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METHODS REPORT

A new strategy for introducing photoactivatable 4-thiouridine (^{4S}U) into specific positions in a long RNA molecule

YI-TAO YU and JOAN A. STEITZ

Department of Molecular Biophysics and Biochemistry, Boyer Center for Molecular Medicine, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06536, USA

ABSTRACT

We describe a new protocol, which does not require ^{4S}UpG, for introducing ^{4S}U into specific sites in a pre-mRNA substrate. A 5′-half and a full-length RNA are first synthesized by phage RNA polymerase. p^{4S}Up, which is derived from ^{4S}UpU and can therefore be ^{32}P-labeled, is then ligated to the 3′ end of the 5′-half RNA with T4 RNA ligase. The 3′ phosphate of the ligated product is removed subsequently by CIP (calf intestinal alkaline phosphatase) to produce a 3′-OH group. The 3′-half RNA with a 5′ phosphate is produced by site-specific RNase H cleavage of the full-length pre-mRNA directed by a 2′-O-methyl RNA–DNA chimera. The two half RNAs are then aligned with a bridging oligonucleotide and ligated with T4 DNA ligase. Our results show that ^{32}P-p^{4S}Up ligation to the 3′ end of the 5′-half RNA is comparable to ^{32}P-pCp ligation. Also, the efficiency of the bridging oligonucleotide-mediated two-piece ligation is quite high, ~30–50%. This strategy has been applied to the P120 pre-mRNA containing an AT-AC intron, but should be applicable to many other RNAs.

Keywords: 2′-O-methyl RNA–DNA chimeras; p^{4S}Up; RNase H; T4 RNA ligase

INTRODUCTION

4-Thiouridine (^{4S}U) site-specific crosslinking is a powerful tool for detailed probing of RNA–RNA and RNA–protein interactions. The use of this technology in pre-mRNA splicing has yielded important information as to how the 5′ and 3′ splice sites and branch site of a splicing substrate are recognized during pre-mRNA splicing (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Gaur et al., 1995; Newman et al., 1995; Reyes et al., 1996; Kim & Abelson, 1996; Maroney et al., 1996).

Except in few cases where a ^{4S}U-containing oligonucleotide was synthesized chemically (Reyes et al., 1996), ^{4S}U has been introduced into specific positions in a long RNA via in vitro transcription coupled with oligonucleotide-mediated two-piece ligation (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Gaur et al., 1995; Newman et al., 1995). Specifically, to incorporate ^{4S}U at a single position near the 5′ or the 3′ splice site or branch site of various splicing substrates, the dinucleotide ^{4S}UpG has been used to initiate transcription of a 3′-half RNA and the resultant transcript ligated to a 5′-half RNA to create a full-length substrate (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Gaur et al., 1995; Newman et al., 1995). Because T3, T7, and SP6 RNA polymerases all require guanosine to initiate transcription, priming of 3′-half transcripts with ^{4S}UpG was essential. As a consequence, this strategy has a major limitation: the uridines that are to be substituted must be followed by guanosines. Clearly, many sites of interest within a substrate RNA do not meet this requirement. Among these is the 5′ splice site of the P120 AT-AC intron (Tarn & Steitz, 1996), where the first three uridines (in positions +2, +4, and +7 of the intron) are not followed by guanosines.

We therefore devised a new strategy to substitute U_{+2}, U_{+4}, or U_{+7} of the P120 AT-AC intron with ^{4S}U. Because there is no flanking sequence requirement, this strategy can be used to introduce ^{4S}U into any position in a long RNA.

Reprint requests to: Joan A. Steitz, Department of Molecular Biophysics and Biochemistry, Boyer Center for Molecular Medicine, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, Connecticut 06536, USA.

RESULTS AND DISCUSSION

Figure 1 summarizes the introduction of ^{4S}U at position +2 of the P120 AT-AC intron. Instead of priming transcription of the 3′-half RNA with ^{4S}UpG, we attached ^{4S}U to the 3′ end of the 5′-half RNA. The DNA template for the 5′-half RNA was generated by PCR and designed to end at the nucleotide preceding the U residue to be substituted. RNA transcribed from the template was then joined by T4 RNA ligase to ^{32}P-p^{4S}Up, which was derived from ^{4S}UpU by phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase, followed by digestion with RNase A (see Materials and Methods). To check the ligation efficiency, equal amounts of 5′-half RNA were ligated to either ^{32}P-p^{4S}Up (Fig. 2, lane 1) or ^{32}P-pCp, which is commercially available (Fig. 2, lane 2). The results indicate that the yield of product with ^{32}P-p^{4S}Up is comparable to that with ^{32}P-pCp. The 3′ phosphate on the 5′-half RNA was removed subsequently by phosphatase (see Materials and Methods).

The 3′-half RNA was created by site-specific RNase H cleavage of a full-length P120 pre-mRNA directed by a 2′-O-methyl RNA–DNA chimera. The chimera was designed to direct phosphodiester bond cleavage 3′ to the uridine to be substituted (Lapham & Crothers, 1996; Yu et al., 1997; and see Fig. 1). After gel electrophoresis, the 3′-half RNA, which contains a phosphate at its 5′ terminus, was isolated. In some cases where a substrate with higher specific radioactivity was desired, the 5′ phosphate of the 3′-half RNA was also replaced with ^{32}P by dephosphorylation–rephosphorylation at this stage (see Materials and Methods).

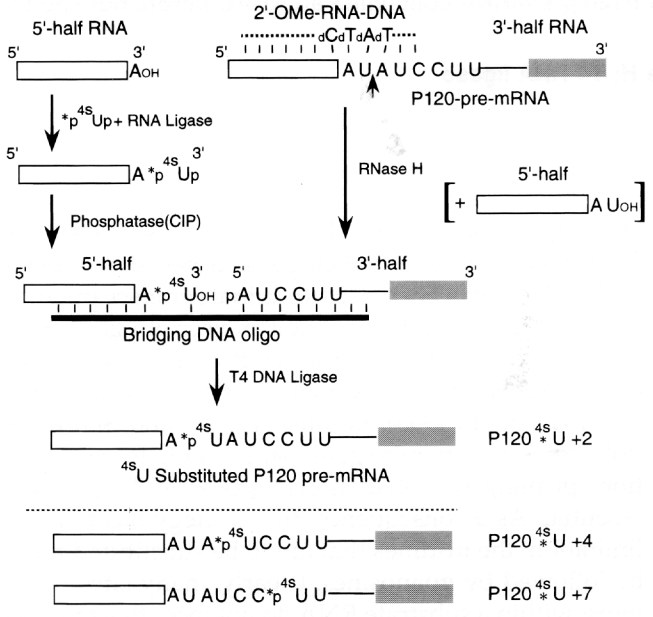

FIGURE 1. Schematic representation of the construction of a ^{4S}U-substituted P120 pre-mRNA splicing substrate (for detailed reaction conditions, see Materials and Methods). Open boxes, shaded boxes, and thin lines represent 5′ exons, 3′ exons, and introns, respectively. For generation of the 3′-half RNA, a 2′-O-methyl RNA–DNA chimera designed to base pair with the 5′ splice site region of the P120 pre-mRNA induces RNase H cleavage at the site indicated by an arrow. In the chimera, the four deoxynucleotides are shown, and 2′-O-methyl nucleotides are represented by a dotted line. The base pairing of the bridging DNA oligonucleotide that mediates the two-piece ligation is represented by a bar. The asterisk indicates the label introduced on the 5′ phosphate of p^{4S}Up.

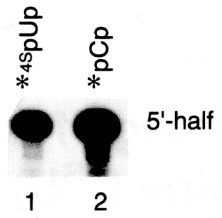

FIGURE 2. Ligation of p^{4S}Up to the 3′ terminus of the 5′-half RNA. 5′ ^{32}P-p^{4S}Up (derived from 10 nmol of ^{4S}UpU and 1.5 mCi of [γ^{32}P]ATP, see Materials and Methods) (lane 1) and commercially available 5′ ^{32}P-pCp (150 μCi) (lane 2) were ligated to 20 pmol of 5′-half RNA with T4 RNA ligase. The position of the ligated RNAs on a 5% polyacrylamide–8 M urea gel is indicated (5′-half). The exposure time for this gel was ~1 s.

The 5′-half and 3′-half RNAs were then ligated together by T4 DNA ligase in the presence of a bridging oligodeoxynucleotide (see Fig. 1) (Moore & Sharp, 1992). Figure 3 shows a typical two-piece ligation experiment using 3′ end-labeled 5′-half RNA and 5′ end-labeled 3′-half RNA (see above). Ligations involving either ^{4S}U (lane 1) or normal U (lane 2) had an estimated efficiency of ~30–50%.

Using the same strategy, we have also successfully introduced ^{4S}U into two other positions (+4 and +7) near the 5′ end of the AT-AC intron in the P120 splicing substrate (Fig. 1). The substrates thus prepared can be labeled with ^{32}P at either the 5′-phosphate or both the 5′- and 3′-phosphates flanking the ^{4S}U residue. Because the requirement for ^{4S}UpG as a transcription primer (Wyatt et al., 1992; Sontheimer & Steitz, 1993; Gaur et al., 1995; Newman et al., 1995) has been elim-

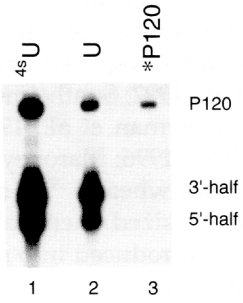

FIGURE 3. Oligonucleotide-mediated two-piece ligation. The 5′ ^{32}P-p^{4S}Up ligated 5′-half RNA (from Fig. 2) (lane 1) or 5′ ^{32}P-pUp ligated 5′-half RNA (synthesized in the same way) (lane 2) were joined to the 3′-half RNA with the T4 DNA ligase and a bridging oligonucleotide. The 3′-half RNA was also labeled radioactively (see Materials and Methods). Lane 3 shows a uniformly [α^{32}P]UTP-labeled P120 transcript that served as a full-length marker. The positions of P120, the 5′-half RNA, and the 3′-half RNA are indicated on the right.

inated, ^{4S}U can be introduced into an RNA molecule at any position. Moreover, because $p^{4S}Up$ can be produced readily from ^{4S}UpU (or from any other ^{4S}UpN dinucleotide), the strategy is applicable for producing a variety of RNA substrates containing a single ^{4S}U residue.

It should be noted that the 5′-half RNAs described here were produced via run-off transcription with phage RNA polymerase. In many cases, phage RNA polymerases have a tendency to incorporate additional nucleotides at the 3′ end of a transcript, causing a serious problem for two-piece ligation mediated by a bridging oligonucleotide. To avoid this problem, site-specific RNase H cleavage directed by 2′-O-methyl RNA–DNA chimeras, which was used here to create the 3′-half RNAs (see above), can be used to produce a clean 3′ end on the 5′-half RNA. Specifically, a 2′-O-methyl RNA–DNA chimera designed to direct phosphodiester bond cleavage 5′ to the uridine to be substituted would be used. This idea can be expanded further for reasons of economy. Instead of transcribing a wild-type RNA, the substrate for RNase H cleavage could be a full-length RNA with a single uridine deletion. Then, the 2′-O-methyl RNA–DNA chimera would direct RNase H cleavage specifically at the deletion site, generating both the 5′-half and the 3′-half RNAs in a single step (see above).

MATERIALS AND METHODS

DNA templates for transcribing 5′-half P120 RNA splicing substrates were created by PCR. The 5′ primer contained a T7 promoter sequence fused to a sequence corresponding to the first 20 nt of the P120 substrate (Tarn & Steitz, 1996). The 3′ primers, 5′-TCCTGCTCCATCTCCCCAGC-3′, 5′-TATCCT GCTCCATCTCCCCA-3′, 5′-GGATATCCTGCTCCATCTCC-3′, were complementary to nucleotides −19 to +1, −17 to +3, −14 to +6 of P120, respectively (numbering relative to the 5′ splice site). Using these templates, trace-labeled, GpppG-capped 5′-half P120 RNAs were synthesized using T7 RNA polymerase as described (Yu et al., 1995).

To generate 5′ ^{32}P-$p^{4S}Up$ (or unlabeled $p^{4S}Up$), ^{4S}UpU (Sigma) was 5′ phosphorylated for 30 min at 37 °C in a 20-μL reaction containing 0.5 mM ^{4S}UpU (or UpU for control substrates), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.5 mCi [$\gamma^{32}P$]ATP (or 10 mM ATP for unlabeled $p^{4S}Up$), and 18 units of polynucleotide kinase (Pharmacia). After inactivating the kinase by exposure to 65 °C for 10 min, 40 μg RNase A were added and the resultant mixture was incubated at 42 °C for 1 h. Digestion was stopped by addition of 100 μg of proteinase K (Boehringer Mannheim) in 80 μL of buffer containing 20 mM Tris-HCl, pH 7.5, 300 mM NaAc, 2 mM EDTA, and 0.25% SDS. After an additional hour at 42 °C, protein was removed by four PCA extraction steps. ^{32}P-$p^{4S}Up$ (or unlabeled $p^{4S}Up$) was precipitated with 400 μL ethanol in the presence of ~ 2 mM ATP. The recovered ^{32}P-$p^{4S}Up$ was ligated immediately to the 3′ terminus of a 5′-half P120 RNA (20 pmol) using T4 RNA ligase (England & Uhlenbeck, 1978).

Following PCA extraction, the ^{32}P-$p^{4S}Up$-ligated 5′-half P120 RNAs were recovered by ethanol precipitation and were treated subsequently with 20 units of calf intestinal alkaline phosphatase (CIP) in a 20-μL reaction at 50 °C for 1 h (as described by the supplier, Boehringer Mannheim), re-extracted with PCA, and retrieved by ethanol precipitation.

3′-Half P120 RNAs were derived by RNase H cleavage of full-length P120 RNA directed by 2′-O-methyl RNA-DNA chimeras (5′-dAdTdCdCU$_m$G$_m$C$_m$U$_m$C$_m$C$_m$A$_m$U$_m$C$_m$U$_m$C$_m$ C$_m$C$_m$C$_m$A$_m$G$_m$-3′ for P120-^{4S}U+2; 5′-A$_m$G$_m$G$_m$dAdTdAd TC$_m$C$_m$U$_m$G$_m$C$_m$U$_m$C$_m$C$_m$A$_m$U$_m$C$_m$-3′ for P120-^{4S}U+4; 5′-dAdGdGdAU$_m$A$_m$U$_m$C$_m$C$_m$U$_m$G$_m$C$_m$U$_m$C$_m$C$_m$A$_m$U$_m$C$_m$U$_m$ C$_m$C$_m$-3′ for P120-^{4S}U+7). The procedure was essentially as described previously (Yu et al., 1997). Briefly, 50 pmol of P120 was mixed with 75 pmol of each of the chimeras in a volume of 5 μL, followed by heating to 95 °C for 3 min and reannealing at 37 °C for 10 min. Fifteen microliters of a mixture containing 26 mM Tris-HCl, pH 7.5, 13 mM $MgCl_2$, 133 mM KCl, 13 mM DTT, 6.6% Sucrose, 40 units RNase inhibitor (Boehringer Mannheim), and 5 units RNase H (Boehringer Mannheim) was then added and the reaction was allowed to proceed at 37 °C for another hour. Following PCA extraction and ethanol precipitation, the 3′-half P120 RNAs were purified from a 5% polyacrylamide–8 M urea gel. To generate ^{4S}U-substituted P120 with higher specific radioactivity, the 5′ phosphate of the 3′-half RNA was removed by CIP and re-phosphorylated with polynucleotide kinase and 2 mCi [$\gamma^{32}P$]ATP.

The 5′-half and 3′-half P120 RNA fragments were then ligated together by bridging-oligonucleotide mediated two-piece ligation as described previously (Moore & Sharp, 1992). Here, the bridging oligonucleotide sequence was complementary to nt −17 to +31 of the P120 RNA. The molar ratio of 5′-half RNA:3′-half RNA:bridging oligonucleotide was 1:1.5:1.5.

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