Ribonucleoprotein Particle Assembly and Modification of U2 Small Nuclear RNA Containing 5-Fluorouridine†

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ABSTRACT: An *in vitro* assembly/modification system was used to study the effect of 5-fluorouridine (5-FU) incorporation on the biosynthesis of the U2 small nuclear ribonucleoprotein particle (U2 snRNP). Labeled U2 RNAs were transcribed *in vitro* with 5-fluoro-UTP either partially supplementing or completely replacing UTP during synthesis. The resulting U2 RNAs have levels of 5-fluorouridine that range from 0 to 100% of the uridine content. When incubated in reactions containing extracts from HeLa cells, these 5-FU U2 RNAs are assembled into RNPs that are recognized by anti-Sm monoclonal antibody even when there is a complete replacement of uridine with 5-FU. However, when the *in vitro* assembled U2 snRNPs are subjected to buoyant density gradient centrifugation, the particles that contain 100% 5-FU are not resistant to salt dissociation. When the *in vitro* assembled U2 snRNPs were analyzed by velocity sedimentation gradient centrifugation, 5-FU incorporation correlated with a shift in the sedimentation rate of the particles. With 100% 5-FU incorporation, the peak of radioactivity shifted to ~15 S (control U2 RNA was at ~12 S). This peak from 5-FU U2 snRNPs was not resistant to dissociation on cesium sulfate gradients. The amount of pseudouridine (Ψ) found in the RNA from snRNP assembled *in vitro* on control and 5-FU-containing U2 RNAs was determined, and even at very low levels of 5-FU incorporation (5% replacement), the formation of Ψ was severely inhibited (36% of control). At higher levels of 5-FU incorporation, there was essentially no Ψ formed.

Fluoropyrimidines (FPys)[1] have been used for decades in the treatment of cancer and are still the drugs of choice for chemotherapeutic treatment of certain cancers (Mayer, 1991; Weckbecker, 1991; Tobias, 1992). A quest to improve the efficacy of the drugs has resulted in a tremendous number of studies concerned with the metabolism and mode of action of these anticancer agents [see Weckbecker (1991)].

A great deal of emphasis has been placed on the study of the effects of FPys on DNA synthesis in general and the inhibition of thymidylate synthase in particular (Heidelberger *et al.*, 1983; Parker & Cheng, 1990; Weckbecker, 1991). These effects on DNA synthesis are necessary for the anticancer activity of the drugs, but FPys affect the metabolism of RNA as well [see Weckbecker (1991) for a review]. These RNA effects may contribute to the death of cells not undergoing DNA synthesis. The processing of 45S preribosomal RNA is inhibited by 5-fluorouridine (5-FU) incorporation (Wilkinson & Pitot, 1973; Wilkinson *et al.*, 1975), and 5-FU incorporation inhibits the modification of tRNAs (Randerath *et al.*, 1983). The modifications that are inhibited are all at the 5 position on uridine and include 5-methyluridine, pseudouridine (Ψ), and 5,6-dihydrouridine (Tseng *et al.*, 1978; Frendewey & Kaiser, 1979). In addition, 5-FU-containing small nuclear RNAs (snRNAs) are very specific inhibitors of the *in vitro* formation of Ψ in these essential splicing cofactors (Patton, 1993).

The splicing of mRNA is also affected by the incorporation of 5-FU, with an increase in pre-mRNA splicing intermediates seen in drug-treated cells (Will & Dolnick, 1987, 1989). When extracts from 5-FU-treated cells were used for *in vitro* splicing assays, the processing of β-globin pre-mRNA was inhibited (Sierakowska *et al.*, 1989). This inhibition appeared to be caused, at least in part, by a decreased level of U2 small nuclear ribonucleoprotein particle (snRNP), a pre-mRNA splicing cofactor (Sierakowska *et al.*, 1989). Armstrong *et al.* (1986) also found changes in the structures and levels of other snRNP splicing cofactors when cells were treated with FPys. A protein core containing the Sm proteins binds to each of these snRNAs (U1, U2, U4/U6, and U5), and most bind specific proteins as well (Bach *et al.*, 1989; Luhrmann *et al.*, 1990; Behrens *et al.*, 1993). These snRNPs interact in the presence of pre-mRNA to form a spliceosome, a large splicing complex [for a review, see Steitz *et al.* (1988)]. These interactions involve RNA–RNA base-pairing and RNA–protein recognition (Steitz *et al.*, 1988; Guthrie, 1991) that could be disrupted by the presence of 5-FU in the snRNAs.

In this report, an *in vitro* assembly/modification system was used to study the effect of 5-FU incorporation on the biosynthesis of U2 snRNP. The results indicate the presence of 5-FU modifies the structure of the RNP particle that results in altered RNA–protein interactions. In addition, the *in vitro* modification of U2 RNA was inhibited by low levels of 5-FU incorporation.

EXPERIMENTAL PROCEDURES

Human snRNAs were transcribed *in vitro* as described (Melton *et al.*, 1984) using *Bam*HI-cut pHU1 (Patton *et al.*, 1987) to generate U1 RNA, *Sma*I-cut pMRG3U2-27 (Jacobson *et al.*, 1993) to synthesize U2 RNA, and *Mae*I-cut pHU5a2 (Patton, 1991) to transcribe U5 RNA. 5-FU-containing RNAs were synthesized as described (Patton, 1993) using either ^{32}P-labeled CTP or GTP (typically 50 μCi per reaction; 650 Ci/mmol) or [2,8-^{3}H]ATP (40 μCi; 38 Ci/mmol) depending on the experiment (see figure legends).

The *in vitro* assembly/modification reactions were carried out as described (Patton *et al.*, 1987; Patton, 1991, 1993)

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[1] Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; Ψ, pseudouridine; 5-FU, 5-fluorouridine; FPys, fluoropyrimidines.

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using either a HeLa S100 cell extract or a combination of S100 and nuclear extracts (Dignam *et al.*, 1983) depending on the experiment (see figure legends for details). The analysis of the assembled particles on cesium sulfate gradients has been described previously (Mayrand & Pederson, 1981; Patton *et al.*, 1987). Samples were layered on a 1.25–1.75 g/cm^3 Cs_2SO_4 gradient and centrifuged at 15 °C for 60 h in an SW50.1 rotor at 32 000 rpm. For glycerol gradient centrifugation, the samples were layered on 10–30% glycerol gradients and centrifuged at 4 °C for 20 h in an SW41 rotor at 40 000 rpm. Other conditions for centrifugation are as described in Patton (1991).

The assay for Ψ formation has been described elsewhere (Nishimura, 1972; Patton, 1991). To measure the formation of Ψ, ^{32}P-labeled U2 RNA was isolated from the reaction, electrophoresed on a denaturing 10% polyacrylamide/8.3 M urea gel, eluted from the gel, and digested with RNase T_2 (Patton, 1991). The digested RNA was chromatographed on thin-layer cellulose (TLC) plates with isobutyric acid/0.5 M NH_3 (5:3, v/v) in the first dimension and 2-propanol/concentrated HCl/water (70:15:15, v/v/v) in the second dimension (Nishimura, 1972). The level of 5-FU incorporation was also determined on TLC plates as described above. 5-FUp migrates slightly faster than Up in the second dimension (R_f of Up is ~0.75 and the R_f of 5-FUp is ~0.88). The spots were scraped from the TLC plates for 5-FUp and Ψp determination and counted in Ecolume scintillant (ICN).

The procedures for the antibody selection of Sm antigen-containing U2 snRNPs and the isolation of RNA from the assembly/modification extracts have been described (Patton *et al.*, 1989).

RESULTS

Effect of 5-FU Incorporation on snRNP Assembly in Vitro. A characteristic of snRNP assembled *in vitro* or snRNP isolated from cells is their resistance to dissociation in high salt. This may be due in part to the presence of the Sm proteins on the snRNP, a set of proteins that binds very tightly to U1, U2, U4, and U5 RNAs (Lelay-Taha et al., 1986). The particles band at characteristic buoyant densities in cesium sulfate or cesium chloride gradients, depending on their RNA to protein ratio (Lelay-Taha *et al.*, 1986; Patton *et al.*, 1987; Kleinschmidt *et al.*, 1989; Patton, 1991). When ^{32}P-labeled U1, U2, or U5 RNAs were incubated in the assembly/modification reactions containing S100 extract, particles with buoyant densities of ~1.4 g/cm^3 were formed (Figure 1A–C). These *in vitro* assembly results have been documented in earlier studies and are typical of snRNAs that do not contain 5-FU (Patton *et al.*, 1987; Kleinschmidt *et al.*, 1989; Patton, 1991). There is also a peak at 1.6 g/cm^3 with each of the RNAs, which is indicative of RNA that did not form a salt-resistant, stable particle *in vitro* (Patton *et al.*, 1987).

When 5-FU was substituted for uridine, the *in vitro* assembly of salt-resistant particles from the exogenously added snRNAs was inhibited (Figure 1A–C). The amount of radioactivity in the ~1.4 g/cm^3 peak was reduced when 5-FU U1 and U5 RNAs (Figure 1A,C) were added to the assembly reaction. The peak was absent when 5-FU U2 RNA was added (Figure 1B). With 5-FU U2 RNA, nearly all of the radioactivity was found in the 1.6 g/cm^3 peak, indicative of protein-free RNA. Since the effect of 5-FU incorporation on the assembly of salt-resistant complexes appeared to be greatest for U2 RNA, subsequent experiments presented in this report will focus on this RNA.

The length of incubation time for the reactions presented in Figure 1 was 30 min. It was possible that salt-resistant

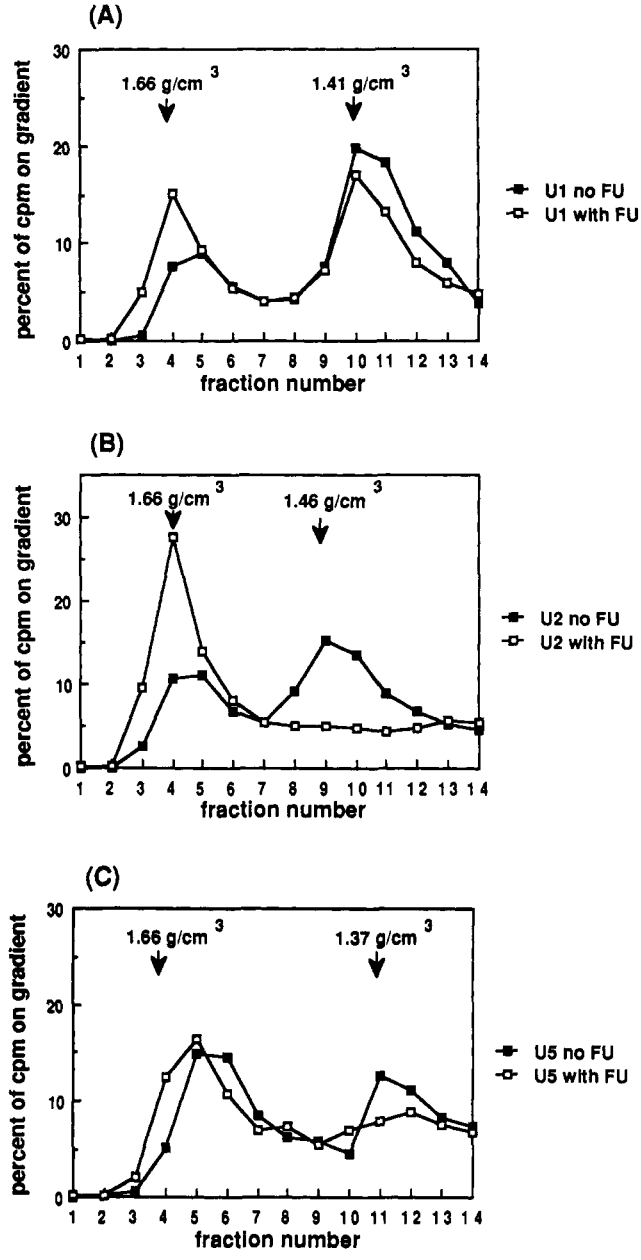

FIGURE 1: Effect of 5-FU incorporation on the assembly of snRNPs *in vitro*. [^{3}H]ATP-labeled U1 (A), U2 (B), and U5 (C) RNAs with uridine (■) or with 5-FU in place of uridine (□) were incubated in assembly/modification reactions containing S100 extract for 30 min and then analyzed by cesium sulfate buoyant density gradient centrifugation as described under Experimental Procedures. The bottoms of the gradients are on the left, and reference densities are given at the top of each panel.

particles did indeed form on 5-FU U2 RNA but subsequently disassembled over the course of the incubation. A time course of assembly of U2 RNA with and without 5-FU is shown in Figure 2. There is a significant 1.4 g/cm^3 peak at 5 min when control U2 RNA was incubated in the assembly/modification reaction (Figure 2A), and it persists during subsequent incubation. However, the 5-FU U2 RNA never forms a 1.4 g/cm^3 peak during the course of incubation; nearly all of the counts are found in the 1.6 gm/cm^3 peak containing protein-free 5-FU U2 RNA.

In the above experiments, the uridine in U2 RNA was completely replaced by 5-FU in the 5-FU-containing RNAs. In cells treated with FPys, the replacement is typically less complete, so U2 RNAs with varying levels of 5-FU incorporation were synthesized by adjusting the ratios of UTP to

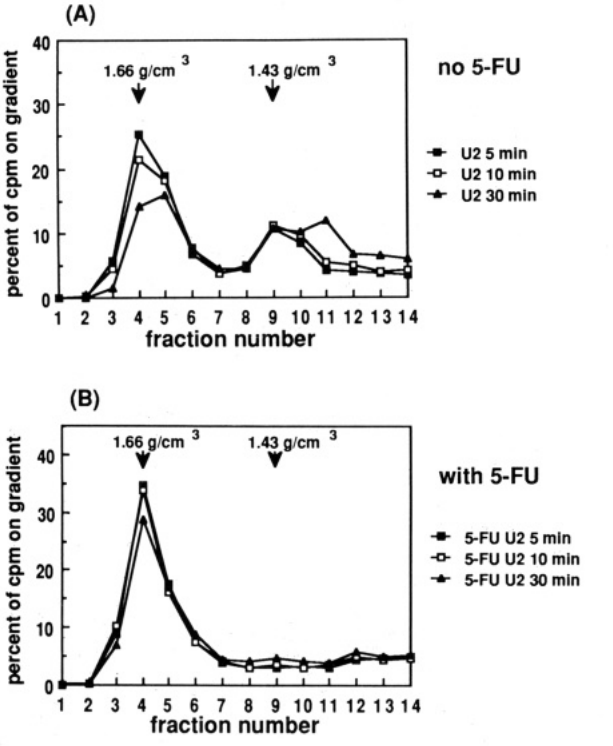

FIGURE 2: Time course of assembly of control and 5-FU-containing U2 RNAs. [^{32}P]GTP-labeled U2 RNAs with uridine (A) or with 5-FU (B) were incubated in assembly/modification reactions that contain S100 extract. Aliquots were taken after 5 (■), 10 (□), and 30 min (▲) of incubation at 37°C. These aliquots were analyzed by cesium sulfate buoyant density gradient centrifugation as described under Experimental Procedures. The bottom of each gradient is on the left in each panel, and reference densities are given at the top.

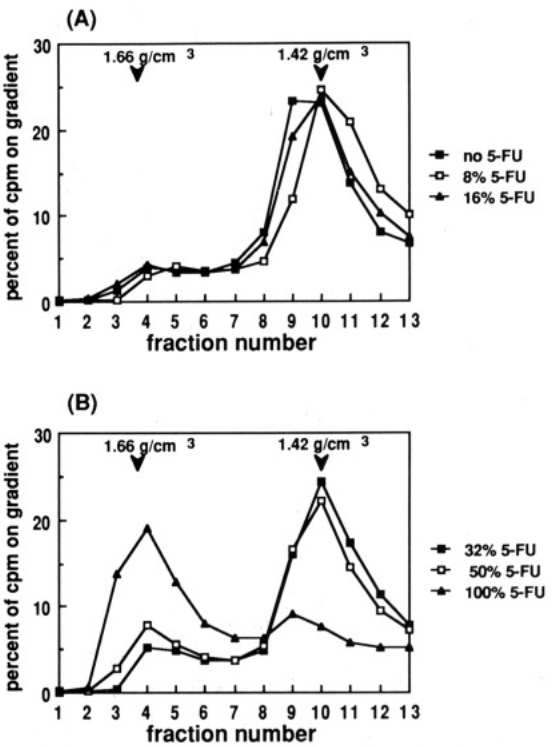

FIGURE 3: Assembly of U2 RNPs on U2 RNAs containing various amounts of 5-FU. [^{32}P]GTP-labeled U2 RNA was synthesized with various amounts of 5-FUTP replacing UTP. The amount of 5-FU incorporated was determined as described under Experimental Procedures. The RNAs were incubated in assembly/modification reactions containing S100 extract for 30 min and analyzed by cesium sulfate buoyant density gradient centrifugation as described under Experimental Procedures. The bottom of each gradient is on the left in each panel, and reference densities are given at the top. The levels of 5-FU incorporation in terms of the percent replacement of uridine for the gradients were for panel A (control U2 RNA) no 5-FU (■), 8% 5-FU U2 RNA (□), and 16% 5-FU U2 RNA (▲) and for panel B 32% 5-FU U2 RNA (■), 50% 5-FU U2 RNA (□), and 100% 5-FU U2 RNA (▲).

5-FUTP in the transcription reactions. The level of 5-FU incorporation was determined as described under Experimental Procedures. These RNAs were incubated in the assembly/modification reactions and analyzed on cesium sulfate gradients. The results (Figure 3) show that there can be up to 50% replacement of the uridine with 5-FU without significant effect on the salt stability of the resulting snRNP. There was an increase in the 1.6 g/cm^3 peak, indicative of protein-free RNA, with increasing levels of 5-FU incorporation, but only the 100% replacement of uridine with 5-FU showed a significant effect (Figure 3B). Interestingly, there was a very small 1.4 g/cm^3 peak with 100% 5-FU U2 RNA, which was not seen in earlier experiments. A different S100 extract was used in this experiment, and the assembly of U2 snRNP was unusually efficient (compare Figures 1B and 2A with Figure 3A). The effect of 5-FU incorporation was consistent with the earlier experiments; nearly all of the 100% 5-FU U2 RNA was found in the 1.6 g/cm^3 peak. This small peak at 1.4 g/cm^3 was not seen with other extracts.

In vitro assembled U2 snRNPs are known to bind Sm proteins (Kleinschmidt *et al.*, 1989), and U2 RNAs that contain different levels of 5-FU reacted with anti-Sm monoclonal antibody (Lerner *et al.*, 1981) after incubation in the assembly reaction (Figure 4). Even a U2 RNA that has complete replacement of uridine with 5-FU (Figure 4, lane 6) bound Sm proteins, but that association was not resistant to high-salt dissociation.

U2 snRNPs assembled in the assembly/modification reactions containing S100 extract sediment at ~10 S on 10–30% glycerol gradients (Kleinschmidt *et al.*, 1989). When nuclear extract was also included (Patton, 1991) and the salt concentration lowered (Bach *et al.*, 1989), the *in vitro*

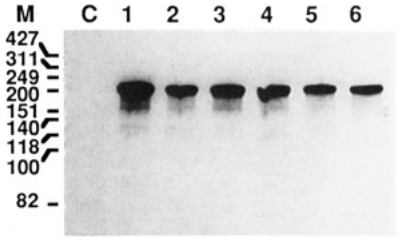

FIGURE 4: Reactivity of *in vitro* assembled control and 5-FU-containing U2 snRNP with monoclonal anti-Sm antibody. *In vitro* transcribed, ^{32}P-labeled, control, and 5-FU-containing U2 RNAs were incubated in assembly/modification reactions containing S100 extract for 30 min as described under Experimental Procedures. The reactions were incubated with protein A–Sepharose-bound anti-Sm monoclonal antibody (Lerner *et al.*, 1981); the bound U2 RNA was isolated and electrophoresed as described (Patton *et al.*, 1989). Lane 1, control U2 RNA (no 5-FU); lane 2, U2 RNA with 5% 5-FU replacement of uridine; lane 3, U2 RNA with 11% 5-FU replacement; lane 4, 30% replacement; lane 5, 49% replacement; lane 6, 100% replacement of uridine with 5-FU. The percent replacement was determined as described under Experimental Procedures. Lane C corresponds to control ^{32}P-U2 RNA incubated without S100 extract and reacted with the anti-Sm antibody and worked up as described above. M denotes the position of *Hinf*I-cut ΦX174 DNA markers.

assembled U2 sedimented at 12–13 S (Figure 5A), possibly due to the binding of additional U2 snRNP-specific proteins found in the nuclear extract (Behrens *et al.*, 1993). As the level of 5-FU incorporation increased in the U2 RNA, the sedimentation rate increased to ~15 S (Figure 5B). A shift

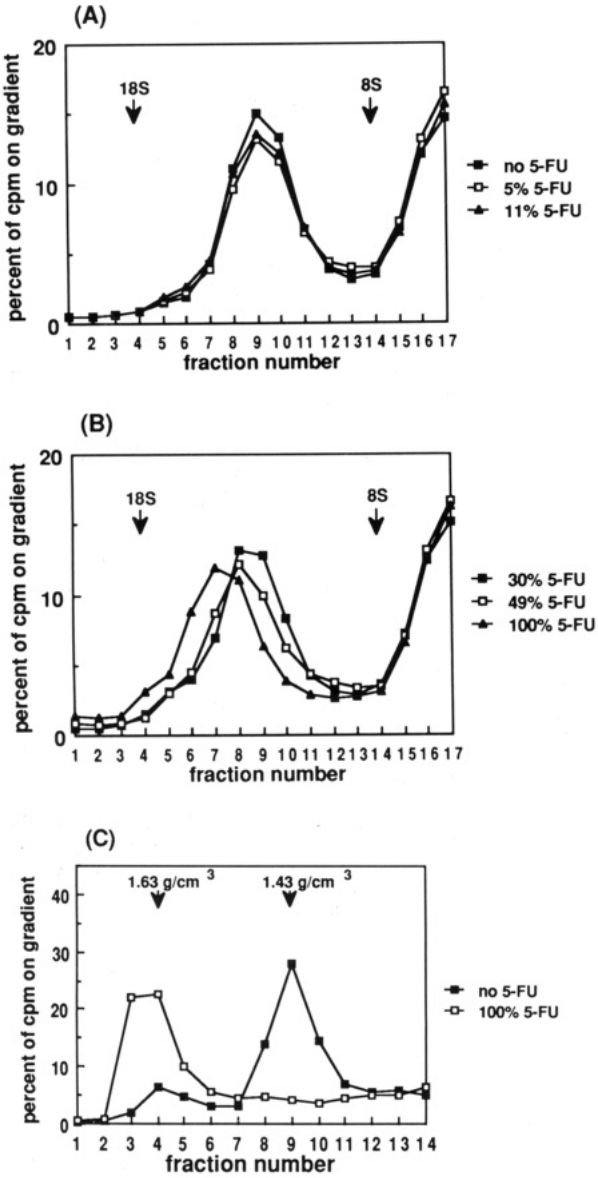

FIGURE 5: Velocity sedimentation of U2 snRNPs assembled *in vitro* on U2 RNAs with various amounts of 5-FU and buoyant densities of selected gradient-purified U2 RNPs. [^{32}P]CTP-labeled U2 RNA was synthesized with various amounts of 5-FUTP replacing UTP. The amount of 5-FU incorporated was determined as described under Experimental Procedures. For panels A and B, the RNAs were incubated in assembly/modification reactions containing S100 for 30 min, then nuclear extract was added, and the reactions were incubated for another 30 min. The samples were then layered on 10–30% glycerol gradients and centrifuged as described under Experimental Procedures. The levels of 5-FU incorporation in terms of the percent replacement of uridine for the RNAs used were for panel A (control U2 RNA) no 5-FU (■), 5% 5-FU U2 RNA (□), and 11% 5-FU U2 RNA (▲) and for panel B 30% 5-FU U2 RNA (■), 49% 5-FU U2 RNA (□), and 100% 5-FU U2 RNA (▲). Panel C: Portions of the peaks from the glycerol gradient for control U2 RNA, no 5-FU (■), and from the gradient for 100% 5-FU U2 RNA (□) were analyzed on cesium sulfate gradients as described under Experimental Procedures. The bottoms of the gradients are on the left, and reference densities are given at the top of the panel.

in the sedimentation rate was observed with as little as 30% 5-FU replacement and was most apparent with the total replacement of uridine with 5-FU (Figure 5B). Therefore, it appears that additional proteins bound to the 5-FU-containing RNAs.

A portion of the glycerol gradient peaks that result from control U2 RNA (no 5-FU) and U2 RNA with 100% 5-FU in place of uridine were analyzed on cesium sulfate gradients.

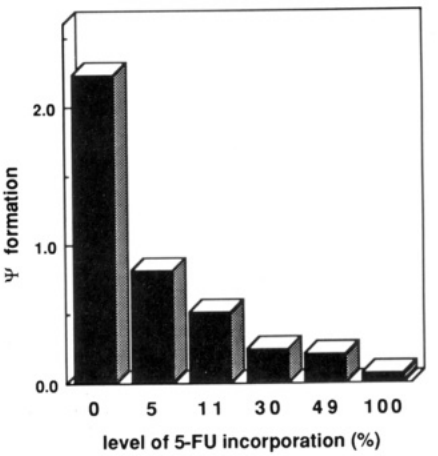

FIGURE 6: Effect of 5-FU incorporation on the formation of Ψ in U2 RNA *in vitro*. Samples of the same unmodified RNAs used in Figure 5 were incubated in assembly/modification reactions containing S100 extract for 2 h at 37 °C. The RNA was isolated from the reaction and gel-purified, and the level of 5-FU incorporation and the extent of Ψ modification were determined as described under Experimental Procedures. The Ψ formation (percent of total cpm in the Ψ spot) was calculated by the formula: Ψ formation = (cpm in Ψp spot)/(cpm in the Up, 5-FUp, and Ψp spots combined) × 100. The largest percent error in the raw counts was 11% on the Ψp spot from 100% 5-FU U2 RNA.

The results (Figure 5C) show that the U2 snRNP containing 5-FU is not resistant to salt dissociation. Nearly all of the counts from the 5-FU-containing U2 snRNP are at 1.6 g/cm^3, indicative of protein-free RNA, whereas the U2 snRNPs assembled on control U2 RNA are resistant to salt dissociation and have a buoyant density of ~1.4 g/cm^3 (Figure 5C). The 5-FU-containing U2 RNAs may have a larger contingent of proteins bound than control U2 RNA, but the interaction between these proteins and the RNA is easily dissociated with salt.

Effect of 5-FU Incorporation on RNA Modification. There have been several reports that formation of Ψ in tRNAs was inhibited when cells were treated with FPys (Tseng *et al.*, 1978; Frendewey & Kaiser, 1979; Frendewey *et al.*, 1982) and that the inhibition was disproportionate to the level of 5-FU incorporation. In other words, the inhibition of Ψ formation was not due to replacement of uridine with 5-FU which cannot form Ψ, but because of the inhibition of Ψ synthase activity in the cells (Tseng et al., 1978; Frendewey *et al.*, 1982).

The *in vitro* assembly/modification system employed in this report supports Ψ formation in exogenously added, unmodified U2 RNA (Kleinschmidt *et al.*, 1989; Patton, 1993) as well as other snRNAs (Patton *et al.*, 1987; Patton, 1991). In order to determine the effect of 5-FU incorporation on the formation of Ψ in snRNAs, the modification of *in vitro* transcribed (unmodified) control U2 and 5-FU-containing U2 RNAs was studied. The control U2 RNA and the U2 RNAs containing 5-FU (see Figures 4 and 5) were incubated in the assembly/modification reactions for 2 h. The RNA was isolated from the reactions and purified on a 10% polyacrylamide/8.3 M urea gel. These RNAs were analyzed for Ψ content and the level of 5-FU replacement, and the results are shown in Figure 6. Even at a low level of 5-FU incorporation (5% of the total uridine content), the formation of Ψ was severely inhibited (36% of control U2 RNA). When the level of 5-FU incorporation was 30% of the total uridine, the amount of Ψ formed was reduced to 11% of control U2 RNA. Of course, when uridine is completely replaced by 5-FU, the level of Ψ is reduced to background (Figure 6).

DISCUSSION

The results presented in this report show that incorporation of 5-fluorouridine (5-FU) into U2 RNA affects RNA–protein interactions and the stability of the ribonucleoprotein particle assembled *in vitro*. In addition, even a low level of 5-FU incorporation inhibits Ψ formation in the U2 RNA, suggesting that these 5-FU-containing RNAs are inhibiting the enzymatic activity(ies) that is (are) responsible for this modification.

The level of 5-FU incorporation required to see a change in the characteristics of the *in vitro* assembled U2 RNPs in these experiments was high relative to the typical incorporation seen with U2 snRNP isolated from 5-FU-treated HeLa cells (Sierakowska *et al.*, 1989). With an 8% replacement of uridine with 5-FU, Sierakowska *et al.* (1989) found there was a decreased level of U2 RNA and snRNP in nuclear extracts made from the 5-FU-treated cells. In addition, the *in vitro* splicing of β-globin pre-mRNA was inhibited. Changes in the structures and levels of other snRNPs were also seen when mouse S-180 cells were treated with FPys (Armstrong *et al.*, 1986). The analytical methods employed in the present investigation have elucidated some of the perturbations that result from 5-FU incorporation. These *in vitro* results suggest what could be happening to snRNP *in vivo* when cells are treated with 5-FU. Additional proteins may be bound to the U2 RNA, and the interaction of Sm proteins or snRNP-specific proteins with the RNA may be affected by the presence of 5-FU. Even if there were only subtle changes in the structures of these essential splicing cofactors, these changes may have profound effects on snRNP–pre-mRNA interactions or snRNP–snRNP associations, resulting in the inhibition of the splicing of pre-mRNA. The effect of 5-FU on DNA synthesis is undoubtedly the major anticancer action of this drug [see Weckbecker (1991)], but the RNA effects may contribute to the death of cells that are not undergoing DNA synthesis.

U2 RNA has 13 Ψ residues (Reddy, 1989), and the presence of 5-FU in the U2 RNA is a potent inhibitor of Ψ formation *in vitro*. When only 5% of the uridine is replaced by 5-FU, there is a 64% decrease in Ψ content in the *in vitro* modified U2 RNA. Therefore, even at the low levels of 5-FU incorporation that would be seen in snRNPs isolated from cells treated with 5-FU, there may be a significant reduction in the level of Ψ formed in the snRNAs. This has already been shown to be true for tRNAs isolated from cells treated with FPys (Tseng *et al.*, 1978; Frendewey & Kaiser, 1979). The function of Ψ in snRNAs is still unknown, but it is possible that Ψ-deficient snRNAs will not function properly in splicing. The absence of Ψ might affect the interaction of snRNAs with the pre-mRNA, other snRNAs, or proteins.

What causes the increase in the sedimentation velocity of U2 snRNP assembled on 5-FU-containing U2 RNA? Most likely, additional proteins bind to the 5-FU U2 RNA, and a likely candidate is Ψ synthase(s). Samuelsson (1991) found that 5-FU tRNAs bound tightly to purified tRNA Ψ synthases. Since the interaction between the U2 RNA substrate and Ψ synthase is usually transient, these proteins would not be expected to be an integral part of the U2 snRNP. However, the presence of 5-FU alters the interaction between the synthase and the unmodified RNA, and the result is a more stable complex (Samuelsson, 1991). This could allow for the long-term interaction of 5-FU U2 RNA with the Ψ synthase and might result in an increase in sedimentation velocity. The formation of Ψ in U2 RNA appears to be catalyzed by several different Ψ synthases (J. Patton, M. Jacobson, and T. Pederson, unpublished results), which together might amount to a significant increase in sedimentation velocity. However, the interaction between Ψ synthases and 5-FU U2 RNA was dissociated by the high salt of the cesium sulfate gradients.

Why have the 5-FU-containing U2 snRNPs lost salt-stability? The answer may lie in the composition of the Sm binding site in snRNAs. This site has the sequence AUU-UUUG in U2 RNA, and the binding of Sm proteins to this site might be particularly sensitive to the replacement of uridine with 5-FU. However, given the results presented here, more than half of the uridines have to be replaced with 5-FU before there is a significant effect on the interaction of these proteins with the binding site.

Future studies concerning the effect of 5-FU incorporation on the splicing of pre-mRNA will focus on whether the functions of snRNPs are compromised. An U2 snRNP containing 5-FU with an altered structure and devoid of modifications might no longer function efficiently in splicing. Verification of that prediction will be one focus of future endeavors.

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