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Incorporation of 5-fluorouracil into U2 snRNA blocks pseudouridylation and pre-mRNA splicing *in vivo*

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ABSTRACT

5-fluorouracil (5FU) is an effective anti-cancer drug, yet its mechanism of action remains unclear. Here, we examine the effect of 5FU on pre-mRNA splicing *in vivo*. Using RT–PCR, we show that the splicing of a number of pre-mRNAs is inhibited in HeLa cells that have been exposed to a low dose of 5FU. It appears that this inhibitory effect is not due to its incorporation into pre-mRNA, because partially or fully 5FU-substituted pre-mRNA, when injected into *Xenopus* oocytes, is spliced just as well as is the unsubstituted pre-mRNA. Detailed analyses of 5FU-treated cells indicate that 5FU is incorporated into U2 snRNA at important naturally occurring pseudouridylation sites. Remarkably, 5FU incorporation effectively blocks the formation of important pseudouridines in U2 snRNA, as only a trace of pseudouridine is detected when cells are exposed to a low dose of 5FU for 5 days. Injection of the hypo-pseudouridylated HeLa U2 snRNA into U2-depleted *Xenopus* oocytes fails to reconstitute pre-mRNA splicing, whereas control U2 isolated from untreated or uracil-treated HeLa cells completely reconstitutes the splicing. Our results demonstrate for the first time that 5FU incorporates into a spliceosomal snRNA at natural pseudouridylation sites *in vivo*, thereby inhibiting snRNA pseudouridylation and splicing. This mechanism may contribute substantially to 5FU-mediated cell death.

INTRODUCTION

5-fluorouracil (5FU), as a potent anti-cancer drug, has been widely used since its discovery in the 1950s (1). However, despite decades of intense study, the mechanism of action of this drug remains unclear (2–4). It has been hypothesized, and in some cases demonstrated, that 5FU directly affects DNA metabolism (2,4). For instance, it has been shown that 5FU, when converted into 5-fluorodeoxyuridine monophosphate (5FdUMP) in the cell, can tightly bind to thymidylate synthase, an enzyme required for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), thereby inhibiting the synthesis of dTMP and its downstream product dTTP and consequently affecting DNA synthesis. As a consequence of thymidylate synthase inactivation, dUMP accumulates, resulting in elevated synthesis of its downstream product dUTP. The incorporation of dUTP into DNA subsequently leads to DNA damage. Puzzlingly, however, when 5FU-exposed cells are treated with thymidine, which can be converted to dTMP through the action of thymidine kinase (a pathway that is independent of the thymidylate synthase pathway), 5FU-mediated cytotoxic and apoptotic effects remain, suggesting that DNA metabolism may not be a primary target of 5FU (5–8).

Given that 5FU can be readily converted into 5-fluorouridine triphosphate (5FUTP), a ribonucleotide analog that can be incorporated into RNAs, it has been proposed that 5FU may directly affect RNA metabolism (2–7). In this regard, past research has focused largely on rRNA synthesis (9–17). When included in culture media, 5FU is readily incorporated into the long single rRNA that is a precursor to 18S, 5.8S and 28S rRNAs, thus posing the question of whether 5FU incorporation directly affects pre-rRNA processing. However, experimental results accumulated thus far are inconclusive. For instance, some experiments suggested that the incorporation of 5FU into pre-rRNA might inhibit rRNA processing at early steps that lead to the production of mature 18S rRNA (13,18–20). In contrast, Jacob and colleagues reported that 5FU-incorporated pre-rRNA, when assayed in a cell-free system, was efficiently and accurately processed at a primary cleavage site (no inhibition was detected), although extracts prepared from 5FU-treated cells exhibited a defect in rRNA processing at this site (3,10). Recently, the Butler group observed that yeast strains heterozygous for deletions of genes required for pre-rRNA processing are more sensitive to 5FU compared with all other heterozygous strains (17). They further detected an accumulation of polyadenylated rRNAs in yeast strains that are defective in the nuclear exosome component Rrp6p, an exonuclease involved in rRNA processing and nuclear RNA degradation, thus demonstrating and verifying the inhibitory

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effect of 5FU on pre-rRNA processing (17). It remains unclear, however, whether the effect is a direct consequence of 5FU incorporation into pre-rRNA.

The effect of 5FU on pre-mRNA splicing has also been studied (2,4). However, the work presented over the years has been largely fragmentary and inconclusive. Only a limited number of genes have been analyzed in 5FU-treated cells. Among them, DHFR (dihydrofolate reductase) is perhaps the most extensively studied. The levels of DHFR pre-mRNA and mature mRNA increase in cultured cells upon 5FU treatment (14,15). Although the increase in mRNA is open to interpretation, the fact that the level of DHFR pre-mRNA is elevated in 5FU-treated cells suggests that 5FU may indeed inhibit the splicing of DHFR pre-mRNA (21–23). To address whether 5FU incorporation into pre-mRNA directly affects the splicing, *in vitro* transcription with various ratios of 5FUTP:UTP was used to generate 5FU-incorporated pre-mRNA, which was then subjected to an *in vitro* splicing assay. However, the results are inconsistent and somewhat contradictory. For instance, using 5FU-incorporated human β-globin pre-mRNA and HeLa nuclear extracts, Doong and Dolnick detected aberrantly spliced products at relatively high pH (7.7 or 8.4) (24). In contrast, using the same splicing system, the Kole group did not detect abnormally spliced products, even when the β-globin pre-mRNA was fully substituted with 5FU (25). This discrepancy remains to be clarified.

Given that the five spliceosomal snRNPs (U1, U2, U4, U5 and U6) participate in pre-mRNA splicing, it is also possible that incorporation of 5FU into spliceosomal snRNAs affects pre-mRNA splicing. Previous work from several groups suggested that 5FU might indeed have inhibitory effect on snRNP biogenesis and function (25–27). However, it remains unclear as to how 5FU affects the assembly/function of snRNPs and whether 5FU is indeed incorporated into snRNAs *in vivo*.

Recently, attention has been drawn to the function of spliceosomal snRNA modifications. All spliceosomal snRNAs contain extensive posttranscriptional modifications, including 2′-*O*-methylation and pseudouridylation (28,29). U2 contains the most modifications. In fact, >10% of the nucleotides in vertebrate U2 snRNA are modified (Figure 1). In particular, all six uridines in or near the U2 branch site recognition region are converted to pseudouridines after initial transcription. Using the *Xenopus* oocyte reconstitution system, we have demonstrated that modified nucleotides, including the pseudouridines in the 5′ end region and the branch site recognition region, are required for U2 function in splicing (30,31). The importance of the pseudouridines in the 5′ end region was recently confirmed in a HeLa splicing reconstitution system (32). Importantly, we also demonstrated that 5FU-containing U2 snRNA is not only non-functional on its own when injected into *Xenopus* oocytes but is also a potent inhibitor that site-specifically blocks the pseudouridylation of an *in vitro* transcribed U2 snRNA injected at a later time (30,31). Thus, we reasoned that 5FU, when incubated with cells, might incorporate into U2 snRNA (and perhaps the other spliceosomal snRNAs) at naturally occurring pseudouridylation sites. The 5FU-incorporated U2 snRNA might in turn block pseudouridylation of newly synthesized U2 snRNA in a site-specific manner (30,31,33), thus inhibiting pre-mRNA splicing.

In the current work, we test the effect of 5FU on U2 snRNA function in HeLa cells. We show that 5FU, even when supplied at low concentrations to the medium, is in fact fairly efficiently incorporated into HeLa U2 snRNA in the branch site recognition region, thereby blocking ∼90% of pseudouridylation at many important pseudouridylation sites in this region. Further functional analysis indicates that hypopseudouridylated U2 snRNA cannot support pre-mRNA splicing in *Xenopus* oocytes. Our results identify a new mechanism by which 5FU affects pre-mRNA splicing.

MATERIALS AND METHODS

Cell culture and total RNA isolation

HeLa cells (ATCC) were cultured at 37°C in Eagle's Minimum Essential Media (EMEM) (Cambrex) supplemented with 15% fetal bovine serum (FBS; Sigma). For uracil or

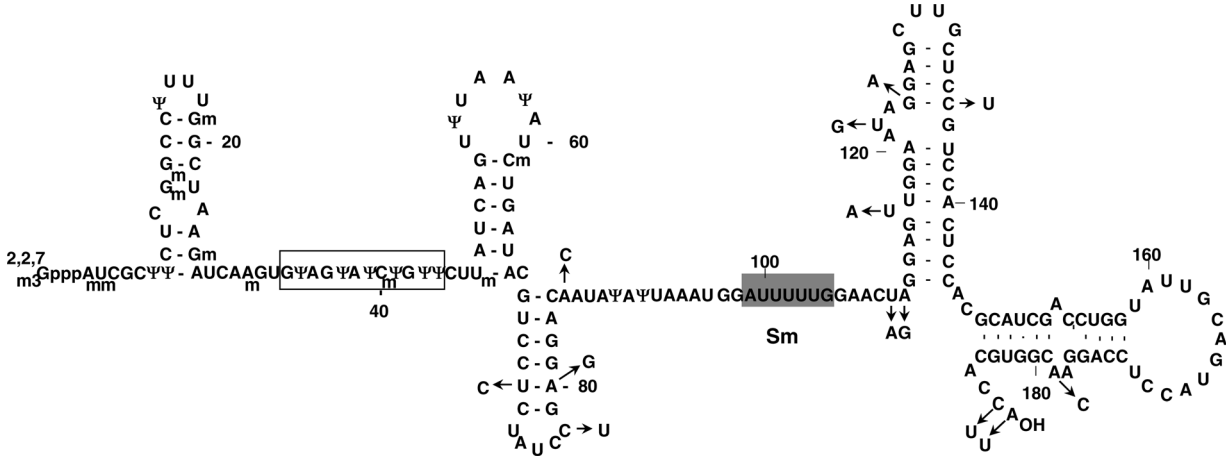

Figure 1. Primary and secondary structure of human and *Xenopus* U2 snRNA. Pseudouridines (Ψ) and 2′-*O*-methyl groups (m) are indicated. The gray box depicts the Sm binding site, and the open box highlights the branch site recognition sequence and its 3′-adjacent sequence (collectively referred to as the branch site recognition region). Arrows indicate the nucleotide differences in *Xenopus* U2 snRNA.

5FU treatment, a final concentration of 10 μM (or 1 mM, as indicated in the section Results) of uracil or 5FU was added freshly to the medium just before culturing. At the end of incubation (1, 3 or 5 days), cells were collected, and total RNA was isolated using TRIzol (Invitrogen), according to manufacturer's protocol. Purified total RNA was treated with ~5 U of RQ1 RNase-free DNase I (Promega) at 37°C for 1 h, extracted with PCA [Tris–HCl–buffered (pH 7.5) phenol:chloroform:isoamyl alcohol (50:49:1)] and then precipitated with ethanol.

RT–PCR

Purified HeLa total RNA (DNA-free) was used for RT–PCR to determine the levels of mRNA and pre-mRNA. Reverse transcription was carried out with a random DNA primer (9mer) and M-MLV reverse transcriptase (Invitrogen), according to manufacturer's instruction. The cDNA was then used for PCR reactions in the presence of pairs of forward and reverse primers, [α-^{32}P]dATP (PerkinElmer) and *Taq* polymerase (Promega). The PCR products were resolved on denaturing gels and quantified by a PhosphorImager (Molecular Dynamics). For nucleolin (GenBank accession no. M60858), the 5′-forward primer specific for spliced mRNA corresponded to nt 3901–3916 and nt 4587–4596 at the boundary of exons 3 and 4, respectively; the 5′-forward primer specific for unspliced pre-mRNA corresponded to nt 4562–4582 located in intron 3; the 3′-reverse primer was complementary to nt 4662–4680 located in exon 4. For GAPDH (GenBank accession no. J04038), the 5′-mRNA-specific primer corresponded to nt 3996–4012 and nt 4206–4215 at the boundary of exons 7 and 8, respectively; the 5′-pre-mRNA-specific primer corresponded to nt 4179–4204 located in intron 7; and the 3′-primer was complementary to nt 4391–4414 in exon 8. For β-globin (GenBank accession no. NG000007), the 5′-mRNA-specific primer corresponded to nt 71 025–71 039 and nt 71 890–71 899 at the boundary of exons 2 and 3, respectively; the 3′-pre-mRNA-specific primer corresponded to nt 71 851–71 870 in intron 2; and the 3′-primer was complementary to nt 71 994–72 017 in exon 3. For 28S rRNA (as an internal control), the 5′-primer corresponded to nt 1605–1623 and the 3′-primer was complementary to nt 1882–1903 (Genbank accession no. M11167).

Pseudouridylation quantification

Assays for pseudouridylation and 5FU incorporation were performed as described previously (34). First, HeLa U2 snRNA was isolated from the purified total RNA (see above), using oligonucleotide affinity chromatography. HeLa total RNA (~400 μg) was mixed with 100 pmol of a biotinylated antisense U2 2′-*O*-methyl oligonucleotide complementary to nt 158–177 of U2 (UmCmCmUmGmGmAmGmGmUmAmCmUmGmCmAmAmUmAmCmBBB, where B stands for biotin-TEG) in 50 μl NET-2-$MgCl_2$ buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) NP-40 and 2 mM-$MgCl_2$. After denaturing, the sample was mixed with 20 μl streptavidin beads (Pierce). Following a 1-h rotation (room temperature), the beads were precipitated and washed with NET-2-$MgCl_2$. U2 was then eluted with 250 μl of dissociation buffer (10 mM Tris–HCl, pH 7.5, 0.1% SDS and 0.5 mM EDTA) at 80°C for 15 min, PCA extracted and ethanol precipitated.

Purified U2 (~10–70 ng) was then subjected to RNase H digestion in the presence of 5 pmol of appropriate 2′-*O*-methyl RNA–DNA chimera (AmCmAmdCdTdTdGAmUmCmUmUmAmGmCmCmAmAm for position 32, UmAmdCdAdCdTUmGmAmUmCmUmUmAmGmCmCm for position 34, AmUmAmdCdTdAdCAmCmUmUmGmAmUmCmUmUmAm for position 37, AmGmAmdTdAdCdTAmCmAmCmUmUmGmAmUmCmUm for position 39 and GmAmAmdCdAdGdAUmAmCmUmAmCmAmCmUmUmGm for position 43) (34). The cleaved 3′-half of U2 was gel-purified, 5′-dephosporylated with calf intestinal phosphatase (Roche), and subsequently rephosphorylated with T4 polynucleotide kinase (Amersham) and [γ-^{32}P]ATP (34).

The 5′-radiolabeled U2 fragment was then treated with nuclease P1 to completion, dotted on a cellulose TLC PEI membrane (EM science), and chromatographed in developing buffer containing isobutyric acid/ammonium/water (50:1:29, v/v/v) [or isopropanol/HCl/water (70:15:15, v/v/v), as indicated in Results]. The percentage of 5-fluoro-uridylate or pseudouridylate was quantified using a PhosphorImager.

Microinjection

Microinjection was carried out essentially as described (30). Antisense U2 DNA oligonucleotide (50 nl of 3 mg/ml, complementary to nt 23–46 of human U2) was injected into *Xenopus* oocytes to knock out the endogenous U2 RNA. After an overnight incubation at 18°C, 36.8 nl of 50 ng/μl U2 RNA, which had been purified from HeLa cells (see above), were injected into the U2-depleted oocytes. Following an overnight incubation, 18 nl of 0.5×10^6 c.p.m./μl adenovirus pre-mRNA (*in vitro* transcribed with [α-^{32}P]GTP) was injected into the nuclei. The nuclei were recovered 10 min later, and RNA was isolated. The total nuclear RNA was then resolved on a 5.5% polyacrylamide-8 M urea gel. Pre-mRNA splicing was visualized by autoradiography.

To verify whether the 5FU-incorporated adenovirus pre-mRNA could be spliced, the pre-mRNA (transcribed *in vitro* in the presence of 5FUTP and [α-^{32}P]GTP) was directly injected into intact *Xenopus* oocytes. Splicing was analyzed by denaturing gel electrophoresis and autoradiography (see above).

RESULTS

Pre-mRNA splicing is inhibited in HeLa cells exposed to 5FU

To assess whether 5FU has a general effect on pre-mRNA splicing, we carried out a quick survey of several randomly chosen genes using RT–PCR. HeLa cells were exposed to either 5FU or uracil (as a control) or were not treated (incubated with regular medium) for various amounts of time. As shown in Figure 2, a low concentration (10 μM) of 5FU, but not uracil (even when its concentration was higher), was sufficient to induce cell death. After a 5-day exposure to 5FU, a large fraction of cells died or became sick (Figure 2).

To test whether pre-mRNA splicing was affected in these cells, we collected cells at several time points and isolated total RNA for RT–PCR analysis, which allowed us to

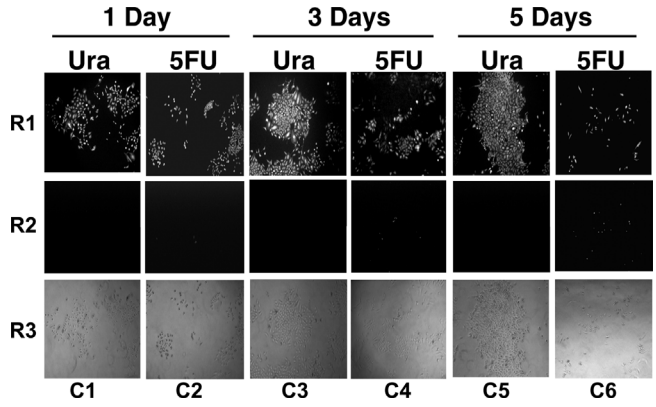

Figure 2. Images of HeLa cells treated with either uracil or 5FU. HeLa cells were cultured in medium containing either 10 μM uracil (C1, C3 and C5) or 10 μM 5FU (C2, C4 and C6) for 1 day (C1 and C2), 3 days (C3 and C4) or 5 days (C5 and C6). After incubation, the cells were stained with fluorescein diacetate for live cells (R1) or propidium iodide dye for damaged/dead cells (R2). Row 3 (R3) shows the phase images.

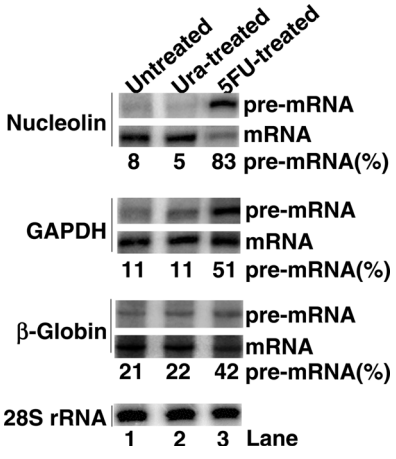

Figure 3. The levels of pre-mRNA and mRNA in uracil- or 5FU-treated cells. After a 5-day incubation in the appropriate medium, HeLa cells were harvested, and pre-mRNA and mRNA levels were assessed, in the same reaction, by RT–PCR (see Materials and Methods). Total RNA used for RT–PCR was isolated either from control HeLa cells (regular medium, lane 1), 10 μM uracil-treated HeLa cells (lane 2), or from 10 μM 5FU–treated HeLa cells (lane 3). The pre-mRNA/mRNA levels of nucleolin, GAPDH and β-globin are shown; 28S rRNA served as the loading control—under the conditions used, i.e. low-dose 5FU, rRNA levels were not affected (or no difference was detected) (43). The percentage of pre-mRNA [pre-mRNA/(pre-mRNA + mRNA)] for each of the three genes is shown at the bottom of each panel. The RT–PCR experiments were repeated twice, and the results were almost identical.

calculate the ratio of levels of corresponding pre-mRNAs and mRNAs. For many genes tested, we found a relatively higher ratio of pre-mRNA:mRNA in 5FU-treated cells than in the control cells, suggesting that the splicing of these pre-mRNAs was inhibited. Figure 3 shows three typical examples of such analyses. Here, cells were harvested after incubation for 5 days. Whereas there was essentially no change in the GAPDH ratio when cells were exposed to uracil (panel GAPDH, lane 2) compared with untreated cells (panel GAPDH, lane 1), a significant increase in the GAPDH pre-mRNA level was observed in cells exposed to 5FU (panel GAPDH, compare lane 3 with lane 1). In fact, the percentage

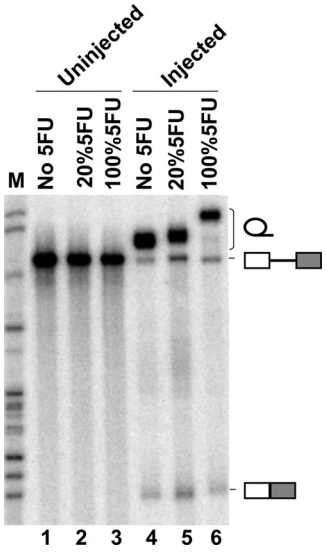

Figure 4. Splicing of pre-mRNA containing 5FU. Uniformly ^{32}P-radiolabeled standard adenovirus pre-mRNAs containing different levels of 5FU were separately injected into *Xenopus* oocytes, and their splicing activity was monitored by electrophoresis on denaturing gels. The percentage of 5FU in the pre-mRNA (relative to uridine) is shown above each lane. The migration positions of pre-mRNA and the spliced products (lariat intron at the top, the mature spliced mRNA at the bottom) are indicated schematically to the right.

of GAPDH pre-mRNA [pre-mRNA/(mRNA+pre-mRNA)] was >4-fold higher in 5FU-treated cells (lane 3) than in uracil-treated cells (lane 2) or untreated cells (lane 1). An even more significant change in the nucleolin ratio was observed when cells were exposed to 5FU; the pre-mRNA level was greatly increased, whereas the mRNA level was dramatically decreased (Figure 3, panel nucleolin, lane 3). In contrast, the ratio remained essentially unchanged when cells were incubated with unsupplemented medium or uracil-containing medium (compare lane 1 with lane 2). The percentage of nucleolin pre-mRNA was ~11-fold higher in cells exposed to 5FU (lane 3) compared with cells treated with uracil (lane 2) or untreated cells (lane 1). Although a rather small difference in the ratio was detected when β-globin was tested, there was still a ~2-fold increase in the percentage of β-globin pre-mRNA when cells treated with 5FU (panel β-globin, lane 3) were compared with the control cells (lanes 1 and 2). These results suggest that 5FU may indeed have a global effect on pre-mRNA splicing.

Incorporation of 5FU into pre-mRNA does not affect splicing in *Xenopus* oocytes

It is possible that 5FU, when converted into 5FUTP, (a ribonucleotide analog) in cells, incorporates into pre-mRNA, thereby affecting splicing. To test this hypothesis, we incorporated 5FU into adenovirus standard pre-mRNA splicing substrate via *in vitro* transcription and then injected the pre-mRNA into *Xenopus* oocytes to assess whether it could be spliced *in vivo*.

As shown in Figure 4, pre-mRNA containing ~20% 5-fluorouridine (derived from T7 *in vitro* transcription using a mixture of 5FUTP and UTP, 20:80) was spliced as efficiently as the unsubstituted pre-mRNA (compare lane 5 with lane 4). Remarkably, splicing was not affected even when the

uridine was fully substituted with 5-fluorouridine (100% 5FUTP); the levels of the lariat intron and the spliced mRNA were comparable to those in the control—in which unmodified pre-mRNA was injected (lane 6). To verify that the splicing was accurate, we excised the spliced mRNA and the lariat intron products and carried out RT–PCR sequencing and primer-extension analyses. The results indicated accurate usage of the branch site, the 5′-splice site and the 3′-splice site (data not shown). Thus, the observed slow migration of the spliced lariat intermediate (lanes 5 and 6) is likely due to the difference in molecular weight (i.e. the extra fluorine increases the molecular weight). To verify that the lack of an effect was not specific/unique to the adenovirus splicing substrate, we also tested the splicing of human β-globin pre-mRNA. Likewise, our results indicated that 5-fluorouridine substitutions did not affect the efficiency and accuracy of β-globin pre-mRNA splicing in *Xenopus* oocytes (data not shown).

From these results, we conclude that 5FU incorporation into pre-mRNA (at least the adenovirus and human β-globin splicing substrates) does not influence the splicing of the pre-mRNA.

5FU incorporates into U2 snRNA at natural pseudouridylation sites *in vivo*

Given that 5FU causes a defect in pre-mRNA splicing in cells, we reasoned that 5FU, after conversion into 5FUTP in the cells, could be incorporated into not only pre-mRNA/mRNA but also into other types of RNA, including spliceosomal snRNAs, possibly at naturally occurring pseudouridylation sites (such as those in the U2 branch recognition region). According to our previous work, U2 snRNA containing 5FU at naturally occurring pseudouridylation sites in the branch site recognition region was by itself not functional in pre-mRNA splicing; additionally, this 5FU-containing U2 snRNA was able to inhibit pseudouridine formation in newly synthesized U2 in a site-specific manner, thereby blocking pre-mRNA splicing (30,31).

To analyze 5FU incorporation and its site-specific inhibitory effect on pseudouridylation of newly synthesized U2 in HeLa cells, we used our previously developed method to examine naturally occurring pseudouridylation sites in the U2 branch site recognition region (34). HeLa cells were exposed to 5FU or uracil for 5 days, and endogenous U2 snRNA was isolated via affinity selection using a biotinylated antisense U2 oligonucleotide and was subsequently analyzed for 5FU incorporation and pseudouridylation through site-specific labeling followed by P1 nuclease digestion and TLC.

We tested all six natural pseudouridine sites (positions 34, 37, 39, 41, 43 and 44) along with one natural uridine site (U32) in the branch site recognition region. Figure 5 shows the TLC results of five of these sites (32, 34, 37, 39 and 43) using a standard cellulose membrane and the developing solution isobutyric acid:ammonium:water (50:1:29, v/v/v); this system can separate uridylate, 5F-uridylate and pseudouridylate. Whereas 5FU incorporation at positions 32 and 34 required a relatively high concentration of 5FU (1 mM) in the medium (panel Positions 32 and 34), all the other natural pseudouridine sites were fairly accessible to

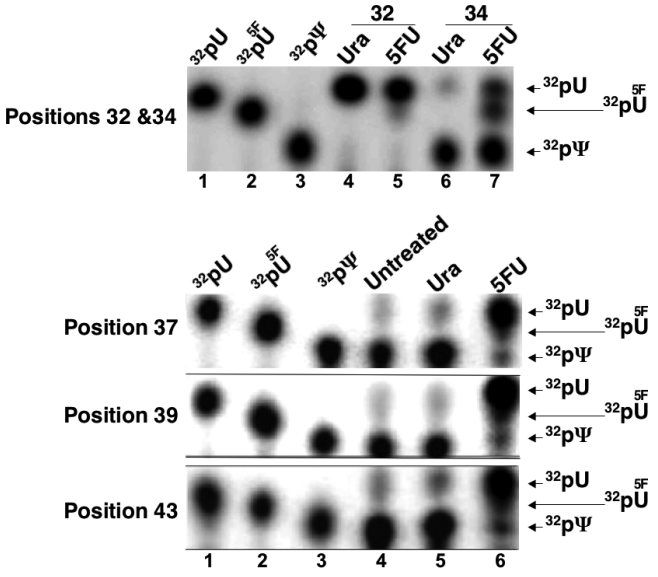

Figure 5. Incorporation of 5FU into U2 snRNA at natural pseudouridylation sites *in vivo*. U2 snRNA was isolated from HeLa cells that had been treated with uracil or 5FU (or untreated) for 5 days. 5FU incorporation and pseudouridylation at natural uridine/pseudouridine sites were subsequently assayed (see Materials and Methods). Shown are the final TLC (thin layer chromatography) analyses of the nucleotides at several such sites (positions 32, 34, 37, 39 and 43). The developing solution for TLC was isobutyric acid:ammonium:water (50:1:29, v/v/v). All but position 32 (a natural uridine site) are natural pseudouridine sites. In the top panel (panel Positions 32 and 34), cells were treated with 1 mM uracil (lanes 4 and 6) or 1 mM 5FU (lanes 5 and 7) and positions 32 (lanes 4 and 5) and 34 (lanes 6 and 7) were analyzed. In the other panels (panels Positions 37, 39 and 43), cells were treated with 10 μM uracil (lanes 5) or 10 μM 5FU (lane 6), or were not treated (lane 4). Lanes 1, 2 and 3 are controls in which the ^{32}P-labeled nucleoside 5′-monophosphate, ^{32}pU, ^{32}p^{5F}U or ^{32}pΨ, respectively, was analyzed. The migration positions of pU, p^{5F}U and pΨ are indicated on the right.

5FU even at the low 5FU concentration (10 μM), although the overall level of 5FU incorporation appeared to be comparatively low (see panel Positions 37, 39 and 43). Remarkably, incorporation of 5FU, although at a low level, effectively blocked the pseudouridylation of newly synthesized U2 at all but one (position 34) naturally occurring pseudouridylation site in the branch site recognition region (positions 37, 39 and 41, 43 and 44); a high level of uridine and a trace of pseudouridine were detected at the respective sites (see panel Positions 37, 39 and 43, and data not shown).

5FU incorporation significantly inhibits U2 pseudouridylation at important sites

To assess, in detail, the inhibitory effect of 5FU incorporation on U2 pseudouridylation in the branch site recognition region, we further quantified the level of pseudouridines at each of the six naturally occurring pseudouridylation sites. Because the TLC system used in Figure 5 developed extremely slowly and sometimes yielded distorted migration patterns, we switched to a cellulose PEI membrane and changed the developing solution to HCl:water:isopropanol (15:15:70, v/v/v). Under these conditions, pseudouridylate (pΨ) can be well separated from uridylate (pU) and 5F-uridylate (p5FU), although uridylate and 5F-uridylate

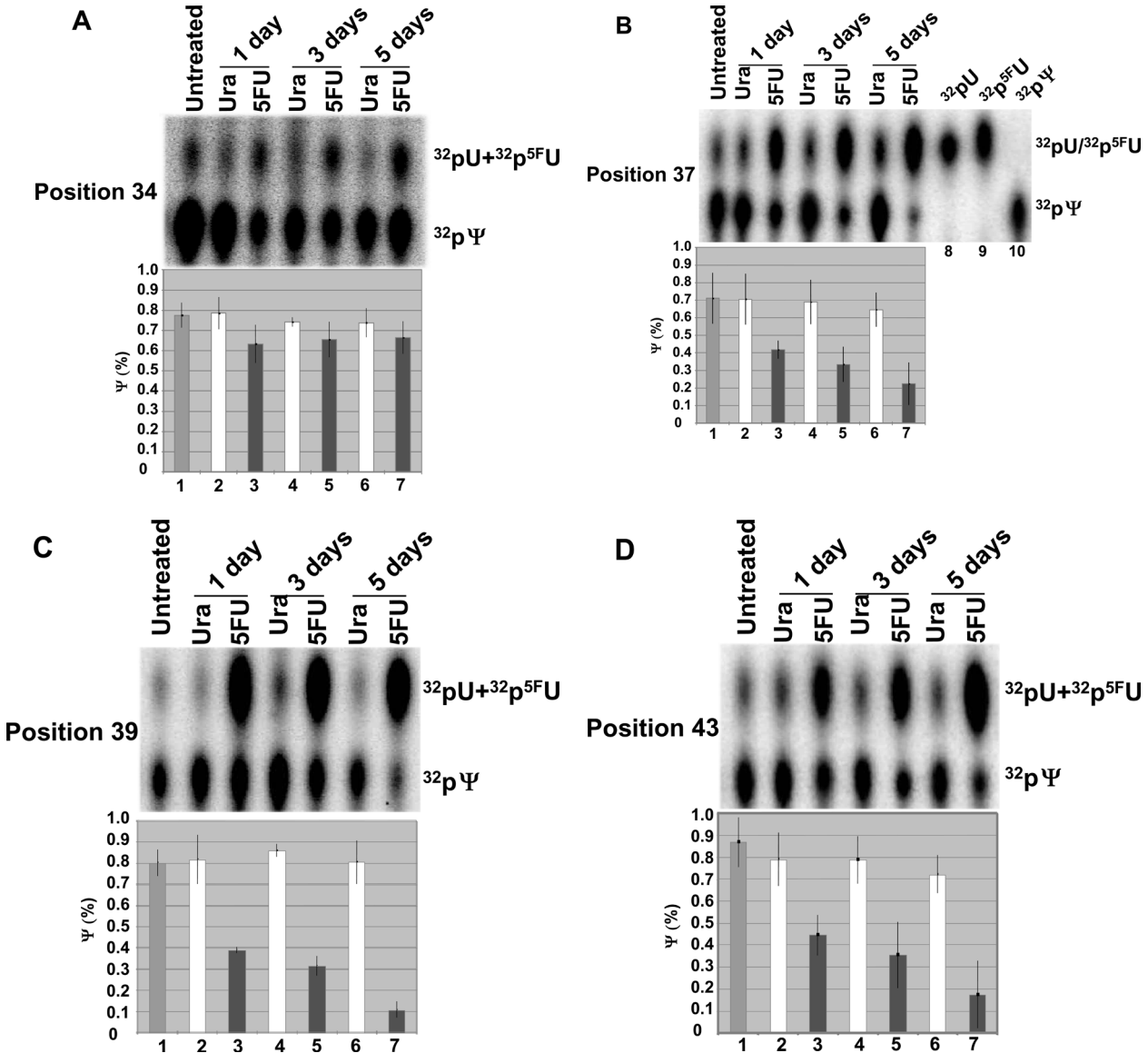

Figure 6. Quantification of pseudouridylation of U2 snRNA at natural pseudouridylation sites. Shown are measurements of pseudouridylation at four natural pseudouridylation sites, positions 34 (panel **A**), 37 (panel **B**), 39 (panel **C**) and 43 (panel **D**). Experiments were carried out exactly as in Figure 5, except that the developing solution for TLC was HCl:water:isopropanol (15:15:70, v/v/v), and that cells were analyzed at three different time points of culturing, i.e. 1 day (lanes 2 and 3), 3 days (lanes 4 and 5) and 5 days (lanes 6 and 7). Lane 1 is a control in which U2 snRNA was isolated from cells cultured in regular medium for 3 h (untreated). In lanes 2, 4 and 6, U2 snRNA isolated from uracil-treated cells was analyzed. In lanes 3, 5 and 7, U2 snRNA isolated from 5FU-treated cells was analyzed. Lanes 8, 9 and 10 in panel B are controls in which the ^{32}P-labeled nucleoside 5′-mono-phosphate, ^{32}pU, ^{32}p5FU or ^{32}pΨ, respectively, was analyzed. The positions of pU, p^{5F}U and pΨ are indicated to the right. Pseudouridylation at every position was repeated at least twice, and was quantified, as shown at the bottom of each panel. The percentage of pseudouridylation was calculated using the formula: pΨ/(pU + p^{35}U + pΨ). The white bars, black bars and grey bars depict the percentage of U2 pseudouridylation in uracil-treated cells, 5FU-treated cells and untreated cells, respectively. The standard deviation (SD) is also indicated.

migrate to the same position (Figure 6, panel Position 37, lanes 8–10). We note that comigration of uridylate and 5F-uridylate does not affect quantification of pseudouridylation. As shown in Figure 6, whereas the level of pseudouridylation at natural modification sites was extremely high in U2 snRNA isolated from uracil-treated cells or untreated cells, pseudouridylation was blocked in U2 snRNA isolated from 5FU-treated cells in a manner dependent on exposure time. For instance, when cells were exposed to 5FU for 1 day, relatively less inhibition of pseudouridylation was observed in U2 at all positions tested. When the exposure time was increased to 3 or 5 days, greater inhibition, ∼70 or 90%, was detected, respectively. Interestingly, the only exception was position 34, the inhibition of which required a significantly higher dose of 5FU. This probably reflects the fact that there are two redundant pseudouridylase activities, U92 sno/scaRNP and the human homolog of yeast Pus7p, both of which can specifically convert U34 to Ψ34 (35–37). To block the two pseudouridylase activities, more 5FU (or longer exposure) might be required.

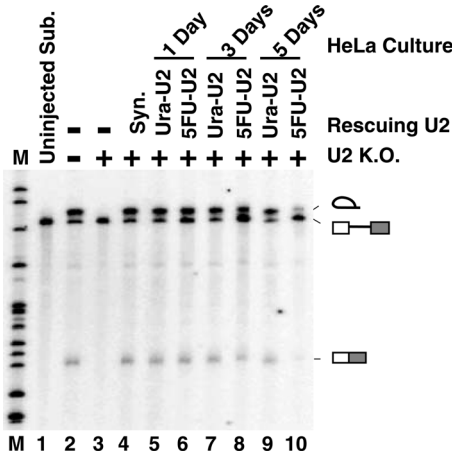

Figure 7. Functional reconstitution of splicing in *Xenopus* oocytes. U2 snRNA isolated from HeLa cells was injected into U2-depleted *Xenopus* oocytes. After a 16-h reconstitution, the oocytes were then injected with ^{32}P-uniformly radiolabeled adenovirus pre-mRNA. The total nuclear RNA was isolated, 10 min after pre-mRNA injection, and assayed for splicing on a denaturing gel. Reconstitution was carried out with U2 snRNA isolated from cells that had been pre-treated with uracil (lanes 5, 7 and 9) or 5FU (lane 6, 8 and 10) for 1 day (lanes 5 and 6), 3 days (lane 7 and 8) or 5 days (lanes 9 and 10). Lanes 3 and 4 are controls where U2-depleted oocytes were reconstituted for 16 h with no RNA or with the *in vitro* transcribed U2 snRNA, respectively. In lane 2, ^{32}P-radiolabeled pre-mRNA was directly injected into U2 mock–depleted oocytes, and no exogenous U2 was supplemented. Lane 1 represents the uninjected adenovirus pre-mRNA. Lane M is a size marker of MspI-digested pBR322 DNA. The positions of unspliced pre-mRNA and spliced products—the lariat intron (top band) and mature mRNA (lower band)—are indicated schematically.

U2 snRNA isolated from 5FU-treated HeLa cells is defective in reconstituting pre-mRNA splicing in *Xenopus* oocytes

Our previous work showed that U2 snRNA lacking pseudouridines in the branch site recognition region could not function in pre-mRNA splicing (30). Thus, we tested whether U2 snRNA isolated from 5FU-treated HeLa cells could support pre-mRNA splicing. Here, since *Xenopus* U2 and human U2 differ in only a few nucleotides in the 3′-half of the molecule (Figure 1) and they are functionally interchangeable (31), we directly injected U2 snRNA (isolated from HeLa cell) into U2-depleted *Xenopus* oocytes and subsequently assessed its ability to reconstitute pre-mRNA splicing. U2 snRNA isolated from 5FU-cultured cells functioned poorly (Figure 7, lanes 6 and 8, 1 and 3 days of 5FU-exposure, respectively) or failed almost completely (Figure 7, lane 10, 5 days of 5FU-exposure) in restoring pre-mRNA splicing. In contrast, U2 snRNA isolated from control cells (exposed to uracil or untreated) fully rescued splicing (Figure 7, lanes 5, 7 and 9). In summary, our results are consistent with the observation that pre-mRNA splicing is globally inhibited in HeLa cells exposed to 5FU, further demonstrating the importance of the pseudouridines in the branch site recognition region of U2 (and perhaps in other regions of other RNAs as well).

DISCUSSION

Using a quantitative method, we have shown that 5FU, when included in HeLa cell cultures, is incorporated to a significant extent into U2 snRNA at naturally occurring pseudouridylation sites. Remarkably, 5FU incorporation almost completely inhibited the formation of pseudouridines at sites that normally undergo this modification (Figures 5 and 6). More importantly, U2 snRNA isolated from 5FU-treated cells failed to reconstitute pre-mRNA splicing in *Xenopus* oocytes, whereas U2 snRNA extracted from control cells was fully functional in this regard. These results are consistent with the finding that 5FU exhibited a global inhibitory effect on pre-mRNA splicing of several genes we tested (Figure 3). Thus, our results suggest a new putative mechanism by which 5FU, as an effective anti-cancer drug, counteracts the proliferation of cancer cells.

Incorporation of 5FU into spliceosomal snRNAs, but not pre-mRNAs, blocks pre-mRNA splicing

Whether 5FU incorporation into a pre-mRNA affects splicing has been a controversial issue. It was reported that a small fraction of β-globin pre-mRNA containing 5FU (randomly incorporated into the pre-mRNA by *in vitro* transcription) was inaccurately spliced *in vitro* at pH > 7 (24). However, using nearly the same system, the Kole group failed to detect any abnormally spliced products (25). In the current work, we incorporated 5FU into two different pre-mRNAs and tested whether they could be accurately spliced in *Xenopus* oocytes. Our results indicate that splicing of the substrates (even with 100% 5FU substitution) occurs accurately, efficiently, and comparable to the splicing of unsubstituted pre-mRNAs that contain no 5FU. Here, the splicing was carried out in *Xenopus* oocytes, thus representing an *in vivo* circumstance. Thus, our results indicate that the ability of 5FU to inhibit pre-mRNA splicing is not due to its incorporation into pre-mRNA, at least for the two genes tested. In contrast, according to our results, the inhibitory effect of 5FU on splicing is due to its incorporation into U2 snRNA (and perhaps the other spliceosomal snRNAs as well) at naturally occurring pseudouridylation sites, thereby inhibiting the formation of pseudouridines that are required for pre-mRNA splicing (Figure 7) (30–32). Interestingly, however, our results have shown that different pre-mRNAs are affected differently. This difference in 5FU's effect is probably due to intrinsic differences among pre-mRNAs. For instance, one pre-mRNA might have an intrinsic enhancer element that helps splicing, whereas another pre-mRNA might have no such enhancer elements. Consequently, 5FU would show a greater effect on the splicing of the latter pre-mRNA than that of the former pre-mRNA.

5FU-containing U2 snRNA efficiently blocks the formation of pseudouridines required for pre-mRNA splicing

We have previously shown that the injection of 5FU-containing U2 into oocytes site-specifically blocks the formation of pseudouridines in U2 snRNA injected at a later time, presumably by sequestering or binding irreversibly with pseudouridylases (30,31). In the current study, we detected 5FU incorporation into HeLa U2 snRNA at these important pseudouridylation sites and observed a substantial blockade of pseudouridine formation in newly synthesized U2 snRNA at the respective sites. Thus, our current results from the

HeLa system are consistent with the previous results obtained from the *Xenopus* oocyte system. Interestingly, we note that, depending on individual sites, the extent of inhibition of pseudouridylation is not always the same. For instance, whereas pseudouridine formation at positions 37, 39, 41, 43 and 44 was greatly inhibited by 5FU, there was relatively weak inhibition at position 34. One explanation for this observation is that perhaps there are multiple or redundant pseudouridylation activities acting on Ψ34, and relatively light incorporation of 5FU at this site may not be sufficient to block all these activities. Alternatively, the redundant activities may have different sensitivities to 5FU-containing U2 snRNA, the pseudouridylation inhibitor. An individual activity that is less sensitive to the inhibitor may still be able to catalyze pseudouridine formation at this site in the presence of the inhibitor. Consistently, Ψ34 formation appears to be catalyzed by two redundant activities, the Pus7p homolog (a protein enzyme) (35) and U92/PugU2-34/44 (a sno/scaRNP) (36,37). In fact, when PugU2-34/44 is depleted, Ψ34 formation is not completely irreversibly blocked. In contrast, Ψ44 formation, which is also catalyzed by PugU2-34/44, is completely abolished (36).

We have previously demonstrated that pseudouridines in the 5′ end region and the branch site recognition region are important for pre-mRNA splicing in *Xenopus* oocytes (30,31). Our current results reinforce this conclusion. HeLa U2 snRNA containing low levels of pseudouridines at the naturally occurring pseudouridylation sites in the U2 branch site recognition region (and perhaps in other regions as well) failed to effectively reconstitute pre-mRNA splicing in *Xenopus* oocytes. Although it is still unclear as to how these pseudouridines contribute to U2 function in splicing, several lines of evidence suggest that pseudouridines may enhance RNA–RNA interactions and may even change the local structure of the interactions that may favor a functionally important configuration (38–40). In this regard, the U2 branch site recognition region is involved in base-pairing interactions with the branch site in the pre-mRNA. A recent NMR study indicated that at least one of the pseudouridines in the U2 branch site recognition region can help maintain the bulge of the branch point adenosine, a configuration believed to be necessary for nucleophilic attack during the first step of the splicing reaction (41). Based on the recent work of the Fournier group that several large-subunit rRNA pseudouridines, which are located at the peptidyl transferase center of the ribosome, synergistically contribute to ribosome function (42), it is possible that the multiple pseudouridines in the U2 branch site recognition region contribute to U2 function in the same synergistic manner. Verification of this hypothesis will require a detailed dissection of the mechanism of action of these pseudouridines.

Our current work also suggests that 5FU might be incorporated into pre-rRNA/rRNAs as well. Although it is still unclear as to whether incorporation into pre-rRNA will inhibit its processing, what is clear (or very possible) is that incorporation of 5FU into the important pseudouridylation sites within rRNAs (e.g. the pseudouridylation sites in the peptidyl transferase center, see above) will inhibit pseudouridylation at these sites, thus impairing ribosome function and protein translation, a scenario supported by strong experimental evidence (42). However, a definitive answer to this problem requires further experiments on rRNA/ribosome biogenesis and function.

Multiple effects of 5FU

It has been widely speculated and in some instances demonstrated that 5FU affects the metabolism of both DNA and RNA (2,4). With respect to the effect on RNA metabolism, efforts have focused primarily on the question of whether incorporation of 5FU into pre-mRNA or pre-rRNA influences the processing efficiency and accuracy of these RNAs. However, despite decades of extensive study, answers to this question remain somewhat ambiguous. Given that 5FU, when converted to 5FUTP in the cell, is a ribonucleotide analog, it is equally possible that the nucleotide analog is incorporated into the other types of RNA (not just pre-mRNA or pre-rRNA), some of which may participate in the processing of pre-mRNA or pre-rRNA, thereby affecting gene expression. Indeed, our current research provides such an example. Given that there are a huge number of cellular RNAs, each exhibiting a unique role in cellular processes, it is not surprising that 5FU can incorporate into these RNAs and perturb cell growth in a number of different ways.

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