***In vitro* splicing of pre-messenger RNA with extracts from 5-fluorouridine-treated cells**

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The effect of 5-fluorouridine (5-FU) treatment of cells on the splicing of pre-mRNA was determined using cellular extracts and splicing *in vitro*. Nuclear extracts from control cells and cells treated with 5-FU were prepared and used to splice pre-mRNAs *in vitro*. The drug treatment resulted in inhibition of cell growth but had little effect on RNA synthesis. The extracts from 5-FU-treated cells showed significant inhibition of splicing. This

inhibition was the result of reduced efficiency and was not caused by a block at a specific step in the splicing pathway. There were no observable changes in the levels or physical properties of the small nuclear ribonucleoprotein particles that are essential cofactors in the splicing process. The deficiency in splicing in the extracts from 5-FU-treated cells could be supplemented by the addition of complementary fractions from a control extract.

INTRODUCTION

Fluoropyrimidines (FPys) are widely used for the chemotherapeutic treatment of certain cancers, including colorectal, cervical and breast cancer [1–4]. The mode of action and metabolism of these drugs have been the subjects of numerous studies and the focus of many review articles [4]. Of the many FPys that have been used in chemotherapy, 5-fluorouracil is the most useful clinically [4]. It is transported into the cell via a low-affinity transport system [4–6] and is retained in the cell after phosphorylation of the nucleoside derivative [4,6]. Transport and accumulation of derivatives such as 5-fluorouridine (5-FU) are also by a facilitated process and have been exhaustively reviewed [4–7].

The effects of FPys on the synthesis of DNA and inhibition of thymidylate synthase have received a great deal of attention [4,6] but these drugs also affect the metabolism of RNA [4]. Treatment of cells with 5-FU results in inhibition of 45 S pre-ribosomal RNA processing and thus the production of 28 and 18 S rRNA [8–10]. In addition, incorporation of 5-FU inhibits modification of tRNA, which includes modifications at the 5 position on uridine, such as 5-methyluridine, pseudouridine and dihydro-uridine [11–13].

Splicing of pre-mRNA *in vivo* and *in vitro* is affected by FPy treatment [4,14]. An increase in dihydrofolate reductase pre-mRNA splicing intermediates has been seen in mRNA isolated from cells treated with 5-FU [15,16]. When 5-FU-containing β -globin pre-mRNA was incubated in extracts from control HeLa cells, the majority of the pre-mRNA was spliced correctly [17,18]. When extracts from cells treated with 2 μ M 5-FU for up to 4 days were used, the splicing of β -globin pre-mRNA was inhibited at all time points tested [18]. This inhibition appeared to be caused by a decreased level, even at 24 h, of the U2 small nuclear ribonucleoprotein particle (snRNP), a pre-mRNA splicing cofactor [18]. Changes in other snRNP pre-mRNA splicing cofactors, including an altered structure of U4/U6 snRNP and reduced turnover of U1 snRNP, have also been seen in cells treated with 5-FU [19].

In the present report, HeLa cells were treated with 5-FU for 24 h and splicing extracts made from the cells. These extracts were

used to study the splicing of pre-mRNAs *in vitro*. Treatment of cells with 5-FU results in nuclear extracts that are inefficient at splicing, but this deficiency does not appear to be due to decreased levels of the spliceosomal snRNPs. Instead, the function of these cofactors may be compromised by treatment of the cells with 5-FU.

MATERIALS AND METHODS

Pre-mRNAs were transcribed in the presence of [α - 32 P]GTP *in vitro* as described [20] using BamHI-digested pSP64-H β Δ6 [21], BglII-digested pRSP1 [22] and BamHI-digested pMINX [23] to synthesize the β -globin, adenovirus major late and MINX RNAs respectively.

HeLa cells were grown in minimum essential medium, Joklik modified for suspension cultures (Sigma) with 3.5% (v/v) calf serum and 3.5% (v/v) newborn calf serum (Gibco). Penicillin (100 units/ml) and streptomycin sulphate (100 μ g/ml) were included in the media and the cells were grown in 5% CO₂ in spinner flasks. The cultures were treated with either uridine or 5-FU or combinations of these nucleosides as 1 mM solutions in water. The cultures were then grown in the presence of 10 μ M thymidine while being treated to minimize cell death [24]. The viability of the cells were determined by the Trypan Blue exclusion method [25]. Measurement of 3 H-labelled uridine incorporation was conducted as described by Sierakowska et al. [18].

Splicing extracts were prepared from HeLa cells as described by Dignam et al. [26], and fractionation of the control nuclear extract on DEAE-cellulose was carried out as described [27]. The assay for the splicing of pre-mRNA *in vitro* and the gel electrophoresis of the splicing products were carried out essentially as described [21,28] except that no poly(vinyl alcohol) was added to the reaction mixtures. Protein concentrations of the extracts were determined by the method of Bradford [29]. Splicing complexes were separated by native gel electrophoresis as described by Ruskin et al. [30].

Caesium sulphate-gradient and velocity-gradient centrifugations were carried out as described [31]. Samples of the fractions from the gradients were blotted on to nitrocellulose, and probes specific for each spliceosomal snRNA were used to detect

snRNAs as described [32]. An LKB Bromma Ultrosan XL laser densitometer was used for densitometry of autoradiographs.

RESULTS

Treatment of cells with 5-FU for 24 h in the presence of 10 μ M thymidine [24] resulted in minimal cell death over the treatment period, as measured by Trypan Blue exclusion [25]. However, the 5-FU treatment did have an effect on cell growth (Figure 1). Growth was defined as the number of additional cells present after 24 h of treatment and is given in terms of percentage of control. The growth of the culture treated with 1 μ M 5-FU was 76 % of control, whereas the 5 μ M 5-FU-treated culture exhibited 34 % of control growth. So, although the treatment with 5-FU at these concentrations did not kill the cells, the growth of the cells was diminished during the 24 h treatment.

When [3 H]uridine incorporation was determined in control and 5-FU-treated cells, a minimal effect of 5-FU concentration on the amount of [3 H]uridine incorporated into acid-insoluble counts was found. Compared with the number of counts incorporated by the control cells, the 1 μ M and 5 μ M 5-FU-treated cultures exhibited 92 and 83 % of control incorporation respectively (Figure 1). These calculations have been corrected for the viable cell density at the time of the incorporation assay (see the legend to Figure 1).

In vitro splicing in extracts from 5-FU-treated cells

Would this 24 h treatment with 5-FU inhibit the splicing of pre-mRNA? In order to answer this question, nuclear extracts (NEs) were prepared [26] from control cells and cells treated with 5-FU and used to assay the splicing of model pre-mRNAs *in vitro*. Nuclear extracts were prepared from cultures at equal cell density that had been treated with 5 μ M uridine (control NE), 4 μ M uridine and 1 μ M 5-FU (1 μ M 5-FU NE) or 5 μ M 5-FU (5 μ M 5-FU NE). Protein concentrations of the extracts were equalized

before use in the splicing assays. Two pre-mRNA transcripts were used as substrates in the reactions: β -globin RNA, which contains the entire first exon, the first intron and most of the second exon of human β -globin RNA [21], and adenovirus RNA from the major late transcription unit of adenovirus 2, which contains the L1 exon, a shortened intron and the L2 exon [22]. These 32 P-labelled pre-mRNAs were incubated in reaction mixtures containing nuclear extract, ATP, phosphocreatine and MgCl_2 . The RNA was extracted, electrophoresed on polyacrylamide/8.3 M urea gels and the gels were autoradiographed. When β -globin pre-mRNA (Figure 2a) or adenovirus pre-mRNA (Figure 2b) was used, the formation of spliced product and splicing intermediates was minimal in the reaction mixture containing extract from the cells treated with 5 μ M 5-FU. *In vitro* splicing activity was also reduced in the reaction mixtures that contained extracts from cells treated with 1 μ M 5-FU. For β -globin pre-mRNA, the amount of exon 1 present after 60 min of incubation was 53 % and 14 % of the control level for reaction mixtures containing extracts from cells treated with 1 μ M and 5 μ M 5-FU respectively. The levels of exon 1 were determined by densitometric scanning of the autoradiograph. The corresponding levels for the adenovirus pre-mRNA were 38 % and 22 % respectively. Therefore *in vitro* splicing of these model pre-mRNAs was affected even after a short treatment of the cells with 5-FU, a treatment that has minimal effect on RNA synthesis.

The 32 P-labelled pre-mRNA used in these assays does not contain 5-FU, so the inhibition of splicing must be due to deficiencies in the extracts taken from the cells treated with 5-FU and not to pre-mRNA-derived inhibition. Both steps of the splicing process are inhibited, as there was no accumulation of any splicing intermediates. There is no evidence of aberrant-length products when splicing reactions are compared between control and 5-FU-treated lanes. There does not appear to be any cryptic splice site activation, only a reduced amount of the spliced product and intermediates in those reaction mixtures that contain the extracts from treated cells.

Inhibition of splicing-complex formation

The assembly of pre-mRNA into a splicing complex *in vitro* was also inhibited in reaction mixtures in which the nuclear extracts were from cells treated with 5-FU. A labelled adenovirus-derived pre-mRNA, MINX [23], which forms splicing complexes very efficiently, was incubated in splicing reaction mixtures containing extracts from control and treated cells and separated on native gels [22,30]. The control nuclear extract assembled the labelled MINX pre-mRNA into splicing complexes quite efficiently (A and B in Figure 3) [18,22,23,30], whereas less of the precursor was assembled into splicing complexes in the reaction mixtures containing extracts from 5-FU-treated cells. The reduction appears to be uniform, the formation of all the complexes being decreased. This suggests that there was a reduction in the efficiency of splicing in general and not just a block at a particular step. This correlates with the results shown in Figure 2, where no accumulation of particular intermediates was seen, only a reduction in all of the products and intermediates.

Figure 1 Growth and [3 H]uridine incorporation of cells treated with 5-FU

Live cells were counted and the amount of [3 H]uridine incorporated was measured as described in the Materials and methods section. The average number of cells/ml ($n = 2$) at the different 5-FU concentrations was determined and the starting cell density subtracted. The difference was used to calculate the percentage of control cell growth (■). The average amount ($n = 3$) of acid-insoluble [3 H]uridine incorporated in samples of cells was determined and the results were corrected for the number of cells present in the different treatment groups. These values were used to calculate the percentage of control radioactivity incorporated (□).

Levels and characteristics of snRNPs in extracts from 5-FU-treated cells

Samples of nuclear extracts from control and treated cells were subjected to buoyant-density and velocity-sedimentation centri-

Figure 2 *In vitro* splicing activity of extracts from 5-FU-treated cells

Extracts from cells treated with 0 μ M (control NE), 1 μ M (1 μ M FU NE) and 5 μ M (5 μ M FU NE) 5-FU were used in splicing reactions with equal amounts of 32 P-labelled β -globin pre-mRNA (a) as the substrate. Incubations were for 30, 60 and 120 min, as indicated, and RNA was isolated, electrophoresed on a 6% polyacrylamide/8 M urea gel, and autoradiographed. Lane M is 32 P end-labelled pBR322 DNA cut with *MspI* and lane P is unincubated pre-mRNA. On the left are the sizes of the markers in nucleotides. On the right are schematic representations showing the positions of the precursor RNA, the lariat-exon 2 intermediate, the spliced product and exon 1 respectively from the top of the panel. In (b) 32 P-labelled adenovirus pre-mRNA (pRSP-1) was incubated in the reaction mixtures and the products were electrophoresed on a 10% polyacrylamide/8 M urea gel. The same splicing reaction products and intermediates as in (a) are denoted with the addition of the excised lariat, second from the top. The lariat-containing intermediates migrate more slowly than the precursor in (b) because of the high-percentage gel (10%) used to analyse these adenovirus mRNA products (see ref. [28]). The lariat-containing intermediates migrate faster than the precursor in the lower-percentage gel (6%) used to analyse the β -globin mRNA products in (a) [28].

Figure 3 Native gel of splicing reactions

Splicing reaction mixtures (10 μ l) containing equal amounts of 32 P-labelled MINX pre-mRNA were incubated for the indicated times (in min) and left on ice; then 2 μ l of 20 mg/ml heparin was added and electrophoresed on a 0.5% agarose/4% acrylamide/bisacrylamide (80:1 w/w) gel in 30 mM Tris/borate/EDTA (pH 8.3) containing 10% glycerol as described [30]. The autoradiograph of the dried gel is shown. P is the MINX probe alone, NS is a non-specific complex, and A and B are other complexes described previously (see the text). The location of the bottom of the well is marked with a w.

fugation to determine if the treatment with 5-FU results in a reduction in the level of any of the snRNPs or in a significant change in size or composition of these essential splicing cofactors. A change in the levels or characteristics of snRNPs might explain the observed effect on *in vitro* splicing. Fractions from the buoyant density (caesium sulphate) and 10–30% glycerol gradients were blotted onto nitrocellulose (see the Materials and methods section) and probed with 32 P-labelled antisense U RNA specific probes for U1, U2, U4 and U6 RNAs [32]. The blots to detect U5 were probed with a 32 P-end-labelled antisense U5 oligodeoxynucleotide [33].

There was no significant change in the levels of any of the snRNPs, nor was there any apparent change in either the buoyant density or the sedimentation velocity of any of these particles when cells were treated with 5-FU. The autoradiographs of the slot-blots for U2 snRNP are shown in Figure 4 as an example of the results that were typical for all the other snRNPs. The U2 snRNP particles from control and treated cells all have buoyant densities of 1.4 g/cm³ (Figure 4a), which reflects a protein/RNA ratio that is characteristic of U2 snRNP [34,35]. If the incorporation of 5-FU into U2 RNA affected the interaction of proteins with the snRNA, then one would expect to detect increased amounts of U2 RNA in the region of the gradient where protein-free RNA would band (1.6 g/cm³). No increase in the levels of protein-free U2 RNA was detected (Figure 4) and the same result was obtained for all of the other snRNAs probed (results not shown).

Likewise, there was no change in the sedimentation velocity of any of these splicing cofactors. The results for U2 snRNP (Figure 4b) show that most of it, whether from control or treated cells, sedimented at approx. 18 S [36]. If the incorporation of

Figure 4 Slot-blots of buoyant-density and velocity-sedimentation gradients probed for U2 snRNP

Portions of each gradient were blotted to nitrocellulose as described in the Materials and methods section. The blot for each gradient is on two lines and each fraction is numbered for each gradient. The blots were probed with ^{32}P -labelled antisense U2 RNA [35]; the autoradiographs are shown. (a) Blots of the caesium sulphate buoyant-density gradients from each extract; (b) blots of the 10–30% glycerol sedimentation-velocity gradients. The position of reference densities or sedimentation markers are indicated for each type of gradient in the panels for the control extract.

5-FU had affected the composition and resulted in a change in the number of proteins associated with the U2 RNA, a shift in the sedimentation velocity of U2 snRNP or a broadening of the peak of radioactivity would be apparent. No shift or broadening of the peak of U2 snRNP concentration was observed when extracts from 5-FU-treated cells were examined. In addition, the other snRNAs probed also showed no shift or broadening of the peaks on the gradients (results not shown).

Therefore, at these levels of 5-FU treatment for 24 h, there is no change in the apparent characteristics of the individual snRNP particles. Associations between snRNPs, such as the formation of the U4/U6–U5 pre-spliceosomal complex [22,37] in the presence of ATP, were also not affected by treatment of the cells with 5-FU (results not shown).

Reconstitution of splicing activity with fractionated control extract

A portion of the control nuclear extract was fractionated on a DEAE-cellulose column into two fractions, non-bound (NB or DEAE I) and bound (B or DEAE II). Neither of these fractions alone will splice the adenovirus pre-mRNA but, when combined, splicing is reconstituted (Figure 5, lanes B, NB, and B + NB). The non-bound fraction contains primarily non-snRNP factors necessary for splicing, and the bound fraction contains primarily the snRNPs [27]. When either the bound or the non-bound fraction was used to supplement the nuclear extract from the 5 μM 5-FU-treated cells, *in vitro* splicing of the adenovirus pre-mRNA was stimulated. This is most easily seen by following the appearance of the lariat-exon 2 intermediate and the excised lariat (Figure 5,

Figure 5 Augmentation of splicing efficiency with control extract fractions

Control (C) extracts and extracts from 5 μM 5-FU-treated cells (FU) were supplemented with the non-bound (NB, DEAE I) or the bound fractions (B, DEAE II) from DEAE-cellulose chromatography of control extract. C and FU are equivalent portions (with respect to protein concentration) of extracts, C + FU is an equal portion (protein) of C and FU extracts mixed in one reaction. FU + NB is 6 μl of FU extract plus 0, 3, 6 or 9 μl of the NB fraction. FU + B is 6 μl of FU extract plus 0, 3, 6 and 9 μl of the B fraction. NB is 9 μl of the non-bound fraction alone and B is 9 μl of the bound fraction alone. B + NB is 7.5 μl of each fraction mixed. The ^{32}P -labelled pre-mRNA used was adenovirus late leader mRNA [22] and the samples were run on a 10% polyacrylamide/8 M urea gel; an autoradiograph of the gel is shown. The positions of precursor, spliced products and splicing intermediates are shown at the right; see Figure 2 for what these schematics represent. M is ^{32}P end-labelled *Hinf*I-cut ϕX174 , and P is unincubated probe.

top of the gel). These results suggest that at least two components of the splicing machinery, probably snRNPs and non-snRNP proteins, are not functioning properly in the extract from 5-FU-treated cells. These results from the 24 h treatment of cells with 5-FU confirm the earlier findings of Sierakowska et al. [18] which showed that long-term (up to 4 days) 5-FU treatment affected both snRNP and the non-snRNP cofactors that are necessary for splicing.

DISCUSSION

Incorporation of 5-FU appeared to affect the function of snRNPs in the splicing process without significantly affecting their levels or characteristics. It is possible that the interaction of snRNPs with pre-mRNA or with other snRNPs was affected. In addition, changes in the metabolism of the snRNPs that do not result in gross changes in the buoyant density or size of the particles may be caused by incorporation of 5-FU. One such metabolic process is the modification of the RNA component of the snRNP particle. 5-FU-containing snRNAs are potent inhibitors of pseudouridine formation *in vitro* [38,39], and pseudouridine formation on U2 RNA from *in vitro* assembled snRNP is severely inhibited when extracts from 5-FU-treated cells are used for *in vitro* assembly of U2 snRNP (J. R. Patton, unpublished work).

How would changes in the level of pre-mRNA splicing affect cellular metabolism? The levels of 5-FU used in this study did have an effect on cell growth, but a significant effect on RNA synthesis was not observed. Splicing in the treated cells was partially inhibited and this may have profound effects on the cell, resulting in a backlog of unspliced or incorrectly spliced mRNAs that will eventually affect protein levels and cell viability. The

processing of ribosomal RNA [8,9] and the modification of tRNA [11–13] are also affected by treatment with FPys. Any one of these effects may not be lethal to the cell by itself, but the sum of all the effects could result in cell death. These RNA effects may be the reason for the cytotoxicity observed in cells that are not dividing.

Inhibition of *in vitro* splicing after exposure (up to 4 days) of cells to 5-FU has been shown previously [18]. Sierakowska et al. [18] found that this inhibition could be attributed in part to a reduced level of U2 snRNP. Non-snRNP factors were also deficient in extracts from 5-FU-treated cells [18]. The current report shows that 5-FU treatment affects *in vitro* splicing after 24 h, without observable effects on the levels or characteristics of snRNP splicing cofactors. The reason for this apparent contradiction is not clear but the methods used to estimate the amount of U2 snRNP are different in the two studies.

Doong and Dolnick [17] observed low-level use of cryptic splice sites when 5-FU-containing β -globin pre-mRNA was spliced *in vitro*. The use of cryptic sites on β -globin pre-mRNA was not observed in the present studies, because the pre-mRNA used in these splicing assays did not contain 5-FU. When 5-FU-containing β -globin pre-mRNA was incubated in control extracts, additional bands that possibly indicate the use of cryptic sites were observed at a low level (results not shown). The majority of the precursor was spliced correctly as was seen previously [17,18].

In vitro RNA-processing assays could be used for the screening of anticancer drugs and types of modulation therapy. Drugs that limit the effects on cellular processes involving RNA metabolism could be tested *in vitro*. These assays may help in the rational design of drugs by testing cells in culture for maximal DNA-synthesis effects with minimal RNA effects and thus improve the efficacy or therapeutic index of the drug.

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