DNA Damage and Cellular Stress Responses

The Incorporation of 5-Fluorouracil into RNA Affects the Ribonucleolytic Activity of the Exosome Subunit Rrp6

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Abstract

5-Fluorouracil (5FU) is a fluoropyrimidine used for the treatment of solid tumors. 5FU is a precursor of dTTP and UTP during biogenesis, and it interferes with both DNA and RNA metabolism. The RNA exosome, a multisubunit complex with ribonucleolytic activity, has been identified as one of the targets of 5FU in yeast. Studies in human cells have shown that the catalytic subunit of the nuclear exosome, Rrp6, is specifically targeted. Here, we have investigated the direct effect of 5FU on the activity of Rrp6 in Drosophila S2 cells, and we have identified two aspects of Rrp6 function that are altered by 5FU. First, gel filtration analysis revealed that the repertoire of multimolecular complexes that contain Rrp6 is modified by exposure to 5FU, which is consistent with the proposal that incorporation of 5FU into RNA leads to the sequestration of Rrp6 in ribonucleoprotein complexes. Second, the incorporation of 5FU into RNA renders the RNA less susceptible to degradation by Rrp6, as shown by Rrp6 activity assays in vitro. Our results imply that aberrant transcripts synthesized in 5FU-treated cells cannot be turned over efficiently by the surveillance machinery. Together with previous results on the mechanisms of action of 5FU, our findings suggest that the cytotoxicity of 5FU at the RNA level is the result of at least three different effects: the increased levels of retroviral transcripts with mutagenic potential, the reduced synthesis of ribosomes, and the inhibition of the nuclear RNA surveillance pathways. Drugs that reinforce any of these effects may boost the cytotoxicity of 5FU.

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Introduction

The pyrimidine analogue 5-fluorouracil (5FU) is widely used in the treatment of solid tumors including colorectal and breast cancers. After penetration into the cell, 5FU is converted into 5-fluoro-oxyzuridine 5'-monophosphate (5FdUMP) and 5-fluorouridine 5'-monophosphate (5FUMP), both of which can be further phosphorylated and incorporated into DNA and RNA, respectively (reviewed by ref. 1). Multiple mechanisms contribute to the antiproliferative activity of 5FU (reviewed by ref. 2). First, 5FUMP associates with 5,10-methylene tetrahydrofolate and with thymidylate synthase (TS) and forms an inactive TS complex. TS is a key enzyme in the de novo synthesis of thymidylate, and the inactivation of TS by 5FUMP leads to reduced levels of dTTP and to inhibition of DNA replication. Second, the reduced activity of TS results in abnormally high levels of dUTP, and both dUTP and 5FdUTP then become misincorporated into DNA, instead of dTTP. Excision of the misincorporated 5FU derivatives by uracil-DNA-glycosylase results in DNA damage and cell death (3). And third, FUTP is extensively incorporated into RNA, which inhibits the processing and maturation of rRNA, tRNA, and mRNA precursors (4–7).

The extent to which each of the pathways mentioned above contributes to the cytotoxicity of 5FU probably varies among cancer types, individual genetic backgrounds and administration schemes. TS is thought to be the main target of 5FU. However, a pharmacological analysis that compiled gene expression profiles for human cancer cell lines treated with different drugs showed that 5FU clusters with inhibitors of RNA synthesis (8), which suggests that RNA-mediated mechanisms contribute significantly to the therapeutic properties of 5FU. A genome-wide screen aimed at discovering the modes of action of several therapeutic compounds using yeast heterozygotes identified 8 potential targets for 5FU and, interestingly, 4 of them were subunits of the exosome (9). The exosome is a multiprotein complex with ribonucleolytic activity that plays several important roles in RNA metabolism. In the cytoplasm, the exosome has degradative functions and is responsible for mRNA turnover. In the cell nucleus, the exosome participates in the processing and maturation of small noncoding RNAs and pre-mRNAs, and in the quality control of mRNA biogenesis (reviewed by refs. 10–12). The exosome associates with different multiprotein complexes and interacts with...
different specific cofactors when carrying out its different functions (13).

The exosome has a ring-shaped core composed of 9 protein subunits. Two additional subunits, Rrp6 and Dis3, interact with the exosome ring and provide the ribonucleolytic activity (14). Structural and biochemical studies have shown that the structure and function of the exosome are highly conserved throughout evolution from yeast to man (15 and references therein). The components of the exosome of *Drosophila melanogaster* have been identified and are very similar to their human counterparts (16).

Studies in *D. melanogaster* revealed that Rrp6 not only works in association with the exosome ring, it also has exosome-independent functions in cell cycle regulation and mitotic progression (17).

A recent study has shown that human cells depleted of the exosome subunit Rrp6 are more sensitive to 5FU than control cells with full Rrp6 activity, and that the levels of natural exosome substrates (such as endogenous retroviral transcripts) are increased by the 5FU treatment. These results confirm that Rrp6 is indeed a target for 5FU in human cells (18). However, we do not know the molecular mechanisms by which 5FU affects the function of Rrp6. We have addressed this question by analyzing the effect of 5FU on 2 aspects of Rrp6 function in order to increase our understanding of the cytotoxicity of 5FU. These aspects are the incorporation of Rrp6 into multiprotein complexes in vivo and the Rrp6 exoribonuclease activity in vitro. We have used *Drosophila* S2 cells as a model system and we have produced epitope-tagged Rrp6 that we have used for activity assays in vitro. Our results show that RNA molecules that contain 5FU instead of uracil are not degraded by Rrp6 as efficiently as normal RNA is degraded. We discuss a revised model for the mechanism of action of 5FU in vivo.

**Materials and Methods**

**Culture of *Drosophila* S2 cells**

*D. melanogaster* S2 cells were cultured at 28°C according to the instructions of the *Drosophila Expression System* manual from Invitrogen. Cells stably transfected with a plasmid that encodes V5-tagged Rrp6 have been described by Hessle and colleagues (19). The mock cells used in this study are S2 cells stably transfected with the hygromycin selection plasmid but without the Rrp6-V5 expression plasmid. The cells were maintained in the presence of 300 μg/mL hygromycin B. Expression of Rrp6-V5 was induced with treatment with CuSO4 for 24 hours.

**Antibodies**

The anti-V5 antibody was a monoclonal antibody from Invitrogen. The anti-Rrp6 antibody was generously provided by E.D. Andrusi (Case Western Reserve University, Cleveland, OH). The anti-Nona Bj6 antibody was generously provided by H. Saumweber (Humboldt University, Berlin, Germany). The anti-Ski6 antibody was a peptide-specific antibody made against the amino acid sequence YNPSRKKRPENKAG found in *D. melanogaster* Sk6. The anti-Trf4 antibody was made against the amino acid sequence YNPSRKKRPENKAG found in *D. melanogaster* Trf4-1. The peptides were conjugated to keyhole limpet hemocyanin and used to immunize rabbits following standard procedures. Immunization was carried out at AgriSera AB (Vännäs, Sweden).

**Preparation of nuclear protein extracts**

S2 cells were washed in PBS and resuspended in lysis buffer (50 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 1 mmol/L MgCl₂, 10% glycerol, 0.1% Nonidet P-40) with Complete EDTA-free Protease Inhibitor Cocktail (Roche). The cells were homogenized in a tight pestle. The homogenate was centrifuged at 4,000 × g for 10 minutes at 4°C. The pellet nuclei were resuspended in 50 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 1 mmol/L MgCl₂, 10% glycerol, 5 μmol/L PMSF, and then sonicated 4 times for 30 seconds each time in a Bioruptor sonication ice bath (Diagenode). The sonicated solution was supplemented with Nonidet P-40 to a final concentration of 0.1%. The extract was finally centrifuged at 16,000 × g for 10 minutes at 4°C.

**Gel filtration analysis**

Rrp6-V5 S2 cells were treated with 20 μmol/L CuSO₄ for 24 hours to induce the expression of Rrp6 and for 6 hours with 20 μmol/L of either uracil or 5FU. Nuclear extracts were prepared as described above with an additional digestion with 0.1 mg/mL RNase A immediately before the final centrifugation. Spun extracts were fractionated on a Superose 12/300 GL column equilibrated in nuclear extraction lysis buffer at 0.5 mL/min using the AKTA FPLC system (GE Healthcare). Fractions of 250 μL were precipitated by acetone, the proteins were separated by SDS-PAGE using the Mini-Protean II system (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in Tris-glycine buffer with 0.02% SDS and 4 mol/L urea using a semidy electrophoretic transfer cell (Bio-Rad). The apparent molecular masses of the detected complexes were estimated by comparison with known protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa; GE Healthcare). The membranes were probed with antibodies following standard procedures. Densitometric analyses of Western blots were done using the Fujiﬁlm Multi Gauge software (Fujiﬁlm).

**Purification of RNA synthesized in vivo**

RNA was purified using TRizol (Invitrogen) from *Drosophila* S2 cells that had been exposed to 20 μmol/L uracil or 5-fluouracil for 24 hours. The resulting RNA was extracted by phenol, precipitated and resuspended in RNase-free water.

**In vitro transcription**

RNA was generated *in vitro* by using T7 polymerase (Invitrogen) to transcribe a PCR amplified fragment of EGFP designed to add the T7 promoter to the 5’ end (T7 Forward: taataagcttctagatgATGAGCAAA-...
Figure 1. Expression of epitope-tagged Rrp6 in S2 cells. S2 cells were stably transinfected with an expression vector that encodes V5-tagged Rrp6 under the control of an inducible promoter. A, induction of the expression of V5-tagged Rrp6 with increasing amounts of copper sulfate, indicated in the figure. Rrp6 was detected by Western blot using an anti-V5 antibody. B, the expression of V5-tagged Rrp6 was induced with 400 μmol/L copper sulfate and the subcellular distribution of the V5-tagged protein was analyzed by immunofluorescence using the anti-V5 antibody. Rrp6 is restricted to the cell nucleus, as expected. An unrelated IgG was used in parallel as a negative control. Phase contrast images are shown in the bottom part of the figure. The magnification bar represents 5 μm. C, the expression of V5-tagged Rrp6 (lane 1) was induced with 20 μmol/L copper sulfate and nuclear protein extracts were extracted and analyzed by Western blot with an anti-Rrp6 antibody that can recognize both the endogenous and the V5-tagged Rrp6 proteins. Mock S2 cells that only express the endogenous Rrp6 protein (lane 2) were processed in parallel for comparison of expression levels. Mock S2 cells treated with 20 μmol/L 5FU for 6 hours were processed and analyzed in parallel (lane 3) to estimate the effect of 5FU treatment on Rrp6 expression. The blot was re-probed with an anti-Nona antibody that served as a loading control. D, protein extracts were prepared from cells expressing Rrp6-V5 after treatment with either uracil or 5FU, as indicated. The levels of Rrp6-V5 were analyzed by Western blot using the anti-V5 antibody. The lower half of the blot was probed with an anti-tubulin antibody that served as a loading control.

GGGCCAGGAGCTG; Reverse: GCCGTCAGAATCCACAGAC. The manufacturer’s conditions for polymerization were followed with the exception that the UTP concentration was lowered to 1 mmol/L (from 2 mmol/L; ATP, CTP, GTP remained at 2 mmol/L each). RNA into which 5F-UTP had been incorporated was generated by resuspending 5-fluorouridine 5'-triphosphate tetraammonium salt (5F-UTP, Moravek Biochemicals Inc.) in water and including it in the reaction (0.35 mmol/L 5F-UTP) while the levels of UTP were adjusted accordingly (0.65 mmol/L UTP). After transcription, the reaction products were digested with Turbo DNase (Ambion) and precipitated with ethanol.

In vitro Rrp6 activity assay

Cells expressing Rrp6-V5 and mock S2 cells were induced for 24 hours with 400 μmol/L CuSO₄ prior to extraction of nuclear proteins as described above, with additional attention paid to keeping the extracts in RNase-free conditions. Extracts were precleared with a mixture of protein A-coupled and protein G-coupled Sepharose beads previously equilibrated in lysis buffer. The precleared extracts were then incubated for 1 hour with 15 μg/mL anti-V5 antibody (Invitrogen). Protein A/G Sepharose bead slurry was added to the extracts, and the mixture was incubated again for 1 hour. The beads with bound Rrp6-V5 were extensively washed with lysis buffer. The washed beads were resuspended in 100 μL lysis buffer containing 50% glycerol and stored at −20°C. The activity of the precipitated Rrp6 was assayed by incubating 250 ng RNA with 1 μL Rrp6-V5 bead slurry in 10 mmol/L TrisCl at pH 8.0, 50 mmol/L KCl, 7.1 mmol/L MgCl₂, 75 μmol/L MnCl₂ for 1 hour at 30°C under agitation. The RNA in the reaction mixtures was immediately phenol-extracted and ethanol-precipitated. The pellet was resuspended in 5 μL water, and the RNA was then immediately analyzed with the Experion automated electrophoresis system using standard sensitivity RNA chips (Bio-Rad).

Results

5FU affects the association of Rrp6 with multimolecular complexes

Rrp6 is a target for 5FU in human cells (18) but the molecular mechanisms of 5FU cytotoxicity have not been fully elucidated. To address this question, we carried out a series of experiments in which we analyzed whether the assembly of Rrp6 into multimolecular complexes in vivo was affected by 5FU. We used Drosophila S2 cells that expressed a V5-tagged version of Rrp6 under the control of a metallothionein promoter (19). The expression of the tagged Rrp6 was induced with copper sulfate, as shown in Figure 1A. Several observations indicated that the presence of the V5 tag in Rrp6 did not affect the function of the tagged Rrp6 protein. Firstly, the growth of S2 cells was not inhibited by the expression of the tagged Rrp6 (data not shown). Secondly, the tagged Rrp6 was predominantly localized in the cell nucleus (Fig. 1B), as described for the endogenous Rrp6 (20). And thirdly, we showed in a previous study that Rrp6-V5 associates in vivo with proteins such as Spt5 and Sp6, which are known to interact with the endogenous exosome (16, 19).

Nuclear protein extracts were prepared from S2 cells after inducing the expression of the V5-tagged Rrp6 with a low concentration of copper sulfate (20 μmol/L) to avoid over-expression artifacts. The level of Rrp6 in the nuclear extracts was only slightly higher than the level in extracts prepared from mock S2 cells (Fig. 1C, compare lanes 1 and 2). The nuclear protein extracts were fractionated by gel filtration chromatography and the proteins in each fraction were analyzed by SDS–PAGE and Coomassie blue staining (Fig. 2A). The presence of Rrp6 in multimolecular complexes of sizes up to 5 MDa was assayed by Western blotting (Fig. 2B). Virtually no free Rrp6 protein was detected in the conditions of our experiment. Rrp6 was detected in a variety of bands.
of complexes of molecular masses between 400 and 800 kDa, and was particularly enriched in complexes of molecular masses approximately 750, 600, and 440 KDa, respectively (arrowheads in Fig. 2B). A low-abundance complex larger than 2 MDa was also present (see Fractions 3–5 in Fig. 2B). This fractionation pattern was reproducible.

We used an antibody against the exosome ring subunit Ski6 to identify the exosome-containing fractions. Figure 2B shows that Ski6 was mostly concentrated in a complex of molecular mass 600 kDa. We concluded, based on this observation and on the expected size of the nuclear exosome in vivo (21), that the 600 kDa complex contained the entire nuclear exosome. Trf4, a subunit of the TRAMP complex, was also found in the fractions in the 600 kDa range. Trf4 is not a component of the exosome but contributes to enhance the hydrolytic activity of the nuclear exosome on highly structured RNA substrates (22), and therefore the Trf4 fractionation pattern is expected to partially overlap with that of the exosome. In
and the core exosome.

molecular mass of the complex that contains both Rrp6

effects of 5FU, we detected a change in the relative

Figure 3. An in vitro assay to study the ribonucleolytic activity of Rrp6. A, isolation of V5-Rrp6 from S2 cells. Nuclear protein extracts were prepared from S2
cells expressing epitope-tagged Rrp6 (induction with 400 µmol/L copper sulfate) and the Rrp6 protein was isolated by immunoprecipitation. The
immunoprecipitated proteins were resolved by SDS–PAGE and stained with Coomassie blue. The asterisk indicates the position of Rrp6. The 2 strong bands at
25 and 55 kDa are the heavy (H) and light (L) chains of the antibody. The mobilities of molecular mass standards in kDa are shown at the left. Mock cells that did
not express Rrp6-V5 were grown and processed in parallel as negative controls. B, in vitro Rrp6 activity assay. Total RNA from S2 cells was purified and used
as substrate in Rrp6 activity assays. Rrp6 was isolated by immunoprecipitation as in (A). The RNA was incubated for 1 hour at 30°C in the presence of
immunoprecipitated Rrp6. A mock reaction was carried out in parallel using mock-transfected cells that did not express V5-Rrp6. The input RNA (lane 1) and
the reaction products (lanes 2 and 3) were resolved by automated electrophoresis using microfluidic chips. The image shows a simulated gel. Molecular mass
standards are shown at the left and their lengths are indicated in nucleotides. The asterisk indicates the position of rRNA. C, the quantitative output
of the automated electrophoresis system. The image shows the electropherogram of the reaction products shown in (B). RNA amounts are given in
relative arbitrary units.

summary, we conclude that a large fraction of Rrp6
cofractionates with the nuclear exosome in a complex
of approximately 600 kDa (Fraction 27 in Fig. 2B and
D). Moreover, Rrp6 is also present in exosome-free com-
plexes of 750 and 440 kDa (Fractions 21 and 33 in Fig. 2B
and D).

The same type of chromatographic analysis was carried
out with nuclear protein extracts prepared from S2 cells
treated with 20 µmol/L 5FU for 6 hours (Fig. 2C and D,
dashed line). Treatment of the cells with 5FU did not have
any significant effect on the levels of Rrp6-V5 (Fig. 1D) but
had a profound effect on the association of Rrp6 with other
nuclear components (Fig. 2C and D). Rrp6 was still
detected in a variety of complexes, but the 600 and 440
kDa complexes were no longer conspicuous. Rrp6 was
instead enriched in complexes in the 500 to 550 kDa range
(Fractions 29–31 in Fig. 2C and D, dashed line). The
nuclear exosome complex, again defined by the presence
of Ski6, had been shifted from 600 kDa to approximately
500 kDa. Trf4 also showed a shift toward the 500 kDa
region (Fraction 31 in Fig. 2C).

The compositions of the different Rrp6-containing com-
plexes are not known, which precludes a detailed
interpretation of the molecular interactions that are
affected by 5FU. However, our present results clearly
demonstrate that the treatment of cells with 5FU has a
direct effect on the association of Rrp6 with multimolec-
ular complexes in the cell nucleus. Among the observed
effects of 5FU, we detected a change in the relative
molecular mass of the complex that contains both Rrp6
and the core exosome.

Establishment of an in vitro system to assay the
ribonucleolytic activity of Rrp6

In another series of experiments, we sought to analyze the
effect of 5FU on the ribonucleolytic activity of Rrp6. For this
purpose, we first established an in vitro assay for Rrp6
activity. We induced the expression of Rrp6 in S2 cells,
we prepared nuclear protein extracts and we isolated Rrp6
using the anti-V5 antibody. The quality of the immuno-
precipitated Rrp6 was analyzed by SDS–PAGE and Coom-
assie staining (Fig. 3A). Apart from the heavy and light
chains of the antibody (H and L in Fig. 3A), Rrp6 was the
main immuno-precipitated protein (asterisk in Fig. 3A).
Mock enzyme preparations were obtained in parallel using
S2 cells that had been transfected with a resistance plasmid
but without the Rrp6 expression plasmid (for details see
Materials and Methods section). The mock enzyme pre-
parations were used in all subsequent assays to discriminate
between Rrp6-mediated ribonucleolysis and unspecific RNA
degradation.

The two catalytic subunits of the exosome, Dis3 and
Rrp6, interact with each other in vivo (23) and we con-
considered the possibility that Dis3 was present as a contami-
nant in our Rrp6 preparations. For this reason, all the
activity assays were carried out in the presence of 7
mmol/L Mg²⁺, which inhibits Dis3 (24).

The enzymatic activity of the immuno-precipitated Rrp6
protein was assayed using total RNA purified from S2 cells
as a substrate. This RNA synthesized in vivo and purified
from S2 cells constituted a source of physiologically relevant
substrate for the in vitro activity assays. The reaction
mixtures were incubated at 30°C for 1 hour, and the
reaction products were purified and analyzed using micro-fluidic chips in an automated electrophoresis system. The results of the electrophoretic analysis were visualized as either simulated gels (Fig. 3B) or electropherograms (Fig. 3C). An example of total RNA digested with Rrp6 is shown in Figure 3B and C. The two intense bands of lengths approximately 2,000 nucleotides (nt) correspond to the rRNAs (asterisk in Fig. 3B). The mock-digested RNA preparations were not significantly digested and their electrophoretic profile was very similar to that of the input undigested RNA (compare lanes 1 and 2 in Fig. 3B). The RNA samples digested with Rrp6, in contrast, were significantly degraded (compare lanes 2 and 3 in Fig. 3B and C). In summary, these results show that the Rrp6 preparations isolated from S2 cells were active in vitro and could be used in activity assays.

rRNA synthesized in vivo in the presence of 5FU is not efficiently degraded by Rrp6

One of the mechanisms of action of 5FU involves the conversion of 5FU into 5FUTP and its subsequent incorporation into RNA (reviewed by ref. 1). The resulting RNA, from now on referred to as “5F-RNA”, is not a proper substrate for some of the enzymes that are involved in pre-rRNA processing (see, for example, ref. 7). Under normal growth conditions, defective rRNA molecules are identified by the surveillance machinery of the cell, and degraded by the nuclear exosome (11). However, the rRNA intermediates accumulate in 5FU-treated cells (25). We therefore sought to assess the ability of Rrp6 to degrade 5F-RNA synthesized in vivo. S2 cells were treated with 20 μmol/L 5FU for 24 hours before purification of the total RNA. Control cells were treated in parallel with the same concentration of uracil (Ura). The RNA made by Ura-treated cells was normal, nonmodified RNA. Ura-RNA and 5F-RNA were used as substrates in the enzymatic assays similar to those described in Figure 3. Figure 4 shows the electropherograms of mock-digested and Rrp6-digested samples. The electropherograms of the RNAs from mock digestions were very similar to those of undigested RNA, as expected, and the RNAs synthesized in the presence of Ura or 5FU were similar to each other (left in Fig. 4A). In contrast, the...
electropherograms of Ura-RNA and 5F-RNA digested with Rrp6 were significantly different. Both the Ura-RNA and the 5F-RNA were digested to some extent, as shown by the reduced abundance of the rRNA peaks (right in Fig. 4A). However, the digestion of 5F-RNA was not complete. 5F-RNA species in the 100 to 1,900 nt range accumulated in the Rrp6-digested samples (see the low molecular mass region of the electropherogram in Fig. 4A), which suggests that the digestion of 5F-rRNA by Rrp6 is not as processive as the digestion of normal Ura-RNA is.

The electropherograms in Figure 4A show results from 1 experiment. We next quantified and compiled the results from 3 independent digestions (Fig. 4B). The amounts of RNA in the low molecular mass region and in the rRNA peak were quantified separately, and Figure 4B shows histograms of the averages. The accumulation of low molecular mass 5F-rRNA was significant and reproducible.

The results presented above support the hypothesis that 5F-rRNA synthesized in vivo is not efficiently degraded by Rrp6. The disappearance of the rRNA peak suggests that 5F-rRNA is recognized as a substrate by Rrp6 and is degraded to some extent. However, the concomitant accumulation of RNA molecules of lower molecular mass indicates that digestion of 5F-rRNA by Rrp6 is not fully processive.

5F-RNA synthesized in vitro is not efficiently degraded by Rrp6

The incomplete digestion of 5F-rRNA reported above may have been caused directly by the presence of 5FU in the rRNA. Alternatively, the incorporation of 5FU into pre-rRNA in vivo may interfere with post-transcriptional modifications (reviewed by ref. 26), which in turn could lead to the formation of aberrant rRNA species less susceptible to digestion by Rrp6. We carried out Rrp6 activity assays on 5F-rRNA synthesized in vitro to determine whether the presence of 5FU in the RNA had a direct impact on the ribonucleolytic activity of Rrp6. We chose to use an mRNA sequence instead of an rRNA to minimize the formation of stable secondary structures that could affect the processivity of Rrp6 in vitro. We synthesized an mRNA molecule of 750 nt by in vitro transcription in the presence of 5FU as described in Materials and Methods section. The same mRNA sequence containing normal uridine was synthesized in parallel and used as a reference. Both the normal RNA and the 5F-RNA were digested by Rrp6 to some extent, but quantification of the reaction products revealed that digestion of the 5F-RNA was less efficient (Fig. 5). We conclude that the presence of 5FU in RNA is sufficient to render the RNA more resistant to digestion by Rrp6.

Discussion

5FU is widely used in combination therapies for the treatment of a variety of cancers including colorectal cancer (reviewed by ref. 27). Resistance to 5FU, however, arises relatively often and limits the use of this drug. Understanding the mechanisms of 5FU cytotoxicity is an important step toward the development of new strategies to minimize drug resistance. 5FU was originally designed to inhibit DNA replication by blocking the de novo synthesis of thymidylate (28). However, 5FU is also incorporated into
RNA and alters RNA metabolism through mechanisms that are not fully understood (reviewed by ref. 2). The human Rrp6 protein has recently been identified as a target for 5FU (18). We have now investigated the direct effect of 5FU on the activity of Rrp6 and we have identified two aspects of Rrp6 function that are altered in 5FU-treated cells. First, the repertoire of multimolecular complexes that contain Rrp6 is modified by the exposure of cells to 5FU. The molecular mass of the nuclear exosome is altered, which suggests that its composition or its association with other factors is modified. This observation is consistent with the suggestion that incorporation of 5FU into RNA leads to sequestration of Rrp6 (7, 18). Second, the ribonucleolytic activity of Rrp6 is less efficient on RNA that contains 5FU than it is on normal RNA. This is a direct effect of the incorporation of 5FU into RNA that can be measured in vitro under conditions in which the influence of other activities or processes, such as RNA post-transcriptional modifications, can be ruled out.

Early studies showed that the exposure of cells to 5FU inhibits the processing of pre-rRNA (29). In vitro experiments based on the use of mouse cell extracts revealed that the incorporation of 5FU into pre-rRNA did not have a direct effect on pre-mRNA processing, but that the ability of cell extracts to catalyze pre-rRNA cleavage reactions was impaired by the 5FU treatment (6). This observation led to the proposal that inhibition of pre-rRNA processing by 5FU was due to the inactivation of a transacting processing factor (6). In a recent study (7), Hoskins and Butler have implicated the activity of the yeast rRNA pseudouridylase Cbf5p in the cytotoxicity of 5FU, and have suggested that Cbf5p forms stable adducts with 5F-RNA in cells treated with 5FU. The toxic effect of 5FU may thus be partly mediated by the sequestration of pseudouridylases in inactive ribonucleoprotein complexes (7). In such a scenario, pseudouridylation reactions would be inhibited and the 5F-RNA bound to pseudouridylases would be degraded by Rrp6 and the nuclear exosome. However, the results presented here predict that the ability of Rrp6 to resolve such adducts is limited and that cells grown in the presence of 5FU accumulate large amounts of stable 5F-RNAs. The presence of cofactors such as the TRAMP complex might enhance the activity of Rrp6 in vitro, but several observations suggest that 5FU-treated cells do accumulate different types of 5F-RNA species. A study aimed at characterizing the effects of 5FU on the transcriptome of yeast cells revealed a significant accumulation of polyadenylated rRNA precursors in cells exposed to the drug (25). Furthermore, a similar study carried out in human cells showed a specific stabilization of Rrp6 RNA substrates upon 5FU treatment (18).

Rapidly growing cells, such as tumor cells, need robust and active RNA biogenesis pathways to continuously build up the large amounts of ribonucleoprotein complexes, mostly ribosomes, that are needed for cell growth. The results presented here together with results of other authors establish that 5FU affects RNA metabolism in multiple ways. On the one hand, the incorporation of 5FU into RNA blocks the processing of RNA precursors, including pre-mRNAs and rRNAs (4, 5, 7), which in itself is detrimental for RNA biogenesis. Moreover, 5F-RNA sequesters pseudouridylases in stable ribonucleoprotein complexes, which further limits the capacity of the cell to modify RNA precursors (7). On the other hand, 5FU makes RNA less susceptible to degradation by the surveillance machinery, as shown here, and reduces the cellular levels of Rrp6 (18), a consequence of which is that aberrant transcripts synthesized by 5FU-treated cells cannot be turned over efficiently. This results in the accumulation of stable 5F-RNA intermediates. Some of the RNAs that are stabilized in human cells treated with 5FU are endogenous retroviruses of the HERV family (18). Most HERV sequences are inactive but some have retained their coding capacity, and their expression may cause chromosomal instability (reviewed by ref. 30).

In summary, the cytotoxic activity of 5FU at the RNA level is the result of at least three effects: the increased levels of retroviral elements with mutagenic potential, the reduced synthesis of ribosomes, and the inhibition of the nuclear RNA surveillance pathway. Drugs that reinforce any of these effects will probably boost the cytotoxic activity of 5FU.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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