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18 S rRNA Degradation Is Not Accompanied by Altered rRNA Transport at Early Times following Irradiation of HeLa Cells

PINHAS FUCHS,* JOHN M. KROLAK,† DAVID MCCLAIN,† AND KENNETH W. MINTON‡¹

*Israel Institute for Biological Research, Ness-Ziona, Israel; †Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814-5145; and

‡Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

FUCHS, P., KROLAK, J. M., MCCLAIN, D., AND MINTON, K. W. 18 S rRNA Degradation Is Not Accompanied by Altered rRNA Transport at Early Times following Irradiation of HeLa Cells. *Radiat Res.* 121, 67-70 (1990).

The effect of ionizing radiation (^{137}Cs) on processing and transport of ribosomal RNA (rRNA) was studied by pulse-labeling HeLa S3 cells with [^3H]uridine immediately prior to irradiation. This approach permits kinetic analysis of processing of 45 S rRNA (radiolabeled predominantly prior to irradiation) into its 28 S and 18 S rRNA daughter species following irradiation. By this technique, we have recently demonstrated an increase in the normal 28 S:18 S rRNA stoichiometric ratio of 1:1 to as high as 1.6:1 during the interval 5 to 20 h following irradiation of HeLa cells at ≥ 7.5 Gy. Alterations in 28 S:18 S ratio were evaluated in greater detail at early times following irradiation, up to 2 h. The 28 S:18 S ratio was found to be maximal at 1 h after radiation, at about 2:1, following 5 or 10 Gy. Using a method for rapid separation of nucleus from cytoplasm, transport of rRNA from nucleus to cytoplasm was also evaluated during this period. Despite an increase in the rate of 45 S rRNA processing, as well as an increased 28 S:18 S ratio, no alterations in transport from nucleus to cytoplasm were detected. This lack of transport alteration suggests that accumulation of excess 28 S rRNA is restricted to the nucleus, where it may represent an early step in the process of radiation-induced cell killing. © 1990

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INTRODUCTION

In recent investigations on the effects of radiation on rRNA processing in HeLa S3 cells, we pulse-labeled the cells with [^3H]uridine immediately prior to irradiation (1). The 45 S rRNA precursor, which undergoes nuclear processing to form one each of its major daughter species, 28 S and 18 S rRNA, was separated from the daughter species by gel electrophoresis and the radiolabel in each species determined at various times after irradiation. By pulse-labeling the cells prior to irradiation, superimposed effects caused by radiation-induced alterations of rRNA transcription ((1)

and Refs. therein) were minimized, permitting selective analysis of the processing of that fraction of 45 S precursor that had been synthesized (radiolabeled) predominantly prior to irradiation. Processing in control cells yielded the normal stoichiometric ratio of 28 S:18 S rRNA of 1:1 (1-4). However, irradiated cells showed two effects: (i) an accelerated conversion of radiolabeled 45 S rRNA to 28 S and 18 S species; (ii) an altered stoichiometry of 28 S:18 S rRNA of about 1.6:1 stably maintained from 5 to 20 h following irradiation of 10 or 20 Gy (1).

We now report more detailed studies on 45 S rRNA processing within the first 2 h following irradiation in which we have found a maximum 28 S:18 S ratio of 2:1 that is observed about 1 h following irradiation of 5 or 10 Gy. Concurrent studies on nuclear export of the daughter species do not provide evidence of an altered 28 S:18 S ratio in the cytoplasm.

MATERIALS AND METHODS

Cell Culture

HeLa S3 cells were cultured as previously described (1). All experiments employed asynchronously growing monolayers at $1-2 \times 10^5$ cells/cm², with a doubling time of 20 h. Cells were seeded 3 days prior to use.

Isotope Labeling of Cells and Exposure to Ionizing Radiation

Cells were labeled in complete conditioned medium containing [5,6- ^3H]uridine (50 Ci/mmol; DuPont NEN) at 70 $\mu\text{Ci}/\text{ml}$ for experiments in which RNA was selectively harvested or 10 $\mu\text{Ci}/\text{ml}$ for experiments in which acid-insoluble incorporation was measured. Incubation in the presence of label was for 15 min, after which the flasks were washed three times with unlabeled prewarmed medium. After washing, unlabeled prewarmed, complete conditioned, and CO_2 -equilibrated medium was added to the flasks which were immediately exposed at 70 Gy/h using a ^{137}Cs source at room temperature (1).

Measurement of Radiolabel in rRNA Species

Total RNA was collected and purified using guanidine isothiocyanate (GI), as previously described (1). Cytoplasmic RNA was collected using a modification of the technique of Butler (5): cells were washed twice with ice-cold culture medium and once with ice-cold phosphate-buffered saline, and then treated for 2 min with reticulocyte sensitizing buffer (RSB; ice-cold Tris-HCl, pH 7.0, 10 mM; NaCl, 10 mM; MgCl_2 , 3 mM) to induce osmotic swelling of the cells. The RSB was removed, and monolayers in

¹ To whom correspondence should be addressed.

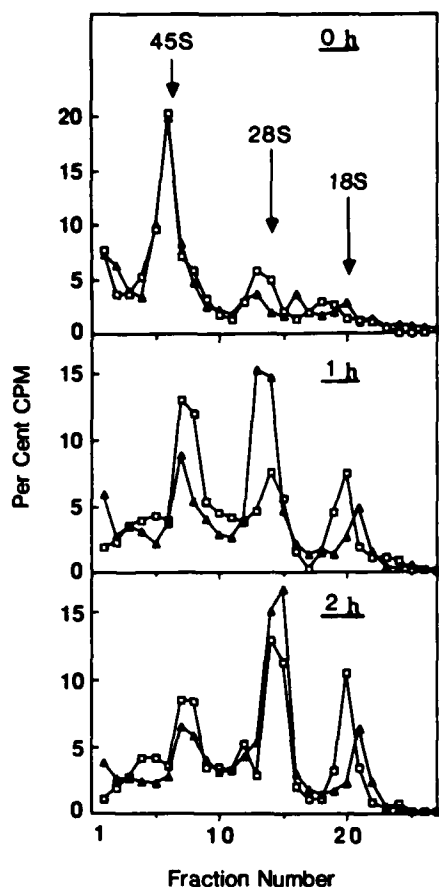


FIG. 1. Gel electrophoresis of whole cell RNA labeled with [^3H]uridine. After 15 min of labeling, flasks were washed in prewarmed conditioned full medium and either exposed to 5 Gy or held at room temperature for the same duration (4.3 min). RNA was then harvested immediately or after further incubation of flasks at 37°C for 1 or 2 h. The arrows indicate the location of rRNA species visualized in the ethidium bromide-stained gels. Control ($\square$); 5 Gy ($\blacktriangle$).

each flask were then dissolved in 0.8 ml of the following: 5 g ethylhexadecyldimethylammonium bromide plus 3 ml glacial acetic acid brought to 100 ml in water. This solution lyses the plasma membrane but not the nuclei (5). Immediately following, 2.3 ml of 10 mM Tris-HCl, pH 7.5, containing 10 mM EDTA and vanadyl ribonucleoside inhibitor (Sigma), 1:20 dilution, was added to each flask, and the resulting solution was centrifuged at room temperature at 1500g for 5 min to pellet the nuclei. The supernatant (cytoplasmic) fraction was ethanol-precipitated twice and the pellet resuspended in GI solution and processed as for whole cells (1). A total of 6.5 μg RNA per lane (either whole cell or cytoplasmic RNA) was taken for agarose-formaldehyde gel electrophoresis, as described (1). The 45 S, 28 S, and 18 S rRNA bands were visualized by ethidium bromide staining of the gel, as described (1). The gels were either sliced into 1-mm fractions and the radioactivity was determined by scintillation counting or blotted onto a nylon membrane; the blot was cut into 2-mm fractions and radioactivity was determined by scintillation counting, as previously described (1).

The modified technique of Butler, described above, consistently yielded undegraded cytoplasmic rRNA, with readily identified, clearly separated 28 S and 18 S peaks (e.g., Fig. 4). In control experiments, the nuclear pellet obtained with this technique was dissolved in GI solution, and RNA was purified as for whole cells (1) and subjected to gel electrophoresis. Unlike

preparations of total or cytoplasmic RNA, the RNA from isolated nuclei using our procedure appeared partially degraded and rRNA species were difficult to identify with confidence.

Measurement of Radiolabel in Acid-Insoluble Fraction

At the desired postirradiation time, monolayers in T-25 flasks were processed into nuclear and cytoplasmic fractions using the modified Butler technique (5) described above. Fifty-microliter aliquots of the cytoplasmic fraction were precipitated on Whatman 3MM filters with 5% trichloroacetic acid. The nuclear pellet was washed twice with ice-cold phosphate-buffered saline, resuspended in 1% SDS, and sheared several times with a 22-gauge needle to reduce the viscosity of the resulting solution, and 50- μl aliquots were precipitated on Whatman 3MM filters with 5% trichloroacetic acid.

RESULTS

HeLa S3 cells were pulse-labeled with [^3H]uridine for 15 min, followed immediately by washing and irradiation. In prior studies under identical conditions (1), we found that (a) the great majority of acid-insoluble incorporation occurred by the end of the 15-min pulse-labeling period; (b) radiation up to 20 Gy did not alter subsequent incorporation of [^3H]uridine into the acid-insoluble fraction; and (c) synthesis of 45 S rRNA precursor following irradiation was unaffected by exposures up to 20 Gy.

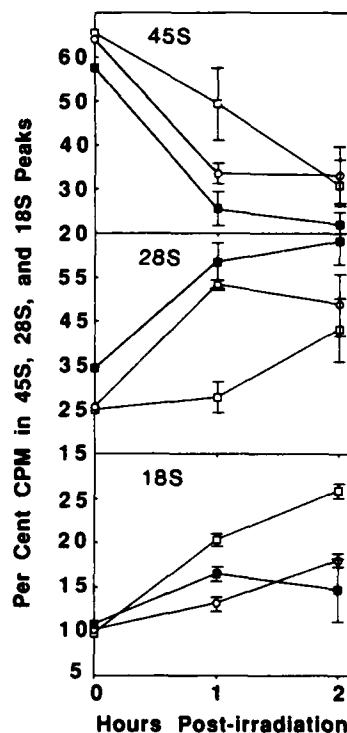


FIG. 2. Relative amounts of 45 S, 28 S, and 18 S rRNA in cells exposed to 5 or 10 Gy. Procedure as in legend to Fig. 1. Two independent experiments on control cells, three on cells receiving 5 Gy, and four on cells receiving 10 Gy were analyzed for the relative areas under the 45 S, 28 S, and 18 S rRNA peaks. Brackets indicate SE. Control ($\square$); 5 Gy ($\circ$); 10 Gy ($\blacksquare$).

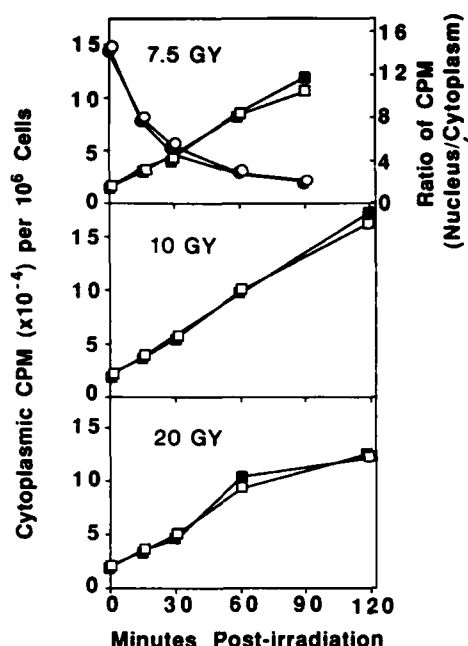


FIG. 3. Transport of acid-insoluble [^3H]uridine from nucleus to cytoplasm. Cells were labeled for 15 min with [^3H]uridine, followed by washing and incubation for indicated duration. All experiments were accompanied by unirradiated controls processed in parallel. Cytoplasmic fraction was analyzed for TCA-precipitable incorporation ($\square$, control; $\blacksquare$, irradiated), and in the case of 7.5 Gy, the nuclear pellet was also analyzed ($\circ$, control; $\bullet$, irradiated). Points represent the means of six determinations. In all cases SE was $<5.2\%$ of the indicated values.

Figure 1 shows electrophoretic profiles of total RNA, pulse-labeled for 15 min with [^3H]uridine prior to an exposure of 5 Gy. RNA was harvested either immediately following irradiation or after further incubation of the flasks for 1 or 2 h at 37°C . Compared to RNA from control cells, at the 1- and 2-h time points there was a decrease in radiolabeled 45 S and 18 S rRNA and an increase in radiolabeled 28 S rRNA. Previous studies (1) focused attention on alterations in the 28 S:18 S ratio that remained the same from 5 to 20 h after irradiation, 20 h being the latest time evaluated. However, studies shown here indicate that alterations in 45 S, 28 S, and 18 S rRNA are even more dramatic at shorter times (Fig. 2). From the experimental data summarized in Fig. 2, it can be calculated that the 28 S:18 S stoichiometric ratio is maximal at 1 h, at about 2:1, following either 5 or 10 Gy, and only about 1:1 in control cells, the expected ratio. These calculations take into account the difference in uracil content in the 28 S and 18 S rRNA species, as previously discussed (1).

Although rRNA species content is altered in total RNA preparations, it is not known whether transport from nucleus to cytoplasm reflects this alteration, yielding perturbations in cytoplasmic rRNA content. It might be expected that the accelerated conversion of 45 S rRNA to smaller species results in a higher rate of transfer of [^3H]uridine-

labeled species to the cytoplasm. However, findings illustrated in Fig. 3 indicate that transfer of acid-soluble label from nucleus to cytoplasm is unaffected following 7.5, 10, or 20 Gy, for up to 2 h. It could be that the increased 28 S:18 S ratio of newly processed rRNA, maximal at 1 h following irradiation, might yield an altered ratio of transport of these daughter species to the cytoplasm without measurably affecting total transport. However, as illustrated in Fig. 4, the content of newly synthesized rRNA in the cytoplasm 60 min following irradiation (approximately 80 min following initiation of 15 min pulse label) is identical to that in control cells. The label in cytoplasmic 18 S rRNA is greater than cytoplasmic 28 S rRNA (Fig. 4); this is consistent with prior findings that the movement of the newly formed 40 S ribosomal subunits from the nuclei in HeLa cells proceeds more swiftly than the movement of the 60 S ribosomal subunit (6).

DISCUSSION

We previously measured the stoichiometric ratio of newly processed 28 S:18 S rRNA in whole HeLa S3 cells at times ≥ 5 h following irradiation (1). The 28 S:18 S ratio was altered in a dose-dependent fashion, deviating from the normal of 1:1 in control cells (1-4) to a maximum of 1.6:1 following 20 Gy (1). The current study extends these observations to earlier times after irradiation, within the first 2 h. We found that the aberrant 28 S:18 S ratio is somewhat larger during the first 2 h (2:1) than at later times (1.6:1), and this difference is detectable following 5 Gy as well as 10 Gy (Fig. 2). At later times (≥ 5 h) an altered 28 S:18 S ratio

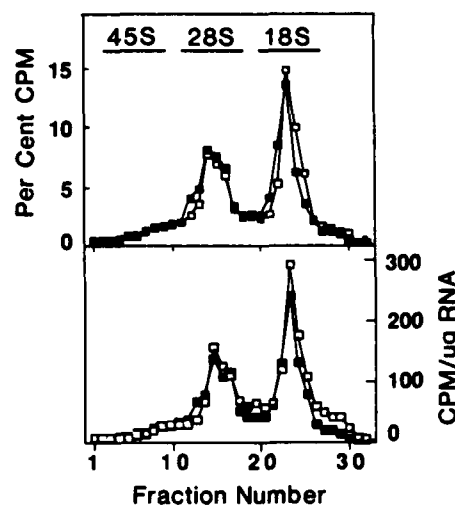


FIG. 4. Labeled rRNA in cytoplasmic fraction 1 h after irradiation. See Materials and Methods for technique and text for further explanation. The location of 28 S and 18 S rRNA species in the ethidium bromide-stained gel are indicated. No 45 S rRNA is present in the cytoplasmic fraction. The location of this species is approximated by comparison to whole cell RNA gels. Control ($\square$); 10 Gy ($\blacksquare$).

was not found following 5 Gy (1), suggesting a degree of cellular recovery following this lower exposure.

As previously, we suggest that the most likely explanation for the altered 28 S:18 S ratio is degradation of the 18 S portion of the 45 S rRNA precursor during or after processing (1). This suggestion is based on the exclusion of three other possible explanations, as follows:

(i) The altered 28 S:18 S ratio is unlikely to be related to alterations in 45 S precursor transcription for two reasons. First, pulse-labeling was completed prior to irradiation, facilitating observation of processing, and minimizing observation of effects caused by postirradiation rRNA synthesis (1). Second, even if the measurements were influenced by residual postirradiation incorporation, the opposite result would be expected: since the 18 S portion of the 45 S precursor is upstream from the 28 S portion, postirradiation inhibition of synthesis, yielding partial transcripts, would increase the relative amount of 18 S rRNA, contrary to what was found.

(ii) Effects caused by radiation-induced uridine pool alterations are also an unlikely explanation, for at least two reasons: First, labeling was performed prior to irradiation. Second, since both the 28 S and 18 S rRNA species derive from a single 45 S precursor, any alteration in the specific activity of intracellular pools would affect both daughter species, failing to alter their measured ratio.

(iii) Direct damage of rRNA by ionizing radiation is unlikely to be responsible for the increased 28 S:18 S ratio for two reasons. First, at doses employed in this study direct damage to 45 S rRNA or its daughter species is expected to be slight (1). Second, if direct effects were occurring, 28 S rRNA would be expected to present a larger target than 18 S rRNA, decreasing the relative amount of 28 S rRNA, the opposite of what was found.

Cooper observed an increased 28 S:18 S rRNA ratio following pulse-labeling of resting lymphocytes, but not in mitogen-stimulated proliferating lymphocytes, and proposed, as we have, that 18 S rRNA was undergoing degradation in the nondividing cells (7). He also proposed that maintenance of cellular integrity would not permit continued accumulation of excess 28 S rRNA in resting lymphocytes, and demonstrated that 28 S rRNA was subsequently degraded, restoring the 28 S:18 S labeling ratio to normal (8). This degradation of 18 S and 28 S rRNA may represent an adaptation to cell cycle arrest, reducing the number of newly synthesized ribosomes that would otherwise be required for cellular proliferation (1, 7, 8). However, we failed to detect any restoration of normal labeling ratio in HeLa

cells for durations up to 20 h following irradiation. Perhaps the orderly degradation of excess rRNA in resting cells, first 18 S followed by 28 S rRNA, does not occur in irradiated cells because of abnormal regulation of cellular rRNA content following lethal exposures.

The current studies show that total transport of RNA from the nucleus to the cytoplasm is not affected by the relative overabundance of 28 S rRNA detected in whole cells (Fig. 3). The 28 S:18 S ratio of newly processed rRNA in the cytoplasm was also normal following 10 Gy (Fig. 4). These measurements were carried out within the first 2 h after irradiation when the altered labeling ratio is maximum. Taken together, they suggest that the relative overabundance of 28 S rRNA is limited to the nucleus. However, direct measurement of 28 S:18 S ratio in the nucleus (in contrast to whole cells and the cytoplasmic fraction) was not accomplished because of technical limitations (Materials and Methods). Studies have not yet been carried out to determine whether an altered ratio in the cytoplasm might occur at later times. The apparent accumulation of excess 28 S rRNA in the nucleus, suggested by the current studies, may represent an early step in the process of radiation-induced cell killing.

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