Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3324–3328, April 1994
Biochemistry

Pseudouridine formation in U2 small nuclear RNA

(5-fluorouridine/U2 RNA mutations/RNase T1 oligonucleotides/independent pseudouridine domains)

JEFFREY R. PATTON*†, MARTY R. JACOBSON‡, AND THORU PEDERSON‡

*Department of Pathology, School of Medicine, University of South Carolina, Columbia, SC 29208; and ‡Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545

Communicated by Sheldon Penman, January 3, 1994

ABSTRACT **U2 small nuclear RNA contains 13 pseudouridine (Ψ) nucleotides, of which 11 are clustered in 5′ regions involved in base-pairing interactions with other RNAs in the spliceosome. As a first step toward understanding the Ψ formation pathway in U2 RNA, we investigated Ψ formation on unmodified human U2 RNA in a HeLa cell extract system. Ψ formation was found to occur specifically within only those RNase T1 oligonucleotide fragments of U2 RNA known to contain Ψ *in vivo*. Using 5-fluorouridine (FUrd)-containing U2 RNAs as specific inhibitors of Ψ formation in non-FUrd-substituted substrate U2 RNA, we found that wild-type FUrd-containing U2 RNA as well as several FUrd-containing mutant U2 RNAs completely inhibited Ψ formation. In contrast, certain other mutant U2 RNAs containing FUrd displayed reduced inhibitory capacity. In these cases Ψ modifications occurred in specific RNase T1 fragments of the substrate U2 RNA only if the FUrd-containing competitor RNA was mutated at or near this site. Formation of Ψ at one site in U2 RNA appeared to be neither dependent on prior Ψ formation at another site or sites nor required for subsequent Ψ formation elsewhere in the molecule. This autonomous mode of Ψ formation may be driven by multiple Ψ synthase enzymes acting independently at different sites in U2 RNA.**

At least five small nuclear RNAs (snRNAs)—U1, U2, U4, U5, and U6—participate in the splicing of pre-mRNA (1, 2). The synthesis of the spliceosomal snRNAs themselves has turned out to be more complex than might have been anticipated, including a cytoplasmic phase of U1, U2, U4, and U5 3′ end processing, 5′ cap hypermethylation, and small nuclear ribonucleoprotein (snRNP) assembly (3).

One of the more enigmatic posttranscriptional modifications of the snRNAs is pseudouridine (Ψ) formation. In the course of developing an *in vitro* assembly system for snRNPs, we observed that Ψ formation occurred (4, 5), and subsequent studies demonstrated that Ψ formation takes place at specific sites in U1, U2, and U5 RNAs in this *in vitro* system (6, 7). Additional experiments with 5-fluorouridine (FUrd)-substituted snRNAs as inhibitors of Ψ formation in the homologous ^{32}P-labeled snRNA substrate revealed that there are multiple snRNA Ψ synthase activities in HeLa cell extracts with distinct specificities for U1, U2, or U5 RNAs (7). For example, FUrd-containing U2 RNA inhibits Ψ formation in ^{32}P-labeled U2 RNA but not in ^{32}P-labeled U1 or U5 RNA (7).

Mammalian U2 RNA contains 13 Ψ nucleotides, the most of any of the known spliceosomal snRNAs (8). We have examined the site specificity of U2 RNA Ψ formation *in vitro* and have asked whether Ψ formation throughout U2 RNA proceeds via a pathway of obligatorily sequential reactions or independently at the various sites.

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MATERIALS AND METHODS

RNA Transcription and *in Vitro* Assembly. The plasmids encoding recombinant U2 RNA have been described (9). Wild-type (wt) U2 RNA was transcribed *in vitro* from *Sma* I-digested plasmid pMRG3U2-27 with T7 RNA polymerase (9). Mutant U2 RNAs (Table 1) were transcribed with T7 RNA polymerase from either *Msp* I- or *Bam*HI-digested plasmid DNAs as indicated. To synthesize [^{32}P]UTP-labeled RNAs, [α-^{32}P]UTP (50 μCi, 600 Ci/mmol; 1 Ci = 37 GBq) was added to standard transcription mixtures containing 250 μM ATP and CTP, 50 μM UTP and GTP, and 1 mM m^{7}GpppG. To synthesize [^{3}H]UTP-labeled RNAs, either [5-^{3}H]UTP (19 Ci/mmol) or [5,6-^{3}H]UTP (23 Ci/mmol) was used as the sole source of UTP (80 μM) in transcription mixtures containing 250 μM ATP and CTP, 50 μM GTP, and 1 mM ^{7}mGpppG. RNAs containing FUrd were synthesized by substituting 5-fluoro-UTP (1 mM; Sierra Bioresearch, Tucson, AZ) for UTP in *in vitro* transcription mixtures containing 250 μM ATP, CTP, and GTP and a small amount of [2,8-^{3}H]ATP (10 μCi, 38–40 Ci/mmol) to facilitate RNA quantitation. All *in vitro* transcribed RNAs were gel purified (10) before use in the Ψ formation assays.

Ψ Formation Reactions. The *in vitro* system for Ψ formation is adapted from the snRNP assembly system (4, 5). A typical 100-μl reaction mixture contained ≈5 ng of gel-purified U2 RNA, 60% (vol/vol) HeLa cell cytoplasmic S100 extract (11), ATP (0.5 mM), creatine phosphate (20 mM), and $MgCl_2$ (3.2 mM). Inhibition of U2 RNA Ψ modification by FUrd-containing RNAs was examined by preincubating the FUrd-containing inhibitor RNA (25 ng/100 μl, constituting a 5-fold molar excess over the U2 RNA substrate; ref. 7) in the reaction mixture at 37°C for 10 min prior to addition of the substrate RNA (either ^{3}H- or ^{32}P-labeled wt U2 RNA). The amount of competitor RNA necessary for maximal inhibition was determined as described (7). The reaction mixture was then incubated for an additional 60 min at 37°C, unless otherwise indicated. In some cases, assembled U2 snRNPs were immunoselected from the reaction mixture (10). Extraction of total RNA, electrophoresis of RNA in polyacrylamide/urea gels, visualization of RNA by autoradiography, and elution of RNA from gels have been described (4–7, 10).

Assays of Ψ Formation. The two assays of Ψ formation used in this study have been described (6). In the first, the ^{3}H at pyrimidine C-5 of [^{3}H]UTP-labeled U2 RNA is exchanged with the bulk solvent when uridine is converted to Ψ (12). To determine the amount of ^{3}H released, 5 vol of charcoal [Norit A, 15% (wt/vol) in 0.2 M HCl] was added to the pseudouridine reaction mixture, mixed, and incubated at 37°C for 30 min. The charcoal was pelleted by centrifugation and the supernatant was filtered through a 0.22-μm-pore cellulose acetate Spin-X centrifuge filter unit (Costar). A portion of the total eluate was

Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; FUrd, 5-fluorouridine; Ψ, pseudouridine; wt, wild type.

†To whom reprint requests should be addressed.

Table 1. Description of U2 RNAs

RNA*	DNA sequence of mutant
wt	$CCT^{189-191} \rightarrow GGG$
2	$ATATTAAAT^{88-96} \rightarrow TATAATTTG$
3	$A^{90} \rightarrow T$
4	$TTTTT^{100-104} \rightarrow AAAAA$
6	$TATCA^{47-51} \rightarrow ATAGT$
7	Deletion of nt 112–144
10	Deletion of nt 15–18
11	$T^{58} \rightarrow A$
12	$T^{58} \rightarrow A$; $A^{90} \rightarrow T$
13	$GGCT^{19-22} \rightarrow ACAC$
14	Deletion of nt 46–49
19	Deletion of nt 34–37
22	$CCTGG^{154-158} \rightarrow GGACC$
24	$GAGGAC^{79-84} \rightarrow CTCCTG$
29	$GTCCTC^{68-73} \rightarrow CAGGAG$
30	Deletion of nt 53–95
31	$GTCCTC^{68-73} \rightarrow CAGGAG$; $GAGGAC^{79-84} \rightarrow CTCCTG$
33	Deletion of nt 63–66
36†	Deletion of nt 123–136; $AG^{110-111} \rightarrow TC$
37‡	Deletion of nt 116–140; $AG^{110-111} \rightarrow TC$
48	Deletion of nt 1–26
50	Deletion of nt 1–104; $AG^{110-111}$ to TC
51	$TCTGTTCT^{39-46} \rightarrow AAAAAAAA$
52	$GGAG^{112-115} \rightarrow CTC$

*Each RNA's number corresponds to the U2 plasmid having the same number in table 1 of ref. 9.
†This mutant has an additional TC at the deletion junction.
‡This mutant has an additional CATG at the deletion junction.

mixed with an aqueous-based scintillation fluid and the amount of ^3H released was determined. The second assay involved [^{32}P]UTP-labeled U2 RNA. After incubation in the reaction mixture (with or without FUrd-containing inhibitor RNA) the ^{32}P-labeled U2 RNA was phenol/chloroform extracted, gel purified, and then digested with RNase T1 (Pharmacia) (6). The digestion products were electrophoresed in a 20% polyacrylamide/8.3 M urea gel and identified by autoradiography. The RNase T1 fragments were eluted from the gel and digested with nuclease P1 (100 μg/ml) in 20 mM sodium acetate (pH 5.2) for 60 min at 37°C. The samples were then chromatographed on cellulose TLC plates (Eastman Kodak) in 2-propanol/concentrated HCl/water, 70:15:15 (vol/vol) (13). The plates were dried overnight at 20–22°C and autoradiographed.

RESULTS

Ψ is a posttranscriptionally modified form of uridine present in a variety of RNA molecules including snRNAs, tRNAs, and rRNAs (for reviews see refs. 8, 14, and 15). Ψ synthases catalyze an isomerization involving cleavage of the uracil N-glycosidic bond and formation of a C-glycosidic bond at C-5. FUrd-substituted RNAs are potent and specific inhibitors of Ψ formation in homologous unsubstituted tRNAs (16, 17) and snRNAs (7). We used FUrd-substituted wt and mutant U2 RNAs (Table 1) as specific inhibitors to study Ψ formation in U2 RNA, which is the most highly modified of the snRNAs, containing 13 Ψ nucleotides (ref. 8; Fig. 1).

Inhibition of ^3H Release from U2 RNA by FUrd-Containing RNAs. Incubation of [^{3}H]UTP-labeled U2 RNA in reaction mixtures containing HeLa cell S100 extract (*Materials and Methods*; refs. 4 and 5) results in an exchange of the ^3H at the C-5 position with water when uridine is converted to Ψ (12). (This is an indirect assay of Ψ formation, useful for screening large numbers of reactions.) As expected, when FUrd-substituted U2 RNA, a specific inhibitor of Ψ formation in U2 RNA (7), was included in the reaction mixture, an inhibition of ^3H release from ^3H-labeled U2 RNA was observed (averaging 28% of control, Table 2). We next examined the capacity of various FUrd-substituted mutant U2 RNAs to act as specific inhibitors of Ψ formation (Table 2). The majority of the FUrd mutant U2 RNAs inhibited ^3H release from the U2 RNA substrate to extents similar to that observed for FUrd-substituted wt U2 RNA. These "no-effect" mutations include both single nucleotide replacements (e.g., mutants 3 and 11) and large deletions (e.g., mutants 7, 36, and 37), indicating that there are discrete regions of U2 RNA that do not play a role in directing Ψ formation. In contrast, nine of the FUrd-substituted mutant RNAs (mutants 4, 12, 14, 19, 22, 30, 48, 51, and 52) showed intermediate levels of inhibition of ^3H release from wt U2 RNA. This indicates that some Ψ formation in the substrate U2 RNA occurred despite the presence of these FUrd-substituted mutant RNAs.

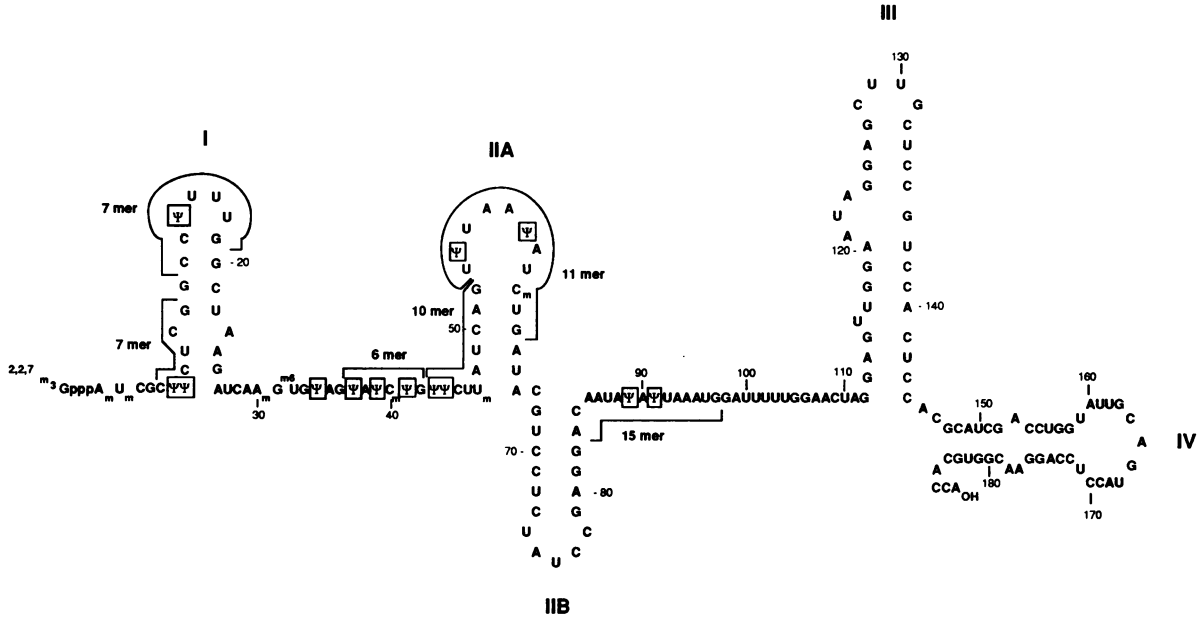

FIG. 1. Rat U2 RNA (8) with the Ψ residues boxed. The RNase T1 oligonucleotides that were analyzed for Ψ content (see Table 3) are identified by solid lines.

Table 2. Inhibition of Ψ formation in wt U2 RNA by FUrd-containing U2 RNA competitors

Inhibitor	% control*	Inhibitor	% control*
tRNA	100		
wt	28 ± 1.0	22	41 ± 3.8
2	26 ± 3.3	24	31 ± 1.1
3	35 ± 1.0	29	28 ± 0.7
4	41 ± 1.0	30	41 ± 1.6
6	35 ± 2.1	31	22 ± 2.6
7	37 ± 1.9	33	33 ± 0.4
10	30 ± 2.1	36	31 ± 1.0
11	33 ± 0.9	37	33 ± 1.5
12	42 ± 2.0	48	43 ± 1.4
13	26 ± 0.7	50	103 ± 3.2
14	46 ± 1.6	51	62 ± 1.8
19	66 ± 1.7	52	41 ± 0.1

*Percent ^{3}H released from wt ^{3}H-labeled U2 RNA in the presence or absence of FUrd RNAs. Each value in the table represents the mean ± SD of three separate assays of the same reaction with an averaged no-extract control value subtracted.

Most of the mutations in these competitor RNAs are localized to regions of U2 RNA that have Ψ, but there are interesting exceptions. For instance mutant 4, in which the uridines in the Sm binding domain are replaced (Table 1), shows intermediate inhibition, suggesting that the binding of Sm proteins may be important for the formation of some Ψ moieties but not all. In addition, mutant 22, which disrupts the fourth stem–loop of U2 RNA, exhibits intermediate inhibition. This result suggests that the structure of this region of the U2 RNA molecule may play a role in substrate recognition by Ψ synthase(s). No inhibition of ^{3}H release was observed with FUrd mutant 50 RNA, which has a deletion of the first 104 nt of the U2 RNA sequence (Table 1). This suggests that the sequences and/or structures in the 3′ half of U2 RNA (nt 105–188, a region which does not include any of the Ψ positions) are not sufficient for inhibition of Ψ formation.

We examined the possibility that the intermediate or lack of inhibition of Ψ formation observed with some of the FUrd mutant U2 RNAs might be due to their different stabilities in the *in vitro* system. RNAs were transcribed in the presence of [α-^{32}P]GTP and 5-fluoro-UTP, gel purified, and incubated in the *in vitro* system at the same concentrations used in the ^{3}H-release assays. After phenol/chloroform extraction and ethanol precipitation, the ^{32}P-labeled, FUrd-substituted RNAs were electrophoresed in a 10% polyacrylamide/8.3 M urea gel and examined by autoradiography. Little if any degradation was observed even after 60 min of incubation (Fig. 2). As another indication of their integrity, we found that all of the FUrd mutant RNAs, with the exception of mutants 4, 50, and 52, were readily assembled into snRNP complexes, as defined by their ability to be immunoselected by Sm monoclonal antibody (data not shown). FUrd mutants 4 and 52 reacted very weakly but consistently with Sm antibody, as did their non-FUrd-substituted counterparts (data not shown). Thus, the intermediate or lack of inhibition of ^{3}H release observed for some of the FUrd mutant RNAs can be attributed to their inherent differing capacities to act as specific inhibitors of Ψ formation.

Specific Inhibition of Ψ Formation in U2 RNA by FUrd-Substituted RNAs. Most of the FUrd RNAs which only partially inhibited Ψ formation are mutated in a location at or near a site of Ψ modification. We therefore wanted to directly examine Ψ formation in U2 RNA. [α-^{32}P]UTP-labeled wt U2 RNA was incubated in the *in vitro* system and FUrd RNAs were included as indicated. The assembled snRNP particles were immunoselected with Sm antibody and the RNAs were isolated and digested with nuclease P1 before TLC and autoradiography. The antibody selection was employed to remove assembled U2 particles from degraded ^{32}P-labeled U2 RNA fragments that might interfere with the assay. A prominent Ψ spot (comprising 10–15% of the theoretical 100% level of Ψ formation) was observed when no FUrd-substituted RNA was included in the reaction mixture (Fig. 3, lane tRNA), in keeping with previous results (7). However, when FUrd-substituted wt U2 RNA was included, Ψ formation was almost completely inhibited, again demonstrating that FUrd-substituted wt U2 RNA is a potent inhibitor of Ψ formation in U2 RNA. These data also suggest that the lack of complete inhibition observed in Table 2 (^{3}H-release assay) when FUrd wt U2 RNA was used as an inhibitor could be due to other reactions involving the C-5 tritium atom that occur in the ^{3}H-release assay.

When various FUrd mutant U2 RNAs were examined as specific inhibitors of Ψ formation in the substrate RNA (Fig. 3), results similar to those obtained by the ^{3}H-release assay were observed. FUrd mutant U2 RNAs which displayed wt inhibition of Ψ formation in the ^{3}H-release assay (mutants 24, 29, and 31) were also potent inhibitors of Ψ formation as measured by direct ^{32}P nucleotide analysis. Similarly, those FUrd mutant RNAs which displayed intermediate inhibition in the ^{3}H-release assay (mutants 3, 4, 14, 19, and 30) only partially inhibited Ψ formation in the ^{32}P-labeled wt U2 RNA (Fig. 3).

It is possible that those mutants which cause only partial inhibition interact less efficiently with the activity responsible for Ψ formation in U2 RNA, resulting in a general reduction in the overall amount of Ψ formation. It is also possible that if the mutation in a FUrd inhibitor RNA were located at or near a site of Ψ modification, it would not be capable of inhibiting Ψ formation at the corresponding site in the substrate RNA. To examine these possibilities, high-specific-activity [α-^{32}P]UTP-labeled wt U2 RNA (substrate RNA) was incubated *in vitro* with competitor FUrd RNAs. Total RNA was isolated from the reaction mixtures and digested to completion with RNase T1. The ^{32}P-labeled RNase T1 reaction products were separated by electrophoresis in a 20% polyacrylamide/8.3 M urea gel and

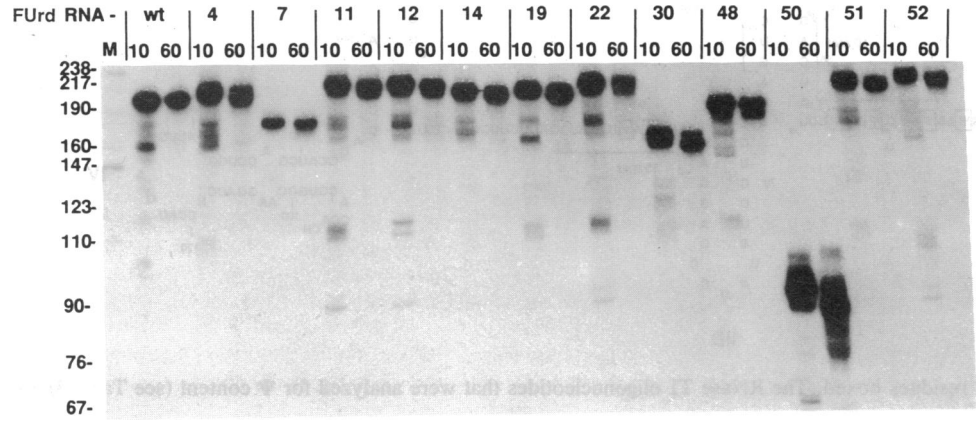

FIG. 2. Stability of FUrd-containing U2 RNAs. [α-^{32}P]GTP-labeled, FUrd-containing wt and mutant U2 RNAs were incubated for either 10 or 60 min in the *in vitro* Ψ formation system and the RNA was isolated and electrophoresed in a 10% polyacrylamide/8.3 M urea gel. Lane M, size markers: ^{32}P-end-labeled DNA fragments from an *Msp* I digest of pBR322; the sizes of the fragments in nucleotides are indicated at left.

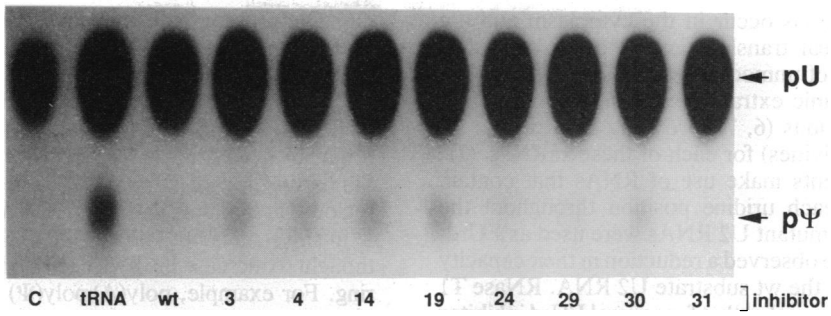

FIG. 3. Inhibition of Ψ formation in ^{32}P-labeled U2 RNA by FUrd-containing wt and mutant U2 RNAs. [α-^{32}P]UTP-labeled U2 RNA was incubated in the *in vitro* system after preincubation of the system with FUrd-containing wt and mutant U2 RNAs. The ^{32}P-labeled substrate U2 RNA was isolated and assayed for Ψ as described in *Materials and Methods*. A portion of the autoradiograph of the resulting TLC plate is shown. Lane C, control (^{32}P-labeled U2 RNA was not incubated). The inhibitor RNAs used in the other reactions are indicated below each lane. The positions of uridine 5′-monophosphate (pU) and Ψ 5′-monophosphate (pΨ) are indicated at right.

identified by autoradiography. The five labeled RNase T1 oligonucleotides which could theoretically contain pΨ (the 15-, 11-, 10-, 7-, and 6-mers; Fig. 1) were subsequently eluted from the gel and digested to completion with nuclease P1 before TLC (13). {The trimer (nt 34–36) which would result from RNase T1 digestion of U2 RNA should contain Ψ (nt 34; see Fig. 1), but this oligonucleotide would not contain label derived from [α-^{32}P]UTP, since RNase T1 digestion produces 3′ phosphates and nuclease P1 leaves 5′ phosphates.}

When no FUrd-substituted RNA was included in the reaction mixture, Ψ was present in all of the expected RNase T1 oligonucleotides (Table 3). Moreover, when FUrd-substituted wt U2 RNA was included as an inhibitor, none of the T1 oligonucleotides from the ^{32}P-labeled substrate U2 RNA contained Ψ, again as expected.

In contrast, when the ^{32}P-labeled substrate RNA was incubated individually with each of the other FUrd mutant RNAs, different amounts and patterns of Ψ formation in the substrate's T1 oligonucleotides were observed for each RNA tested (Table 3). For example, FUrd mutant 30 RNA, which has a deletion of nt 53–95 (which includes the region corresponding to the 11-mer and 15-mer T1 oligonucleotides), was not able to inhibit Ψ formation in the 15-mer and 11-mer T1 oligonucleotides of the substrate RNA. However, complete inhibition was observed in the 10-mer, 7-mers, and 6-mer, which are regions not mutated in FUrd mutant 30 RNA. Similarly, FUrd mutant 48 RNA was not capable of inhibiting Ψ formation in the 7-mer T1 oligonucleotides of the substrate RNA (the region mutated in mutant 48; see Table 1), whereas complete inhibition was observed in the other T1 fragments.

Table 3. Location of Ψ in ^{32}P-labeled wt U2 RNA after incubation with FUrd-containing U2 RNAs

Inhibitor	Presence of Ψ in RNase T1 fragments*				
	15-mer	11-mer	10-mer	7-mer	6-mer
tRNA (no FUrd)	+++	+++	+++	+++	+++
wt	−	−	−	−	−
4	−	++	+	+	+
12	−	++	−	−	−
14	−	−	+	−	+
19	+	+	++	−	++
22	−	−	−	−	+
30	+++	++	−	−	−
48	−	+	−	+++	−

Amount of ^{32}P-labeled substrate wt U2 RNA in the reaction mixture was 10 ng. Amount of each inhibitor RNA added was 50 ng. *+++, Ψ level approximately the same as the tRNA (no FUrd) control; ++, Ψ significantly lower than control; +, Ψ level very low; −, no Ψ detected.

FUrd mutant 12 RNA contains two point mutations ($U^{58} \rightarrow$ A and $A^{90} \rightarrow$ U). U^{58} is normally modified to Ψ whereas A^{90} is located between two uridines which are normally modified (Fig. 1). When FUrd 12 RNA was used as a competitive inhibitor, Ψ formation was observed only in the 11-mer T1 oligonucleotide of the substrate RNA (Table 3), suggesting that the presence of a uridine residue at position 58 in the FUrd RNA is important for specific inhibition of Ψ formation in this region of U2 RNA. In contrast, the mutation $A^{90} \rightarrow$ U had no affect on the ability of FUrd 12 RNA to specifically inhibit Ψ formation at positions 89 and 91 (which are in the 15-mer T1 oligonucleotide of the substrate RNA).

Interpretation of the results from the other mutant inhibitors used (nos. 4, 14, and 19) is not as straightforward. FUrd 4 (a mutation of the Sm binding site) partially inhibited Ψ formation in four of the T1 oligonucleotides. This partial inhibition may be due to a role for Sm binding in substrate recognition, similar to the requirement for Sm proteins in Ψ modification of U5 RNA (6). However, this requirement is apparently not absolute, since partial inhibition was observed. In the FUrd 14 and 19 inhibitor RNAs, the mutations are in the highly modified region between stem–loops I and IIA. The FUrd 14 and 19 RNAs inhibited Ψ formation in the 10-mer and 6-mer T1 oligonucleotides, which are sites closest to the areas of mutation. In addition, FUrd 19 only partially inhibited Ψ formation in the 15-mer and 11-mer, even though the mutation is a deletion of nt 34–37. These results suggest that the deletions in these mutant RNAs are large enough to affect the Ψ-inhibitory capacity but not extensive enough to allow complete Ψ formation in the substrate RNA.

When FUrd mutant 22 RNA (CCUGG → GGACC at nt 154–158; a stem IV mutant) was included in the reaction as an inhibitor, Ψ formation was observed in the 6-mer T1 oligonucleotide of the substrate RNA. Since the mutation in this FUrd RNA is in the fourth stem–loop it is not readily apparent why there was incomplete inhibition of Ψ formation in the 6-mer. This mutant would be expected to exhibit wt inhibition, but it does not in either this assay or the ^{3}H-release assay (Table 2).

In general, therefore, Ψ modifications occurred (to different degrees) in specific RNase T1 oligonucleotides of the substrate RNA only when the FUrd-containing competitor RNA was mutated at or near the corresponding location; i.e., FUrd mutant RNAs were less able to inhibit specific Ψ formation in the substrate RNA at positions corresponding to the site of their mutation. In contrast, sequences not mutated in the FUrd RNAs appeared to specifically inhibit Ψ formation at the corresponding sites in the substrate RNA.

DISCUSSION

Posttranscriptional nucleotide modifications have been identified in all of the U snRNAs examined, preferentially located in single-stranded regions within the 5′ half of the molecules (8).

Most of these modifications occur in the cytoplasm and are completed prior to nuclear translocation of the assembled U snRNPs (3). Incubation of unmodified U1, U2, or U5 snRNA in a HeLa cell cytoplasmic extract results in Ψ formation at specific nucleotide positions (6, 7), probably by a distinct Ψ synthase activity (or activities) for each of these snRNAs (7).

The present experiments make use of RNAs that contain FUrd substitutions at each uridine position throughout the molecule. When certain mutant U2 RNAs were used as FUrd-substituted inhibitors, we observed a reduction in their capacity to inhibit Ψ formation in the wt substrate U2 RNA. RNase T1 oligonucleotide analysis revealed that for several FUrd inhibitor U2 RNAs, Ψ formation in the substrate U2 RNA was not inhibited at the sites corresponding to the inhibitor's mutation, whereas Ψ formation was specifically inhibited at the other regions in the substrate. Thus, Ψ formation at discrete sites in U2 RNA does not proceed via an ordered pathway of sequential reactions, at least under the conditions of the *in vitro* system we have employed. Rather, Ψ formation proceeds independently at apparently autonomous domains in the U2 RNA molecule. A similar situation has been reported for tRNA (17).

Is Ψ formation at all 13 sites in U2 RNA catalyzed by a single or multiple Ψ synthases? If there were but a single enzyme, Ψ formation would be expected to be inhibited at all sites whenever the (single) enzyme was inhibited by any FUrd RNA, acting as a substrate-analog competitive inhibitor of the enzyme's uracil binding site. Since this was not observed, multiple U2 RNA Ψ synthases seem more likely, though we cannot exclude the possibility of a single enzyme possessing a constellation of several (13?) catalytic sites for the multiple Ψ domains in U2 RNA. The existence of multiple Ψ synthases that specifically modify different snRNAs, and individual Ψ sites within a given snRNA, would indicate a substantial enzymatic commitment by the cell to this aspect of snRNA maturation.

As mentioned earlier, several posttranscriptional modifications of U1, U2, U4, and U5 snRNAs take place in the cytoplasm before the mature snRNA returns to the nucleus as a functional snRNP (3). These include both covalent modifications such as 5′ cap hypermethylation, 3′ end processing, and Ψ formation, as well as the association of these RNAs with both common and specific sets of proteins (1). Since both trimethylguanosine and snRNP protein antibodies immunoselect precursor forms of U1, U2, U4, and U5 snRNAs from HeLa cytoplasmic extracts (18–20), it is clear that both 5′ cap hypermethylation and snRNP assembly precede 3′ end processing. However, it is not known where Ψ formation fits into this sequence of events. In the *in vitro* system employed in the present investigation, Ψ formation in U5 RNA was dependent on binding of the Sm snRNP proteins (6). However, in the case of U2 RNA an earlier study suggested that Ψ formation did not require binding of the Sm snRNP proteins (5). The results obtained here suggest that U2 RNA can indeed be a substrate for Ψ formation without Sm proteins being bound, since FUrd mutant 4 RNA, which lacks the Sm binding site, inhibits Ψ formation and therefore must be recognized by the Ψ synthase(s). Thus, U2 and U5 RNAs appear to differ in their dependence on Sm protein binding for Ψ formation. It is likely that the processes of Ψ formation and snRNP assembly can proceed concurrently and without mutual interference, since neither the two Ψ nucleotides in U1 RNA nor the 13 in U2 RNA are located in regions that are known snRNP protein binding sites (refs. 21 and 22; reviewed in ref. 1).

5-Fluorouracil was one of the first cancer chemotherapeutic drugs and is still used effectively today. Its antitumor activity is generally attributed to the inhibition of DNA synthesis, but incorporation of FUrd into cellular RNA can obviously be expected to have serious effects. Indeed, 5-fluorouracil-treated cells display aberrant pre-mRNA splicing (23–25) and FUrd-substituted U2 RNA assembles into altered snRNP particles (26). Our present findings suggest that inhibition of Ψ formation in the spliceosomal snRNAs may be among the factors in the observed inhibition of pre-mRNA splicing in 5-fluorouracil-treated cells (23–25). In the case of U2 RNA, several of the Ψ nucleotides are located in regions known to interact either with pre-mRNA (U2 nt 33–38) or with U6 RNA (U2 nt 3–12). Similarly, the two Ψ nucleotides in U1 RNA are located in a region that interacts with pre-mRNA. While the precise role of Ψ in snRNAs is not known, the C-5 glycoside configuration is thought to increase the hydrogen-bonding potential of the uracil ring. For example, poly(A)·poly(Ψ) duplexes are more stable than poly(A)·poly(U) duplexes (27). Ψ can form hydrogen bonds to bridging water molecules or to vicinal ribose 2′-hydroxyl groups (28). Inhibition of a Ψ-based repertoire of hydrogen bonding in snRNAs, presumably essential for their role in mRNA splicing, may be among the factors at play when cells or patients are treated with 5-fluorouracil.

We gratefully acknowledge perspectives on Ψ chemistry from Daniel M. Brown (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). This investigation was supported by an institutional grant from the American Cancer Society (J.R.P.), the Stefan Mironescu Research Award from the University of South Carolina (J.R.P.), National Institutes of Health Grant GM21595-18 (T.P.), and the G. Harold and Leila Y. Mathers Foundation (T.P.).

1. Luhrmann, R., Kastner, B. & Bach, M. (1990) *Biochim. Biophys. Acta* **1087**, 265–292.
2. Baserga, S. J. & Steitz, J. A. (1993) in *The RNA World*, eds. Gesteland, R. F. & Atkins, J. F. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 359–381.
3. Zieve, G. W. & Sauterer, R. A. (1990) *CRC Rev. Biochem. Mol. Biol.* **25**, 1–46.
4. Patton, J. R., Patterson, R. J. & Pederson, T. (1987) *Mol. Cell. Biol.* **7**, 4030–4037.
5. Kleinschmidt, A. M., Patton, J. R. & Pederson, T. (1989) *Nucleic Acids Res.* **17**, 4817–4828.
6. Patton, J. R. (1991) *Mol. Cell. Biol.* **11**, 5998–6006.
7. Patton, J. R. (1993) *Biochem. J.* **290**, 595–600.
8. Reddy, R. & Busch, H. (1988) in *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*, ed. Birnstiel, M. L. (Springer, Berlin), pp. 1–37.
9. Jacobson, M. R., Rhoadhouse, M. & Pederson, T. (1993) *Mol. Cell. Biol.* **13**, 1119–1129.
10. Patton, J. R., Habets, W., van Venrooij, W. J. & Pederson, T. (1989) *Mol. Cell. Biol.* **9**, 3360–3368.
11. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
12. Mullenbach, G. T., Kammen, H. O. & Penhoet, E. E. (1976) *J. Biol. Chem.* **251**, 4570–4578.
13. Nishimura, S. (1972) *Prog. Nucleic Acids Res. Mol. Biol.* **12**, 49–85.
14. Bjork, G. R., Ericson, J. U., Gustafsson, C. E. D., Hagervall, T. G., Jonsson, Y. H. & Wikstrom, P. M. (1987) *Annu. Rev. Biochem.* **56**, 263–287.
15. Maden, B. E. H. (1990) *Prog. Nucleic Acids Res. Mol. Biol.* **39**, 241–303.
16. Kammen, H. O., Marvel, C. C., Hardy, L. & Penhoet, E. E. (1988) *J. Biol. Chem.* **263**, 2255–2263.
17. Samuelsson, T. (1991) *Nucleic Acids Res.* **19**, 6139–6144.
18. Madore, S. J., Wieben, E. D. & Pederson, T. (1984) *J. Cell Biol.* **98**, 188–192.
19. Madore, S. J., Wieben, E. D., Kunkel, G. R. & Pederson, T. (1984) *J. Cell Biol.* **99**, 1140–1144.
20. Wieben, E. D., Nenninger, J. M. & Pederson, T. (1985) *J. Mol. Biol.* **183**, 69–78.
21. Patton, J. R. & Pederson, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 747–751.
22. Mattaj, I. W. & DeRobertis, E. M. (1985) *Cell* **40**, 111–118.
23. Will, C. L. & Dolnick, B. J. (1987) *J. Biol. Chem.* **262**, 5433–5436.
24. Will, C. L. & Dolnick, B. J. (1989) *J. Biol. Chem.* **264**, 21413–21421.
25. Sierakowska, H., Shukla, R. R., Dominski, Z. & Kole, R. (1989) *J. Biol. Chem.* **264**, 19185–19191.
26. Patton, J. R. (1993) *Biochemistry* **32**, 8939–8944.
27. Pochon, F., Michelson, A. M., Grunberg-Manago, M., Cohn, W. E. & Dondon, L. (1964) *Biochim. Biophys. Acta* **80**, 441–447.
28. Griffey, R. H., Davis, D., Yamaizumi, Z., Nishimura, S., Bax, A., Hawkins, B. & Poulter, C. D. (1985) *J. Biol. Chem.* **260**, 9734–9741.