of ca. 50,000 daltons through UV irradiation. Exposure of our transcription reactions to UV light caused the appearance of a major radioactive band which migrated more slowly than VA RNA in an SDS-polyacrylamide gel (Fig. 5A). This material is identified as the La antigen-VA RNA complex by the experiments described below. Several minor RNP bands of lower mobility were also generated, but their origin has not been explored in detail.

The major RNP band (as well as the minor bands) disappeared upon incubation of the reaction mixture with pronase (Fig. 5B) or RNase A (Fig. 5C). Furthermore, the band failed to appear during irradiation of reaction mixtures lacking VA RNA (owing to omission of template; Fig. 5D) or lacking the La antigen (removed by immunoaffinity chromatography; Fig. 6A). These results demonstrate that the band is RNP in nature and suggest that it is composed of VA RNA and the La antigen. As expected for an RNP complex, the material partitioned into the organic layer during phenol extraction (Fig. 6C), and it was fully precipitable by anti-La antibody (Fig. 6B), showing that the covalently attached protein is indeed the La antigen. After degradation of the RNA compo-

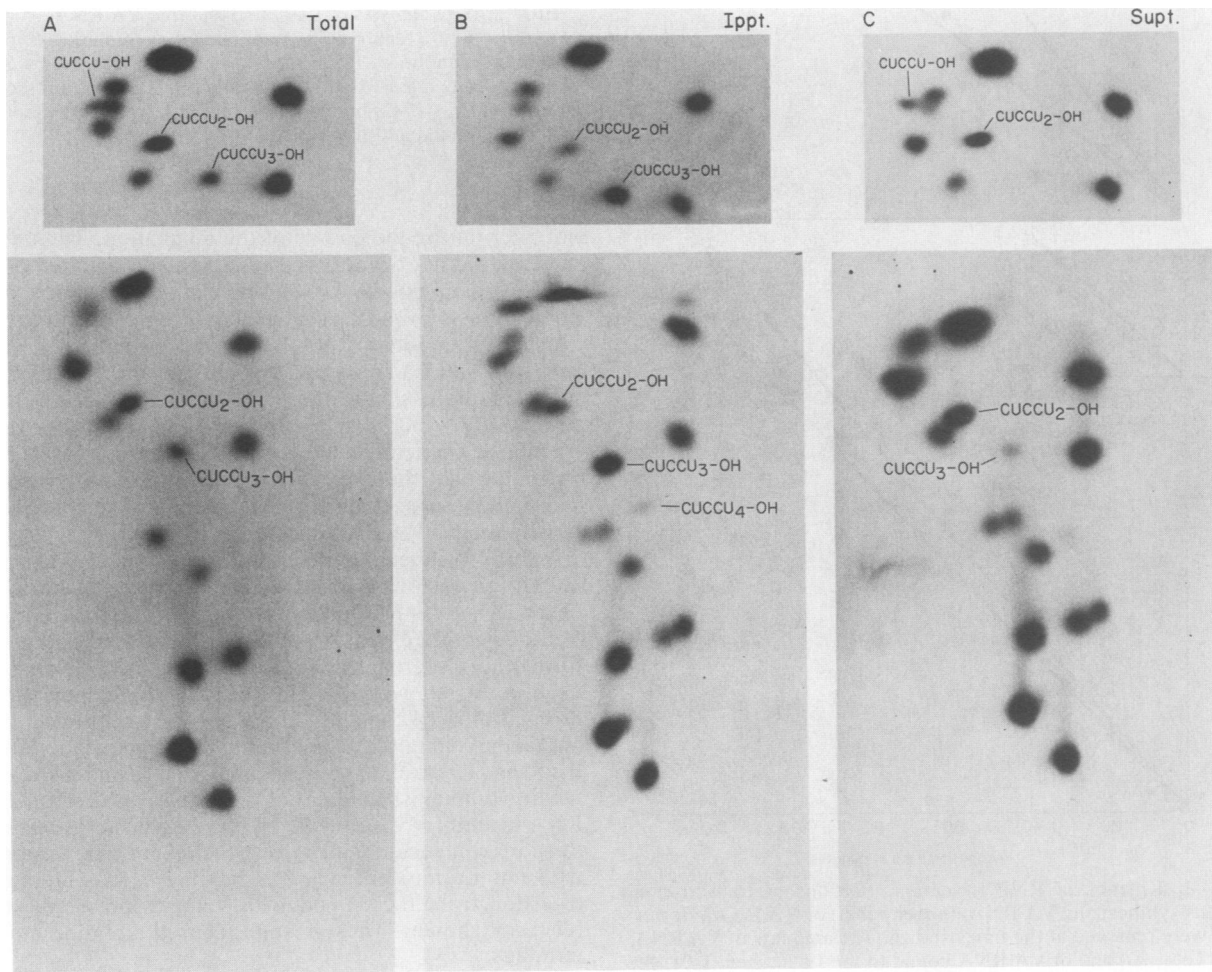

FIG. 3. Fingerprints of La-bound and free VA RNA. VA RNA_I was labeled with UTP, fractionated by immunoprecipitation, purified by gel electrophoresis, digested with RNase T_1, and fingerprinted. Lower panels show complete fingerprints; upper panels show a detail from separate fingerprints with better resolution of 3′-end spots. (A) unfractionated RNA (Total), (B) La-bound RNA (Ippt.), (C) free RNA (Supt.).

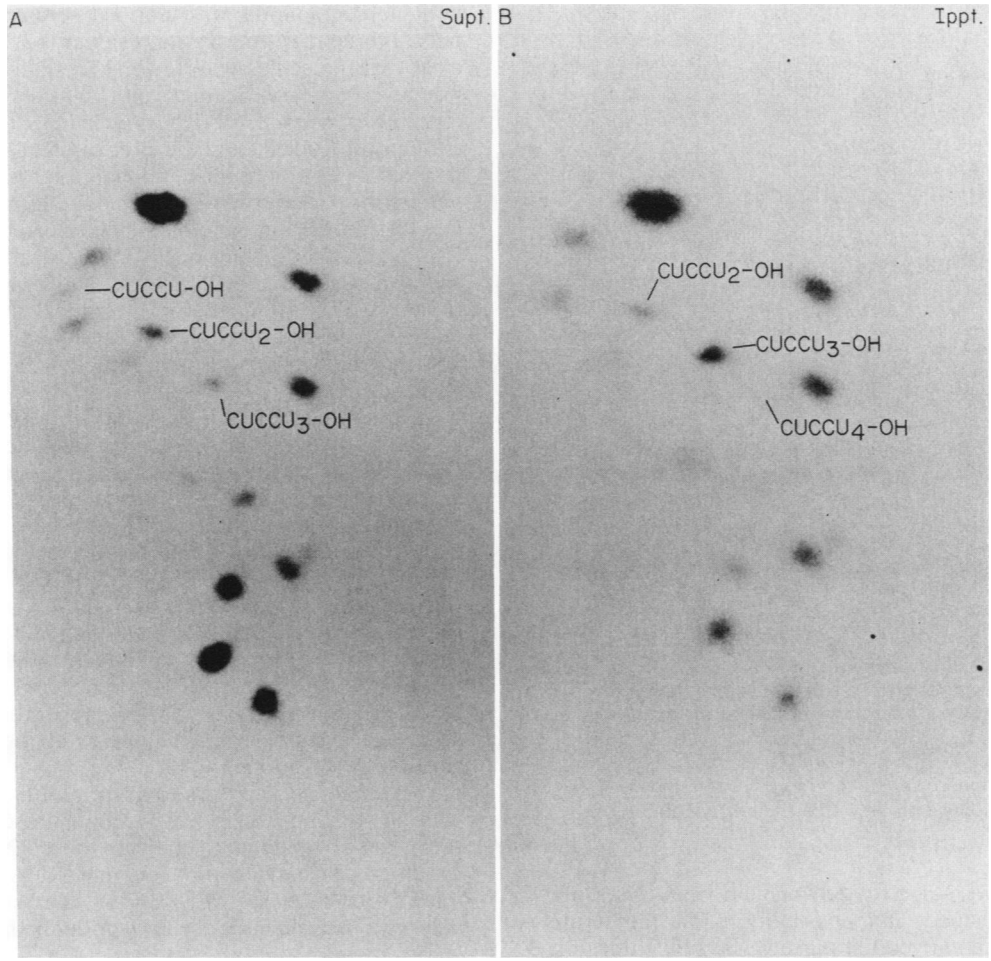

FIG. 4. Reassociation of VA RNA with the La antigen. VA RNA_I labeled with UTP was deproteinized and then incubated with a source of La antigen. The reconstituted RNP complexes were precipitated with anti-La antibody, and RNA fingerprints were prepared. (A) Unbound RNA (Supt.), (B) La-bound RNA (Ippt.).

nent of the complex by RNase A or T_1, the protein had an apparent M_r slightly higher than that of the La antigen, as would be expected if a small RNA fragment remained attached (data not shown). Likewise, the RNA liberated by pronase treatment migrated slightly slower that naked VA RNA, presumably because of a covalently bound peptide residue (Fig. 6D). These data establish that irradiation of the transcription reaction leads to formation of a covalent VA-RNP complex containing the La antigen.

La antigen cross-linked to 3′ end of VA RNA. To locate the protein-binding site on the RNA, we examined the fingerprint of the VA RNA present in the RNP complex. When labeled with [α-^{32}P]GTP, which fails to label the 3′-terminal oligonucleotides, its fingerprint was indistinguishable from that of the control (data not shown). However, when labeled with [α-^{32}P]UTP, the 3′-terminal spots were conspicuously missing from the VA-RNP fingerprint (Fig. 7B). These spots were also missing when the RNP was subjected to pronase digestion and phenol extraction before fingerprinting (data not shown). The fingerprint of UV-irradiated VA RNA isolated from the immunoprecipitate but not cross-linked to protein (Fig. 7A) contained the expected 3′-end spots (chiefly $CUCCU_3$-OH) and was indistinguishable from that of immunoprecipitated VA RNA that had not been irradiated (data not shown), indicating that UV light alone had not caused the disappearance of the 3′-end spots from the fingerprint. Thus the antigen binds the oligonucleotide structure at the 3′ end of VA RNA_I.

DISCUSSION

We have shown that the La antigen selectively binds forms of VA RNA carrying at least two 3′-terminal uridylate residues. Molecules with three or four such residues are very efficiently bound. Furthermore, after UV irradiation the protein is attached to the 3′ terminus of the RNA, which typically has the structure $CUCCU_3$-OH. Although the 3′ terminus clearly constitutes the recognition site, we do not know which of these bases is linked to the protein or, indeed, whether attachment is to a nearby location such as the neighboring G residue. Nevertheless, the data show that the protein is in intimate contact with the 3′ end of molecules carrying a minimum of two terminal U residues. Most likely the antigen is bound to the U-rich tail itself.

These results extend our previous conclusion that the La antigen recognizes a constant feature of the termini of RNA polymerase III products (5). In particular, they are fully consistent with our earlier finding that a 22- to 26-nucleotide fragment from the 3′ end of VA RNA_I can bind the antigen.

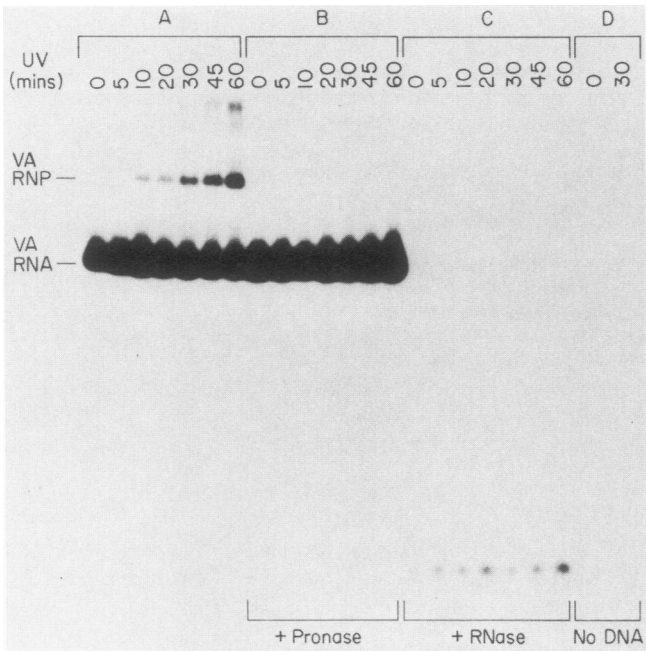

FIG. 5. Kinetics of cross-linking of VA RNA_I to the La antigen. After transcription, portions of a reaction were exposed to UV light for the periods shown. Aliquots were heated in sample buffer and examined directly in an SDS-polyacrylamide gel (A). Further aliquots were digested with pronase (B) or pancreatic RNase (C) before gel electrophoresis. In the absence of template DNA, no bands appeared either with or without UV irradiation (D).

The binding of a 13-nucleotide fragment from the 5′ end is less readily explained. One possibility is that this terminus interacts with the antigen but more weakly than does the 3′ end. In connection with this, it should be noted that we have not proved here that the 3′ end is the only site of La antigen binding. Alternatively, as discussed previously, it is possible that the 5′ end pairs with the 3′ end or with some other unlabeled RNA in the extract and interacts with the La antigen only indirectly.

The preference for 3′-terminal runs of U residues also provides an explanation for the low efficiency of VA RNA precipitation from infected cell extracts and *Xenopus* oocytes (F. A. M. Asselbergs, A. M. Francoeur, and M. B. Mathews, manuscript in preparation). In both of these cases, less than 5% of the RNA is bound to the La antigen, compared with over 50% for transcripts synthesized in vitro. This distinction is paralleled by the relative efficiencies with which these molecules reform RNP complexes after deproteinization, showing that it is a property of the RNAs themselves. In vivo the predominant forms of the 3′ end of VA RNA_I molecules carry only one or two terminal U residues and are, therefore, too short to bind the antigen efficiently.

The length of the oligo U tail may play an important part in regulating the interaction between polymerase III products and the La antigen in a variety of situations. This factor may be largely responsible for the differential efficiencies seen with VA RNA_I, VA RNA_{II}, and other derivatives (5). Differences in the number of terminal U residues may also explain variations among cell types in the efficiency of 7S RNA precipitation by anti-La antibody (4). Findings in other systems are consistent with this idea. First, the Epstein-Barr virus RNAs EBER I and II purified from cell extracts by immunoprecipitation with anti-La serum have 3′-terminal tails of three to five uridylate residues (17), although it is not clear that molecules with fewer U residues exist in the cell free of La antigen. Second, anti-La antibody precipitates a form of ribosomal 5S RNA called 5S* from HeLa cell extracts or nuclei (16): the precursor has three to five U residues at its 3′ terminus, compared to two or three for the mature form, and trimming of the 3′ end is accompanied by loss of antibody precipitability. Third, precursors to tRNAs (16), and possibly other newly synthesized small RNAs (6), are also associated with the La antigen, whereas mature tRNAs are not. Removal of 3′-terminal runs of U residues during processing may therefore account for the transient association of the antigen observed with several types of RNA.

The identification of a cross-linked RNP particle containing the La antigen and VA RNA strengthens the identification of the antigen made previously (5). The data presented here make it very likely that the cellular polypeptide studied by van Eekelen et al. (22) is the La antigen. Their protein had a molecular weight of about 50,000, bound VA RNA, and could be cross-linked to VA RNA in UV light, although it was not proven to interact with anti-La antibodies and its site of binding to the RNA was not determined. Further studies have shown that the antigen exists in a number of phosphorylated forms (A. M. Francoeur, J. I. Garrels, and M. B. Mathews, manuscript in preparation) and that the degree of phosphorylation appears to be increased after adenovirus infection (14).

The La antigen appears to associate, at least transiently, with all RNAs synthesized by polymerase III. Translation seems to be a common theme with these RNAs: 5S RNA is a structural ribosomal component and tRNA plays an adaptor role. More recently, VA RNA_I has been implicated in polypeptide chain initiation (21), possibly through its interac-

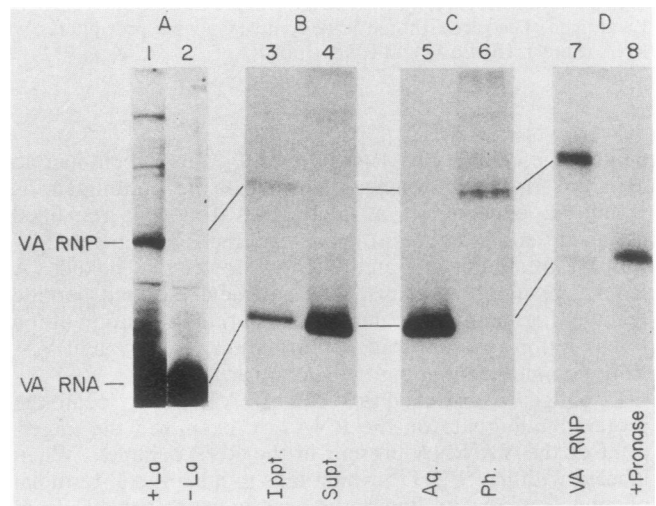

FIG. 6. Properties of cross-linked VA RNP complex. (A) A standard reaction (lane 1) and a reaction from which the La antigen had been removed by immunoadsorption (5) before transcription (lane 2) were irradiated and displayed in an SDS-polyacrylamide gel. (B and C) After transcription and UV irradiation, the products of a standard reaction were fractionated by immunoprecipitation with anti-La antibody, giving an immunoprecipitate (lane 3) and supernatant (lane 4), or by phenol extraction, giving aqueous (lane 5) and phenol (lane 6) phases. (D) the cross-linked RNP complex eluted from a gel was rerun without further treatment (lane 7) or after pronase digestion (lane 8).

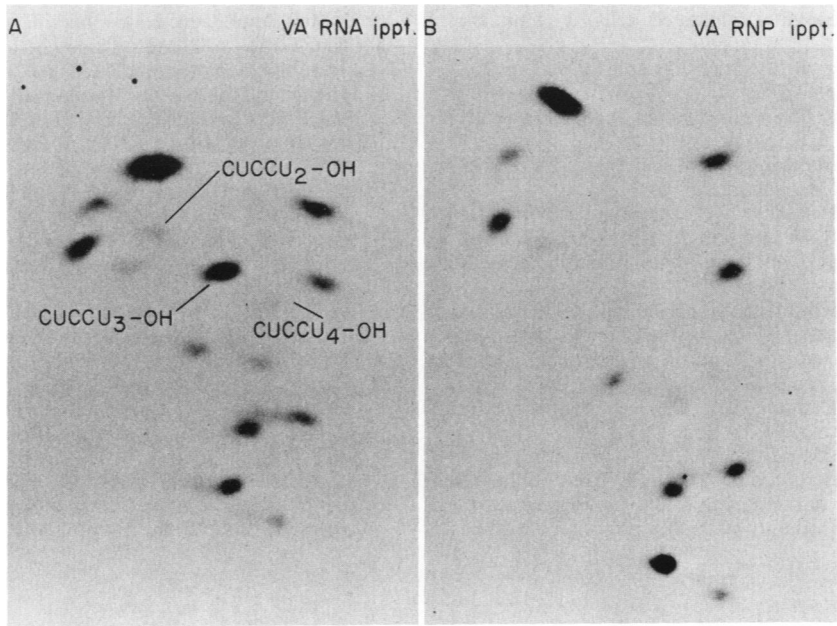

FIG. 7. Fingerprint of cross-linked VA RNA. VA RNA_I was labeled with UTP, cross-linked by UV irradiation, and immunoprecipitated with anti-La antibody. After electrophoresis in an SDS-polyacrylamide gel, the RNA (A) and RNP (B) fractions were eluted and fingerprinted.

tion with mRNA (12). The 7S RNA component of the signal recognition particle is also involved in protein synthesis (24) and binds the La antigen at least in some cell lines (4). However, other cases are less clear-cut. Hamster 4.5S RNA binds to the La antigen (6) and is made by polymerase III, but its role is unknown, although it does associate with mRNA and heterogeneous nuclear RNA (7). Similarly, the Ro RNAs are probably made by this enzyme and interact transiently with the La antigen (6), although their functions are unknown. The diversity of these RNAs makes it seem unlikely that the La antigen is important in their functioning. Rather, a role in their synthesis, processing, transport, or degradation seems more plausible. Transcription, at least in vitro, proceeds in the absence of the antigen, and deproteinized RNA can bind the antigen independently of transcription (5) with the same specificity for 3'-terminal runs of U residues (Fig. 4). These results argue against a role in RNA synthesis. Similarly the interaction of the La antigen with precursor forms of RNA would argue against involvement in degradation. The processing and transport of RNA remain as likely possibilities. We conjecture that the La antigen may play a role in one or both of these processes and that recognition of the oligouridylate stretch at the 3' terminus of RNA polymerase III products is an important feature of this function.

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