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Pathway of B1-*Alu* Expression in Microinjected Oocytes: *Xenopus laevis* Proteins Associated with Nuclear Precursor and Processed Cytoplasmic RNAs

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We have previously characterized *B1-Alu* gene expression by microinjected *Xenopus laevis* oocytes. The transcription, endonucleolytic processing and its kinetics, nuclear transport kinetics, and subsequent cellular compartmentalization have been described previously (Adeniyi-Jones and Zasloff, Nature 317:81–84, 1985). Briefly, a *B1-Alu* gene is transcribed by RNA polymerase III to a 210-nucleotide (210nt) primary transcript which is processed to yield 135nt and 75nt RNAs. After processing, the 135nt RNA enters the cytoplasmic compartment, where it remains stable, while the 75nt RNA is degraded. In this report we characterize this pathway further and show that the RNAs involved are complexed with specific *X. laevis* proteins. The primary transcript was associated with an *X. laevis* protein of 63 kilodaltons (p63) as well as La, a protein known to be associated with RNA polymerase III transcripts. After processing, the cytoplasmic 135nt RNA remained associated only with the *X. laevis* p63 in the form of a small ribonucleoprotein. Human autoimmune antibodies were purified by affinity chromatography to *X. laevis* p63 and used to immunoprecipitate human ribonucleoprotein containing a 63-kilodalton polypeptide and small RNAs. These data suggest that *Alu*-analogous ribonucleoproteins and their metabolic pathways are conserved across species and provide insight as to their possible functions.

The *Alu* sequence represents an RNA polymerase III (pol III)-dependent gene which constitutes the most abundant family of middle repetitive sequences and provides a substantial portion of the mammalian genome (27). Although, as originally noted by Britten and Davidson, such sequences might be likely candidates in the regulation of coordinated genetic programs (5), little is known about their expression in vivo or their cellular function.

We recently described a pathway involving transcription, nuclear processing, and transport of a *B1*-type *Alu* RNA from within the first intron of the mouse α-fetoprotein (AFP) gene (39) with the *Xenopus laevis* oocyte microinjection system (2). This *Alu* gene lies in an antisense orientation to the AFP sequence and is transcribed by RNA pol III to a 210-nucleotide (210nt) primary transcript (2). The RNA is then processed by a 3′ endonucleolytic event to yield a 135nt species which becomes a stable cytoplasmic RNA (see diagram in Fig. 8). The 135nt 5′ RNA corresponds to the consensus "core *B1-Alu*" sequence, whereas the 75nt 3′ RNA is derived from a nonconserved "trailer" region (2). By using *Alu*-specific probes, a distinct 3′ processed 135nt RNA was detected in RNA from fetal mouse liver and the cytoplasm of a cell line that expresses AFP. This RNA was found to be approximately 10 times more abundant in fetal liver than in other fetal or adult mouse tissues. These observations suggested the possibility that the small RNA in AFP-producing cells was derived from the *B1-Alu* sequence located within the AFP gene and furthermore that this could represent a coordinated system, since high-level expression of the processed pol III product was concordant with expression of the pol II product, AFP (2).

It is now well accepted that most small RNAs exist in eucaryotic cells as ribonucleoprotein (RNP) complexes and that certain human autoimmune sera have provided powerful means of probing the structure and function of these (9, 10, 12, 17–20, 26, 35, 36). Two well-characterized sera, anti-La and anti-Ro, recognize pol III-derived RNAs. The La protein binds at least transiently to all primary transcripts of pol III, while Ro binds to a cytoplasmic subset of these (12). Knowing that the *X. laevis* oocyte faithfully and efficiently transcribes and processes the *B1* gene and that the microinjection system lends itself well to nuclear and cytoplasmic separation (23, 40), we have begun to dissect the *B1-Alu* expression pathway with the help of autoimmune sera. After screening several sera (approximately 15), we identified one, which we refer to as anti-Be, that specifically precipitates the *B1-Alu* made in the oocyte. Using this serum, which recognizes *Xenopus* antigens, we show that the primary *B1-Alu* RNA is associated with at least two *Xenopus* proteins: a 63-kilodalton (kDa) protein (p63) and La. In contrast, the processed RNA is associated with p63 but not with La. Antibodies to p63 were affinity purified and used to immunoprecipitate human RNP containing a protein of approximately 63 kDa and small RNAs. These studies describe a unique cellular pathway of expression of an *Alu* sequence involving specific protein interactions and provide insight as to their possible functions.

MATERIALS AND METHODS

Sera. Anti-Ro, anti-La, anti-Sm, and anti-RNP were obtained as reference sera from the Centers for Disease Control, Atlanta, Ga. The anti-La serum used for the immunoblot in Fig. 3B was kindly provided by James Stefano; this serum is designated Y356 (31). Anti-Be is the serum from a patient with systemic lupus erythematosus under the care of the Arthritis and Rheumatism Branch at the National Institutes of Health, Bethesda, Md.

Oocyte injections and immunoprecipitation. Oocytes were

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coinjected with plasmid DNA, as indicated in the appropriate figure legends, and [α-^{32}P]GTP as described previously (2). At various times thereafter, as specified in the appropriate figure legends, they were homogenized in a buffer containing 150 mM NaCl and 50 mM Tris hydrochloride (pH 7.6) (NT buffer), and the supernatant was collected after centrifugation at 2,000 × *g* for 15 min. Antisera or affinity-purified antibody were incubated with protein A-Sepharose (Pharmacia), and the beads were washed and added to 50 μl of oocyte extract. The suspension was incubated at room temperature for 1 h, washed three times with NT buffer and resuspended in NT buffer containing proteinase K (1 mg/ml), sodium dodecyl sulfate (SDS) (1%), and 20 μg of carrier tRNA. The nucleic acids were extracted and analyzed by polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels containing 8 M urea as described previously (2).

Immunoblot of *Xenopus* and HeLa proteins. To each lane of a 10% Laemmli gel (16), 300 μg of soluble (S-100) mature *Xenopus* ovary or HeLa cell extract was applied. Immunoblotting has been described previously (6). After electrophoresis, the proteins were transferred onto nitrocellulose in 25 mM Tris (pH 8.3)–192 mM glycine containing 20% methanol by using a Bio-Rad transblot cell at 80 mA overnight. The nitrocellulose was preincubated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and subsequently incubated with 1:10 dilutions of sera or anti-La serum Y356, which was used at a 1:50 dilution. The filter was then washed once for 15 min in PBS, twice for 15 min each in PBS containing 0.05% Nonidet P40 (NP-40), and once with PBS. After washing, the nitrocellulose was incubated with 0.1 μCi of ^{125}I-protein A (30 mCi/mg; Amersham) per ml in PBS containing 5% BSA. After the washing protocol was repeated, the filter was exposed to Kodak XAR film overnight at −70°C.

Preparation and characterization of affinity-purified monospecific antiserum. Affinity purification of antibodies was done by the previously described method of Olmstead (24). A preparative 10% polyacrylamide–SDS Laemmli gel (12 cm by 15 cm × 1.5 mm) was loaded with 3 to 5 mg of *Xenopus* oocyte S-100, subjected to electrophoresis, and transferred onto nitrocellulose as described above for immunoblotting. Vertical strips from the edges of the nitrocellulose were incubated with anti-Be and ^{125}I-protein A as described above. The resulting band pattern was used to locate the strip of nitrocellulose corresponding to the 63,000-molecular-weight (63K) band. This strip was incubated with anti-Be serum for 2 h at room temperature, washed for 15 min with PBS, washed twice for 15 min each with PBS containing 0.05% NP-40, and washed again for 15 min with PBS. Bound immunoglobin was eluted from the nitrocellulose with 0.2 M glycine, pH 2.8, for 5 min, immediately neutralized to pH 7.5 with 1.5 M Tris hydrochloride (pH 8.8), dialyzed against PBS, and used for immunoblot or immunoprecipitations. Antibodies eluted from other regions of the preparative blot which did not contain the 63-kDa band were similarly prepared for use as controls.

Sedimentation velocity analyses of RNP structures. Oocytes were injected with the *B1-AFP* minigene plasmid (39) and [α-^{32}P]GTP and dissected in J buffer as described previously (2). Fractions dispersed in J buffer were cleared of insoluble material by centrifugation at 10,000 × *g* for 5 min. The supernatants were then layered onto 15 to 30% sucrose gradients containing 50 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol and centrifuged at 275,000 × *g* for 19 h in an SW41 rotor (Beckman); 0.5-ml fractions were collected, and the nucleic acids were extracted from each fraction following addition of yeast carrier tRNA (30 μg) as described previously (2). The samples were then electrophoresed through 10% polyacrylamide gels containing 8 M urea. *Escherichia coli* rRNA and tRNA (Sigma) provided sedimentation markers.

Immunoprecipitation of [^{35}S]methionine-labeled KB cell extract. KB cells were obtained from the American Type Culture Collection (Rockville, Md.) and grown in monolayers to approximately 60% confluency. The monolayers were washed with methionine-free medium and labeled with 180 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.; 1,800 Ci/mmol) per ml. The labeling medium was one part Dulbecco modified Eagle medium (DMEM; Gibco) with methionine and containing 5% fetal bovine serum to 5 parts DMEM without methionine for 14 h. (We find that in order to achieve high specific labeling of the 63K protein, the cells must increase in number by at least 50% during labeling.) The cells were washed three times with PBS and solubilized with buffer SB (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.5% NP-40, 0.02% SDS, 1 mM phenylmethanesulfonyl fluoride [Sigma]) by Dounce homogenization at 4°C. The homogenate was centrifuged at 145,000 × *g* in a Beckman 65 rotor for 2 h, and the supernatant used for immunoprecipitation. A 200-μl amount of a 10% suspension of protein A-Sepharose (Pharmacia) in SB was added to 20 μl of serum or antibody and incubated at 4°C for 1 h and then washed three times with SB. To the pellet, 500 μl of KB cell supernatant was added, incubated for 1 h at 4°C, and then washed three times with SB buffer. An equal volume of 2× Laemmli sample buffer (16) was added, mixed, and heated at 100°C for 2 min, and the beads were centrifuged out. The proteins were analyzed on a 10% polyacrylamide Laemmli gel. After electrophoresis, the gel was fixed, treated with En^{3}Hance, and autoradiographed overnight.

Immunoprecipitation of KB cell [^{32}P]RNA. KB cells were grown in monolayers and labeled with $^{32}P_i$ as described before (19). The cells were harvested and homogenized in a buffer containing 10 mM Tris hydrochloride (pH 7.6), 10 mM NaCl, and 0.05% NP-40. The postnuclear supernatants were obtained by centrifugation at 20,000 × *g*, and RNP species were immunoprecipitated as described above.

Partial proteolysis of ^{35}S-labeled KB cell immunoprecipitates. Immunoprecipitates were digested with the *Staphylococcus aureus* V8 protease (Boehringer Mannheim) by a modification of a procedure described by Cleveland et al. (7). Immunoprecipitates of ^{35}S-labeled KB cell extract described above were incubated with V8 protease in V8 buffer (7) containing 0.1% SDS at 37°C for 30 min with gentle shaking. The reaction was stopped by the addition of SDS and β-mercaptoethanol and boiling (7). The samples were analyzed on 15% polyacrylamide Laemmli gels by electrophoresis, treatment with En^{3}Hance, and autoradiography. To compare anti-Ro and anti-p63 immunoprecipitates, a V8 concentration of 5 μg/ml was used. The immunoglobulin G (IgG) concentration of anti-p63 is about 100 times less than that of the anti-Ro serum (data not shown). Therefore, after the final washing of the anti-p63 precipitate and just prior to the addition of V8 protease, the protein concentration of the anti-p63 reaction mix was adjusted by the addition of human IgG (Sigma). All reactions were carried out in 50-μl volumes.

RESULTS

Novel autoimmune serum immunoprecipitates both the *B1-Alu* primary transcript and the processed cytoplasmic form.

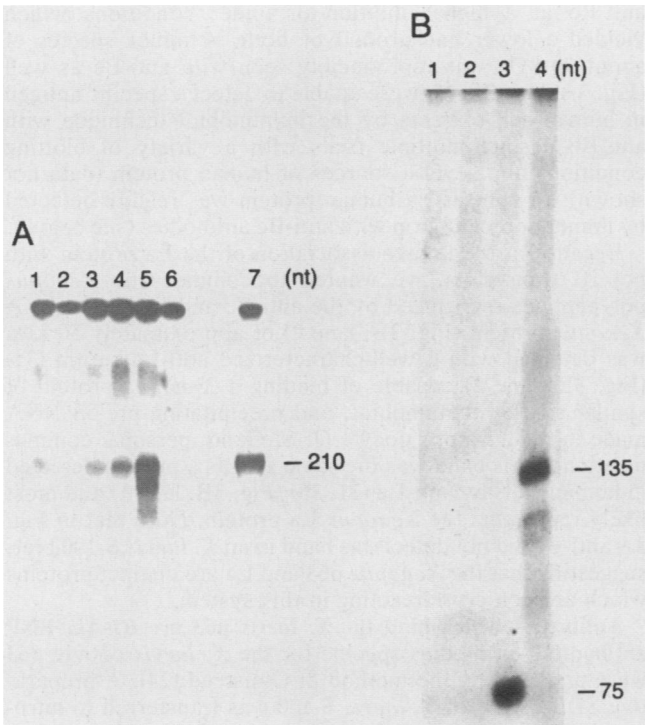

FIG. 1. Immunoprecipitation of *B1-Alu* RNA transcripts generated in microinjected *X. laevis* oocytes. (A) 210nt primary transcript of the *B1-Alu* gene. Pooled oocytes were immunoprecipitated 70 min after injection of the AFP minigene. Lanes: 1, anti-RNP; 2, anti-Sm; 3 and 4, two different anti-La sera; 5, anti-Be; 6, normal serum; 7, ^{32}P-labeled *B1* 210nt RNA, serving as species marker. (B) Processed 135nt RNA from the cytoplasmic fraction. Nuclei and cyctoplasm were separated and RNA was immunoprecipitated from the cytoplasmic fraction 24 h after gene injection. Lanes: 1, anti-RNP; 2, anti-Sm; 3, anti-La; 4, anti-Be.

Figure 1A shows in vivo ^{32}P-labeled RNAs extracted from immunoprecipitates 70 min after injection of the *B1-Alu*-containing AFP minigene (39) into the nucleus of the *Xenopus* oocyte. The predominant RNA at this time is the 210nt primary *B1-Alu* transcript (2), and it was strongly immunoprecipitated from the microinjected oocyte by the autoimmune serum obtained from a patient with systemic lupus erythematosus (lane 5). We discovered this specificity in only one of several autoimmune sera tested for the ability to specifically precipitate the *B1-Alu* transcript. We refer to this serum as anti-Be. Anti-RNP (lane 1) and two different anti-La sera (lanes 3 and 4) reproducibly immunoprecipitated this transcript less efficiently. The transcript was essentially unrecognized by anti-Sm (lane 2) and normal human serum (lane 6). Lane 7 provides a size marker showing ^{32}P-labeled RNA following injection of the minigene and RNA extraction without immunoprecipitation.

It was previously shown that the primary *B1-Alu* transcript undergoes intranuclear processing to generate a 135nt species which appears in the cytoplasm within 1 h after introduction of the precursor into the nucleus (2). The 135nt RNA could be immunoprecipitated from a manually isolated nucleus after gene injection by anti-Be but not by anti-RNP, anti-La, or anti-Sm (data not shown).

Figure 1B shows immunoprecipitated [^{32}P]RNA from the cytoplasm of oocytes previously injected with the *B1-Alu-AFP* minigene into the nucleus. Anti-Be (lane 4) precipitated the 135nt species, whereas anti-RNP (lane 1) and anti-Sm (lane 2) did not precipitate an RNA from the cytoplasm. Anti-La serum immunoprecipitated the 3′ trailer sequence comprising the 75nt fragment (lane 3) generated by the endonucleolytic cleavage of the 210nt primary transcript described previously (2). Thus, both anti-La and anti-Be precipitated the primary transcript, but anti-Be precipitated the 5′ core and anti-La precipitated the 75nt trailer RNA.

These data suggest that (i) a determinant on or bound to the 5′ portion of the primary transcript which is recognized by anti-Be remains associated with the core-*Alu* RNA during nuclear processing and transport into the cytoplasm, (ii) the La determinant is on or bound to the 3′ portion of the primary transcript, a finding which appears to be characteristic of La-pol III primary transcript interactions (21, 31), and (iii) La and its associated trailer RNA are separated from the p63-core-*Alu* by the specific endonucleolytic cleavage described previously (2).

We should note that the slower-migrating band seen above the 210nt species in some of the autoimmune precipitates in Fig. 1 (lanes 4 and 5) has been found to be sensitive to α-amanitin (data not shown). A band with the same relative mobility has been detected on Northern (RNA) blots of *B1-Alu-AFP* minigene-microinjected oocytes with an AFP exon probe (data not shown). Therefore, we think that this larger species seen in Fig. 1 represents AFP minigene pre-mRNA based on its size. In contrast, this band did not coprecipitate with the *Alu* RNA from the cytoplasm (lane 4, Fig. 1B).

Anti-Be recognized *Xenopus* protein determinants (see below) rather than naked RNA structures. Following proteinase K digestion and phenol extraction, neither the primary *B1-Alu* transcript nor the 135nt RNA generated in the microinjected *X. laevis* oocyte were immunoprecipitated (data not shown).

Anti-Be determinant specific for *B1-Alu* RNA. In order to determine whether the anti-Be determinant was carried by other pol III gene products expressed in the oocyte, we attempted immunoprecipitation of other RNAs known to be pol III dependent. Figure 2 shows that while 5S RNA, adenovirus VA I RNA, and tRNA^{Met} were faithfully and efficiently expressed in the oocyte (lanes 7, 10, and 13) at levels comparable to those of the *B1-Alu* RNA (lane 4), none of them were precipitated by anti-Be (lanes 6, 9, and 12) or normal human serum (lanes 5, 8, and 11). Figure 2 also shows that the 135nt *B1-Alu* was not precipitated by anti-Ro (lane 2) or normal serum (lane 1). Thus, in the microinjection system, the interaction with anti-Be was specific for the *B1-Alu* RNA over the range of substrates tested. We should note that native stage VI oocytes yielded two anti-Be-specific RNAs which migrated between 5S and 5.8S in urea-polyacrylamide gels. These RNAs were not efficiently accumulated, however, and attempts are under way to characterize anti-Be-precipitable RNPs from early embryos.

Identification of *Xenopus* proteins recognized by anti-Be. A high-speed supernatant (S-100) of *X. laevis* ovary was analyzed by the immunoblot method of Burnette (6) with anti-Be serum. Antibodies present bound to a major band corresponding to a polypeptide with a molecular mass of about 63 kDa (p63) (Fig. 3A, lane 2), approximately the size reported for the mammalian Ro protein (35, 36). However, several anti-Ro sera, including a standard reference anti-Ro (Fig. 3A, lane 3), did not detect this band, nor did normal human serum (Fig. 3A, lane 5). As seen in Fig. 3A, the dilution of anti-Be that produced a good signal-to-noise ratio yielded a high background when used for anti-Ro serum. However, we never observed specific bands above the control when using

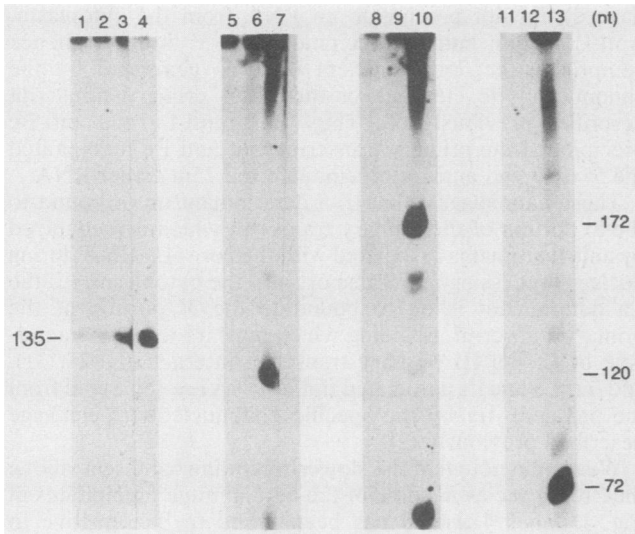

FIG. 2. Anti-Be specificity for the *B1-Alu* transcript: immunoprecipitations of microinjected oocytes. Lanes 1–4, *B1-Alu*: 1, normal serum; 2, anti-Ro; 3, anti-Be; 4, ^{32}P-labeled 135nt species transcribed in oocyte without immunoprecipitation. Lanes 5–7, *Xenopus borealis* 5S gene (pXbs) was injected (gift of Eric Ackerman): 5, normal serum; 6, anti-Be; 7, ^{32}P-labeled 5S RNA transcribed in oocyte without immunoprecipitation. Lanes 8–10, adenovirus type 2 DNA (Bethesda Research Laboratories) was injected: 8, normal serum; 9, anti-Be; 10, ^{32}P-labeled adenovirus VA I transcribed in oocyte without immunoprecipitation. Lanes 11–13, human tRNA^{Met} plasmid phH2D (40) was injected: 11, normal serum; 12, anti-Be; 13, ^{32}P-labeled tRNA^{Met} transcribed in oocyte without immunoprecipitation. To demonstrate equivalent amounts of each transcript present, an equal volume was removed from each oocyte extract prior to immunoprecipitations and the RNA was extracted and applied on control lanes 4, 7, 10, and 13.

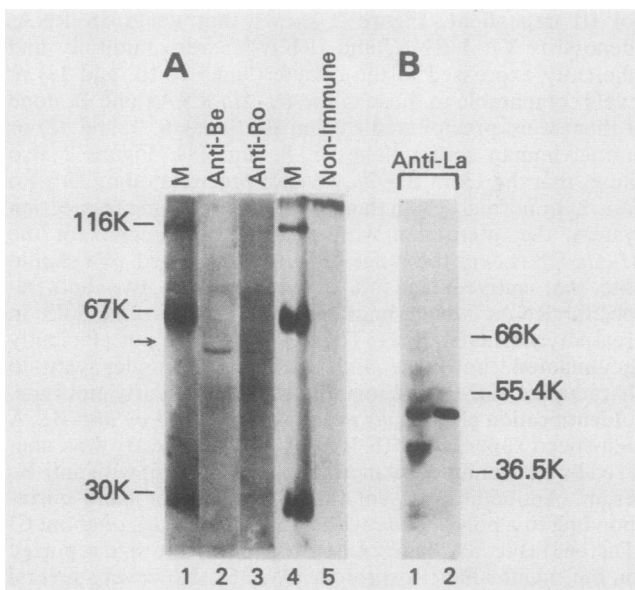

FIG. 3. Immunoblot of *Xenopus* proteins with autoimmune sera. Soluble (S-100) extract (300 μg) was applied to each lane of a 10% SDS gel and immunoblotted. (A) *X. laevis* ovary. Lane 2, anti-Be; lane 3, anti-Ro; lane 5, normal human serum. Lanes 1 and 4 are ^{125}I-labeled m.w. markers. (B) Anti-La immunoblot of HeLa (lane 1) and *X. laevis* ovary (lane 2).

anti-Ro at a higher dilution or under conditions which yielded a lower background or both. A minor species of about 90 kDa was reproducibly seen with anti-Be as well (Fig. 3A, lane 2). We were unable to detect a specific antigen in human cell extracts by the immunoblot technique with anti-Be despite multiple trials with a variety of blotting conditions and several sources of human protein (data not shown). In contrast, a human protein was readily detected by immunoprecipitation with anti-Be antibodies (see below).

Because of the known association of the La protein with pol III transcripts, we wanted to compare the *Xenopus* polypeptides recognized by the anti-Be and anti-La sera. A *Xenopus* protein (Fig. 3B, lane 2) of approximately 50 kDa was detected with a well-characterized anti-La serum (31) (Fig. 3B, lane 2) capable of binding a *Xenopus* protein of similar size by immunoblot, and precipitating pre-5S RNA made in the *Xenopus* oocyte (J. Steffano, personal communication). This band was the same size as a protein detected in human cells by anti-La (31, 35) (Fig. 3B, lane 1) and most likely represents the *Xenopus* La protein. Note that in Fig. 3A anti-Be did not detect this band in an *X. laevis* S-100 blot, suggesting that the *Xenopus* p63 and La are distinct proteins which are non-cross-reacting in this system.

Antibodies which bind the *X. laevis* p63 are *B1-Alu* RNP antibodies. Antibodies specific for the *X. laevis* oocyte p63 were prepared by the method of Olmstead (24). A preparative SDS-PAGE of *X. laevis* S-100 was transferred to nitrocellulose, the location of p63 was identified, and a narrow strip of nitrocellulose containing the p63 band was incubated with anti-Be serum. After extensive washing, the antibodies were eluted at low pH and immediately neutralized. Preparations made this way reproducibly bound only to the p63 region of immunoblots and did not bind to any of the minor bands recognized by the whole serum (Fig. 4A, lane 1). This affinity-purified antibody preparation, which we refer to as anti-p63, efficiently precipitated the 135nt *B1-Alu* RNA generated in the *X. laevis* oocyte, while antibody from normal serum did not (Fig. 4B). We conclude that p63 and the *B1-Alu* RNAs are in the form of RNPs in the oocyte which anti-p63 antibodies recognize.

***B1-Alu* RNA forms small RNP complexes in the oocyte.** To determine the size of the RNP complexes which contained the *Alu* RNAs, we performed velocity sedimentation analyses of the RNA species generated in *X. laevis* oocytes. After injection of the *B1-Alu* gene, nuclei and cytoplasm were analyzed separately on 15 to 30% sucrose gradients. The RNA was extracted from the resulting fractions and analyzed by denaturing gel electrophoresis. Analysis of nuclei (Fig. 5A) showed that most of the RNA corresponding to the 210nt primary transcript sedimented between 5S and 16S. Although there was some faster-sedimenting ^{32}P-labeled nucleic acid (>23S) in the nucleus as well (Fig. 5A), this represented high-molecular-weight (m.w.) species and not the 210nt or 135nt transcript. Figure 5B demonstrates that virtually all of the 135nt RNA existed in the *Xenopus* cytoplasm as slowly sedimenting species of approximately 7S, comparable in size to that found in nuclei. The gradient fractions which contained the 135nt RNA from both nucleus and cytoplasm were specifically immunoprecipitable by anti-Be (data not shown), demonstrating that they were in the form of small RNPs. Although the sucrose gradient sedimentation conditions used did not allow assignment of precise S values for the *B1-Alu* RNPs, it did localize them to the slowly sedimenting region apparently excluded from large complexes. Inclusion in small discrete RNPs is consistent with a specific *Alu* RNA-p63 interaction, as this decreases

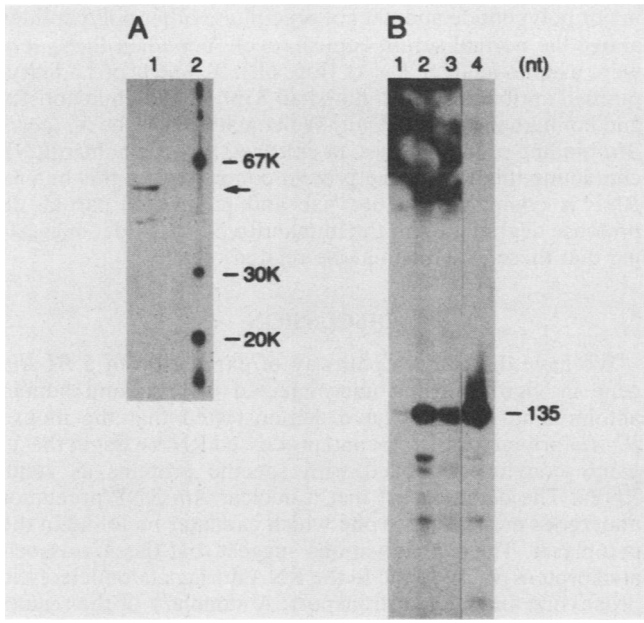

FIG. 4. Characterization of antibody affinity-purified to the 63-kDa *Xenopus* oocyte protein (anti-p63). Antibody bound only to the 63-kDa band was eluted by the method of Olmstead (24) and used to detect *Xenopus* protein and RNA following microinjection. (A) Immunoblot with anti-p63. Lane 1, *Xenopus* ovary; lane 2, m.w. markers. The arrow denotes the position of p63. (B) Immunoprecipitation of the *B1-Alu* RNA with anti-p63. Oocytes were injected with the AFP minigene plasmid and [α-^{32}P]GTP, incubated, and subsequently immunoprecipitated, and RNA was extracted. Lanes: 1, normal serum; 2, anti-Be serum; 3, affinity-purified anti-p63; 4, RNA extraction without immunoprecipitation.

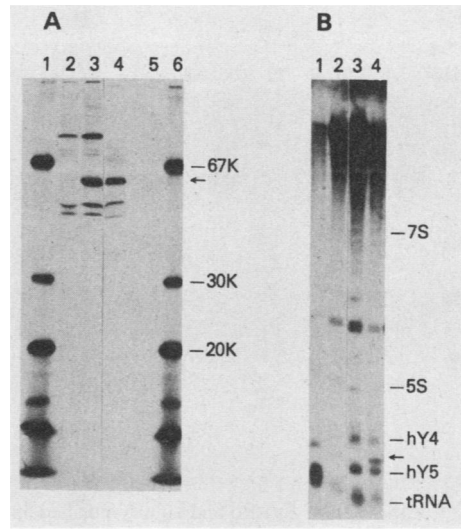

FIG. 6. Human RNP immunoprecipitated by anti-p63. (A) Immunoprecipitations of in vivo [^{35}S]methionine-labeled KB cell extract. Lanes: 1 and 6, m.w. standards; lane 2, normal human serum; lane 3, anti-Ro serum; lane 4, anti-p63; lane 5, control affinity-purified antibody. (B) KB in vivo ^{32}P-labeled RNA species recognized by anti-p63. Lanes: 1, total RNA extracted from KB cells; 2, normal serum; 3, anti-Ro (hY4 and hY5 are 90 and 80 nt, respectively); 4, anti-p63. Arrow denotes 85nt RNA species immunoprecipitated by anti-p63.

the possibility that anti-Be precipitability occurs via complementary base-pairing with larger RNAs, specifically the AFP RNA generated in our system (see above).

Antibodies which bind *X. laevis* p63 recognize human RNP. In order to determine which if any human RNPs were recognized by anti-p63, the protein and RNAs from immunoprecipitates of KB cells were analyzed separately by PAGE (10% polyacrylamide). Because it is known that the human Ro RNP contains pol III-derived RNAs and a cytoplasmic polypeptide of 60 kDa (35), approximately the same size as *Xenopus* p63, we included anti-Ro immunoprecipitations for comparison and as a positive control. Figure 6A shows the products of immunoprecipitation of the soluble fraction of [^{35}S]methionine-labeled KB cells. A major band corresponding to a protein of approximately 63 kDa was

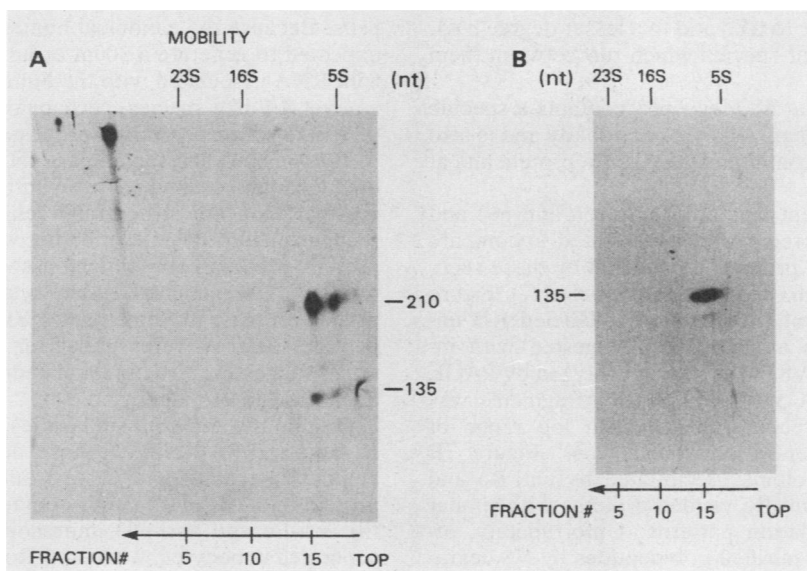

FIG. 5. Sedimentation velocity analysis of RNP structures containing *Alu* transcripts. Sucrose gradient (15 to 30%) sedimentation of *B1-Alu*-microinjected oocyte extract. RNA from fractions was extracted, electrophoresed through 8 M urea–10% polyacrylamide gels, and autoradiographed. *E. coli* rRNA and tRNA served as sedimentation markers. (A) Nuclear contents, fractionated 60 min after gene microinjection. (B) Cytoplasmic contents, 24 h after gene microinjection.

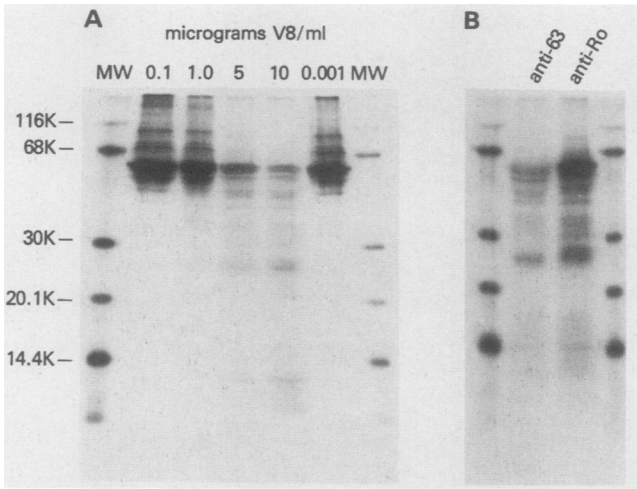

FIG. 7. Comparison of anti-Ro and affinity-purified anti-Be (anti-p63) ^{35}S-labeled KB immunoprecipitates by partial proteolysis. After immunoprecipitation, the RNPs were subjected to *S. aureus* V8 protease in the presence of SDS (7) and analyzed by SDS-PAGE (15% polyacrylamide). (A) Anti-Ro immunoprecipitates subjected to increasing concentrations of V8 as indicated. Lanes 1 and 6, ^{125}I-labeled protein m.w. standards. (B) Anti-p63 (lane 2) and anti-Ro (lane 3) immunoprecipitates after V8 protease (5 μg/ml) treatment.

precipitated by anti-p63 (lane 4). A similar size band was found with anti-Ro reference serum (35, 36) (lane 3). The affinity-purified anti-p63 did not precipitate a higher-m.w. species recognized by anti-Ro (compare lanes 3 and 4). Lane 5 shows a control affinity preparation utilizing eluate from a non-63-kDa region of the nitrocellulose as described above; as expected, it did not precipitate significant amounts of labeled material. Figure 6B illustrates the human in vivo ^{32}P-labeled RNAs immunoprecipitated by anti-Ro, anti-p63, and normal serum compared with total RNA. The hY5 and hY4 species were the RNAs immunoprecipitated most strongly by anti-Ro (12) and can be seen in lane 3 with reference anti-Ro serum. Anti-p63 immunoprecipitated RNAs comparable in size to hY5 and to a lesser degree hY4, in addition to a prominent species which ran between them (arrow, lane 4).

These data indicate that *X. laevis* p63 contains a specific determinant which is recognized by our antibody and is also present on human RNP containing the 63-kDa protein and at least two small RNAs.

Because of the apparent similarity between anti-p63 and anti-Ro human immunoprecipitates, we wanted to compare more directly the human proteins recognized by these specificities by partial *S. aureus* protease V8 digestion (7). Figure 7A shows the products of [^{35}S]methionine-labeled KB immunoprecipitations with anti-Ro serum digested with increasing amounts of the V8 protease and analyzed by PAGE (15% polyacrylamide). A pattern of peptide fragments was generated which was recognizable over a log range of protease-substrate concentrations (lanes 2–6). Figure 7B compares the human proteins precipitated by anti-Ro and anti-p63. Anti-p63 and anti-Ro yielded proteins with similar but not identical degradation patterns. Unfortunately, attempts at analysis of gel-purified polypeptides by V8 degradation were unsuccessful due to a limited supply of affinity-purified antibody.

The protein immunoprecipitation results in Fig. 6A show that the affinity-purified anti-p63 antibodies precipitated one major polypeptide and did not precipitate other polypeptides above the normal serum control level. Yet when these sera were used to analyze RNAs (Fig. 6B), *X. laevis* p63 affinity-purified antibodies precipitated an 85nt RNA, while anti-Ro and nonimmune sera did not. We conclude that the *X. laevis* *Alu*-binding protein shares an epitope(s) with a human RNP containing this RNA. The protein component of this human RNP is comparable in its size and pattern of partial V8 protease degradation to the human Ro polypeptide, suggesting that these proteins may be related.

DISCUSSION

We have dissected a pathway of expression of a *B1-Alu* gene in vivo by using microinjected oocytes and human autoimmune sera and have demonstrated that the mouse *B1-Alu* primary transcript and processed RNA exist in the *X. laevis* oocyte complexed with specific proteins as small RNPs. The data suggest that a nuclear *Alu*-RNP precursor undergoes processing to one which can later be found in the cytoplasm. These observations suggest that the *Alu*-associated protein p63 is bound to the RNA during endonucleolytic processing and nuclear transport. A summary of the results of this and previous studies (2) is presented in Fig. 8.

Because human autoimmune sera can be quite variable in their specificities (12, 22), we characterized and used affinity-purified autoimmune antibodies to a discrete polypeptide to study a *B1-Alu* expression pathway in vivo. In doing so, it became necessary to partially characterize these antibodies in a human system as well, although this was not our primary goal. We have demonstrated that, as expected, these antibodies were monospecific; they bound a single band on immunoblots of *Xenopus* oocytes and specifically precipitated a single polypeptide band from ^{35}S-labeled human cells.

Use of affinity-purified autoimmune antibodies revealed that a 63-kDa RNA-binding protein of *X. laevis* appears to have a human counterpart. The presence of a protein in human cells which cross-reacts with an autoantibody affinity-purified to *X. laevis* *B1-Alu*-associated protein of similar size suggests that an analogous class of RNP exists in human cells. Because the canonical human *Alu* sequence would be expected to generate a 300nt or larger RNA species (8), the 85nt RNA associated with the human protein may represent a novel *Alu*-like species, perhaps related either by sequence or structure, or possibly a processed *Alu* transcript.

Xenopus p63 and the human RNA-associated polypeptide are of similar size and share an antigenic epitope(s), suggesting that they are structurally related. The human protein recognized by anti-p63 antibodies was compared with the Ro protein, a human autoantigen associated with at least three to four different small stable cytoplasmic RNAs (12), by partial protease treatment and SDS-PAGE. Although the peptide patterns were similar for the two immunoprecipitates, suggesting structural relatedness, such an experiment does not address identity.

Despite the apparent similarities between the human anti-Be and anti-Ro RNPs we have demonstrated, we wish to emphasize that (i) anti-Be and anti-p63 detected a 63-kDa protein on blots of *X. laevis* oocytes, while anti-Ro did not, (ii) anti-Be and anti-p63 immunoprecipitated *B1-Alu* RNA generated in oocytes, while anti-Ro did not, and (iii) anti-p63 immunoprecipitated a human RNP containing an 85nt RNA, whereas anti-Ro did not. These results clearly indicate that the specificities of anti-p63 and anti-Ro are different. Therefore, although both anti-p63 and anti-Ro are autoimmune

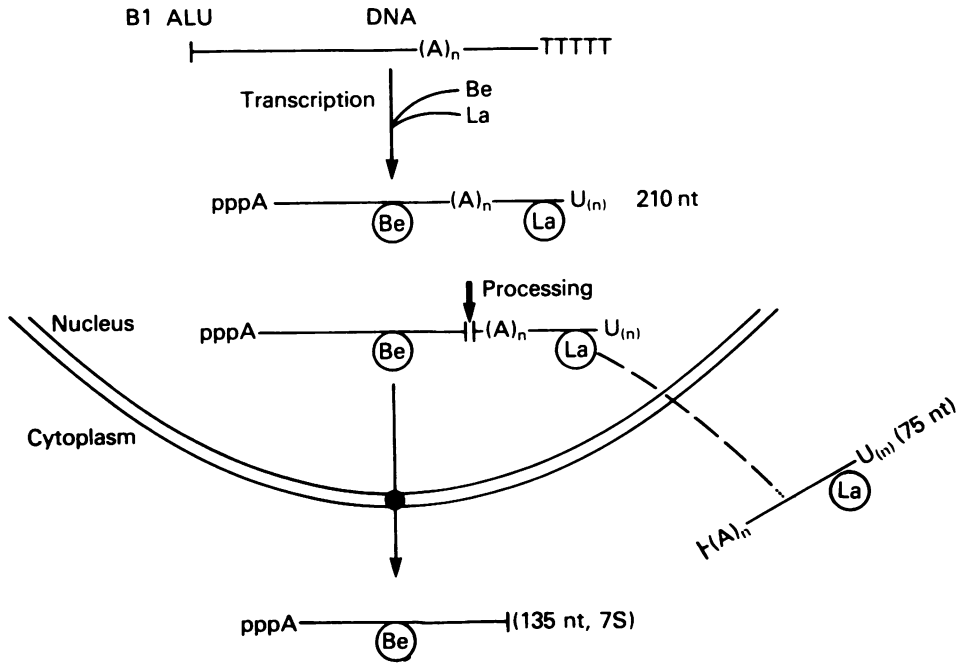

FIG. 8. Schematic of the *B1-Alu* processing pathway in *X. laevis* oocytes.

antibodies that precipitate human proteins of similar mass and partial protease degradation pattern, the precise nature of this apparent relatedness remains to be elucidated.

X. laevis p63 and Ro polypeptides do seem to have limited functional homology, since both are found in cells in the cytoplasmic compartment complexed with small stable RNAs of pol III origin (2, 12). In contrast to the mammalian Ro polypeptide, which has been demonstrated to be a cytoplasmic protein associated with pol III products (35), the *X. laevis* p63 bound both the primary transcript intranuclearly and the processed cytoplasmic RNA. Thus, the data in this report suggest an important nuclear role in transcription, processing, or transport in addition to a cytoplasmic function for the *Xenopus* p63. Indeed, we can demonstrate significant decrease in *B1-Alu* expression in the oocyte by preinjecting anti-Be IgG (unpublished data).

So far, the only role which can clearly be ascribed to an *Alu* sequence-containing RNA is the translational elongation arrest activity mediated by the signal recognition particle (SRP), an RNP complex of six polypeptides and one RNA, which conveys three functions (signal recognition, elongation arrest, and translocation promotion) necessary for protein secretion (for review, see references 30 and 34). SRP-RNA (also known as 7SL RNA) is 300nt long and contains a 140nt *Alu* sequence divided into two regions terminating each end of the SRP-RNA (11, 32). As a result of physical and biochemical dissection of SRP by multiple methods, it has been shown that alteration (28, 30) or deletion (29) of the "*Alu* domain" (30) of SRP severely compromises elongation arrest activity, while signal recognition and translocation activities remain. It seems possible that *Alu* sequences play a role in other cellular functions. It seems unlikely that an RNA domain with potential for regulation of gene expression would be limited in nature to one RNP, considering that (i) the mammalian genome contains many functional *Alu* transcription units (8, 25), (ii) *Alu* sequences are abundant in heteronuclear RNA (8, 27), (iii) *Alu* sequences have been found on certain mRNAs (8, 15, 37), and (iv) discrete core-*Alu* transcripts are detectable in vivo (2, 14). Recently a novel autoantibody has been described which immunoprecipitates human *Alu* and SRP (7SL) RNA generated in vitro and appears to bind the human 68-kDa polypeptide component of SRP (4). The relationship if any between this 68-kDa protein and the p63 described here remains to be established. It should be noted in this regard that our antibodies to p63 did not precipitate the 7S SRP RNA (Fig. 6B, lane 4). Thus, the antibodies described in this report should be helpful in further identifying and dissecting the cellular functions of *Alu*-RNPs. For example, non-SRP *Alu*-RNPs may be involved in macromolecule transport within cells, as is SRP, or may modulate translation of certain mRNAs (29). Also, interaction of *X. laevis* p63 with oocyte repetitive sequence RNAs which are abundant, associated with cytoplasmic polyadenylated RNA, and developmentally regulated (3, 13, 27) can be studied.

Although our system is a heterologous one, employing amphibian oocyte expression of mouse-derived DNA and human autoimmune sera, it is encouraging that evolutionarily conserved factors are involved. The validity of using such a system is strengthened by the finding of an *Alu* RNA in mouse tissue which corresponds to the core-*Alu* generated in the oocyte (2). Thus, it would seem that this system should be useful for further probing of cellular functions of *Alu*-RNPs.

The presence of a processing and transport system (2) and association of a specific protein with a *B1-Alu* further demonstrates that the eucaryotic cell has evolved a specific pathway for handling these transcripts and provides further evidence that *Alu* RNA sequences may be playing a role in cellular metabolism.

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