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# The inhibitory effects of 5-fluorouracil on the metabolism of preribosomal and ribosomal RNA in L-1210 cells in vitro

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**Summary.** Addition of 5FU to the culture medium of mouse L-1210 cells resulted in inhibition of the maturation process of ribosomal RNA precursors in vitro. In the presence of $10^{-6}M$ 5FU for 2 h, the 45S preribosomal RNA was processed to 32S preribosomal RNA, but 28S rRNA was not produced. The processing to 18S rRNA was intact at this drug concentration. Higher concentrations of 5FU for a longer incubation period affected the RNA processing more severely. At $10^{-5}M$ of the drug for 24 h the processing to 18S rRNA was also inhibited, in addition to the processing to 28S rRNA and 32S preribosomal RNA. When the cells were labeled with $^{14}$C-UR for 2 h following $^{3}$H-5FU at $10^{-6}M$ for 24 h, the radioactivities of newly synthesized RNA labeled with $^{14}$C-UR accumulated in the region of 45S and 32S preribosomal RNA, and no processing to 28S rRNA was observed. Radioactivity corresponding to $^{3}$H-5FU did not persist in the preribosomal RNA region, because further maturation proceeded in the condition of depletion of 5FU after the long incubation period. Thus, inhibition of the processing of preribosomal RNA to 28S rRNA was not brought about by the accumulation of 5FU-substituted 45S preribosomal RNA, but by some other, yet unknown, mechanism.

## Introduction

Since 5-fluorouracil (5FU) was first introduced in 1957 [4], it has been one of the most commonly used anticancer drugs in the clinical treatment of certain types of cancer [1, 10, 11, 13]. The main mechanism of the effect has been considered to be inhibition of DNA synthesis by one of the metabolites, FdUMP, through its covalent binding to thymidilate synthetase in the presence of *N*-methylene-tetrahydrofolate [8, 9]. On the other hand, 5FU was shown to be incorporated to various classes of cellular RNA at a substantial rate and produce structurally and functionally defective RNAs [3, 5–7, 10, 12, 16]. A number of reports have shown that 5FU also inhibits the metabolism of rRNA, especially the processing of rRNA [2, 17–19]. Wilkinson et al. reported that the inhibition of ribosomal RNA maturation by 5FU in Novikoff hepatoma cells appeared to depend upon the incorporation of the analog into 45S rRNA precursor [17]. In mammalian cells, mature 28S and 18S rRNA are produced in the nucleolus from a common precursor by a common complex maturation process [18, 20]. The initial rRNA transcript product is a 45S molecule, which contains the sequence of both 28S and 18S rRNA and additional sequences whose functions have not yet been defined [19]. The 45S molecule is methylated in both sugar and base moieties either during or soon after its synthesis. After methylation, the 45S molecule is converted to a 41S molecule, which also contains the sequence for both 28S and 18S rRNA. The 41S molecule is cleaved to a 32S molecule and a 20S molecule, which are the immediate precursors of mature 28S and 18S rRNA, respectively [14]. 5FU is metabolized, phosphorylated, converted to FUTP, and incorporated into 45S ribosomal precursor tion of uracil, and this precursor appears to be processed finally to mature 28S and 18S ribosomal RNA.

However, it has been reported that maturation of 5FU-containing RNA is severely inhibited at the 45S and 32S regions causing congestion of preribosomal RNA to occur [17–19].

The present experiments were designed to investigate the effect of 5FU on the processing of preribosomal RNA, particularly that of 5FU incorporation into the precursor molecule.

## Materials and methods

*Cells and incubation medium.* L-1210 cells maintained in BDF1 mice were obtained 3 days after inoculation and introduced into the RPMI 1640 incubation medium supplemented with 10% fetal bovine serum and 10 $\mu M$ 2-mercaptoethanol. The cells were grown at 37°C in suspension culture under an atmosphere of 5% $CO_2$: 95% air.

*Incorporation of 5FU into nucleic acids.* L-1210 cells were cultured by plating $1 \times 10^6$ cells into Falcon dishes 60 mm in diameter and containing 5 ml medium in the presence of 1 μCi $^{3}$H-UR in various concentrations of 5FU, or $10^{-6}M$ $^{3}$H-5FU for 24 h, followed by 1 μCi $^{14}$C-UR for an additional 2 h. After the incubation, the cells were collected by centrifugation at 500 rpm for 10 min, and washed

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*Abbreviations used.* 5FU, 5-fluorouracil; rRNA, ribosomal RNA; FdUMP, 5-fluoro-2-'deoxyuridine 5'-monophosphate; FUTP, 5-fluorouridine 5'-triphosphate; UR, uridine

twice with ice-cold saline. The cells thus treated were used for RNA extraction.

*Extraction of RNA.* RNA was extracted by sodium dodesyl sulfate methods directly from the labeled cells without prior preparation of the nuclear or nucleolar fraction as an RNA source [19]. Briefly, the cell pellet was suspended by vortexing in buffer containing 10 μ*M* sodium acetate (pH 5.1), 0.14 *M* NaCl, 0.1 *M* ethylene diamine tetraacetic acid-sodium salt (EDTA-Na) and polyvinyl sulfate-K (20 μg/ml). Then 0.1 ml 3% sodium dodesyl sulfate was added to the cell mixture to give a final concentration of 0.3%. An equal volume of water-saturated phenol solution (phenol : water : *m*-cresol, 7 : 2 : 1,8-hydroxyquinoline 1 g/liter) was added, and the mixture was shaken vigorously at 60°C for 5 min.

After cooling in an ice bath, the phases were separated by centrifugation and the phenol phase was removed and discarded. The aqueous phase and interphase were extracted again with another equal volume of phenol solution. After the centrifugation, the aqueous phase was transferred to an another tube and extracted again with an equal volume of the phenol phase. RNA was precipitated from the final aqueous phase with 2 vol 95% ethanol containing 2% potassium acetate at −20°C overnight. Precipitated RNA was collected by centrifugation, washed twice with 75% ethanol containing 2% potassium acetate, and dissolved in distilled water.

*Sucrose density gradient centrifugation.* RNA precipitated from the aqueous phase was collected by centrifugation, washed with 95% ethanol, and dissolved in a small amount of 0.05 *M* sodium acetate solution at pH 5.1. RNA thus prepared was placed gently on a 15-ml, 5%–30% linear sucrose gradient containing 0.01 *M* sodium acetate, 0.1 *M* NaCl, and 1 m*M* EDTA, and centrifuged in a Hitachi RPS 25.3 rotor at 22000 rpm for 18 h at 4°C. The gradient was fractionated with the aid of an ISCO Model UA5 density gradient fractionator with monitoring absorbance at 254 nm. The radioactivity was determined for each fraction.

*Determination of radioactivity.* To each 0.6 ml sucrose density fraction an equal volume of distilled water was added, after which 10 ml ACS II aqueous counting scintillant (Amersham Corp., UK) was added in a scintillation vial. The radioactivity was counted by means of separation windows for each isotope in a Beckman LS 150 liquid scintillation spectrometer. Appropriate corrections were made for the contamination of $^{14}C$ radioactivity in the $^{3}H$ window. With this technique, counting efficiency was almost constant throughout the gradient for each isotope.

## Results

The effects of 5FU on the processing of preribosomal RNA of L-1210 cells are shown in Figs. 1 and 2. As indicated in Fig. 1, when the cells were incubated with $^{3}H$-UR for 2 h it was evident that 45S preribosomal RNA was well processed to 28S and 18S rRNA. Although separation of either the nuclear or the nucleolar fraction was omitted before the RNA extraction, our extraction procedure is considered to be satisfactory for the analysis of RNA metabolism.

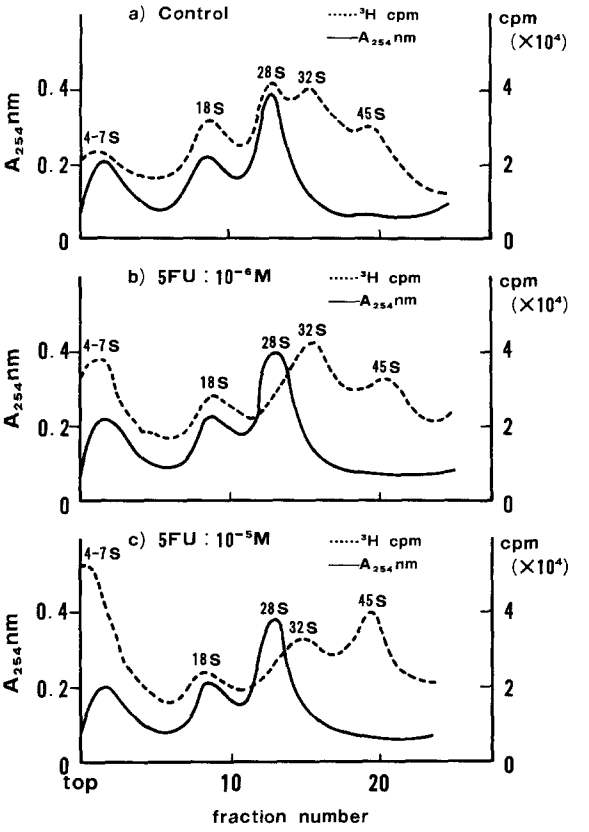


**Fig. 1a–c.** Processing of preribosomal RNA of L-1210 cells labeled with $^{3}H$-UR for 2 h in the absence or presence of 5FU. After incubation, the RNA was extracted from the whole cells as described in the text. RNA was extracted from *a* control cells labeled with $^{3}H$-UR: . . ., cpm ($^{3}H$); ———, optical density (A254 nm); *b* cells labeled with $^{3}H$-UR in the presence of $10^{-6}$ *M* 5FU: . . ., cpm ($^{3}H$); ———, optical density (A254 nm); and *c* cells labeled with $10^{-5}$ *M* 5FU: . . ., cpm ($^{3}H$); ———, optical density (A254 nm). The repetitive experiments were carried out independently and a similar result was obtained in the second experiment

In the presence of 5FU at $10^{-6}M$, the peak of radioactivity was not seen in 28S rRNA, but 18S rRNA was unaffected (Fig. 1 b). This tendency was more marked in the presence of 5FU at $10^{-5}M$. The 45S preribosomal RNA was most prominent among classes of rRNAs, suggesting that both the conversion of 45S to 32S preribosomal RNA and the cleavage to 28S rRNA were inhibited.

When the cells were incubated with 5FU for 24 h before $^{3}H$-UR for 2 h, the inhibition of the processing was more evident. As shown in Fig. 2b, 5FU at even $10^{-6}M$ inhibited the conversion of 45S preribosomal RNA to 32S preribosomal RNA. Moreover, in the presence of $10^{-5}M$ 5FU the conversion of 45S preribosomal RNA to 18S ribosomal RNA was also inhibited (Fig. 2c).

When the cells were treated with $^{3}H$-5FU at $10^{-6}M$ for 24 h to see the distribution of false RNA after the long incubation period, the radioactivity peak corresponding to 28S rRNA was lower than that to 18S rRNA, and the peak of 4–7S RNA was higher than the control peak. The high peak in the 4–7S region might be due to degradation products coming from 28S or to larger preribosomal RNA (Fig. 1c), suggesting that RNA larger than 28S rRNA degraded via 28S and 18S rRNA or by other ways, as seen in

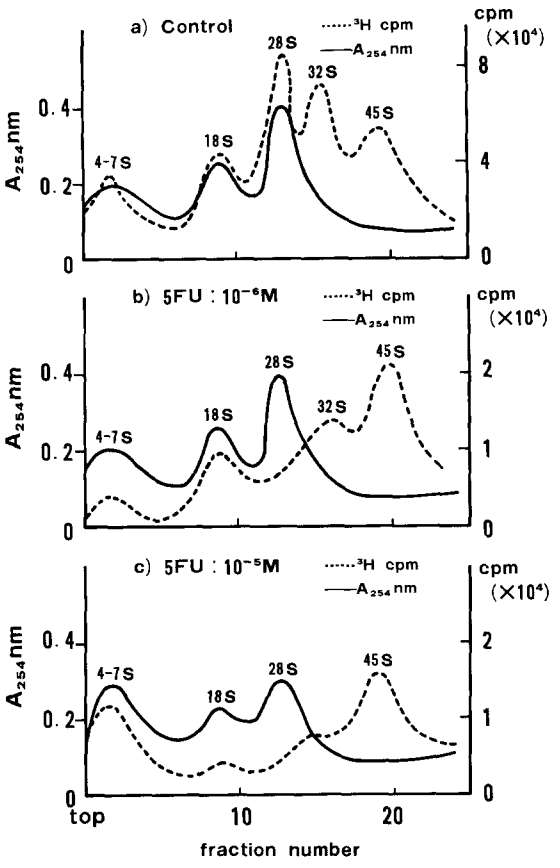


**Fig. 2a–c.** Processing of preribosomal RNA of L-1210 cells labeled with $^3$H-UR for 2 h following 5FU for 24 h. After incubation, the RNA was extracted from whole cells as described in the text. RNA was extracted from *a* control cells labeled with $^3$H-UR: . . ., cpm ($^3$H); ———, optical density (A254 nm); *b* cells incubated in the presence of $10^{-6}$ *M* 5FU for 24 h followed by $^3$H-UR for 2 h: . . ., cpm ($^3$H); ———, optical density (A254 nm); and *c* $10^{-5}$ *M* 5FU for 24 h followed by $^3$H-UR for 2 h: . . ., cpm ($^3$H); ———, optical density (A254 nm). Repeat experiments were carried out independently and a similar result was obtained in the second experiment

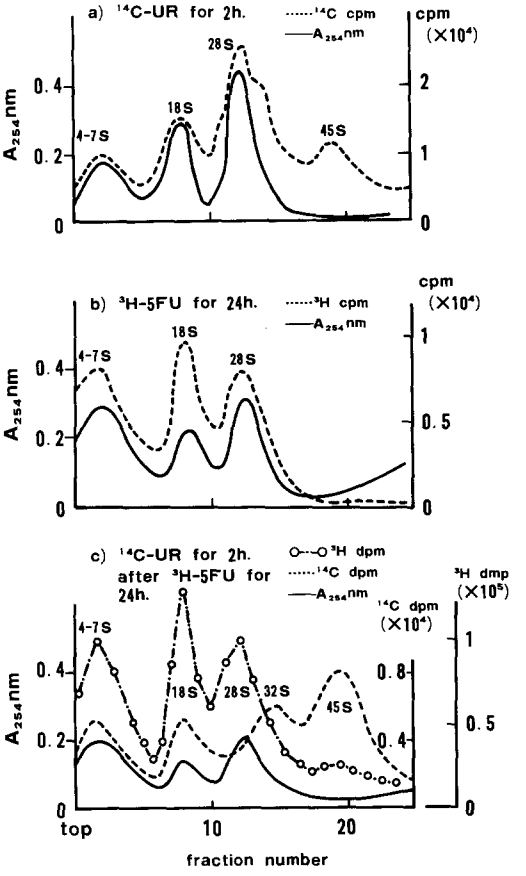


**Fig. 3.** Processing of preribosomal RNA of L-1210 cells treated with $^3$H-5FU for 24 h followed by $^{14}$C-UR for 2 h. After the incubation, the RNA was extracted from whole cells as described in the text. RNA was extracted from cells labeled with *a* $^{14}$C-UR for 2 h: . . ., cpm ($^{14}$C): ———, optical density (A254 nm); *b* $^3$H-5FU for 24 h: . . ., cpm ($^3$H); ———, optical density (A254 nm); and *c* $^{14}$C-UR for 2 h after $^3$H-5FU for 24 h: O– – –O, cpm ($^3$H); . . ., cpm ($^{14}$C); ———, optical density (A254 nm). Repeat experiments were carried out independently and a similar result was obtained in the second experiment

Fig. 3b. The 5FU molecule was not contained in the preribosomal regions owing to maturation of rRNA under the condition of 5FU depletion in the incubation medium.

Figure 3c shows the sedimentation profile of the RNA obtained from the cells treated with 1 μCi $^{14}$C-UR for 2 h after $^3$H-5FU at $10^{-6}M$ for 24 h. The tritium peak produced by $^3$H-5FU for 24 h is identical with that seen in Fig. 3b. However, for peaks produced by $^{14}$C-UR for 2 h after $^3$H-5FU for 24 h, the radioactivity of $^{14}$C accumlated in the regions of 45S and 32S preribosomal RNA. Their processing to 28S rRNA was not observed (Fig. 3c) although no 5FU molecule was contained in preribosomal RNA regions.

## Discussion

In eukaryotic organisms, the ribosomal RNA transcriptional unit has the general form spacer – 18S rRNA – spacer – 28S rRNA. According to many workers, in mouse cell lines 45S preribosomal RNA has four principal cleavage sites numbered 1–4 from the 5'end. Assuming that 18S rRNA is formed by the second cleavage before the fourth cleavage, by which 28S rRNA is produced [19], the formation of 28S rRNA might be affected more severly than that of 18S rRNA by 5FU. This might be why processing of the precursor containing 5FU to 28 rRNA is inhibited more easily and earlier than that to 18S rRNA.

The processing of preribosomal RNA of Novikoff hepatoma cells grown in suspension culture was inhibited in a manner similar to the inhibition of the processing in rat liver by 5-fluoro-orotic acid. An adenosine analogue, toyokamycin, completely inhibited 28S and 18S rRNA synthesis in L cells growing in suspension culture [15]. This compound permitted the synthesis of 45S preribosomal RNA but inhibited further processing. It was postulated that in the case of toyokamycin, drug-substituted 45S preribosomal RNA had altered physicochemical characteristics, which inhibited its maturation [20]. This could also be the case for maturation inhibition of 5FU-substituted 45S preribosomal RNA. Wilkinson et al. reported that the ribosomal RNA molecules, both precursor and mature, produced in the presence of 5FU appeared to have normal

molecular weight, and suggested that the mechanism of inhibition of ribosomal RNA maturation by the analogue did not depend on premature chain termination [17, 18]. It has been postulated that the ability of analogues to inhibit rRNA maturation may be related to their ability to be incorporated into 45S rRNA precursor and the extent to which that incorporation alters the physicochemical characteristics of the molecule. HeLa cell 45S rRNA precursor has a series of hairpin loops, which is due to the base pairing. It is possible that the arrangement of loops is one of the signals that the processing enzymes can recognize. The analogue substitution could alter the conformation of the loops and maturation enzymes would not recognize [18]. Wilkinson et al. concluded that the inhibition of rRNA maturation by 5FU did depend on the incorporation of the analogue into 45S rRNA precursor, and that inhibited processing of 5FU-substituted 45S precursor decreased the synthesis of new 45S molecule [17, 18]. The inhibition of rRNA maturation by 5FU has been reported to persist even after prolonged incubations following removal of the drug from medium [17]. Wilkinson et al. considered that this finding might be related to the extremely slow disappearance of 5FU-substituted rRNA precursors from the cells [17].

However, our experiment, as shown in Fig. 3c, clearly indicated that the processing of newly synthesized preribosomal RNA was markedly inhibited in the absence of 5FU-substituted preribosomal RNA. With incubation for longer periods, i.e., 24 h or longer, there is no accumulation of 5FU-substituted 45S precursors owing to further maturation of RNA in the condition of 5FU depletion in the incubation medium. In addition, it is possible that nonspecific nucleases are constantly degrading the false RNA, especially that of the 45S moiety.

In conclusion, we do not consider that 5FU-substituted preribosomal RNA is responsible for the retardation of the processing of preribosomal RNA. So far, the precise mechanism of the inhibition of RNA processing is unkown, and further investigation is in progress in our laboratory.

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