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Specific Inhibition of Pre-Ribosomal RNA Processing in Extracts from the Lymphosarcoma Cells Treated with 5-Fluorouracil

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Abstract

To elucidate the molecular mechanism by which the potent anticancer drug, 5-fluorouracil (5-FUra), inhibits cell proliferation, the effect of its metabolite, 5-fluorouridine triphosphate, on transcription of rat rRNA gene and processing of pre-rRNA was investigated in S-100 extract from the mouse lymphosarcoma cells. The in vitro processing of pre-rRNA substrate synthesized from the T3 promoter occurred at the correct primary processing site. Replacement of UMP with 5-fluorouridine monophosphate in the rRNA substrate did not affect the pre-rRNA processing. Similar result was obtained when coupled transcription-processing was studied. When the coupled reaction was examined using extracts from the cells treated with 5-FUra, rRNA processing was abolished whereas transcription of rRNA gene was unaffected. Treatment of cells with thymidine along with 5-FUra did not reverse the inhibitory effect of the drug on rRNA processing. In contrast to the effect on rRNA processing, treatment of cells with 5-FUra did not impede the 3' end processing of pre-mRNA. These data show that inhibition of pre-rRNA processing is a major mechanism of action of 5-FUra and suggest that the activity and/or synthesis of a trans-acting factor(s) involved in this reaction is altered by the anticancer drug.

Introduction

The pyrimidine analogue 5-FUra is widely used in the treatment of a variety of solid tumors such as colorectal, breast, and liver carcinomas. The primary mechanism of action of this potent drug used for cancer chemotherapy since the 1950s is still being debated. Although inhibition of thymidylate synthetase is generally accepted as the major mechanism of its cytotoxic action (1), other modes of action mediated by its incorporation into RNAs have received attention in recent years. These include alterations in mRNA expression (2), inhibition of pre-rRNA processing (4), and interference with tRNA (5) modification. These data show that inhibition of pre-rRNA processing is a major mechanism of action of 5-FUra and suggest that the activity and/or synthesis of a trans-acting factor(s) involved in this reaction is altered by the anticancer drug.

Materials and Methods

Materials. 5-FUra and thymidine were purchased from Sigma Chemical Company, St. Louis, MO. 5-FUTP was a generous gift from Dr. B. J. Dollnick, University of Wisconsin, Madison, WI.

Plasmid Constructs. The plasmid, pB 7-2.0 spanning —167 to +2000 base pairs of rat rDNA gene linearized with EcoRI, was used to study coupled transcription and processing assay. The region +636 to +2000 base pairs spanning the primary processing site was generated by digestion with the

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1 The abbreviations used are: 5-FUra, 5-fluorouracil; rDNA, ribosomal RNA gene; nt, nucleotide; FUTP, fluorouridine triphosphate; DTT, dithiothreitol; FUMP, fluorouridylic acid; poly(A), polyadenylate.
restriction enzymes Xho I and Sal I and cloned into the same restriction sites of pBluescript KS(−) (Promega). This construct, pT3(X-S) linearized with ApaL I, was used to study uncoupled processing. A schematic representation of the plasmid spanning the pre-rRNA processing site and the processing reaction is presented in Fig. 1A.

**Cell Culture and Preparation of S-100 Extract.** Mouse lymphosarcoma cells, P1798 (a generous gift from Dr. Aubrey E. Thompson), were grown in RPMI 1640 supplemented with penicillin, streptomycin, and fetal bovine serum (5%) (18). The cells grown at a density of 2.5 × 10^6 cells/ml were treated with thymidine (10 μM), thymidine plus 5-FUra (10 μM each), or 5-FUra alone (10 or 20 μM).

Cells were cultured in spinner flasks for 24 h in the presence or absence of the drug and harvested by spinning at 2000 rpm for 10 min at 4°C in a Sorval GS3 rotor. The cell pellet was washed twice with phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 1.2 mM KH₂PO₄, and 8.1 mM NaH₂PO₄) in a Beckman Accuspin centrifuge at 2000 rpm for 5 min at 4°C. S-100 extract was then prepared according to the protocol of Mahajan and Thompson (18). The extract was then dialyzed overnight against buffer D (20 mM-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.9), 20% glycerol, 5 mM MgCl₂, 0.2 mM EDTA, 120 mM KCl, and 0.5 mM DTT) with two changes of the buffer. Protein concentrations in the extracts were measured by bicinchoninic acid reagent (Pierce) according to the manufacturer’s protocol.

**Coupled rDNA Transcription and Processing at the Primary Site.** The coupled transcription and processing assay was done essentially by the method of Craig et al. (19). To study coupled transcription and processing, pB7-2.0 was linearized at the ApaL I site just 3’ to nt +1149. The 50-μl reaction mixture contained 120 mM KCl; 5 mM MgCl₂; 1 mM DTT; 0.1 mM EDTA; 10% glycerol; 0.5 mM concentrations each of ATP, GTP, and UTP; 0.03 mM CTP; and 1.5 μl of [α-32P]CTP (specific activity, 800 Ci/mmol); 100 ng of the linearized template DNA; and 5–10 μl of the extract. After 30 min incubation at 30°C, the reaction mixture was split into two 25-μl halves. One half of the reaction was stopped (“pulse”) with 185 μl of the stop buffer (160 mM Tris (pH 8.0), 300 mM NaCl, 1 mM EDTA, 300 mM sodium acetate (pH 5.2), 0.4% sodium dodecyl sulfate, and 1 μg/ml tRNA). To the other half, 1 μl of 100 mM CTP was added and was incubated immediately at 30°C for another 30 min ("chase"). The reactions were stopped and RNA was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). The supernatant (200 μl) was precipitated with ethanol and the precipitated RNA was analyzed on polyacrylamide (4% acrylamide)/8.33 M urea gel and detected by autoradiography. To study the effect of 5-FUTP on transcription and processing, UTP in the reaction mixture was replaced by 5-FUTP.

**Uncoupled Processing of Pre-rRNA at the Primary Site.** Labeled pre-rRNA was synthesized from Apal I linearized pT3(X-S) by using [α-32P]CTP and T3 polymerase as specified by the supplier (Promega). To prepare 5-FUMP-containing RNA, UTP in the reaction mixture was completely replaced by an equal amount of 5-FUTP. T3 polymerase can efficiently utilize 5-FUTP as substrate. Labeled RNA was then extracted, ethanol precipitated, and purified by polyacrylamide (4% acrylamide)/8.33 M urea gel electrophoresis. For processing, 2 to 5 fmol of the [α-32P]CMP-labeled RNA produced under the direction of T3 promoter and T3 polymerase were added to a 25-μl reaction mixture containing 5–10 μl of S-100 extract, 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.9), 120 mM KCl, 2 mM MgCl₂, 1.5 mM ATP, 1 mM EDTA, 0.1 mM EDTA, and 10% glycerol. The reaction mixture was incubated for 60 min at 30°C. The RNA products were analyzed as described earlier for coupled transcription and processing assay.

**Preparation of Nuclear Extract and Assay of 3’ End Processing Activity.** Nuclear extract was prepared according to the method of Terns and Jacob (20). Cleavage and polyadenylation reactions were studied with adenyl L3 pre-mRNA as the substrate, which was produced from the plasmid pSP3L3MD (21) using SP6 RNA polymerase. This plasmid was generously provided by Dr. James Stefano.

**Results and Discussion**

**Efficient and Accurate Cleavage of Rat Pre-rRNA Substrate in the Mouse Lymphosarcoma Cell Extract.** The construct, pT3(X-S), spanning the primary processing site upon transcription by T3 polymerase yielded a product of 341 nt (28 nt of vector that is transcribed plus 513 nt of rRNA). Cleavage at the primary processing site of this RNA produced a 3’ processed product of 354 nt (see Fig. 1A for the schematic representation of the process) based on the migration of the molecular weight markers (Fig. 1B, Lanes 2–5). The size of the product was consistent with that predicted from the in vivo processing of rat rRNA precursor at the primary site (18) that spans between 787 and 797 nt in the external transcribed spacer region of rat rRNA gene (17). It should be noted that unlike pre-mRNA processing, pre-rRNA processing at the primary site involves cleavage at two sites spanning a narrow range of 11 nts (17, 22). When the substrate was incubated alone, no such specific cleavage was observed (Fig. 1B, Lane 1). The optimal cleavage was obtained with 3 μl of the extract (compare Lane 3 with Lane 2, and Lane 7 with Lane 6) and reached the plateau at this concentration. The 5’ product (187 nt long) was not detected in this assay, which was consistent with the known rapid degradation of this product in vivo and in vitro (23). These data show that rat pre-rRNA can be efficiently processed in the mouse cell extract and like pre-rRNA
mRNA processing (24), pre-rRNA processing can occur in vitro independent of transcription.

**FUMP-containing Pre-rRNA is Processed Efficiently in S-100 Extract from the Mouse Lymphosarcoma Cells.** There are at least two explanations for the inhibition of rRNA processing in vivo following 5-FUra treatment of the cells: (a), incorporation of 5-FUMP into pre-rRNA may alter its secondary structure and consequently affect its interaction with the processing machinery; (b), 5-FUra or its metabolites may inactivate one or more of the trans-acting factors involved in pre-rRNA processing reaction. We explored these possibilities using the in vitro processing reaction. When the RNA substrate containing 5-FUMP was incubated in the S-100 extract (see “Materials and Methods” for the preparation of the extract and in vitro processing reaction), it was efficiently and accurately processed (compare Lanes 6–9 with Lanes 2–5 in Fig. 1B), which ruled out the direct effect of 5-FUMP-incorporated pre-rRNA on the processing activity. These data are consistent with the in vivo observation (8) and also suggest that a trans-acting factor(s) is (are) modified by 5-FUra treatment, which leads to a block in rRNA processing.

**Transcription of rRNA Gene and Its Subsequent Processing (Coupled Transcription-Processing) Are Not Inhibited by FUTP.** S-100 extract could efficiently transcribe the rDNA when linearized with ApaL I (Fig. 2, Lane 1). A 1149-nt-long transcript was synthesized during “pulse” (indicated by “p”). During “chase” period (indicated by “c”), in the presence of excess cold CTP, the transcript was processed at the primary processing site to give the 5’ product of 795 nt and the 3’ product of 354 nt. The 5’ product was rapidly degraded by the nuclease present in the extract and, therefore, only the 3’ end product was detected (Fig. 2, Lane 2). It should be noted that the processing of the nascent transcript is not as efficient as the uncoupled processing (independent of transcription) in the same extract (compare Fig. 2 with Fig. 1) When UTP in the reaction mixture was replaced by 5-FUTP, neither transcription (Fig. 2, Lane 3) nor processing (Fig. 2, Lane 4) was affected. These data support the in vivo observation that 5-FUra can be efficiently incorporated into rRNA (6).

**Coupled or Uncoupled Processing Is Inhibited in S-100 Extract of the Mouse Lymphosarcoma Cells Treated with 5-FUra.** Since 5-FUTP did not inhibit processing of rRNA directly, an alternative possibility is the inactivation of a trans-acting factor(s) that is involved in the processing reaction. To test this idea, S-100 extract was prepared from the cells treated for 24 h with 10 μM 5-FUra. Under this condition, cell growth was inhibited by 30% (data not shown). When this extract (equal amount of protein) was used for coupled transcription and processing, transcription proceeded unabated (Fig. 2, compare Lane 5 with Lane 1) when UTP was used as the substrate, but processing was inhibited almost completely (compare Lane 6 with Lane 2). The same result was obtained when 5-FUTP replaced UTP in the reaction mixture. In presence of 5-FUTP, transcription of rRNA gene or processing was comparable to that obtained with UTP as the substrate (Lane 7 versus Lane 5); transcription was unaffected whereas processing of pre-rRNA was inhibited almost completely (compare Lane 8 with Lane 4). These data indicate that 5-FUra treatment results in the alteration of a factor(s) responsible for rRNA processing at the primary processing site.

To determine whether treatment of cells with the pyrimidine analogue also inhibits rRNA processing independent of transcription (uncoupled reaction), T3 polymerase-transcribed rRNA spanning the processing site was incubated with S-100 extract under different conditions. As a control, cells were treated with thymidine (10 μM) instead of 5-FUra (Fig. 3, Lane 2). When cells were incubated with 5-FUra, processing of pre-rRNA at the primary site was significantly inhibited (compare Lanes 4 and 5 with the control Lane 2). At the higher concentration (20 μM) of 5-FUra, inhibition was 60% of the...
incubated with adenosine 3' end, a poly(A) tail of (see "Materials and Methods" for details). The pre-mRNA (188 nt) is first cleaved under the cleavage condition, there was no decrease in the cleavage activity as judged by the formation of almost equal amounts of the cleaved RNA (149 nt), (Fig. 4B, Lane 4 versus Lane 5). The cleaved product was also efficiently polyadenylated in the control as well as 5-FUra-treated cell extracts (Lane 2 versus Lane 3). These data suggest that unlike rRNA processing, treatment of the cells with 5-FUra does not affect mRNA 3' end processing.

The present study has demonstrated directly that 5-FUra, a potent anticancer drug, inhibits pre-rRNA processing at the primary processing site without affecting ribosomal gene transcription. The 3' end processing by polyadenylation is not altered by the drug. The persistent inhibition of rRNA processing in vitro and of cell growth following combined treatment of cells with 5-FUra and thymidine suggests that the cytotoxic action of 5-FUra is not solely attributed to inhibition of DNA replication, which is consistent with the in vivo data. Alteration in the activity and/or synthesis of a trans-acting factor rather than inefficient utilization of 5-FUMP-containing RNA is responsible for the drug-induced inhibition of pre-rRNA processing. The nature of the factor affected by 5-FUra is not evident. 5-FUra may inactivate this factor or inhibit synthesis of the factor via inhibition of splicing or other modification of mRNA for the specific factor. Since none of the protein factors involved in pre-rRNA processing has been characterized in the animal systems, purification of these factors should be the first step in resolving this issue. Studies along these lines are in progress.

The 3' end processing activity of pre-mRNA in the nuclear extract of lymphosarcoma cells is not affected by 5-FUra treatment. When [32P]-labeled pre-mRNA spanning the poly(A) site is incubated with adenosine 3' end, a poly(A) tail of 250-300 adenylate residues is added. B, 3' end processing (cleavage and polyadenylation) of adenine 3' end pre-mRNA in the nuclear extract from 5-FUra-treated lymphosarcoma cells. Nuclear extracts (25 ug of protein) from the mouse lymphosarcoma cells treated with thymidine (Lanes 2 and 4) and thymidine plus 5-FUra (Lanes 3 and 5) were incubated under either polyadenylation (Lanes 2 and 3) or cleavage (Lanes 4 and 5) condition as described in the text. The products were analyzed by polyacrylamide (6% acrylamide) -8.33 M urea gel electrophoresis followed by autoradiography. Lane 1, pre-mRNA substrate incubated in the absence of the nuclear extract; Lane M, molecular weights of DNA marker; on left ordinate, sizes in nucleotides.

control value. To prove that the inhibition of rRNA processing occurs independent of DNA replication, cells were incubated with 10 mm concentrations each of 5-FUra and thymidine (Lane 3). Inhibition of rRNA processing under this condition was identical to that observed with 5-FUra (10 mm) alone (Lane 4). Transcription of rRNA gene under this condition remained unaltered (data not shown). Inhibition of cell growth could not be reversed significantly by the combined treatment of 5-FUra and thymidine (data not shown), which further proves that repression of rRNA processing by the anticancer drug is not contingent upon its inhibition of DNA replication.

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MOLECULAR MECHANISM OF ACTION OF 5-FLUOROURACIL


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