Nucleic Acids Research, Vol. 20, No. 20 5375–5382

A new interaction between the mouse 5′ external transcribed spacer of pre-rRNA and U3 snRNA detected by psoralen crosslinking

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Received July 14, 1992; Revised and Accepted September 18, 1992

ABSTRACT

The first cleavage in mammalian pre-rRNA processing occurs within the 5′ external transcribed spacer (ETS). We have recently shown that the U3 snRNP is required for this cleavage reaction, binds to the rRNA precursor, and remains complexed with the downstream processing product after the reaction has been completed (1). Using psoralen crosslinking in mouse cell extract we have detected a new interaction between U3 RNA and the mouse ETS processing substrate and its processed product. The crosslinked sites on both U3 and ETS RNAs have been mapped by RNase H cleavage and primer extension analyses. The crosslinked sites in U3 RNA map to C_5, U_6, and U_8. U_8 lies within and C_5 and U_6 are adjacent to an evolutionarily conserved U3 sequence termed box A′. In the ETS the crosslinked sites are U_{1012} and U_{1013}, 362 nucleotides downstream from the processing site. Although the crosslinked site is dispensable for the primary processing reaction *in vitro*, a short conserved sequence just 3′ to the cleavage site (nucleotides 650–668) is absolutely required for crosslink formation. We conclude that the interaction between U3 RNA and the 5′ ETS detected by psoralen crosslinking may play a role in subsequent step(s) of pre-rRNA processing.

INTRODUCTION

The 18S, 5.8S and 28S ribosomal RNAs in eukaryotes are initially synthesized as a single precursor molecule which undergoes a series of processing reactions to produce the mature species (for review see 2). The primary ribosomal RNA transcript begins with a 5′ external transcribed spacer (5′ ETS), followed by the 18S rRNA sequence, an internal transcribed spacer (ITS1), the 5.8S rRNA region, an ITS2, the 28S rRNA and a 3′ ETS.

The earliest processing of the primary transcript occurs within the 5′ ETS; in mouse cells the cleavage site is located ~650 nucleotides from the transcription start and ~3000 nucleotides from the 5′ end of the 18S rRNA (3,4). An analogous processing event has been described in human cells; the downstream ~200 nucleotides beyond the processing site of mouse and human transcripts are 85% identical, while the surrounding regions show no detectable sequence similarity (3,4). The primary processing reaction has been successfully reproduced *in vitro* in mouse cell extracts (5,6). Analyses of various 5′ and 3′ deleted and internally mutated ETS transcripts showed that highly conserved nucleotides +655 to +666 are most critical for processing, while the remainder of the downstream ~200 nucleotide conserved region stimulates the processing efficiency about 30-fold and sequences beyond only slightly increase processing efficiency (6,7). Substrate RNAs form a specific complex with factors of the mouse cell extract, including the U3 snRNP, which is required for the cleavage reaction and stays bound to the downstream cleavage product (1,8); the upstream fragment is rapidly degraded (1,3). Recently, it has been shown that the 5′ ETS of yeast *S.cerevisiae* also undergoes processing which is abolished in strains deleted for U3 snRNA genes (9).

Psoralen crosslinking has been successfully used to detect a number of base pairing interactions between various RNAs (for review see 10). Psoralen intercalates into nucleic acid helices and forms a covalent linkage between juxtaposed pyrimidine residues, preferentially uridines, on opposite strands upon irradiation with 365 nm light. Helices containing G·U base pairs or bulged nucleotides and the ends of helices are hot spots for psoralen crosslinking. Psoralen can also crosslink non-base paired, but closely positioned residues in complex macromolecular assemblies (10,11). A valuable property of psoralen crosslinks is that they can be reversed with 254 nm light (10).

Using psoralen crosslinking, *in vitro* interactions between U4 and U6 snRNAs (12) and between U2 and U6 snRNAs (13) as well as *in vivo* interactions between heterogenous nuclear RNA and U1 and U2 snRNAs (14,15) have been characterized. Also, *in vivo* psoralen crosslinks have been detected between U3 RNA and the 5′ ETS in human (16) and rat cells (17). Although the exact sites of the U3 crosslinks have not been identified, the region of the ETS crosslinked to U3 includes the primary processing site in rat and is adjacent to it in human cells. Recently, two *in vivo* psoralen crosslinks between yeast U3 RNA and the 5′ ETS have been characterized (18).

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In this paper we describe a novel interaction between U3 RNA and 5′ ETS transcripts detected by psoralen crosslinking *in vitro* in mouse cell extracts. This interaction occurs 362 nucleotides downstream from the processing site, far beyond the previously characterized ~200 nucleotides conserved between mammalian species. Rather, it is located 15 nucleotides upstream from another ETS conserved region, called M4, which has been proposed to contribute to a conserved folding pattern for mammalian ETS RNA sequences (19).

MATERIALS AND METHODS

Plasmids and *in vitro* transcription

Templates for synthesizing the ETS processing substrates (generous gifts from S.Kass and B.Sollner-Webb, Johns Hopkins University, Baltimore) contain mouse rDNA sequences between positions 645 and 1290 or 665 and 1290, isolated from a 5′ deletion series (6) and cloned between the EcoRI and PstI sites of the pGEM3 plasmid. To obtain substrates extending to position 1290, the plasmid DNA was linearized with PstI and transcribed by T7 RNA polymerase (Pharmacia) in the presence of [α-^{32}P]UTP as described by Kass et al. (3). To synthesize the 645−997 and 645−1015 substrates, the plasmid containing 645−1290 rDNA was linearized with ApaLI or HgaI, respectively. All transcripts were gel purified before use.

Processing and psoralen crosslinking reactions

Mouse S-100 cell extracts were prepared as described (5) from mid-log phase L1210 tissue culture cells treated for 45 min with 0.3 μg/ml actinomycin D before harvesting. Processing substrates were incubated in L1210 S-100 extracts under conditions described by Kass et al. (1). An equal amount of a buffer containing 20 mM Hepes pH 7.9, 100 mM KCl, 1 mM $MgCl_2$, and 5 μg/ml AMT psoralen (HRI Associates) was then added to the processing reaction, and the mixture was divided into 15 μl drops placed on a sheet of parafilm on ice and irradiated for 10 min with 365 nm light as described by Hausner et al. (13). 1 μl of 40 μg/ml AMT psoralen was then added and irradiation was continued for another 10 min. The irradiated fractions were pooled, supplemented with SDS to 0.1% and EDTA to 5 mM, and digested with 200 μg/ml proteinase K (Beckman) for 15 min at 37°C. RNA was extracted with PCA (phenol:chloroform:isoamyl alcohol; 25:24:1), ethanol precipitated and used for reverse transcription or RNase H analysis.

Micrococcal nuclease digestion

Ten microliter reactions containing 6 μl of S-100 extract plus 10 mM Hepes pH 7.9, 1 mM $CaCl_2$ (and 4 mM EGTA, where indicated) were pretreated with 10 units of micrococcal nuclease

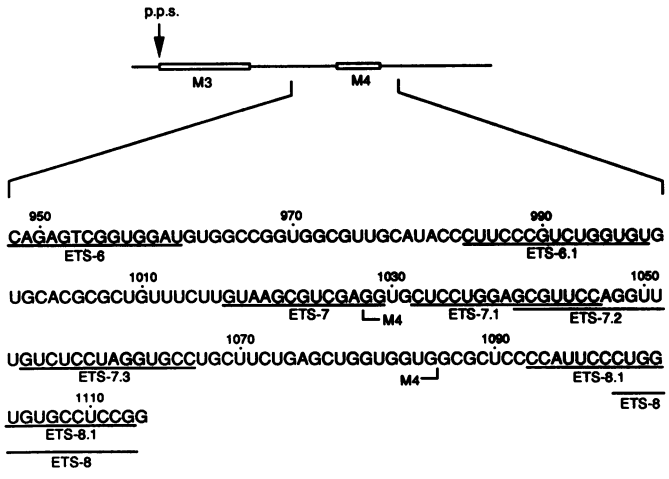

Figure 1. Composition of the ETS transcript used for *in vitro* psoralen crosslinking. The transcript begins with 28 nucleotides derived from the pGEM3 polylinker followed by pre-rRNA sequences spanning nucleotides 645−1290 of the mouse 5′ ETS. P.p.s. stands for the primary processing site (position ~650). The region M3 is ~200 nucleotides long and exhibits high conservation between mammalian species (3,19). Further downstream is another conserved region, M4, spanning sequences 1028−1085 of the ETS. The sequence of nucleotides 948−1114 of the ETS is shown below, indicating regions complementary to the oligonucleotides used for mapping of the crosslinked site in the experiments shown in Fig. 5B and 6.

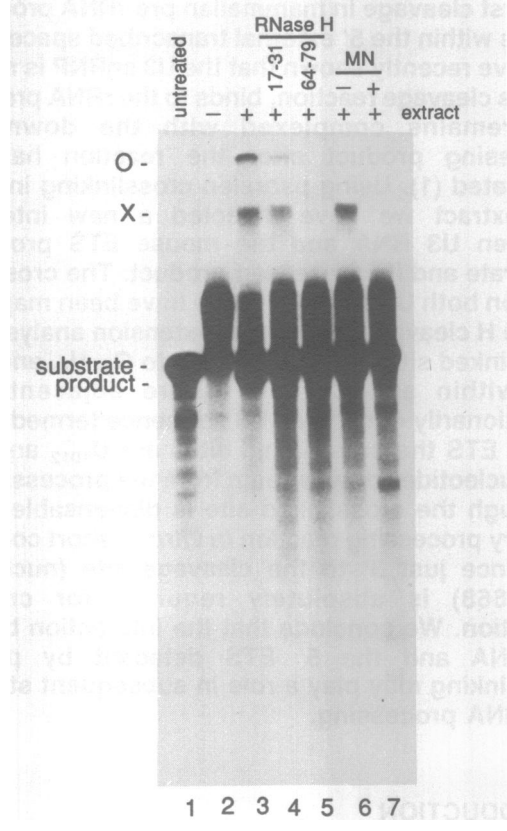

Figure 2. Identification of a psoralen crosslink between the ETS transcript and a small RNA present in S-100 extract. A substrate containing nucleotides 645−1290 of the mouse ETS (lane 1) was incubated under processing conditions in the absence (lane 2) or presence (lane 3) of S-100 extract and irradiated with 365 nm light in the presence of AMT psoralen under standard conditions described in Materials and Methods. Lane 4 and 5: S-100 extract was pretreated with RNase H and U3 specific oligonucleotide 17−31 or 64−79 prior to the processing reaction, respectively. Lanes 6 and 7: S-100 extract was pretreated with $CaCl_2$ and EGTA or $CaCl_2$ and micrococcal nuclease, respectively. A crosslinked RNA species formed only upon UV irradiation of the processing reaction containing S-100 extract is marked X. O: gel origin. The band that does not enter the gel in lane 3 does not seem to represent a functionally relevant interaction since it does not appear reproducibly in all experiments carried out under the same conditions.

(Worthington) at 30°C for 10 min. After incubation, 1 μl of 40 mM EGTA was added to inhibit the nuclease.

Oligonucleotides and RNase H digestion

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Yale University) and ethanol precipitated before use. The sequence of ETS-5 oligonucleotide is 5'ACGCG-AACCACTGAG3' The sequences of other oligonucleotides complementary to ETS or U3 RNAs are indicated in Fig. 1 and Fig. 3C, respectively.

S-100 extract was pretreated with RNase H and U3 oligonucleotides as described by Kass et al. (1). RNase H digestions of naked RNA were carried out in 10 μl containing 40 mM Tris HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.5 μg of oligonucleotide, 4 μg of *E.coli* tRNA, and 1unit of RNase H (Boehringer-Manheim or Pharmacia) at 37°C for 30 min.

Reverse transcription

Reverse transcription was performed as described by Parker and Steitz (20) except that primers were hybridized to the RNA for 30 min.

RESULTS

Psoralen produces a specific crosslink between the 5' ETS transcript and U3 RNA

Mouse 5' ETS transcripts containing the 650 processing site and extending to position 1290 form a specific complex with the U3 snRNP upon incubation in mouse cell extracts under processing conditions (1). To ask whether base pairing interactions between the ETS and U3 RNA occur in such a complex, we irradiated a processing reaction containing uniformly labeled transcript spanning the 645−1290 region of the mouse ETS (Fig. 1) with 365 nm light in the presence of the psoralen derivative AMT [4'-amino-methyl-4,5',8-trimethylpsoralen] (Fig. 2, lane 3). Formation of an RNA species called X, containing ETS sequences, was observed. Band X does not appear when the substrate is irradiated in the absence of S-100 extract (lane 2) or when the extract is pretreated with micrococcal nuclease (lane 7), which abolishes processing. Moreover, the formation of species X is prevented by pretreatment of the extract with RNase H in the presence of an deoxyoligonucleotide complementary to nucleotides 64−79 of U3 RNA (lane 5). The 64−79

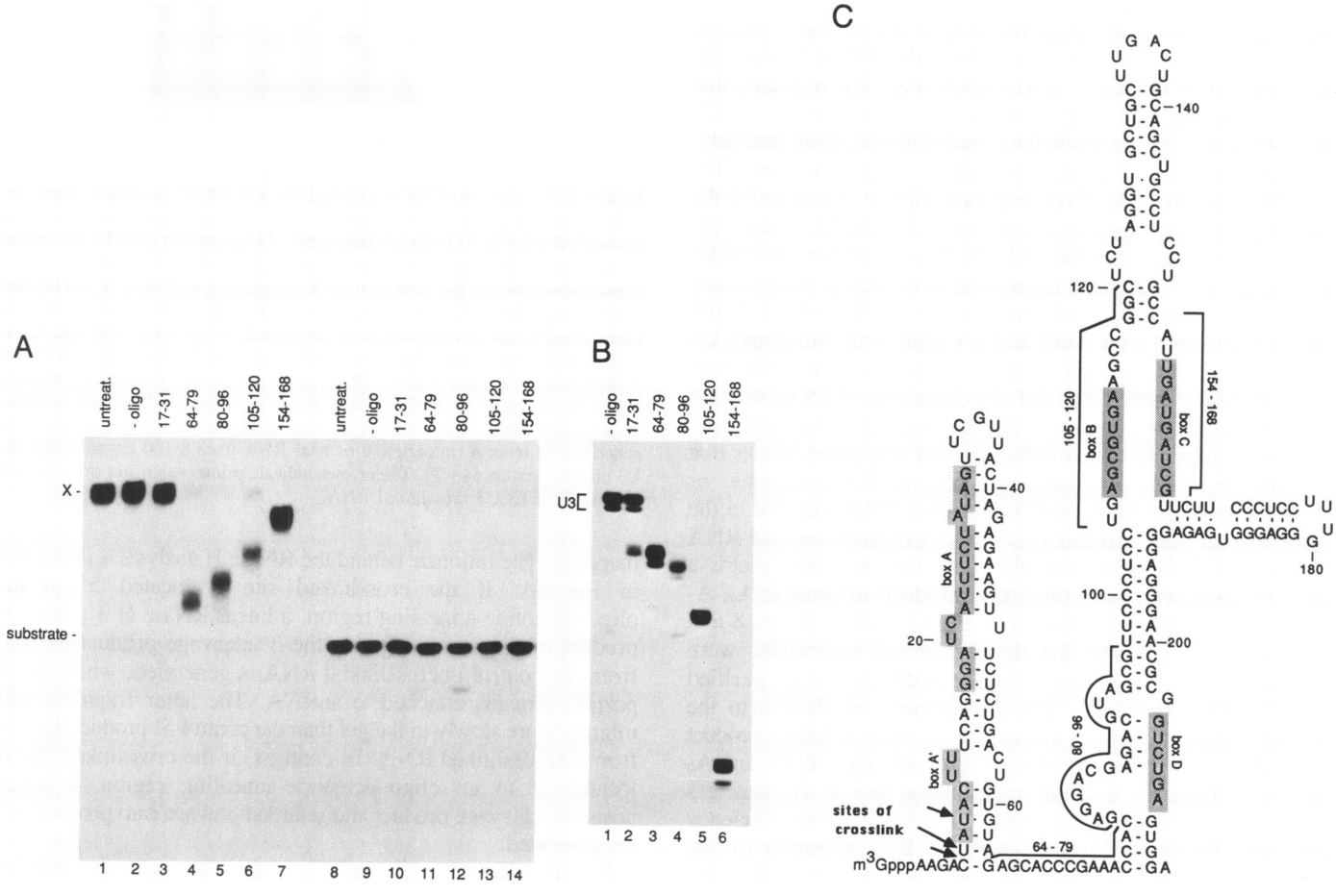

Figure 3. The crosslinked ETS complex contains U3 RNA. **A.** Gel purified band X (lane 1) was digested with RNase H in the absence of an oligonucleotide (lane 2) or the presence of U3 specific oligonucleotides (lanes 3−7). To control for nonspecific RNase H cleavages within the ETS, the processing substrate was also incubated with RNase H and U3 specific oligonucleotides (lanes 10−14). The oligonucleotides, named for the complementary nucleotides in U3 RNA , are indicated at the top. **B.** RNase H digestion of naked, 3' end labeled U3 RNA using U3 specific oligonucleotides. **C.** The primary and secondary structure of mouse U3B RNA taken from (26). Evolutionarily conserved sequences, called boxes A', A, B, C, and D, are shaded. Oligonucleotides complementary to the indicated sequences were used for the RNase H experiments in (A) and (B) and for the primer extensions shown in Fig. 4.

oligonucleotide, but not a control oligonucleotide (17–31) used in lane 4, promotes efficient cleavage of U3 RNA in cell extracts (1). We conclude that species X contains U3 RNA crosslinked either to the 645–1290 processing substrate or to its downstream processing product. Since the cut occurs at several positions around nucleotide 650 (3), cleaving off only about 35 nucleotides (see Fig.1), the crosslinked substrate and processing product would not be expected to resolve in a 4% polyacrylamide gel.

To confirm that species X contains U3 RNA, it was isolated from the gel and subjected to RNase H cleavage in the presence of U3 specific oligonucleotides (Fig. 3A). All oligonucleotides [64–79, 80–96, 105–120 and 154–168 (see Fig. 3C)] that elicit efficient cleavage of naked U3 RNA (Fig. 3B, lanes 3–6) also promote cleavage of RNA X (Fig. 3A, lanes 4–7). The increase in the gel mobility of the digested species X is not due to nonspecific RNase H cleavage within the ETS since none of the oligonucleotides promotes digestion of the uncrosslinked substrate (Fig. 3A, lanes 11–14). Note that oligonucleotide 17–31, which does not promote cleavage of U3 RNA (Fig. 3B, lane 2), also does not elicit cleavage of species X (Fig. 3A, lane 3). This experiment further supports the initial conclusion that band X represents a crosslink between ETS and U3 RNA.

Mapping the crosslinked site on U3 RNA

The sequential increase in gel mobility of the RNase H cleaved ETS-U3 crosslinked RNA (Fig. 3A) as the RNase H cleavage site was moved further 5′ in U3 RNA (Fig. 3B) indicated that the crosslinking site might be located within or 5′ to U3 nucleotides 64–79. To locate the crosslinking site more precisely, we performed primer extension analyses on gel purified ETS-U3 RNA; the progress of reverse transcriptase is arrested at the residue preceding a psoralen crosslink (21). Primers used were complementary to nucleotides 64–79 (Fig. 4) and to nucleotides 80–96 and 105–120 (data not shown) of U3 RNA. As a control, primer extension was performed on total RNA of the S-100 extract not treated with AMT and UV light; only full-length U3 cDNA is generated (lane 1). Primer extension on total RNA from a crosslinking reaction that did not contain the ETS processing substrate yields, in addition to the full-size U3 cDNA, an array of shorter products which reflect primer extension blocks due to either psoralen monoadducts, internal U3 crosslinks, or possible crosslinks between U3 and other RNAs present in the S-100 extract (lanes 10 and 11). Primer extension on total RNA from a crosslinking reaction that contained substrate yields a similar pattern with more prominent products arrested at A_9, A_7 and U_6, reflecting modification of U_8, U_6 and C_5 (lanes 8 and 9). Finally, to confirm that these modified nucleotides were crosslinked sites, we performed primer extension on gel purified ETS-U3 RNA (band X). The appearance, in addition to the background pattern present in lanes 8–11, of an abundant product arrested at A_7 and less abundant cDNAs ending with U_6 and A_9 (lane 6) establishes a major crosslinking site at U_6 and less abundant crosslinks at C_5 and U_8. Other lower-efficiency crosslinks between U3 RNA and the ETS substrate or the processing product would be obscured by the high background of primer extension products caused by psoralen monoadducts and/or intramolecular crosslinks within the U3 RNA structure.

Localization of the crosslinked site in the ETS

The crosslinked site in the ETS RNA was determined by a two-step analysis involving localization by oligonucleotide-directed RNase H cleavage followed by more precise primer extension

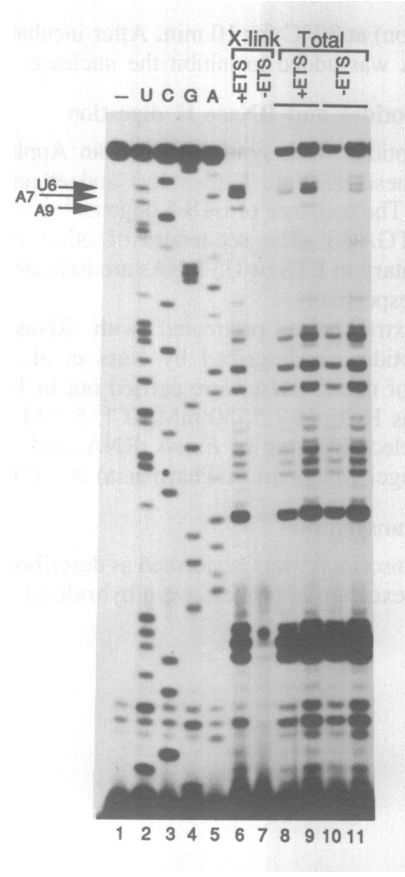

Figure 4. Localization of the crosslinked site in U3 RNA by primer extension. The crosslinked ETS-U3 RNA complex (band X) was gel purified and reverse transcribed using the U3 oligonucleotide 64–79 as a primer (lane 6). To control for nonspecific priming from other RNAs present in the S-100 extract, reverse transcription was also performed on RNA comigrating with band X, but isolated from a crosslinking reaction which did not contain the ETS transcript (lane 7). Lanes 8 and 9 show primer extensions performed on 1/100 and 1/40 total RNA, respectively, from a crosslinking reaction; lanes 10 and 11 show primer extension performed on 1/100 and 1/40 total RNA, respectively, of a crosslinking reaction which did not contain the ETS transcript. Primer extension products were separated on a 8% PAGE alongside dideoxynucleotide sequencing ladders (lanes 1–5) generated by reverse transcription of total RNA from S-100 extract using the U3 oligonucleotide 64–79. The arrows indicate primer extension stops present only in the ETS-U3 crosslinked RNA.

mapping. The rationale behind the RNase H analysis is illustrated in Fig 5A. If the crosslinked site is located 5′ to the oligonucleotide-annealing region, a linear RNase H 3′ cleavage product of the same length as the 3′ cleavage product derived from the control uncrosslinked RNA is generated, while the 5′ portion remains attached to snRNA. The latter fragment will migrate more slowly in the gel than the control 5′ product derived from uncrosslinked RNA. In contrast, if the crosslinked site is located 3′ to an oligonucleotide-annealing region, a linear upstream cleavage product and retarded downstream product will be generated.

Initially, RNase H analysis using ten oligonucleotides complementary to regions distributed along the entire length of the 645–1290 substrate located the crosslinked site between nucleotides 947 and 1029 (data not shown). The region was further narrowed using oligonucleotides spanning position 947–1029 of the ETS (Fig. 1 and 5A). These analyses revealed that the crosslinked ETS-U3 RNA represents a mixture of species with U3 crosslinked to either the ETS substrate or the processing

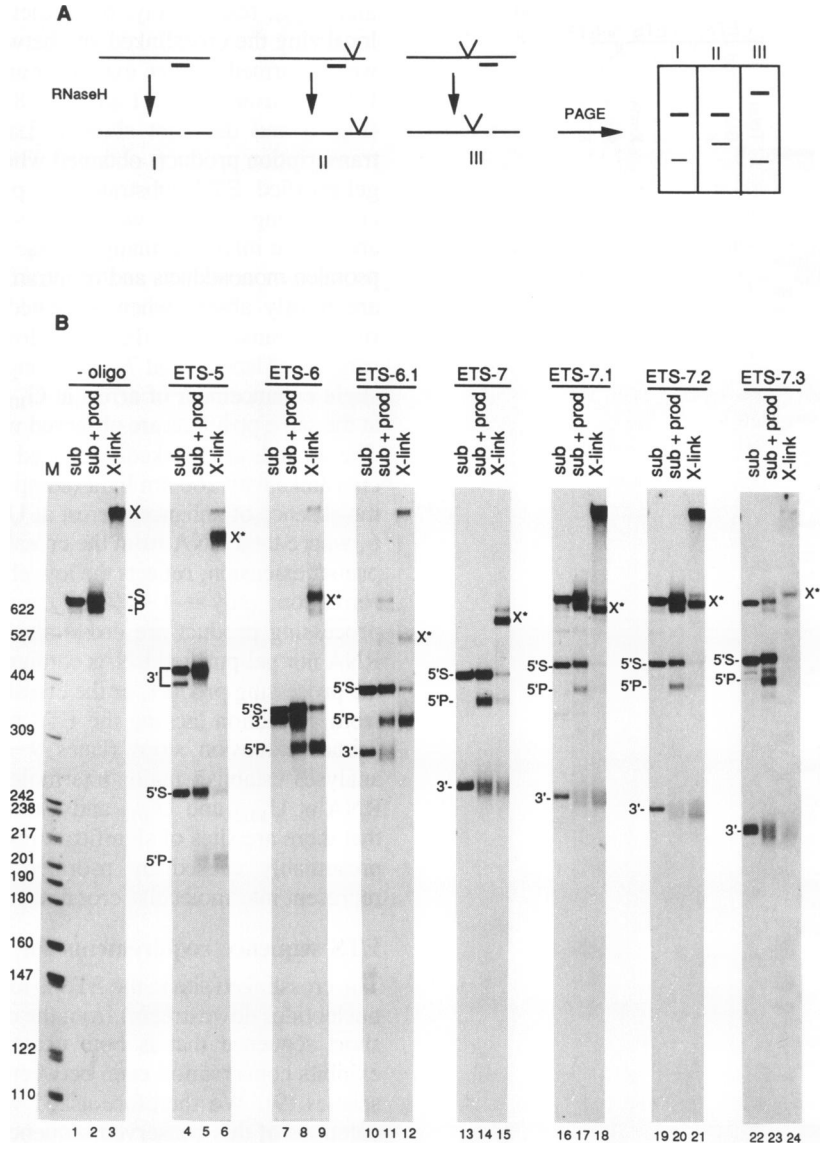

Figure 5. Localization of the crosslinked site in the ETS by RNase H cleavage analysis. **A.** Schematic representation of the RNase H analysis strategy. **B.** Gel purified ETS-U3 crosslinked RNA (band X) was digested with RNase H in the presence of ETS specific oligonucleotides (lanes 3, 6, 9, 12, 15, 18, 21, and 24) and run on a 5% PAGE alongside RNase H digested untreated ETS processing substrate (lanes 1, 4, 7, 10, 13, 16, 19, and 22) and the RNase H digested mixture of the ETS substrate and the processing product, gel-purified from a crosslinking reaction (lanes 2, 5, 8, 11, 14, 17, 20, and 23). The specificities of the oligonucleotides are indicated at the top (also see Fig. 1). 5'S and 5'P stand for the linear 5' RNase H digestion products derived from the ETS precursor and the processing product, respectively. 3' stands for the linear 3' RNase H digestion fragment derived from both the ETS precursor and the processing product. X*: RNase H digested crosslinked ETS-U3 RNA. M: MspI digestion fragments of pBR322.

product, since linear fragments corresponding to the 5' RNase H digestion products of both are observed after targeting with oligonucleotides ETS-5, ETS-6 and ETS-6.1 (Fig. 5B, lanes 6, 9, and 12). Note that the crosslink between U3 and the processing product appears to form more efficiently, since the product/substrate ratio of the 5' RNase H digestion fragments of the ETS is always higher in the crosslinked ETS-U3 RNA than in the uncrosslinked ETS substrate and product mixture isolated from the crosslinking reaction (compare lanes X-link with lanes sub+prod). Thus, these results confirm previous data indicating that the U3 snRNP remains bound to the processing product after cleavage has been completed (1). The 5' RNase H cleavage products derived from the isolated ETS-U3 RNA are the same lengths as the 5' RNase H cleavage products of the substrate and processing product (lanes 4 and 5, 7 and 8, 10 and 11, respectively). The 3' portion in every case is retarded in the gel relative to the control 3' fragment derived from either the ETS substrate or the processing product (compare lanes 4 and 5 with 6, lanes 7 and 8 with 9, and lanes 10 and 11 with 12). Cleavage of the ETS-U3 species with RNase H and oligonucleotides ETS-7, ETS-7.1, ETS-7.2, and ETS-7.3 generates linear 3' fragments and gel retarded 5' fragments (lanes 15, 18, 21, and 24). This analysis established that the crosslinked site is located between oligonucleotides ETS-6.1 and ETS-7.

We then mapped the 5' ends of the 3' fragments generated by RNase H and oligonucleotides ETS-6.1 and ETS-7 to U_{1013}

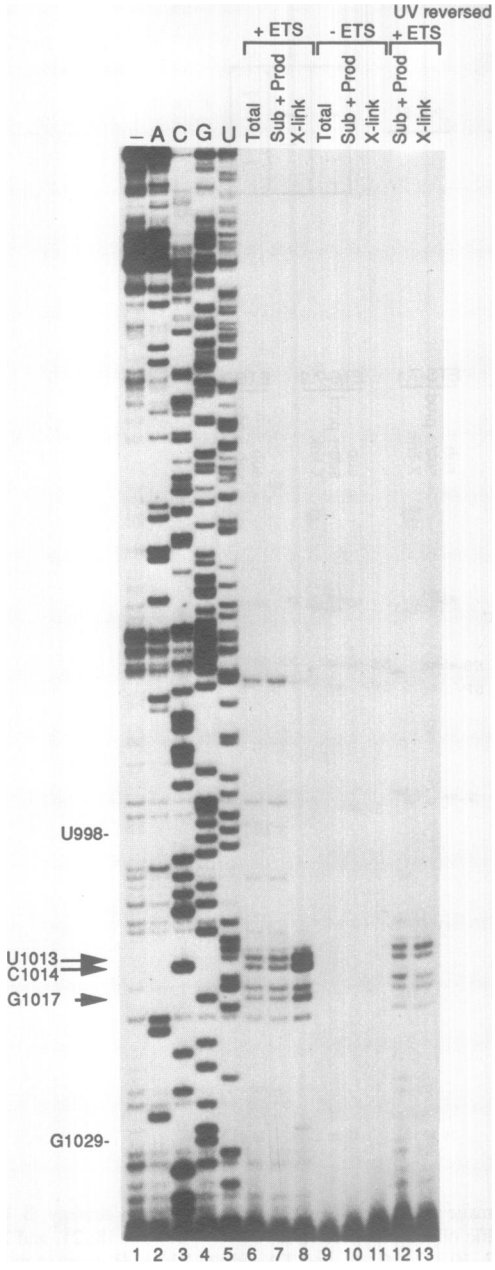

Figure 6. Mapping the crosslinked nucleotides on the ETS by primer extension. Gel purified ETS-U3 crosslinked RNA was reverse transcribed using oligonucleotide ETS-7.1 as a primer (see Fig. 1), and the reaction products were separated on a 8% PAGE (lane 8) alongside the products of primer extension from total RNA isolated from a crosslinking reaction (lane 6) or from a mixture of the ETS precursor and the processing product, gel-purified from a crosslinking reaction (lane 7). Equal molar amounts of the ETS fragments were used in each primer extension reaction. To control for nonspecific priming from other RNAs present in the crosslinking reaction, primer extensions were also performed on total RNA (lane 9), gel purified RNA comigrating with the ETS substrate and the processing product (lane 10) and RNA comigrating with the crosslinked ETS-U3 complex (lane 11) isolated from a crosslinking reaction which did not contain ETS substrate. Lanes 12 and 13 show primer extension performed on the gel-purified mixture of the ETS substrate and the processing product (lane 12) and the crosslinked ETS-U3 RNA (lane 13) irradiated with 260 nm light in the purifying gel before elution. Lane 1 shows primer extension performed on untreated ETS processing substrate and lanes 2–5 show dideoxynucleotide sequencing ladders generated by reverse transcription of the ETS substrate.The arrows indicate reverse transcription stops enhanced in the ETS-U3 RNA relative to stops in the ETS substrate and the processing product.

and G_{1029}, respectively, by primer extension (data not shown), localizing the crosslinked site between these positions. Finally, we performed primer extension analysis on gel purified ETS-U3 RNA using ETS-7.1 and ETS-8.1 oligonucleotides as primers (Fig. 6 and data not shown). Lanes 6 and 7 show reverse transcription products obtained when total RNA or a mixture of gel-purified ETS substrate and processing product from the crosslinking reaction were used as template, respectively. As above, we infer that many reverse transcription stops represent psoralen monoadducts and/or intramolecular crosslinks since they are mostly absent when untreated ETS substrate is used for reverse transcription (lane 1). However, superimposed on the patterns of lanes 6 and 7, are strong stops at U_{1013} and C_{1014} and slight enhancement of arrest at G_{1017} in lane 8. Enhanced stops at the same positions are observed when ETS-8.1 primer is used. These bands are markedly reduced after reversal of the psoralen crosslinks with 260 nm light (compare lane 13 with 8). Note that the absence of enhanced arrest at U_{1013}, C_{1014}, and G_{1017} in lane 6, where total RNA from the crosslinking reaction was used for primer extension, reflects the low efficiency of psoralen crosslink formation; only ~1–2% of the total ETS substrate and the processing product are crosslinked to U3 RNA. Neither total RNA nor gel-purified RNAs comigrating with the ETS substrate, the processing product, or the crosslinked ETS-U3 RNA isolated from a reaction lacking the ETS transcript produce detectable primer extension stops (lanes 9–11). The primer extension analyses establish major intermolecular crosslinks in the ETS RNA at U_{1012} and U_{1013} and a minor crosslink at U_{1016}. Note that there are sites of significant reverse transcription blockage, presumably caused by psoralen monoadducts, that do not represent intermolecular crosslinks (Fig. 6 and data not shown).

ETS sequence requirements for crosslink formation

The crosslinked site in the ETS is located surprisingly far (~360 nucleotides downstream) from the cleavage site and the adjacent short sequence that is both critical for processing (6,7) and exhibits conservation even between distantly related eukaryotic species (9). We therefore asked whether substrates harboring deletions of this conserved sequence can crosslink to U3 RNA. As shown in Fig. 8, lanes 3 and 4, when the entire conserved sequence (nucleotides 645–668) is deleted from the parental 645–1290 substrate, no detectable crosslinks with U3 RNA are formed. In contrast, the processing product (lanes 1 and 2)

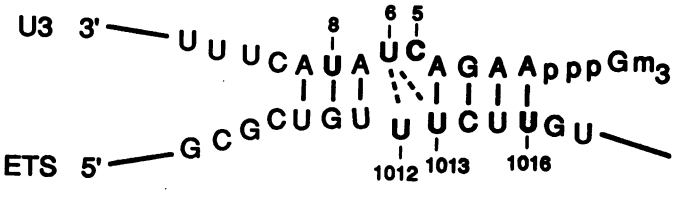

Figure 7. Possible base pairing between the ETS and the 5′ end of U3 RNA. The U residues involved in the major crosslinks (dashed lines) are outlined. In bold are shown additional minor crosslinked nucleotides in U3 RNA (C_5 and U_8) and in the ETS (U_{1016}). The C_5 residue of U3 has potential to form a psoralen crosslink with U_{1012} and U_{1013} of the ETS, while U_8 could theoretically crosslink to U_{1009} or U_{1011} of the ETS, however, modification of neither U_{1009} nor U_{1011} was detectable by primer extension. Nucleotide U_{1016} of the ETS is sequestered in a configuration which does not favor crosslink formation.

crosslinks with efficiency similar to the standard 645−1290 substrate (lanes 5 and 6). Thus, the short conserved sequence downstream of the cleavage site is crucial for crosslink formation, while sequences upstream of the cut site are not.

Since the crosslinked site is located only 15 nucleotides upstream of an ~50 nucleotide region, called M4, that is conserved between mammalian species, we asked whether deletion of this sequence would affect crosslink formation. As shown in Fig. 8, lanes 9 and 10, the 645-ETS-7 substrate, which ends just before the M4 sequence (see Fig. 1), crosslinks to U3 with comparable efficiency to the standard 645−1290 substrate (lanes 5, 6) and 645-ETS-8, which contains the M4 region but lacks additional 3′ sequence (lanes 7 and 8). [Note that the crosslink formed with the 645-ETS-7 substrate is resolved from the crosslink formed with its processed product (lane 9)]. A more extensive 3′ deletion, that generates the substrate that ends with U_{1015} and thus eliminates the last base-pair in the interaction shown in Fig. 7 prevents crosslink formation (lanes 17 and 18). Mutants that delete the crosslinking site, as expected, also do not form detectable crosslinks with U3 RNA (lanes 11−14), although they are processed as efficiently as the parental 645−1290 substrate.

DISCUSSION

Using mouse cell extract, we have identified and mapped a novel psoralen crosslink between the mouse 5′ ETS processing substrate and U3 RNA (Fig. 2−6). The ETS nucleotides involved in crosslink formation, U_{1012} and U_{1013}, are contained in a 8 nucleotide long sequence that exhibits complementarity to the 5′ end of U3 RNA (Fig. 7). Indeed, the crosslinked site in U3 RNA maps within the complementary region, the major crosslinked residue being U_6 and the minor being C_5 and U_8.

The base pairing interactions shown in Fig. 7 predict that C_5 and U_6 of U3 RNA should crosslink to either U_{1012} or U_{1013} of the ETS. The fact that U_{1012}, but not U_{1011}, is the favored crosslink to U_6 of U3 RNA is unexpected, since it is positioned out of the helical path. If an efficient crosslink at U_{1011} existed, it should have been detected by primer extension analysis (Fig. 6) even if it lies 5′ to the major crosslinking sites at U_{1012} and U_{1013}. In that case, primer extension should not be arrested at U_{1012} and U_{1013} since formation of a crosslink at U_{1011} would preclude crosslinks at U_{1012} and U_{1013}, because all of them involve the same U3 RNA residue, U_6. U_8 of U3 RNA resides in a configuration that should promote crosslinking to either U_{1009} or U_{1011} of the ETS. However, modification of neither

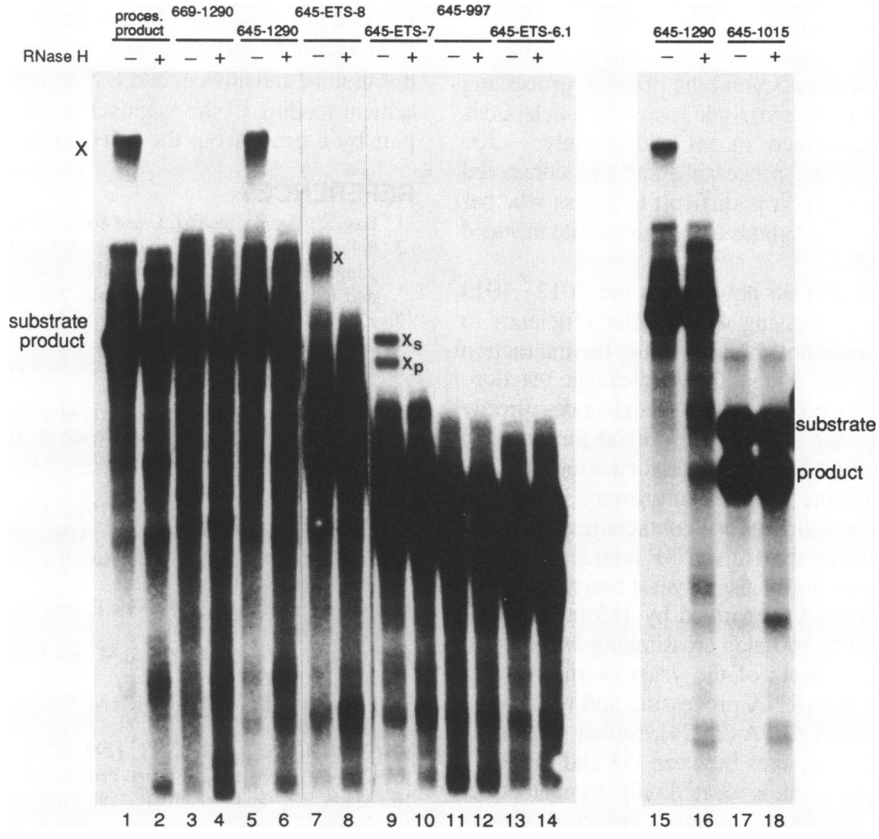

Figure 8. Psoralen crosslinking of U3 RNA and ETS deletion mutants. Transcripts containing the ETS sequences indicated at the top were incubated under processing conditions and irradiated with 365 nm light in the presence of AMT psoralen under standard conditions. After crosslinking, RNA was isolated, divided into two equal parts, and either digested with RNase H in the presence of U3 oligonucleotide 64−79 (even lanes) or not treated (odd lanes). RNA was separated in a 4% denaturing polyacrylamide gel. The processing product used for crosslinking extended to position 1290 and was gel purified from a processing reaction. The 645-ETS-6.1, 645-ETS-7, and 645-ETS-8 substrates were generated by RNase H digestion of the 645−1290 substrate in the presence of appropriate oligonucleotides (see Fig. 1). The precise 3′ ends of these substrates were not defined; they can map to any nucleotide within the region complementary to the oligonucleotide used for RNase H digestion. X indicates the ETS-U3 crosslinked RNAs obtained with the ETS transcript extending to position 1290 (lanes 1, 5, and15) or to the ETS-8 oligonucleotide-annealing region (lane 7). X_s and X_p stand for crosslinks between U3 and the 645-ETS-7 substrate or its processed product, respectively.

U_{1009} nor U_{1011} was detectable by primer extension (see Fig. 6), probably because in this case reverse transcription could be arrested at major crosslinking sites (U_{1012} and U_{1013}) more proximal to the primer. The model (Fig. 7) also does not explain the minor crosslink at U_{1016} of the ETS, since this residue is in a configuration which does not favor psoralen crosslinking. The increased modification of U_{1016} in the isolated ETS-U3 crosslinked RNA compared to the ETS substrate and the processing product may reflect a more favored stereochemical configuration for the formation of a psoralen monoadduct or an intramolecular crosslink at this site after an intermolecular crosslink with U3 RNA has occurred at U_{1012} or U_{1013}.

The crosslinking residues in U3 RNA are located within (U_8) and 5′ to (U_6 and C_5) an evolutionarily conserved sequence designated by Myslinski et al. (22) as box A′ (also see Fig. 3C). According to the secondary structure model for vertebrate U3 RNA (20), all three crosslinking nucleotides are sequestered in a helix. The base pairing interactions with the ETS shown in Fig. 7 include five nucleotides of this U3 helix, suggesting that its end is either melted or that a triple-stranded structure is formed during U3 interaction with the ETS. The crosslinked site in mouse U3 is situated only a few nucleotides 5′ to the crosslinked sites found *in vivo* in rat (17) and in yeast (18).

The crosslinked site in the mouse ETS is located 362 nucleotides downstream from the primary processing site. The previously studied *in vivo* crosslinks in human (16) and rat (17) cells localized the ETS-U3 interaction to regions extending to ~280 and to ~370 nucleotides beyond the primary processing site, respectively. Our mouse crosslink maps 13 nucleotides beyond the region characterized in rat. Since only ~200 nucleotides downstream of the processing site are conserved between mouse and human (3), it is difficult to assess whether the mouse crosslinking site corresponds to the human site included in the mapped region (16).

Shorter ETS transcripts that do not contain the 1012–1013 crosslinking site undergo processing with similar efficiency to the 645–1290 substrate (data not shown). Thus, the interaction with U3 RNA at this site is not crucial for the cleavage reaction. On the other hand, the highly conserved ~15 nucleotides directly adjacent to the 650 processing site that are crucial for cleavage (6,7) are absolutely required for crosslink formation (Fig.8). Therefore, they may constitute a site for a primary interaction with the U3 snRNP that is followed by contacts near position 1012–1013. This implies that the U3 snRNP interacts with the ETS at multiple sites. Interestingly, also in yeast two interactions between the ETS and U3 RNA, separated by 185 nucleotides, have recently been detected by psoralen crosslinking *in vivo* (18). Moreover, deleting various parts of the yeast or mouse ETS invariably interferes with pre-rRNA processing and results in a lack of accumulation of the18S rRNA (23,24), further supporting the idea of multiple interaction sites between U3 and the ETS. Interestingly, the mouse crosslink is located only 15 nucleotides upstream of an ~50 nucleotide region conserved among mammalian species (19), although deleting this conserved region does not influence crosslink formation (Fig. 8).

All ETS substrates that are competent for processing *in vitro* form a large ribonucleoprotein complex which seems to be a prerequisite for the cleavage reaction. This complex is detectable by gel shift and velocity centrifugation analyses (8). Six proteins have been identified by UV crosslinking to contact ETS sequences. The processing complex assembled on substrates lacking the 1004–1062 region (therefore also lacking our crosslinked site) does not stably bind the U3 particle (C.Enright and B.Sollner-Webb, pers. comm.); yet U3 RNA is required for the cleavage reaction itself. We suspect that the interaction pinpointed by psoralen crosslinking at U_{1012} and U_{1013} is responsible for this stable binding of U3 RNP to the longer ETS transcripts. Taken together with previous results, our data indicate that short substrates can complex with specific proteins and transiently associate with the U3 snRNP to achieve cleavage, but that stable binding requires additional contacts with U3 occurring at least 360 nucleotides downstream from the processing site.

Although the interaction between the 5′ end of U3 RNA and the ETS region around the position 1012–1013 is not crucial for primary processing *in vitro*, it may play an important role in stabilizing interactions between the U3 snRNP and the pre-rRNA *in vivo*. After primary processing has occurred, this region may help deliver the U3 snRNP to another site(s) critical for the multiple-step pathway of pre-rRNA processing. Interestingly, the U3 particle has been implicated in the processing event at the boundary between ITS1 and 5.8S sequences in the *Xenopus* oocyte (25). Our findings also show that the 5′ end of U3 RNA is available for interaction(s) with the pre-rRNA during processing.

ACKNOWLEDGMENTS

We are grateful to B.Sollner-Webb and C.Enright for communicating unpublished results and for advice. We would like to thank M.-D.Shu for preparation of the S-100 cell extract, D.Wassarman and other members of the Steitz lab for helpful discussions and advice, and E.Sontheimer and D.Wassarman for critical reading of the manuscript. This work was supported in part by a grant from the NIH, GM26154.

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