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Three New Small Nucleolar RNAs That Are Psoralen Cross-Linked In Vivo to Unique Regions of Pre-rRNA

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We have recently described three novel human small nucleolar RNA species with unique nucleotide sequences, which were named E1, E2, and E3. The present article describes specific psoralen photocross-linking in whole HeLa cells of E1, E2, and E3 RNAs to nucleolar pre-rRNA. These small RNAs were cross-linked to different sections of pre-rRNA. E1 RNA was cross-linked to two segments of nucleolar pre-rRNA; one was within residues 697 to 1163 of the 5′ external transcribed spacer, and the other one was between nucleotides 664 and 1021 of the 18S rRNA sequence. E2 RNA was cross-linked to a region within residues 3282 to 3667 of the 28S rRNA sequence. E3 RNA was cross-linked to a sequence between positions 1021 and 1639 of the 18S rRNA sequence. Primer extension analysis located psoralen adducts in E1, E2, and E3 RNAs that were enriched in high-molecular-weight fractions of nucleolar RNA. Some of these psoralen adducts might be cross-links of E1, E2, and E3 RNAs to large nucleolar RNA. Antisense oligodeoxynucleotide-targeted RNase H digestion of nucleolar extracts revealed accessible segments in these three small RNAs. The accessible regions were within nucleotide positions 106 to 130 of E1 RNA, positions 24 to 48 and 42 to 66 of E2 RNA, and positions 7 to 16 and about 116 to 122 of E3 RNA. Some of the molecules of these small nucleolar RNAs sedimented as if associated with larger structures when both nondenatured RNA and a nucleolar extract were analyzed.

The maturation of pre-rRNA requires some small nucleolar RNA (snoRNA) species, such as U3 RNA (12, 13, 23) and *Saccharomyces cerevisiae* snR10 (27) and U14/snR128 (15). We recently synthesized and cloned human cDNA probes for three new snoRNAs, which were named E1, E2, and E3 (21). Their unique nucleotide sequences suggest that they may belong to a new class of snoRNAs, their 5′ ends are not capped, they are low-abundance species that are relatively conserved between humans and frogs, and their presence in every tissue tested suggests that they are housekeeping RNAs (21). Toward the final goal of determining the function of these snoRNAs, it would be beneficial to have some basic information about their structure and associations. Exposure of cells to psoralen derivatives, such as 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT), and long-wave UV light can produce covalent cross-links between base-paired RNA strands (reviewed in reference 6). U1 and U2 small nucleoplasmic RNAs can be psoralen cross-linked in vivo to heterogeneous nuclear RNA (3, 4), and U3 snoRNA can be psoralen cross-linked to pre-rRNA (18, 25), correctly suggesting that these small RNAs are involved in the processing of pre-mRNA (24) and pre-rRNA, respectively. Antisense oligodeoxynucleotide-targeted degradation by RNase H has been used to locate accessible sequences of small nuclear RNAs assembled in ribonucleoprotein (RNP) particles (24). In the present study, psoralen photocross-linking in whole cells and specific RNase H digestion directed by complementary oligonucleotides were used to study E1, E2, and E3 RNAs. Our results indicate that E1, E2, and E3 RNAs are psoralen cross-linked in vivo to different unique segments of nucleolar pre-rRNA.

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MATERIALS AND METHODS

General methods. The following procedures were done as described before: preparation of nuclear and cytoplasmic fractions from HeLa cells (5); isolation of nucleoli (20); isolation of RNA (26); hybrid selection of RNA (5); 10% polyacrylamide gel electrophoresis (5); electrophoresis of RNA in 1% agarose and 2.2 M formaldehyde (22); and RNA sequencing by the dideoxynucleotide method using HeLa cell nucleolar RNA, avian myeloblastosis virus reverse transcriptase, and sequence-specific, antisense radioactive primers (10). Hybridization with ZetaProbe membranes (Bio-Rad) was performed according to the recommendations of the manufacturer. RNA was transferred to ZetaProbe membranes by electroblotting from polyacrylamide gels and by capillary blotting from agarose gels.

Antibodies. Polyclonal antibodies to the U3 snRNP were kindly supplied by J. Craft (Yale University). Monoclonal antibody H57 specific for the human B/B′ polypeptides of U small nuclear RNPs (snRNPs) was a gift from R. Lührmann (Philipps University, Marburg, Germany). Hybridoma cells for preparing monoclonal antibody to the Sm antigen were kindly provided by E. A. Lerner and C. A. Janeway (Yale University).

Release of small RNAs in a nuclear extract. HeLa cell nuclei were resuspended in 50 mM Tris-HCl (pH 7.5) supplemented with 0.2 to 0.5 M NaCl. The nuclei were broken by sonication, and the sonicate was centrifuged at 20,000 × *g* for 10 min. RNA extracted from the supernatants and pellets was analyzed by 10% polyacrylamide Northern (RNA) blotting.

Glycerol gradient analysis of cell extracts. Glycerol gradient analysis was done essentially as described in reference 30. Cell sonic extracts were prepared by resuspension of washed HeLa cells in 50 mM Tris-HCl (pH 7.5)–0.3 M NaCl–0.05% Nonidet P-40 at 5×10^7 cells per ml and sonication with a probe unit in 20-s pulses on ice until all nuclei were broken. The sample was centrifuged four times at 10,000 × *g* for 30

min each time, and the last supernatant was used as cell sonic extract. A portion of the extract was loaded onto a 10 to 30% glycerol linear gradient containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9)–0.1 M KCl–1 mM $MgCl_2$. Gradients were spun at 32,000 rpm for 20 h at 4°C in a Beckman SW40 rotor and fractionated into 19 samples. Samples were deproteinized and analyzed by 10% polyacrylamide gel Northern blotting. A known amount of ^{3}H-labeled RNA was added to the glycerol gradient fractions immediately after they were collected, and the trichloroacetic acid-precipitable counts in aliquots of each sample were measured before the sample was loaded onto the gel, to verify that the sample recoveries were very similar among various fractions.

Sucrose gradient analysis of nondenatured RNA. RNA was extracted from a nucleolar pellet with phenol and chloroform at room temperature in the absence of denaturing agents. A portion was loaded onto a 15 to 30% sucrose linear gradient in 0.5% sodium dodecyl sulfate (SDS)–2 mM EDTA–10 mM Tris-HCl, pH 7.4. Gradients were centrifuged at 17,500 rpm for 16 h at 20°C in a Beckman SW40 rotor and fractionated into 19 samples. RNA was removed from the fractions by ethanol precipitation and analyzed by 10% polyacrylamide gel Northern blotting.

Psoralen cross-linking in vivo and analysis of cross-linked RNA. Washed, packed HeLa cells were resuspended in 1 volume of 0.15 M NaCl–10 mM Tris-HCl, pH 7.2, with or without 0.5 mg of AMT per ml and were incubated on ice for 10 min (4). Then, they were irradiated in microcentrifuge tubes with long-wave UV light (peak emission at 365 nm, 100 mW/cm^2) for 4.3 or 13 min at 4°C. Nucleoli were isolated from these cells, and their RNA was deproteinized, as indicated above. Two different hybridization protocols were used.

(i) Hybridization protocol I. Nucleolar RNA was partially hydrolyzed to an average size of about 0.5 kb by incubation in 50 mM Tris-HCl, pH 9.5, at 90°C (17, 25). The alkali-treated RNA was hybrid selected with the specified filter-immobilized fragment of human pre-rRNA gene, as indicated above. The specific hybrid was eluted, fractionated by formaldehyde-agarose gel electrophoresis, and blotted, and the blot was hybridized with a radioactive DNA probe for E1, E2, or E3 RNA.

(ii) Hybridization protocol II. Alternatively, a modification of a sandwich blot technique (18, 25) was used. Nucleolar RNA was hybridized with 2.4-cm-diameter discs of GeneScreen (DuPont-NEN) that carried the specified fragment of the human pre-rRNA gene, as indicated above. The discs were washed with 10 mM Tris-HCl (pH 7.4)–2 mM EDTA three times at 50°C and three times at 60°C. They were then hybridized with a radioactive DNA probe for E1, E2, or E3 RNA overnight at 37°C in 50% formamide–50 mM sodium phosphate (pH 6.5)–5× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0)–0.1% SDS–1 mM EDTA. Then, the filters were washed twice at 37°C with 0.1× SSC–0.1% SDS, once with 0.1× SSC, and once with 50 mM sodium acetate, pH 5.5. The discs were incubated with RNase T_1 (100 U/ml) in 50 mM sodium acetate, pH 5.5, at 37°C for 10 min, and then they were washed three times with 2× SSC–0.1% SDS at 37°C for 10 min each time. The specific hybrid was eluted at 85°C for 3 min with 2 mM EDTA–0.1% SDS–*Escherichia coli* tRNA (20 μg/ml), the eluate was precipitated with ethanol and loaded as dots onto ZetaProbe as recommended by the manufacturer, and the membrane was exposed to X-ray film. For primer extension analysis of RNA, nucleolar RNA was incubated at 100°C for 2 min, chilled on ice, and fractionated by sucrose gradient centrifugation as described above, and then the fractions were incubated with snoRNA sequence-specific, antisense, radioactive primers that were extended with reverse transcriptase (10).

DNA clones. Plasmid pHU1-1, which carries a human U1 RNA gene including 6 bp of the upstream flanking sequence and 35 bp of the 3'-flanking sequence, was kindly supplied by E. Lund and J. E. Dahlberg (University of Wisconsin). Plasmid pU3B.4 bears a rat U3 RNA sequence and was a gift from R. Reddy (Baylor College of Medicine). Plasmids pB, pA, and pD7 carry large fragments of a human pre-rRNA gene and were kindly provided by D. Schlessinger (Washington University). The insert in plasmid pB spans from 513 nucleotides 5' to the transcription start site to 230 bases upstream of the 3' end of 18S rRNA. The insert in plasmid pA spans from the latter site to 587 bp upstream of the 3' end of 28S rRNA. The insert in plasmid pD7 spans from the latter site to ~4.3 kb downstream of the transcription termination site (7). These DNA segments and smaller fragments cleaved from them were subcloned in plasmid pT7/T3α-18 (GIBCO BRL) and are summarized in Fig. 3.

Radioactive hybridization probes. The E1, E2, and E3 sequences in the corresponding cDNA clones, minus the tails that had been added for cDNA synthesis and cloning, were amplified by the polymerase chain reaction (19). The amplified products were then used as templates for extension of snoRNA sequence-specific, antisense 3'-end primers with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP, to generate labeled E1-, E2-, and E3-specific DNA probes. The radioactive DNA probe for U1 RNA was made by extension of a U1-specific primer by using as a template the human U1 gene plasmid, linearized near the 5' end of the U1 gene. The radiolabeled DNA probe for U3 RNA was made by random primer labeling (9) using the rat U3 RNA sequence plasmid as a template.

RNase H digestion. A nucleolar extract was prepared by resuspension of a nucleolar pellet in 10 mM Tris-HCl (pH 7.0)–0.15 M NaCl–1 mM phenylmethylsulfonyl fluoride–0.1 mM leupeptin, followed by sonication (8). The nucleolar extract was incubated with the indicated oligodeoxynucleotide (1 μg/10 μl) at 37°C for 5 min. RNase H (2 U/10 μl) was added, and the incubation was continued for 10 min at 37°C and then 20 min at 30°C.

RESULTS

Our first question was whether E1, E2, and E3 RNAs present in cell extracts are associated with other molecules or structures. Preliminary experiments showed that cell extracts prepared in isotonic buffers contained very low levels of E1, E2, and E3 RNAs. This prompted an ionic-strength test. Most of the E1, E2, and E3 RNA molecules present in a nuclear extract, when exposed to 0.2 M NaCl, remained associated with very large structures (that were pelleted when centrifuged at 20,000 × *g* for 10 min [Fig. 1A]). The majority of the E1, E2, and E3 RNA molecules were released to the supernatant when the NaCl concentration reached 0.3 to 0.4 M (Fig. 1A). In the same Northern blot, most of U1 RNA was in the supernatant in the presence of 0.2 M NaCl (Fig. 1A). On the basis of these results, whole-cell extracts were prepared in the presence of 0.3 M NaCl for the next experiment. Most of the E1, E2, and E3 RNA molecules present in a whole-cell extract sedimented in a glycerol gradient faster than the naked snoRNAs but more slowly than deproteinized 18S rRNA (Fig. 1B). These

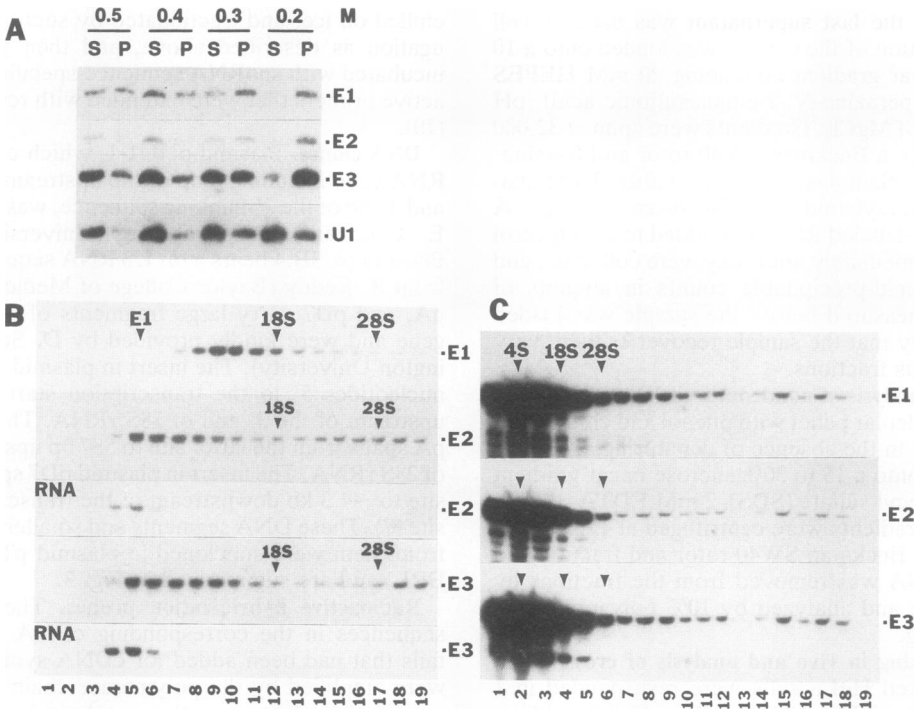

FIG. 1. Release of E1, E2, and E3 RNAs in a nuclear extract with increasing NaCl concentrations (A), their glycerol gradient sedimentation in a HeLa cell sonic extract (B), and their sucrose gradient sedimentation in nondenatured RNA (C). (A) Polyacrylamide Northern blot analysis of supernatants (lanes S) and pellets (lanes P) of a HeLa cell nuclear sonic extract that was adjusted to 0.2 to 0.5 M NaCl before centrifugation. The blot was hybridized with radioactive DNA probes for E1, E2, E3, and U1 RNAs. The X-ray film exposure for the E1, E2, and E3 probes was longer than for the U1 probe. (B) Northern blot analysis of glycerol gradient sedimentation of RNP particles in a HeLa cell sonic extract. Sedimentation was from left to right. The polyacrylamide gel blot was hybridized with radioactive DNA probes for E1, E2, and E3 RNAs. The sedimentation in parallel gradients of naked E1 RNA and 18S and 28S rRNAs is indicated. The electrophoretic mobilities of intact E1, E2, and E3 RNAs are indicated on the right in panels B and C. The glycerol gradient sedimentation of naked nuclear RNA from HeLa cells is shown in the two sections labeled "RNA"; all other conditions were as for the rest of panel B. (C) Northern blot analysis of sucrose gradient sedimentation of nondenatured nucleolar RNA. The blot was hybridized with ^{32}P-labeled DNA probes for E1, E2, and E3 RNAs. Sedimentation was from left to right. Fraction 16 was underloaded relative to the other fractions. The sedimentation in parallel gradients of 4S RNA, 18S rRNA, and 28S rRNA is indicated (arrowheads).

results suggest that E1, E2, and E3 RNAs are present in snRNPs and that the E1 snRNP is larger, with a sedimentation comparable to those of most of the more abundant snRNPs, which sediment at about 10 to 12S (reviewed in reference 16). Some of the E2 and E3 RNA molecules in the extract sedimented faster than 28S rRNA (Fig. 1B), suggesting their presence also in larger complexes. Some of the E1, E2, and E3 RNA molecules in deproteinized, nondenatured nucleolar RNA sedimented much faster than 28S rRNA in a sucrose gradient (Fig. 1C), implying association with large RNA, possibly base pairing with pre-rRNA.

The associations of these snoRNAs with large RNA in whole cells were analyzed next by psoralen photocross-linking. Northern blot analysis of nucleolar RNA after formaldehyde-agarose gel electrophoresis showed that some molecules of E1, E2, and E3 snoRNAs were psoralen-cross-linked in vivo to large nucleolar RNA (Fig. 2). Cross-linking required the simultaneous exposure of the cells to both AMT and long-wave UV light and was comparable to that of U3 RNA (Fig. 2). The high-molecular-weight hybridization signals represented RNA, instead of DNA, because they were not affected by incubation of the samples with RNase-free DNase I before gel electrophoresis (not shown).

The next experiments tested whether some E1, E2, and E3 RNA molecules are psoralen cross-linked to pre-rRNA and, if so, to which segments of pre-rRNA. A human pre-rRNA

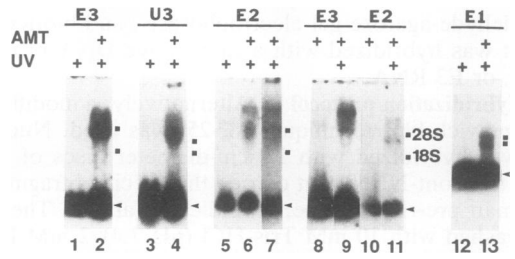

FIG. 2. Psoralen cross-linking of E1, E2, and E3 RNAs to nucleolar large RNA. HeLa cells were preincubated with (+) or without (−) AMT and then were (+) or were not (−) irradiated with long-wave UV light. Nucleolar RNA was fractionated by formaldehyde-agarose gel electrophoresis and blotted, and the blot was hybridized with radioactive DNA probes for E3, U3, E2, and E1 RNAs, as indicated. The RNA samples in lanes 1 and 2, 3 and 4, 5 to 7, 8 to 11, and 12 and 13 were from five separate experiments. The same sample was loaded onto lanes 6 and 7, but in lane 7 the amount of RNA loaded was less and the X-ray film exposure was proportionately longer, to show that the high-molecular-weight radioactive signals tend to form bands. The migration of free snoRNAs is indicated (arrowheads). The upper and lower dots indicate the electrophoretic mobilities of 28S and 18S rRNAs, respectively. The higher electrophoretic mobility of the high-molecular-weight radioactive signals in some lanes (e.g., lane 11 compared with lane 7 and lane 2 compared with lane 9) probably reflects partial RNA degradation in some experiments (for example, during isolation of nucleoli).

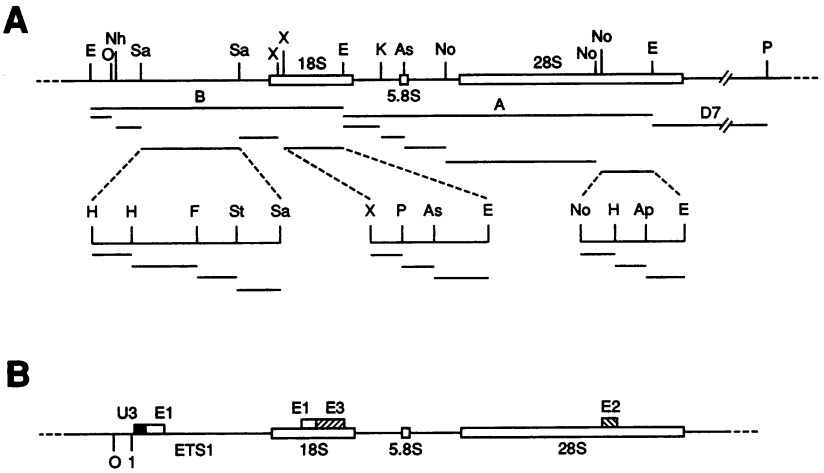

FIG. 3. Subcloned DNA fragments of a human rRNA transcription unit that were used in this study (A) and a map of the E1, E2, and E3 RNA cross-linking sites in pre-rRNA (B). (A) The DNA fragments used span from the *Eco*RI site that is located 513 nucleotides upstream of the transcription start site to the *Pst*I site that is located ~4.3 kb downstream of the transcription termination site (7). The mature rRNA regions are indicated by open boxes. E, *Eco*RI; Nh, *Nhe*I; Sa, *Sal*I; X, *Xba*I; K, *Kpn*I; As, *Ase*I; No, *Not*I; P, *Pst*I; H, *Hin*cII; F, *Fok*I; St, *Sty*I; Ap, *Apa*I; O, transcription initiation site. (B) The site of human pre-rRNA that cross-links to U3 RNA in vivo is from reference 18. 1, the ETS1 processing site.

gene was cleaved and subcloned into a series of progressively shorter DNA fragments (Fig. 3A). The low cell concentrations of E1, E2, and E3 RNAs (21) made it impossible to detect psoralen cross-links in vivo by previous procedures (18, 25). It was necessary to hybrid select nucleolar RNA from large numbers of cells. Nucleolar RNA from cells that had been exposed to AMT and long-wave UV light was analyzed by two methods that gave comparable results. Nucleolar RNA was first hydrolyzed with alkali to an average size of about 0.5 kb (25), then it was hybrid selected with fragments of a human pre-rRNA gene, and finally it was analyzed in Northern blots that were hybridized with a labeled DNA probe for E1, E2, or E3 RNA. Alternatively, nucleolar RNA was hybridized with filter-immobilized DNA fragments of a human gene for pre-rRNA; then the filters were washed, hybridized with a ^{32}P-labeled DNA probe for E1, E2, or E3 RNA, washed, incubated with RNase T_1, washed again, and eluted, and the eluates were loaded as dots onto another membrane. Some of the E1, E2, and E3 RNA molecules were psoralen cross-linked in vivo to pre-rRNA. E1 RNA (Fig. 4A) and E3 RNA (Fig. 4G) were cross-linked to a region between 0.5 kb upstream of the pre-rRNA transcription start site and 0.2 kb upstream of the 3′ end of the 18S rRNA sequence. E2 RNA was cross-linked to a segment between ~0.2 kb upstream of the 3′ terminus of the 18S rRNA sequence and ~0.6 kb upstream of the 3′ end of the 28S rRNA sequence (Fig. 4D). Further analysis with plasmid subclones of smaller DNA fragments of that human rRNA transcription unit indicated that (i) E1 RNA was cross-linked to two regions in pre-rRNA, one between positions 695 and 2923 of the 5′ external transcribed spacer (ETS1) (*Sal*I-*Sal*I fragment) and the other between nucleotides 294 and 1639 of the 18S rRNA sequence (*Xba*I-*Eco*RI fragment) (Fig. 4B); (ii) E3 RNA was cross-linked to a segment in pre-rRNA between positions 294 and 1639 of the 18S rRNA sequence (*Xba*I-*Eco*RI fragment) (Fig. 4H); and (iii) E2 RNA was cross-linked to a section in pre-rRNA between positions 3282 and 4456 of the 28S rRNA sequence (*Not*I-*Eco*RI fragment) (Fig. 4E). Additional probing with subclones of even shorter ribosomal DNA segments narrowed down the regions in pre-rRNA that were cross-linked with these snoRNAs: (i) E1 RNA to a section within positions 697 to 1163 of ETS1 (*Hin*cII-*Hin*cII fragment) and to another segment within residues 664 to 1021 of the 18S rRNA sequence (*Pst*I-*Ase*I segment) (Fig. 4C), (ii) E3 RNA to a region within residues 1021 to 1639 of the 18S rRNA sequence (*Ase*I-*Eco*RI segment) (Fig. 4I), and (iii) E2 RNA to a segment between residues 3282 and 3667 of the 28S rRNA sequence (*Not*I-*Hin*cII segment) (Fig. 4F). These results are summarized in Fig. 3B. The hybridization signals

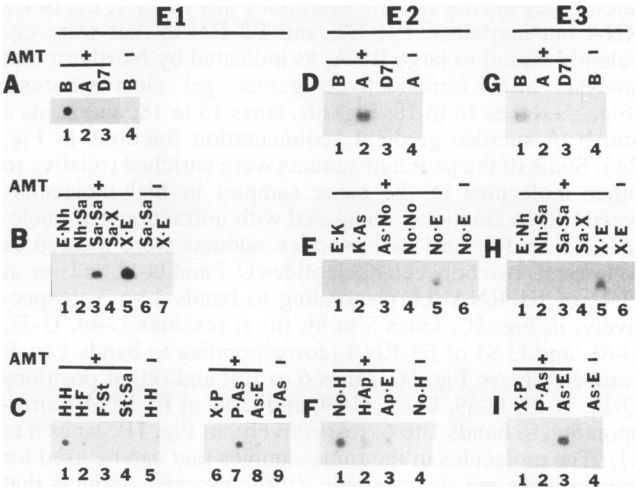

FIG. 4. Psoralen cross-linking in vivo of E1, E2, and E3 RNAs to nucleolar rRNA and location of the regions involved in pre-rRNA. HeLa cells were preincubated with (+) or without (−) AMT and then were irradiated with long-wave UV light. Their nucleolar RNA was analyzed by hybridization protocol I (B, D, G, H, and I) or II (A, C, E, and F), as indicated in Materials and Methods. The radioactive DNA probes (for E1, E2, and E3 RNAs) are indicated at the top. The DNA fragments of the human rRNA gene (Fig. 3) used are indicated above each lane. For abbreviations, see the legend to Fig. 3.

were caused by RNA, instead of cloned DNA released from the membranes during hybrid selection, since they were eliminated by pretreatment with RNase A and were unaffected by preincubation with DNase I before gel electrophoresis (not shown). Cross-linking of E1 RNA to two regions of pre-rRNA appears to be real rather than cross-linking to only one portion of a long pre-rRNA molecule that in turn spans several segments of the pre-rRNA gene and somehow resists digestion with alkali or RNase T_1, because there was no hybridization to the sections located in between (Fig. 4B and C). We wondered about the extents of possible psoralen cross-linking of E1, E2, and E3 RNAs to specific DNA segments, compared with pre-rRNA. Nucleic acid samples that had not been treated with DNase I were digested with *Hin*dIII or *Pst*I, electrophoresed, blotted, and hybridized with labeled DNA probes for E1, E2, and E3 RNAs. Bands of DNA cross-linked to E1, E2, or E3 RNA were not detected (not shown). Then, psoralen cross-linking of E1, E2, or E3 RNA to specific DNA sites does not appear to be a major effect. These results do not support the proposal that E1, E2, or E3 RNA might be involved in some aspect of rRNA gene function, such as transcription.

For a more detailed study of psoralen cross-linking, E1, E2, and E3 RNAs were analyzed by primer extension, since reverse transcriptase stops at the nucleotide downstream of a psoralen adduct (6). The primer extension stops that were detected indicated psoralen adducts (i) at nucleotides U-7, A-10, U-11, C-13, U-42, U-91, C-92, U-95, and U-142 of E1 RNA (Fig. 5A, lanes 2 and 8); (ii) at residues U-10, U-11, U-17, U-45, C-46, U-48, U-49, U-77, U-81, and U-83 of E3 RNA (Fig. 6A, lane 5); and (iii) at nucleotides U-3, U-10, U-12, U-13, U-39, U-58, U-60, U-64, A-73, C-74, U-89, U-90, C-91, C-93, and U-94 of E2 RNA (Fig. 7B, lane 6). Some of these may be psoralen monoadducts, some may be intramolecular cross-links, and others might be cross-links to other small RNAs. Next, nucleolar RNA was heat denatured and then was fractionated by sucrose gradient sedimentation. High-molecular-weight RNA fractions were isolated. They did not contain essentially any free E1, E2, or E3 RNA but contained E1, E2, and E3 RNAs that were covalently bound to large RNA, as indicated by Northern blot analysis after formaldehyde-agarose gel electrophoresis (Fig. 5B, lanes 16 to 18; Fig. 6B, lanes 15 to 18; and pools a and b of sucrose gradient sedimentation fractions in Fig. 7A). Some of the psoralen adducts were enriched (relative to other molecules in the same sample) in high-molecular-weight RNA fractions, compared with unfractionated nucleolar RNA. The enriched psoralen adducts were located as follows: (i) two between nucleotides U-7 and U-11 and one at U-42 of E1 RNA (corresponding to bands 1 to 3, respectively, in Fig. 5C, lanes 5 to 8); (ii) at residues U-49, U-77, U-81, and U-83 of E3 RNA (corresponding to bands 1 to 4, respectively, in Fig. 6C, lanes 6 to 10); and (iii) at positions U-12, U-13, U-39, U-60, U-89, and U-90 of E2 RNA (corresponding to bands 1 to 6, respectively, in Fig. 7B, lanes 8 to 11). The molecules in the same samples that can be used for comparisons are, for example, (i) the psoralen adducts that correspond to the bands indicated by the lower arrowhead in Fig. 6A, lane 5 (for E3 RNA), and in Fig. 7B, lane 6 (for E2 RNA), and (ii) the partially degraded E1 RNA molecules indicated by the lower arrowhead in Fig. 5A, lanes 1 and 2, and Fig. 5C, lanes 5 to 8 (for E1 RNA). Some of the psoralen adducts in the high-molecular-weight RNA fractions (summarized in Fig. 9) might be cross-links to large RNA. The detection of psoralen-specific interruptions of primer extension was not impaired by the presence of some partially

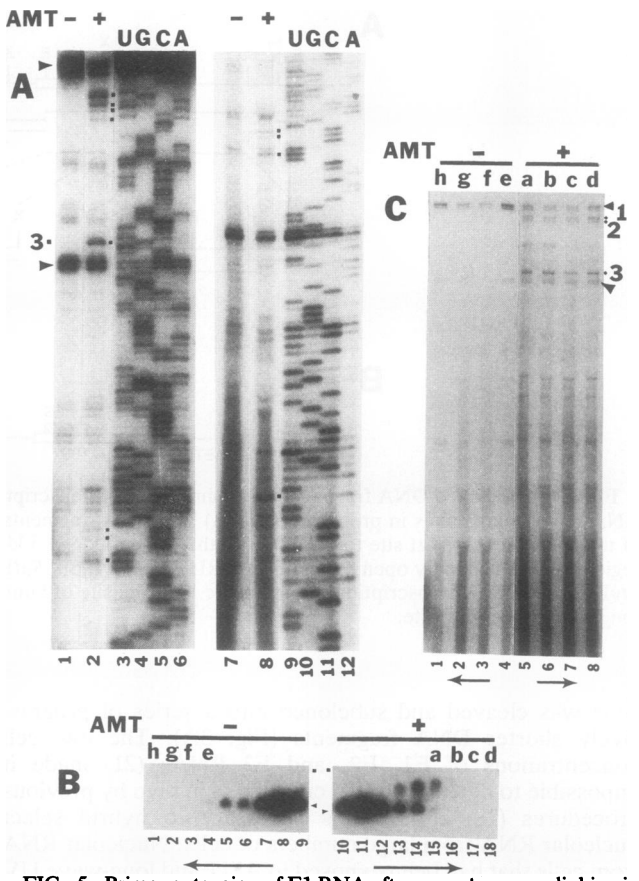

FIG. 5. Primer extension of E1 RNA after psoralen cross-linking in vivo and sucrose gradient centrifugation. (A) HeLa cells were preincubated with (+) or without (−) AMT and then were irradiated with long-wave UV light. Their nuclear RNA was used as a template to extend a 5′-end-labeled oligodeoxynucleotide complementary to the 3′ end of E1 RNA (lanes 1, 2, 7, and 8). Some of this radioactive primer was used to sequence E1 RNA of nuclear RNA from untreated HeLa cells (lanes 3 to 6). The samples in lanes 1 to 6 were electrophoresed for a shorter time in lanes 7 to 12, respectively. The eight dots in lane 2 show the psoralen-specific interruptions in primer extension that correspond to psoralen adducts at the following nucleotides (from top to bottom): U-7, A-10, U-11, C-13, U-42 ("3"), U-91, C-92, and U-95. The four dots in lane 8 indicate psoralen adducts at positions U-91, C-92, U-95, and U-142 (from top to bottom). In panels A and C, the upper arrowheads indicate the full-length E1 sequence and the lower arrowheads indicate two partially degraded E1 RNA molecules. (B) Northern blot analysis of nucleolar RNA from HeLa cells that were preincubated with (+) or without (−) AMT before UV irradiation, incubated at 100°C, and then fractionated by sucrose gradient centrifugation; aliquots of the fractions were analyzed by formaldehyde–1% agarose gel electrophoresis and blotted, and the blot was hybridized with a radioactive DNA probe for E1 RNA. The direction of sucrose gradient centrifugation is indicated (arrows). In a parallel sucrose gradient, 4S RNA was detected in lanes 9 and 10, 18S rRNA was detected in lanes 7 and 12, and 28S rRNA was detected in lanes 5 and 15. The electrophoretic mobilities of 28S and 18S rRNAs were about halfway between the origin (dots) and the migration of free small RNAs, such as E1 RNA (arrowheads). (C) Primer extension analysis of the RNA samples fractionated by sucrose gradient centrifugation in panel B using the radioactive primer indicated in panel A. Lanes 1 to 4 correspond to lanes 1 to 4 in panel B, respectively. Lanes 5 to 8 correspond to lanes 15 to 18 in panel B, respectively. Each sucrose gradient fraction is identified by the same lowercase letter in panels B and C. Three primer extension stops are marked in lanes 5 to 8; the top two dots ("1" and "2") indicate psoralen adducts within nucleotides U-7 and U-11, and the bottom dot ("3") marks a psoralen adduct at position U-42.

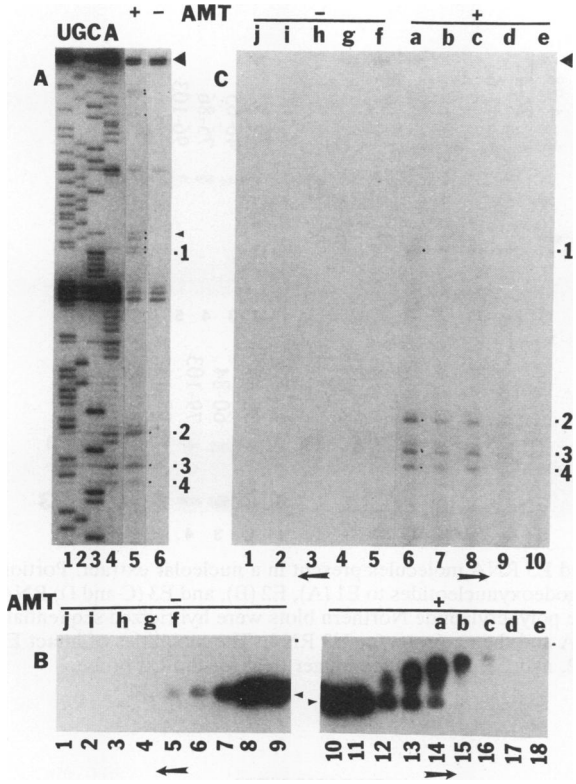

FIG. 6. Primer extension of E3 RNA after psoralen cross-linking in vivo and sucrose gradient centrifugation of RNA. (A) Primer extension analysis of nuclear RNA from HeLa cells that were preincubated with (+) (lane 5) or without (−) (lane 6) AMT and then were irradiated with UV light. A radioactive primer complementary to the 3′ end of E3 RNA was used for this primer extension analysis and for sequencing E3 RNA of nuclear RNA from untreated HeLa cells (lanes 1 to 4). The ten dots in lane 5 mark primer extension stops that indicate psoralen adducts at (from top to bottom) nucleotides U-10, U-11, U-17, U-45 (lower arrowhead), C-46, U-48, U-49, U-77, U-81, and U-83 (the last four are numbered 1 to 4). The upper arrowhead indicates the full-length E3 sequence. (B) Northern blot analysis of nucleolar RNA from HeLa cells that had been preincubated with (+) or without (−) AMT before UV irradiation followed by sucrose gradient centrifugation, gel electrophoresis, and blotting, as for the analysis whose results are shown in Fig. 5B. The direction of sucrose gradient centrifugation is indicated (arrows). The sedimentation of 4S, 18S, and 28S RNAs in a parallel sucrose gradient was as for the analysis whose results are shown in Fig. 5B. The blot was hybridized with a probe for E3 RNA. The electrophoretic mobility of free E3 RNA is indicated (arrowheads). The electrophoretic mobilities of 28S and 18S rRNAs were as indicated in Fig. 5B. (C) Primer extension analysis of the RNA samples fractionated by sucrose gradient sedimentation shown in panel B using the radiolabeled primer used for the analysis whose results are shown in panel A. Each sucrose gradient fraction is identified by the same lowercase letter in panels B and C. In panel B, lane 6, the four dots numbered 1 to 4 mark primer extension stops that indicate psoralen adducts at nucleotides U-49, U-77, U-81, and U-83, respectively. Arrowhead, the electrophoretic mobility of the full-length E3 sequence.

degraded E1, E2, and E3 RNA molecules in the RNA preparations used. Primer extension analysis showed some full-length sequences of E1, E3, and E2 RNAs (indicated by the upper arrowheads in Fig. 5C, 6C, and 7B, respectively) at and near the bottoms of the sucrose gradients of nucleolar RNA from cells that had or had not been exposed to AMT,

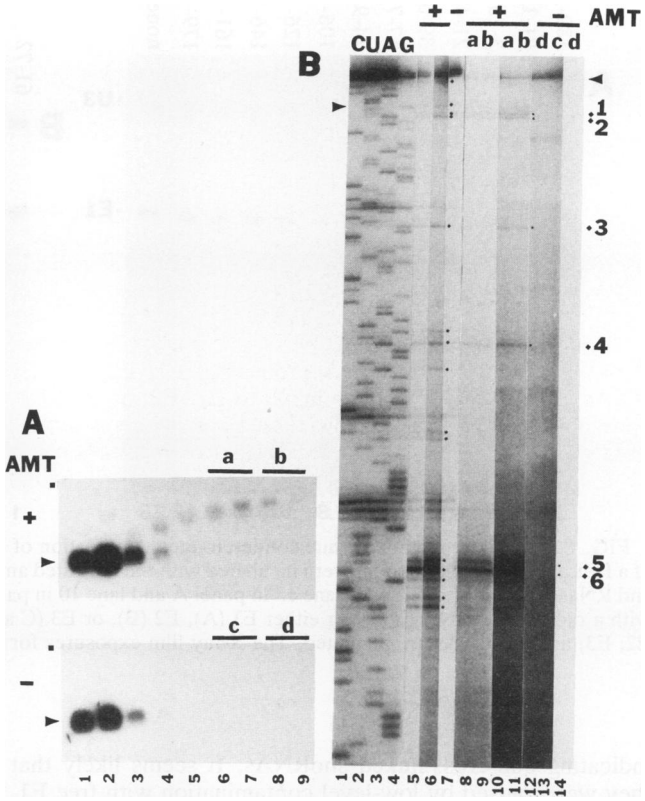

FIG. 7. Primer extension of E2 RNA after psoralen cross-linking in vivo and sucrose gradient centrifugation of RNA. (A) Northern blot analysis of nucleolar RNA that was fractionated by sucrose gradient centrifugation (sedimentation is from left to right). HeLa cells were preincubated either with (+) or without (−) AMT and then were irradiated with UV light. After centrifugation, the RNA was fractionated by formaldehyde-agarose gel electrophoresis (the mobility of free E2 RNA is indicated [arrowheads]). The Northern blot was hybridized with a radioactive DNA probe for E2 RNA. The electrophoretic mobilities of 28S and 18S rRNAs were as indicated in Fig. 5B. (B) A 5′-end-labeled oligodeoxynucleotide complementary to the 3′ terminus of E2 RNA was used to sequence E2 RNA of nucleolar RNA from untreated HeLa cells (lanes 1 to 4). Total nucleolar RNA (lanes 5 to 7) and size-fractionated samples of nucleolar RNA (lanes 8 to 14) were used as templates to extend some of this radiolabeled primer. Total nucleolar RNA from cells incubated with (+) (lane 6) or without (−) (lane 7) AMT was analyzed; lane 5 is a shorter X-ray film exposure of lane 6, allowing better visualization of the more intense bands. Sucrose gradient fractions 6 and 7 and fractions 8 and 9 in panel A were pooled separately and analyzed in lanes 8 and 9, respectively, for RNA from AMT-treated cells. Each pool of sucrose gradient RNA fractions is identified by the same lowercase letter in panels A and B. Lanes 10 and 11 are longer X-ray film exposures of lanes 8 and 9, respectively, allowing better visualization of the fainter bands. Lanes 12 to 14 contain RNA sedimentation fractions from cells that were not exposed to AMT. In terms of both sucrose gradient RNA fractions and X-ray film exposures, lanes 12 to 14 are equivalent to lanes 11, 10, and 9, respectively. The migration of the full-length E2 sequence is indicated by the arrowhead on the right. The 15 dots in lane 6 mark primer extension stops that indicate psoralen adducts at nucleotides U-3, U-10 (the arrowhead on the left), U-12, U-13, U-39, U-58, U-60, U-64, A-73, C-74, U-89, U-90, C-91, C-93, and U-94 (from top to bottom). Six dots mark four upper bands in lane 11 and two lower bands in lane 9; they show primer extension stops that indicate psoralen adducts at positions U-12, U-13, U-39, U-60, U-89, and U-90 (numbered 1 to 6, respectively).

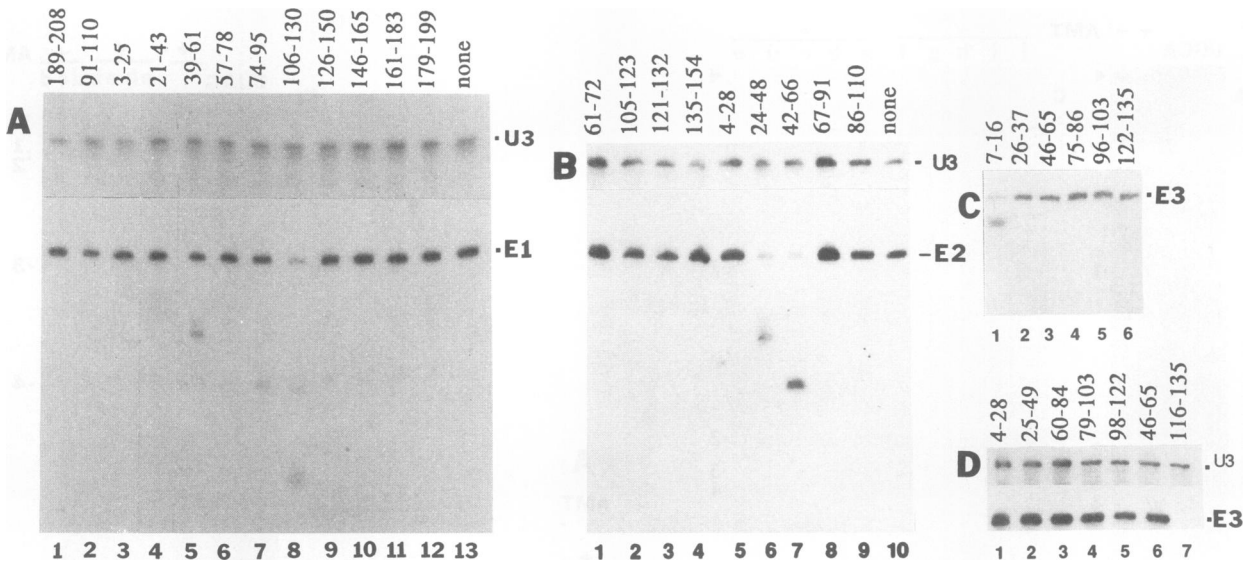

FIG. 8. Antisense oligodeoxynucleotide-targeted degradation of E1, E2, and E3 RNA molecules present in a nucleolar extract. Portions of a HeLa cell nucleolar extract were incubated with the indicated antisense oligodeoxynucleotides to E1 (A), E2 (B), and E3 (C and D) RNAs and RNase H or only RNase H (lane 13 in panel A and lane 10 in panel B). The polyacrylamide Northern blots were hybridized sequentially with a radioactive DNA probe for either E1 (A), E2 (B), or E3 (C and D) RNA and then a probe for U3 RNA. The mobilities of intact E1, E2, E3, and U3 RNAs are indicated. The X-ray film exposures for the E1, E2, and E3 probes were longer than for the U3 probe.

indicating non-cross-linked snoRNAs. It seems likely that they were caused by low-level contamination with free E1, E2, and E3 RNAs (incomplete RNA denaturation before sucrose gradient sedimentation), which was not detected by these X-ray film exposures of the Northern blots (Fig. 5B, 6B, and 7A).

It would be important to know whether E1, E2, and E3 RNAs, while in their putative RNP particles, have any accessible sections and, if they do, to locate those regions. This was examined next by antisense oligodeoxynucleotide-targeted RNase H digestion. A series of complementary oligodeoxynucleotides spanning virtually the whole lengths of E1, E2, and E3 RNAs were tested. Incubation with the antisense oligonucleotides corresponding to positions 106 to 130 in E1 RNA, 24 to 48 and 42 to 66 in E2 RNA, and 7 to 16 and 116 to 135 in E3 RNA resulted in the specific degradation of E1, E2, and E3 RNAs, respectively, present in a nucleolar extract (Fig. 8). There were similar levels of U3 RNA in most of the lanes of the same Northern blots (Fig. 8), representing another example of the specificity of RNase H digestion and indicating that comparable numbers of cell equivalents were loaded onto each lane. The accessible segments at residues 24 to 48 and 42 to 66 of E2 RNA are two separate sites, as opposed to a single region at positions 42 to 48, because different degradation products were generated with each complementary oligonucleotide (Fig. 8). The accessible site near the 5′ end of E3 RNA can be targeted by a relatively short oligonucleotide (complementary to positions 7 to 16) but not with a longer oligonucleotide (antisense to residues 4 to 28) (Fig. 8), implying steric constraints in that region. The oligonucleotide complementary to bases 116 to 135 of E3 RNA targeted degradation, while the antisense oligonucleotide to positions 122 to 135 did not (Fig. 8), suggesting that the accessible site may be approximately between residues 116 and 122 of E3 RNA. These results are summarized in Fig. 9.

DISCUSSION

E1, E2, and E3 RNAs appear to be associated with nucleolar structures quite tightly on the basis of the ionic strength needed to dissociate them. E1, E2, and E3 RNA molecules present in a whole-cell extract sedimented faster than the naked snoRNAs in glycerol gradients. In addition, only one or two segments of the E1, E2, and E3 RNAs present in a nucleolar extract are substantially accessible to oligonucleotide-directed RNase H digestion, as opposed to most of the small RNA sequence, when naked RNA is tested (for example, see reference 30). These data suggest that E1, E2, and E3 RNAs are not free in the cell but are associated with proteins in snRNPs. We have observed that E1, E2, and E3 RNAs present in a nuclear extract are not immunoprecipitated by Y12 monoclonal antibody to the Sm antigen (14), monoclonal antibody H57 specific for the human B/B′ polypeptides of U snRNPs (28), or polyclonal antibodies to the U3 snRNP (data not shown). Then, the proteins of the putative E1, E2, and E3 snRNPs may be quite unique, since they do not include some of the known snRNP proteins. In addition, E1, E2, and E3 RNAs do not have the nucleotide sequence needed to bind fibrillarin, the protein present in the more-abundant nucleolar snRNP species (1, 21).

The present psoralen cross-linking results in vivo indicate that some of the E1, E2, and E3 RNA molecules interact with pre-rRNA in whole cells. These data plus the detection of E1, E2, and E3 RNAs only in the nucleolar fraction (21) imply that these three snoRNAs may function in some aspect of ribosome formation, possibly pre-rRNA processing or nucleolar assembly of ribosomal precursor particles. These results also suggest that the functions of E1, E2, and E3 RNAs may differ from that of U3 RNA, since human U3 RNA psoralen cross-links in vivo to a sequence between nucleotides 438 and 695 of ETS1 (18), downstream of the 414 human early processing site (Fig. 3B). E1, E2, and E3 RNAs may have different functions, since their sequences do not

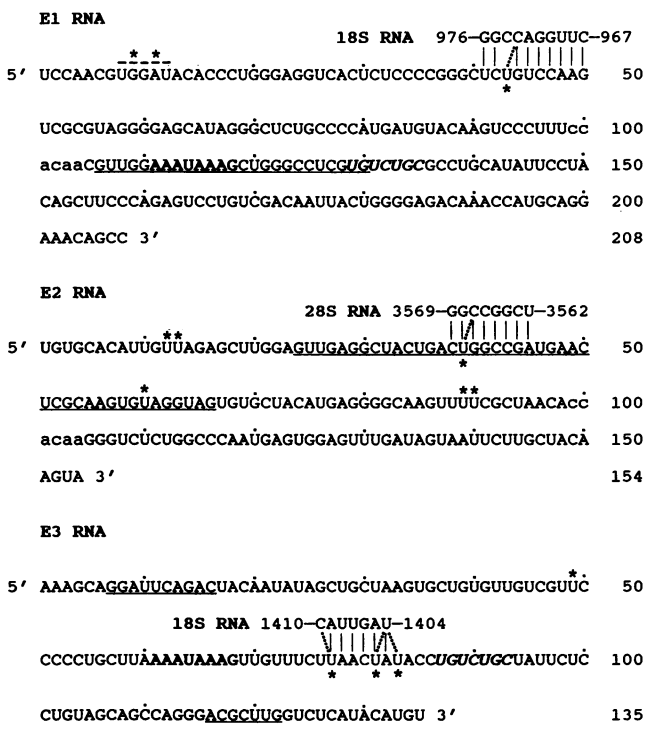

FIG. 9. Summary of the locations of the psoralen adducts that may be cross-linking sites (asterisks) in vivo and of the accessible segments (underlined) in E1, E2, and E3 RNAs present in a cell extract. Hypothetical sites of cross-linking (diagonal dotted lines) and base pairing (vertical lines) between snoRNAs and pre-rRNA are shown. The location of two psoralen adducts between nucleotides U-7 and U-11 of E1 RNA is indicated by the horizontal dashed line above the sequence. In this model, different E3 RNA molecules would be cross-linked at either nucleotide U-77, U-81, or U-83. The three homologous sequences in E1, E2, and E3 RNAs, CCACAA (lowercase), AAAUAAAG (boldface), and UGUCUGC (boldface italic) (21), are indicated. Thin-layer chromatography of HeLa cell E1 RNA that had been 5′ end labeled with ^{32}P and then digested completely with nuclease P1 showed a U residue at the 5′ terminus of E1 RNA (23a). An extra U residue has been added to the 5′ end of the E1 RNA sequence.

show any substantial homology (21) and these snoRNAs are psoralen cross-linked to distinct regions of pre-rRNA in vivo. The functions of E1, E2, and E3 may involve direct interaction or base pairing with pre-rRNA instead of indirect contact. Mouse U3 RNA is psoralen cross-linked in vitro to a second site in ETS1, which is located 362 residues downstream from the early processing site (29); yeast U3 RNA is psoralen cross-linked in vivo to two sites in ETS1 that are 185 residues apart (2). It is surprising that E1 RNA cross-links to two distant segments of pre-rRNA. It is possible that E1 RNA interacts with each site sequentially. Alternatively, the tertiary structure of pre-rRNA might (perhaps transiently) allow one E1 RNA molecule to bind to both sites simultaneously. It is also conceivable that some E1 RNA molecules interact with the ETS1 site while others (possibly in another function) associate with the 18S rRNA sequence. Psoralen cross-links in RNA occur mainly at U residues, and secondarily at C residues, located at ends of base-paired segments (11). Some nucleotide sequences meet these conditions and are within the sites detected experimentally in pre-rRNA: (i) residues U-42 of E1 RNA, U-39 of E2 RNA, and U-77, U-81, and U-83 of E3 RNA might be psoralen cross-linked to large nucleolar RNA (Fig. 5 to 7), and (ii) Fig. 9 shows segments of potential base pairing and possible cross-linking sites between pre-rRNA and these three snoRNAs. Two possible cross-linking sites in E2 RNA, U-39 and U-60, are in accessible regions (Fig. 9). Human E1, E2, and E3 RNAs share three short sequence motifs (21); (i) the sequence AAAUAAAG is in an accessible segment in E1 RNA but not in E3 RNA (Fig. 9), and (ii) the sequence CCACAA, located at the same distance from the 5′ ends of E1 and E2 RNAs, is in an apparently inaccessible site (Fig. 9). If E1, E2, and E3 RNAs function in pre-rRNA maturation, it is interesting that they cross-link to regions in pre-rRNA that are located quite far in primary structure from any known pre-rRNA processing site.

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