Multiple pseudouridine synthase activities for small nuclear RNAs

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The formation of pseudouridine (Ψ) in human U1, U2 and U5 small nuclear RNAs (snRNAs) was investigated using HeLa cell extracts. Unmodified snRNAs were synthesized in vitro and the extent of Ψ formation was determined after incubation in cell extracts. The formation of Ψ on labelled substrates was monitored in the presence of 5-fluorouracil (5-FU)-containing snRNAs as inhibitors of Ψ formation. The conversion of uridine to Ψ was inhibited only when the cognate 5-FU-containing inhibitor snRNA was included in the reaction. For example, 5-FU-containing U1 RNA inhibited Ψ formation in unmodified U1 RNA, but not in (unmodified) U2 or U5 RNAs. The results suggest that there are at least three activities that form Ψ in these snRNAs. The 5-FU-containing RNAs were stable during incubation in the cell extracts. A 12-fold molar excess of unlabelled U1 RNA did not inhibit Ψ formation on a labelled U1 RNA substrate, whereas a 3-fold molar excess of 5-FU-containing U1 RNA nearly abolished Ψ formation on the U1 substrate. The fact that 5-FU-containing snRNAs are potent inhibitors of Ψ formation in these pre-mRNA splicing cofactors raises the possibility that this is related to the cytotoxicity of fluoropyrimidines in cancer chemotherapy.

INTRODUCTION

The splicing of pre-mRNA requires several small nuclear ribonucleoprotein particles (snRNPs) [1,2]. These include U1, U2, U5 and U4/U6 snRNPs, which are composed of at least one highly conserved small nuclear RNA (snRNA) and a cohort of proteins that include both common (Sm) proteins and snRNA-specific proteins [3–5].

These snRNAs are highly modified post-transcriptionally, and pseudouridine (Ψ) represents by far the most abundant of these modifications [3]. The positions of Ψ in snRNAs from different species are highly conserved. Moreover, there are Ψs in regions of U1 and U2 RNAs that are known to be necessary for the function of these cofactors in splicing [1,2]. However, the actual function of Ψ in snRNAs is yet not known. Ψ is necessary both for the interaction of tRNAs with the ribosome [6,7] and for the efficient reading of codons during translation [8]. Studies of his T mutants (his T codes for Ψ synthase) in bacteria suggest that the presence of Ψ in the anticodon region of tRNA is not required for cell growth [9,10]. Recently Tsui et al. [11] found that his T function was required for normal growth of Escherichia coli K-12 on minimal media. The mutation causes a uracil requirement that interferes with cell division [11].

Enzymes that convert uridine to Ψ in tRNAs have been purified from a number of sources [12–14]. There appear to be multiple enzymes that modify tRNAs [12,13]. Several inhibitors of Ψ synthases have been identified, notably 5-fluorouracil (5-FU)-substituted tRNAs [12,15,16]. Enzymes that form Ψ in snRNAs have not been purified, but the formation of Ψ in U5 RNA has been studied in HeLa cell extracts [17]. The modifications are site-specific, and the binding of Sm proteins to the U5 RNA is required for Ψ formation [17].

In the present paper an in vitro assembly system for snRNPs is combined with the use of specific inhibitors of Ψ synthase to study the formation of Ψ in U1, U2 and U5 snRNAs. The results show that there are at least three activities that modify these snRNAs and that each is specific for a particular snRNA. In addition, these studies show that 5-FU-containing snRNAs are potent inhibitors of Ψ formation in snRNAs.

MATERIALS AND METHODS

Human U1, U2 and U5 RNAs were transcribed as described [18] using BamHI-cut pHU1 DNA [19], Mael-cut pHU5a2 DNA [17] and Smal-cut pMRG3U2-27 DNA (a gift from Dr. T. Pederson). In the latter case the resulting U2 RNA has an additional three G residues at the 5’ end of the transcript. The length of in vitro-transcribed U1 and U5 RNAs have been described [17,19]. For the snRNA stability experiment (Figure 3), BamHI-cut pG2U2ser (a gift from Dr. A. Kleinschmidt) was used. The resulting U2 RNA transcript has an additional 18 nucleotides on the 5’ end and an additional 11 nucleotides on the 3’ end [20]. The U1 and U5 RNAs were transcribed with SP6 RNA polymerase, whereas U2 RNA was transcribed by T7 RNA polymerase. The human pre-tRNA 5’ end was transcribed with T7 RNA polymerase using Aael-cut pUC19Ser (a gift from Dr. C. Guerrier-Takada and Dr. S. Altman). The full description of the pUC19Ser clone is unpublished, but the pre-tRNA 5’ transcript is 123 nucleotides in length and has extensions on both the 5’ and 3’ ends of mature tRNA 5’. When [3H]UTP-labelled substrate was required, [5-3H]UTP (23 Ci/mmol) was used as the only source of UTP in the reaction (80 μM). ATP and CTP concentrations were 250 μM, and GTP was present at 50 μM. The rest of the components were as detailed previously [17,18]. When [3H]ATP-labelled RNAs were synthesized, a small amount of [2,8-3H]ATP (40 μCi; 38–40 Ci/mmol) was added to the reaction containing 250 μM each of ATP, CTP and UTP, and 50 μM GTP. When [32P]GTP-labelled RNAs were synthesized, a small amount of [α-32P]GTP (10 μCi; 600 Ci/mmol) was added instead of [3H]ATP. 5-FU-containing RNA was synthesized by substituting 1 mM 5-fluoro-UTP (Sierra Bioresearch, Tucson, AZ, U.S.A.) for UTP. [32P]GTP-labelled RNA was synthesized using [α-32P]GTP (50 μCi; 600 Ci/mmol), and the UTP concentration was decreased to 25 μM. All RNAs were gel-purified before use [17].

The in vitro snRNP assembly reactions were carried out as described [19,20] using a HeLa S100 cell extract [21]. The reaction mixtures were incubated with inhibitors at 37 °C for 30 min prior to the addition of substrate ([3H]UTP- or [32P]GTP-labelled)
RNAs and then incubated at 37 °C for an additional 60 min unless otherwise noted.

Glycerol gradient centrifugation of the assembled snRNPs, antibody selection of Sm antigen-containing snRNPs, RNase T1 digestion of U1 and U2 RNAs and the assays for Ψ formation were carried out as described previously [17]. Procedures for gel electrophoresis and elution of the RNAs from the gel have been described previously [19,20].

RESULTS

Location of Ψ residues in snRNAs assembled in vitro

Ψ is found at nucleotides 5 and 6 in human U1 RNA, nucleotides 43, 46 and 53 in human U5 RNA, and at 13 positions in rat U2 RNA (Figure 1; see also [3]). The extract (S100) used for the in vitro assembly of these snRNPs is known to support the conversion of uridine to Ψ [17,19,20]. In the case of U5 RNA, two of the three positions (at nucleotides 43 and 46) are modified in vitro [17]. The actual sites of modification in U1 and U2 RNAs assembled in vitro have not been defined [19,20]. To determine the specificity of Ψ formation in U1 and U2 RNAs, [32P]UTP-labelled U1 and U2 RNAs were incubated in extracts and RNA from glycerol-gradient-purified snRNPs was isolated. The RNA was digested with RNase T1 and the resulting fragments were electrophoresed on a 20% polyacrylamide/8 M urea gel. The appropriately sized bands, identified by autoradiography, were eluted from the gel, and the RNA was isolated and digested with nuclease P1 [19,20]. The reaction products were chromatographed on TLC plates (cellulose) in one dimension (propan-2-ol/conc. HCl/water, 70:15:15, by vol.) and autoradiographed [17,23].

When U1 RNA was incubated in the reaction, only the 5' end RNAase T1 fragment contained Ψ (Figure 2a). This is the only T1 fragment that is predicted to contain Ψ, based on the known sequence of human U1 RNA [3]. Therefore, as with U5 RNA [17], the formation of Ψ in U1 RNA in vitro mimics the reaction in vivo.

The positions of Ψ in human U2 RNA are not known, but they are predicted to be the same as in rat U2 RNA (see Figure 1; [3]). When human U2 RNA was incubated in the extracts, the bands at 15, 11, 10, 7 and 6 nucleotides all contained Ψ (Figure 2b) and, given the location of Ψ in rat U2 RNA, these would be the T1 fragments that are predicted to contain Ψ in human U2 RNA. There is no Ψ in the 5-nucleotide fragment, as one would predict.

Most importantly, this snRNP assembly system supports Ψ formation in human U1, U2 and U5 RNAs, and the modifications occur at specific positions. The extracts contain highly specific enzymic activities that appear to be modifying only those uridine residues that are normally modified in vivo [17].

Multiple Ψ synthases for the modification of snRNAs

Although U1, U2 and U5 RNAs have a number of characteristics in common, the positions of Ψ residues with respect to the sequence and secondary structure of the snRNAs are very different (see Figure 1). Does the same snRNA Ψ synthase act on all snRNAs, or are there separate synthases for each snRNA? In order to answer this question, very specific inhibitors of the activities were needed. Samuelsson [15] found that tRNAs containing 5-FU were very specific and potent inhibitors of tRNA Ψ synthases. Two types of RNA, one transcribed in the presence of UTP and the other in the presence of 5-fluoro-UTP, were made for each snRNA. A small amount of [32P]GTP (or [3H]ATP) was included in the reaction in order to estimate the amount of RNA produced.

Several studies have shown that in vitro-transcribed snRNAs assemble into stable RNPs during incubation in these extracts [17,19,20], but the effect of substituting 5-FU for uridine on particle assembly or RNA stability has not been studied. To determine if the stability of the RNAs was affected by the presence of 5-FU, both types of RNA were incubated in the extracts for up to 1 h. Figure 3 shows that the 5-FU-containing snRNAs were quite stable in the extracts, with only a slight increase in turnover when compared with transcripts without 5-FU. The U2 RNA used in this particular experiment was processed at the 3' end in these extracts, since it has a 11-nucleotide extension on the 3' end [20]. The presence of the smaller band is quite evident for U2 RNA without 5-FU. The 5-FU-containing U2 RNA is processed, albeit less efficiently since precursor U2 RNA is evident even after 1 h of incubation. The additional band between U2 and U1 RNAs at zero time is U1 RNA transcript that has not been processed by RNAse H due to lack of incubation at 37 °C. An antisense oligodeoxynucleotide that hybridizes to the last 25 nucleotides of the U1 RNA in vitro transcript is included in all reactions in order to obtain U1 RNA that has a correct 3' end [19].

These same 5-FU-containing RNAs, as well as pre-tRNA^ser with and without 5-FU, were used to determine the Ψ synthase activity in extracts. In Table 1, the amount of [3H] released [17,22] in 2 h from gel-purified U1, U2 and U5 RNAs was determined after incubation of these [3H]-labelled substrates in the assembly reaction in the presence of possible inhibitors. The 5-FU-containing inhibitor RNAs were incubated in the reactions for 10 min prior to the addition of [3H]-labelled substrate RNAs. [3H] is released from the C-5 position of [5,6-3H]UTP-labelled RNAs when uridine is converted to Ψ [22]. These gel-purified inhibitors were present in 5-fold molar excess over the substrate. The control contains yeast tRNA (0.7 μg; ~30 pmol), which is used in the gel purification of the U1, U2 and U5 RNAs, and is therefore present in all of the preparations of inhibitor RNA. The amount of yeast tRNA in the inhibitor RNAs added to the reactions was always less than 30 pmol.

Table 1 shows that only the cognate 5-FU-containing inhibitor RNAs have a significant effect on the release of [3H] from the substrate RNAs. Thus Ψ formation on U1 RNA was only inhibited by 5-FU-containing U1 RNA and not by any of the other potential inhibitor RNAs. 5-FU-containing U2 RNA was the strongest inhibitor of [3H] release from the [3H]-labelled U2 RNA substrate. With [3H]-labelled U5 RNA, the [3H] released was only 42% of the control when 5-FU-containing U5 RNA was used as the inhibitor RNA. A small inhibition of [3H] release from U5 RNA was seen with all the other inhibitor RNAs (for instance when 5-FU-containing U1 and U2 RNAs were used as inhibitors), but the greatest amount of inhibition was seen when 5-FU-containing U5 RNA was used as the inhibitor RNA. Neither pre-tRNA^ser nor 5-FU-containing pre-tRNA^ser significantly inhibited [3H] release from any of the snRNA substrates, another indication that these activities are specific for snRNAs.

These data suggest that there are distinct Ψ synthases, since only the cognate 5-FU-containing snRNA inhibited Ψ formation with a particular substrate snRNA. In vitro, all the snRNAs are present and being modified concurrently, but in the Ψ formation experiment Ψ formation (or its inhibition) is monitored with only one substrate RNA in a given reaction. If all three substrates were present at the same time, would there be the same pattern of inhibition? To answer this question [32P]-labelled U1, U2 and U5 RNAs were mixed together and the inhibition of Ψ formation was determined in the presence of each of the 5-FU-containing
The proposed secondary structures for human U1 RNA (a), rat U2 RNA (b) and human U5 RNA (c) [3] are shown, and the locations of \( \Psi \) moieties are boxed.

RNAs. After incubation the assembled snRNPs were subjected to centrifugation in glycerol gradients. \[^{32}P\]UTP-labelled RNAs from the snRNP peak of each gradient were separated in a 10% polyacrylamide/8 M urea gel [17,19,20]. Bands corresponding to the \[^{32}P\]-labelled U1, U2 and U5 RNAs were isolated from each reaction, since they migrate at different rates (see Figure 3), and the RNA was eluted from the gel, digested with nuclease P1 and chromatographed on t.l.c. plates [17,23] to assess the relative amounts of \[^{32}P\] in 5'-UMP and 5'-\( \Psi \)MP. A separate t.l.c. plate was used for each of the snRNAs.

It can be seen that \( \Psi \) formation in a given snRNA is significantly inhibited by only the cognate 5-FU-containing snRNA (Figure 4). For example, with 5-FU-containing U1 RNA as inhibitor, U1 RNA does not contain \( \Psi \) (Figure 4a, lane 4), but the t.l.c.s for U2 and U5 RNAs do indicate the presence of \( \Psi \) (Figures 4b and 4c, lane 4). Similarly, with U2 RNA, there was

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**Figure 1** Locations of \( \Psi \) in U1, U2 and U5 RNAs

The proposed secondary structures for human U1 RNA (a), rat U2 RNA (b) and human U5 RNA (c) [3] are shown, and the locations of \( \Psi \) moieties are boxed.
Table 1  \( \Psi \) formation in U1, U2 and U5 RNAs in the presence of 5-FU-containing inhibitor RNAs

<table>
<thead>
<tr>
<th>Inhibitor RNA</th>
<th>(^{3}H)-labelled U1 RNA (corrected c.p.m.)</th>
<th>(^{3}H)-labelled U2 RNA (corrected c.p.m.)</th>
<th>(^{3}H)-labelled U5 RNA (corrected c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>362 ± 14</td>
<td>277 ± 18</td>
<td>202 ± 6</td>
</tr>
<tr>
<td>U1 RNA</td>
<td>309 ± 10</td>
<td>255 ± 11</td>
<td>177 ± 22</td>
</tr>
<tr>
<td>5-FU U1 RNA</td>
<td>127 ± 8</td>
<td>264 ± 3</td>
<td>162 ± 10</td>
</tr>
<tr>
<td>U2 RNA</td>
<td>319 ± 14</td>
<td>285 ± 8</td>
<td>163 ± 5</td>
</tr>
<tr>
<td>5-FU U2 RNA</td>
<td>336 ± 14</td>
<td>148 ± 6</td>
<td>190 ± 4</td>
</tr>
<tr>
<td>U5 RNA</td>
<td>334 ± 7</td>
<td>219 ± 7</td>
<td>162 ± 10</td>
</tr>
<tr>
<td>5-FU U5 RNA</td>
<td>326 ± 12</td>
<td>236 ± 10</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Pre-RNA(^{30})</td>
<td>352 ± 55</td>
<td>241 ± 10</td>
<td>171 ± 9</td>
</tr>
<tr>
<td>5-FU pre-RNA(^{30})</td>
<td>352 ± 5</td>
<td>242 ± 18</td>
<td>176 ± 4</td>
</tr>
</tbody>
</table>

Results are means ± S.D. of three separate assays of the same reaction. The counts were corrected for \(^{3}H\) release in the absence of extract. The counting efficiency in the \(^{3}H\) window was 57% and Ecolise (d) was used as the scintillant. A total of 30 ng (0.6 pmol of U1, 0.5 pmol of U2, 0.8 pmol of U5) of \(^{3}H\) substrate was added per 300 \( \mu \)l reaction. All of the inhibitor RNAs were labelled with \(^{32}P\)-GTP during transcription and this label bound to the NorI-A. The counts in the \(^{32}P\) window were not significantly above background in any sample. A 150 ng portion of each inhibitor RNA was added per 300 \( \mu \)l reaction.

Figure 2  Location of \( \Psi \) moieties in U1 and U2 RNAs assembled in vitro

Details of the experiment are given in the text. (a) Autoradiograph of T.L.C. of nuclease P1 digest of RNAase T1 fragments from U1 RNA incubated in cell extracts. (b) Autoradiograph of T.L.C. of nuclease P1 digest of RNAase T1 fragments from U2 RNA incubated in cell extracts. The sizes of the fragments, in nucleotides, are indicated below the lanes. The positions of \( \Psi \)MP (p\( \Psi \)) and UMP (pU) are indicated on the right.

Figure 3  Effect of the presence of 5-FU on the stability of the snRNAs in extracts

\([^{32}P\)-GTP]-labelled snRNAs were incubated for the times (min) indicated above the lanes, the RNA was isolated, electrophoresed on a 10% polyacrylamide/urea gel and the gel autoradiographed. The heading 'without 5-FU' refers to RNAs transcribed with UTP and 'with 5-FU' refers to RNAs transcribed in the presence of 5-FUTP instead of UTP. The positions of U1, U2 and U5 RNAs are indicated on the right and the length (in nucleotides) of the \(^{32}P\) end-labelled Mspl digest of pHU1 [19] are indicated on the left. Lane M contains molecular size markers.

a complete lack of \( \Psi \) in a reaction with 5-FU-containing U2 RNA (Figure 4b, lane 6), but \( \Psi \) was found in the U1 and U5 RNAs (Figures 4a and 4c, lane 6). Thus 5-FU-containing U2 RNA did not inhibit the formation of \( \Psi \) in the two other substrates, even though these other substrates were present in the reaction. Likewise, in the reaction with 5-FU-containing U5 RNA, there was a complete lack of \( \Psi \) in the t.l.c. for U5 RNA (Figure 4c, lane 8), whereas there was a prominent spot of \( \Psi \) in the t.l.c.s for U1 and U2 RNAs (Figures 4a and 4b, lane 8). It appears that all of the inhibitor RNAs caused some inhibition of \( \Psi \) formation in U5 RNA, but the inhibition with 5-FU-containing U5 RNA was nearly complete. Therefore, even though all three substrates were present in the reaction, the inhibitors were specific for their cognate snRNA, suggesting again that these inhibitors are reacting with separate \( \Psi \) synthases. Although every effort was made to load equal amounts of radioactivity in each lane, it is obvious that some lanes contain slightly more than others. When the spots for pU and p\( \Psi \) were scraped from the t.l.c. plate, counted and the ratios of p\( \Psi \) to pU + p\( \Psi \) calculated, the level of \( \Psi \) formation was comparable between samples, except in the cases of the cognate 5-FU-containing RNAs which showed inhibition (results not shown).

The above experiments were done with a 5-fold molar excess of inhibitor RNA over substrate. Figure 5 shows that there was almost complete inhibition of \( \Psi \) formation in \([^{32}P]\)UTP-labelled
results rule out the possibility that the inhibitors are sequestering a non-enzymic component of the snRNP assembly machinery, such as Sm proteins that subsequently inhibit Ψ formation. This conclusion could also be drawn from the results in Table 1 and Figure 4, since the non-5-FU-containing cognate RNAs did not significantly inhibit Ψ formation.

**DISCUSSION**

These experiments strongly support the hypothesis that there are distinct snRNA Ψ synthase activities. Why would the cell need to have multiple activities that perform this modification? If there is a need for Ψ at certain positions in different snRNAs, then it would obviously be necessary for the snRNA Ψ synthase to be highly site-specific in each case. Although U1, U2 and U5 RNAs are all small RNAs, their predicted secondary structures (see Figure 1) are very different, and the positions at which Ψ residues occur are unique to each particular snRNA molecule. If a single Ψ synthase were to make all these modifications, it would have to be site-restricted by each snRNA’s secondary structure and/or bound proteins. Yet neither their respective secondary structures nor the known sites of snRNP protein binding to U1, U2 and U5 RNAs [24–28] provide an obvious explanation for such site restriction. For example, in U1 RNA the uridines at positions 106 and 108 are single-stranded and known to be protein-free in the U1 snRNP, yet they are not converted to Ψ. Likewise, in U2 RNA there are many unmodified uridines in the 5′ half of the snRNA that are in single-stranded regions, loops and stems. This notwithstanding, it is certainly possible that long-range features of a given snRNP’s structure influence Ψ formation by its cognate synthase. For example, deletions made in the Sm domain of U5 RNA inhibited Ψ formation [17], suggesting that Sm proteins may influence recognition of U5 RNA by its synthase even though no pseudouridine is formed in this region.

It is also possible that more than one activity is modifying a single snRNA. A hint that this may be the case comes from the modification of human U5 RNA in vitro, since only two of the three positions where Ψ has been identified [3] are actually converted to Ψ [17]. The two Ψs (at positions 43 and 46) that are found in the in vitro-assembled U5 snRNP are located in the terminal loop of the large hairpin loop. The position that is not modified in vitro is located in the stem. Thus the three Ψ moieties are located in different types of secondary structure, and this may require separate synthase activities with different specificities. In the case of U2 RNA one might speculate that there are even more than two synthase activities because there are 13 Ψ residues [3]. Some of these Ψ residues are in loops, others are in stems, and still others are in single-stranded regions that are not a part of hairpin loops [3]. The experiments presented in this paper do not reveal if more than one activity is modifying a single snRNA, since 5-FU was incorporated throughout the inhibitor RNAs. These types of questions await purification of the enzymes.

Separate Ψ synthases that modify different positions in a single tRNA have been purified from yeast [13,15]. Three separate activities were found that modified uridine at positions 13, 32 and 55 in the major glycine tRNA of Saccharomyces cerevisiae. In addition, other groups have found evidence that more than one tRNA Ψ synthase activity modifies a single tRNA [22], and references cited therein). Thus there is a strong precedent for multiple Ψ synthases that recognize a single RNA molecule. These tRNA Ψ synthases are also able to modify more than one type of tRNA [12,13,22], probably due to the structural similarity of most tRNAs.

The function of Ψ in snRNAs is not known, but pseudouridine appears to be necessary for the function of tRNA during
translation [6–8]. Now that it is possible to produce Ψ-deficient snRNPs in vitro, experiments designed to elucidate the function of Ψ in these splicing cofactors will be possible. Understanding the requirement for bound Sm proteins is also a current interest, and the purification of enzymes that form Ψ in these splicing cofactors is under way.

Beyond their bearing on the multiplicity of snRNA Ψ syntheses, the present experiments also reveal that 5-FU-containing snRNAs are highly specific and potent inhibitors of Ψ formation in human cell extracts. Fluoropyrimidines are used in the treatment of certain cancers [29] and their toxicity is assumed to derive from their effect on thymidylate synthase and also their incorporation in cellular RNA [29]. Effects of fluoropyrimidines on the metabolism of snRNAs [30] and on the in vitro splicing of pre-mRNA [31] have been reported. FU incorporation into long-lived species such as snRNAs could have profound consequences for the cell by producing inactive snRNPs and thereby inhibiting mRNA splicing. Such a possibility now deserves serious consideration in the clinical use of fluoropyrimidines.

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