THE EFFECT OF ACTINOMYCIN ON RIBOSOME FORMATION IN HELA CELLS*

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Communicated by John M. Buchanan, December 19, 1963

Although the weight of experimental evidence is decidedly in favor of the view that ribonucleic acid (RNA) in animal cells is made in the nucleus and transported to the cytoplasm,[1-6] Harris and collaborators[7-9] have pointed out that no direct experiment demonstrating such a transfer has been performed. When the action of actinomycin in suppressing all normal cellular RNA synthesis was discovered,[10] several groups immediately attempted to use this agent to demonstrate that nuclear RNA, labeled by incorporation of radioisotopes prior to the addition of actinomycin, was moved into the cell cytoplasm after addition of the drug. Although several of these studies revealed the transfer of a small amount of RNA to the cytoplasm, the striking finding was that the majority of the labeled RNA remained in the nucleus.[5, 7, 11] In addition, Harris[7] demonstrated the conversion to acid-soluble products of a large fraction of the RNA in the nucleus of the HeLa cell which had been labeled for 10 min with adenine before the addition of actinomycin to the culture.

The present experiments extend our earlier observations[12] that in HeLa cells the radioactivity in RNA is largely conserved after a 30-min uridine label followed by actinomycin treatment. Although only a small fraction of the total activity is transferred to the cytoplasm, subsequent to the addition of actinomycin there is a large increase of labeled cytoplasmic ribosomal RNA. In addition, examination of nuclear extracts reveals an explanation of why the transfer is not more extensive.

Materials and Methods.—Cells: Suspension cultures of HeLa cells (doubling time about 24 hr) were grown, labeled, and harvested as previously described.[13, 14]

Radioisotopes: H^3-uridine (1.2–2.0 mC/μmole) and 2-C^{14}-uridine (28–32 μC/μmole) were purchased from New England Nuclear Corp. and were used as sterile solutions added directly to the cultures at final concentrations of 0.001–0.003 mM. The higher concentration was used for labeling periods in excess of 100 min. Previous experiments indicated that these concentrations were satisfactory to maintain linear incorporation for about 200 min.

Cell fractionation: Cytoplasmic extracts were prepared as previously described[15] by the use of the Dounce homogenizer after the cells were swelled in a hypotonic buffer (RSB = 0.01 *M* HCl or NaCl, 0.0015 *M* Mg Cl_2, and 0.01 *M* tris pH 7.4 at 25°). Nuclei were separated by centrifugation at 800 *g* for 10 min, and desoxycholate was added to the supernatant fluid at a final concentration of 0.5%. Nuclear extracts were prepared from the nuclear pellet after resuspension in RSB by treatment for 20 sec in a Mullard ultrasonic vibrator. Desoxycholate (final concentration 0.5%) was added to the resulting opalescent suspension before sedimentation analysis.

RNA extractions: RNA from various cytoplasmic fractions was either extracted with hot phenol-sodium dodecyl sulfate (Fisher, once recrystallized) by a technique previously described[14] or by the technique of Gilbert,[16] which utilizes the detergent sodium dodecyl sulfate alone. The release of RNA from ribosomes is complete by Gilbert's technique, which in addition offers the significant advantages of speed and protection against enzymatic degradation of the RNA. The sucrose gradient patterns of RNA obtained by this method correspond exactly to those previously seen with phenol-prepared RNA, so the two methods have been used interchangeably for cytoplasmic extractions. Only the phenol technique was used when the nuclei or whole cells were to be extracted.

Sucrose gradient analyses: Several different types of sucrose gradients were utilized for separat-

ing particles or extracted RNA, and details are presented in the legends of the figures. In the gradients containing sodium dodecyl sulfate the buffer was that described by Gilbert,[16] and the centrifuge chamber temperature was set at 10°C. For the precipitation of radioactive RNA from the sodium dodecyl sulfate-containing gradients, the following procedure was employed. Fractions of 0.4–0.6 ml were collected from the gradient at room temperature through a recording spectrophotometer.[15] Two ml of cold (4°) 20% trichloroacetic acid were added to each fraction, and immediately the tubes were placed in an ice bath. Collection and assay of radioactivity in the precipitates have been described elsewhere.[17]

Results.—The design of the present experiments was based on three previous observations: (1) The addition of actinomycin to a culture of HeLa cells prevents immediately any further synthesis of high molecular weight RNA.[12] (2) The predominant species of rapidly labeled RNA (30-min label with uridine) in the HeLa cells is a very high molecular weight (45S) nuclear component with base composition similar to ribosomal RNA. After actinomycin treatment 60–70% of the labeled nuclear RNA, is conserved and converted to 28 and 16S RNA, which is the size of ribosomal RNA.[12] (3) Protein synthesis in the cytoplasm of HeLa cells has been demonstrated to occur in groups of ribosomes (polyribosomes) attached to mRNA and after a 35-min exposure to P^{32} orthophosphate the only labeled RNA in the polysomes was mRNA.[15] The majority (over 80%) of the total cytoplasmic activity after a 30-min uridine label, however, was in 4S material as has been reported by other workers.[8, 11, 18]

The following plan then has been followed in all the experiments to be described. Cells were labeled for 30 min with uridine and then treated with actinomycin for various periods of time (actinomycin chase). The labeled RNA in the cytoplasm was then investigated in various ways.

It was first found (Fig. 1) that in spite of the conversion of nuclear RNA to the appropriate size for ribosomal RNA during an actinomycin chase (ref. 12 and Fig. 2*A*), there was only a small increase in radioactivity in the cytoplasm. Although the brief uridine label (30 min) resulted in labeling predominantly 4S material in the cytoplasm, the labeled cytoplasmic RNA after the actinomycin chase is largely 28 and 16S, as is the nuclear RNA (Fig. 2*B*). Also, the labeled 28 and 16S cytoplasmic RNA is obviously in functioning ribosomes, since a large fraction (Fig. 2*C*) of the cytoplasmic label is associated with the rapidly sedimenting polysome structures. It is apparent from these experiments, therefore, that although the total increase in cytoplasmic radioactivity is only twofold after the actinomycin chase, the increase in rRNA is greater. In order to measure the actual increase in rRNA and also to observe the relative entrance of mRNA and rRNA into cytoplasmic particulate structures, the experiment shown in Figure 3 was performed. After a 30-min label a sample was removed, actinomycin was added to the remainder of a culture, and other samples were taken

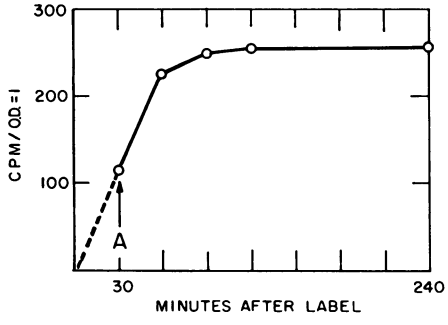

FIG. 1.—4×10^7 HeLa cells were labeled with uridine 2-C^{14} for 30 min, and actinomycin (5 γ/ml) was added to the culture. Samples were taken just before addition of the antibiotic and at intervals after, and the total acid-precipitable radioactivity in the cytoplasm was compared to the absorbancy at 260 mμ

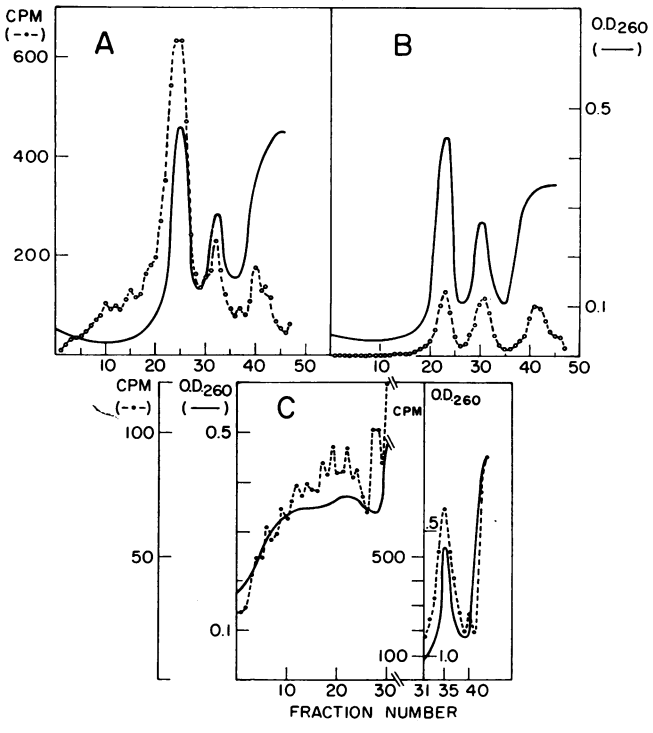

FIG. 2.—Cellular distribution of radioactive RNA after exposure to uridine and actinomycin chase. 16×10^8 HeLa cells were labeled for 30 min with uridine 2-C^{14} and then treated for 3.5 hr with actinomycin D (5 γ/ml). At the end of this time the cells were fractionated into nuclear and cytoplasmic fractions. A sample of the cytoplasm was examined on a 15–30% sucrose RSB gradient (*C*) immediately (110 min, 25,000 rpm, 4°) while from another cytoplasmic sample (*B*) and a nuclear sample (*A*) the RNA was extracted with phenol and examined on another sucrose gradient as previously described.[14]

at intervals. Each sample was fractionated into polysomes and material sedimenting approximately as single ribosomes. (A separate communication will describe the details in growing cells of the entry of labeled mRNA and rRNA into polysomes and structures sedimenting with S values of 50–74S, Girard *et al.*, in preparation.)

The sedimentation pattern of the RNA from each source was determined and is shown in Figure 3. It can be seen that the only label in polysomes after a 30-min uridine label has the sedimentation characteristics previously described for mRNA and that the label from the lighter region of the gradient (50–75S region) appears to be a mixture of emerging 16S rRNA and some mRNA. At later time periods after the addition of actinomycin more and more rRNA can be seen to be entering the particulates of the cytoplasm so that the majority of radioactivity in both polysomes and smaller structures (50–75S) is rRNA. The earlier appearance of 16S RNA compared to 28S RNA is striking and has also been observed in growing HeLa cells (Girard *et al.*, in preparation) as well as in bacteria[19] and liver cells.[18]

By subtracting the approximated contribution of mRNA to the label in particulates at various times, the total amount of ribosomal RNA in the cytoplasm as a function of time after the actinomycin chase can be determined. This is plotted in Figure 4.

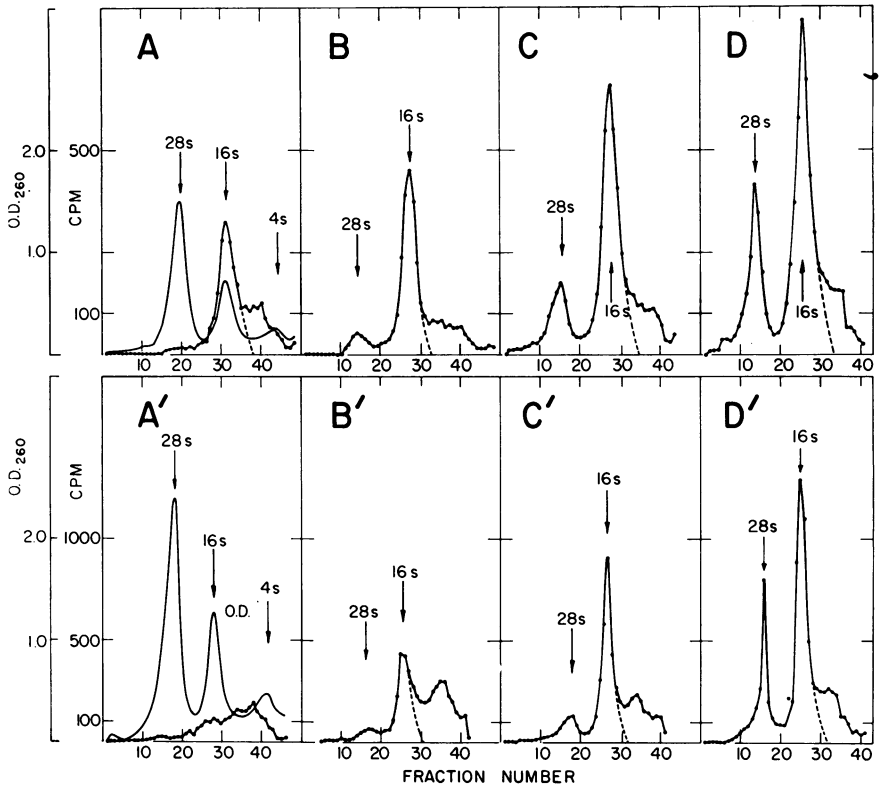

FIG. 3.—Appearance of radioactive RNA in cytoplasmic "ribosomes" and polysomes after actinomycin treatment. 3.2×10^8 growing HeLa cells were labeled for 30 min with H^3-uridine, and a sample was removed (A, A'). To the remainder of the culture actinomycin (5 γ/ml) was added, and samples were removed after 30 (B, B'), 60 (C, C'), or 90 (D, D') min. The cytoplasm of the cells was first fractionated on sucrose gradients into 50–75S particles (A, B, C, D) and polysomes (A', B', C', D'). These particulate fractions were then sedimented as previously described,[15] and the RNA of each fraction was separately analyzed by sucrose gradient sedimentation. Radioactivity to the left of the dotted lines is considered to be ribosomal RNA.

There is a sixfold increase in labeled ribosomal RNA in the cytoplasm which takes place within the first 60 min after actinomycin is added to the culture. Thus, although it seems unquestionable from the above experiments that ribosomal RNA made in the nucleus had appeared in the cytoplasm after actinomycin treatment, the question still remained—why did radioactivity not continue to flow into the cytoplasm?

The nucleus was examined to determine whether the intranuclear radioactivity which was known to be in extractable 28 and 16S RNA (see Fig. 2) was in fact in finished ribosomes. Figure 5 demonstrates that when a sonicated nuclear extract was examined, a large majority of the radioactivity was in small structures with S values less than about 50S. There was a definite peak around 50S and a few 70S particles which could represent contamination from the cytoplasm. Further work will be required to establish whether the labeled RNA in ruptured nuclei is in unfinished precursor particles to whole ribosomes, but since the labeled nuclear RNA is 28 and 16S, this seems likely. (The sonication treatment employed had no effect either on the sedimentation pattern of RNA extracted from the nuclei in the

above experiment or on the sedimentation of added 70S ribosomes from an unlabeled cytoplasmic extract.)

Discussion.—The demonstration that after a short exposure of HeLa cells to isotopically labeled uridine and a subsequent actinomycin treatment, there is both an increase in total radioactivity in cytoplasmic RNA and a sixfold increase of radioactivity in cytoplasmic rRNA, provides direct evidence of the transport of high molecular weight RNA from the nucleus to the cytoplasm. The previous lack of such an experiment was the major reason to question the hypothesis that in animal cells the nucleus serves as the source of all cellular RNA. Harris and coworkers[7–9] have questioned this concept repeatedly in recent years chiefly because they observe in HeLa cells the loss of a certain proportion of radioactive RNA into acid-soluble products when either the radioisotope is withdrawn and replaced by unlabeled RNA precursors, or when actinomycin is added to a growing culture after a 10-min exposure to C^{14}-adenine. Two points should be made concerning their experiments in light of the present results. First, although they observe a loss to acid-soluble form of *some* of the radioactive nuclear RNA after a pulse and chase with nonradioactive precursors, the majority of the total label in RNA is conserved if the nuclear and cytoplasmic radioactivities are added together. In order to account for this conservation without accepting transfer of macromolecular RNA from nucleus to cytoplasm, they are forced to postulate a cytoplasmic pool of RNA precursors which is not accessible except to break down products of nuclear RNA, and thus is not diluted out by unlabeled RNA precursors in the medium. If this hypothesis were true, the formation of cytoplasmic RNA would have to be accomplished in the cytoplasm without the direction of DNA. The second experiment which Harris has cited as evidence against nuclear RNA synthesis and subsequent transport to the cytoplasm is the observation that in HeLa cells a 10-min label with adenine followed by actinomycin treatment causes a loss to acid-precipitable form of a large fraction of the radioactive nuclear RNA with only a small transfer to the cytoplasm.

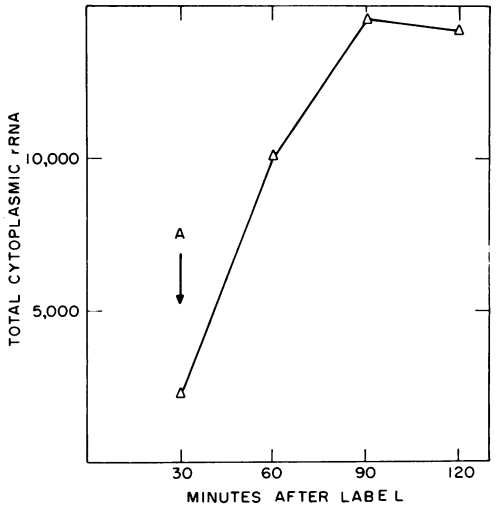

FIG. 4.—Increase in radioactivity in rRNA in cytoplasm after actinomycin treatment. The total amount of radioactive ribosomal RNA in the samples of polysomes and 50–75S particles from the pulse-labeled actinomycin-treated culture analyzed in Fig. 3 is plotted as a function of time after the addition of actinomycin (arrow).

Our experiments show clearly that extensive degradation of labeled nuclear RNA after actinomycin treatment is not an invariable result, because if a 30-min uridine label is followed by actinomycin treatment, then *most* of the radioactive nuclear RNA is stabilized after conversion to 28 and 16S RNA (ref. 12 and Fig. 2). It apparently takes about 30–45 min for the rRNA to pass through the 45 and 35S to 28 and 16S sequence. In addition, incorporation into whole ribosomes appears to require a process which is actinomycin-sensitive. The 10-min adenine label

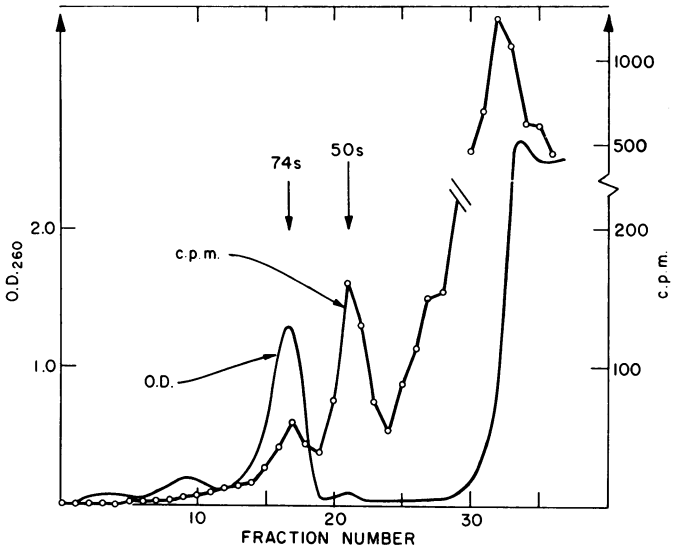

FIG. 5.—Sedimentation analysis of nuclear extract. 1.2 × 10^7 HeLa cells (30 ml of culture) were labeled with uridine 2-C^{14} for 30 min and then treated with 5 γ/ml of actinomycin D for 3.5 hr. The nuclei were prepared from the labeled cells and added to cytoplasmic extract from nonlabeled cells which furnished a 74S OD marker. The material was then subjected to sonic vibration as described in *Materials and Methods*. The sedimentation pattern of radioactivity in extracted nuclear RNA after a comparable labeling period and actinomycin treatment can be seen in Fig. 2. It was found that the sonication procedure used here did not affect this pattern.

employed by Harris might well not allow the newly formed RNA enough time to reach a stable configuration, and when actinomycin is added to the culture, the RNA is attacked by the existing nuclear enzymes capable of destroying RNA.[20] This does not infer that actinomycin is *inducing* an instability in RNA, since again the majority of a 30-min uridine label is stable to actinomycin treatment.

It could be argued that in our present experiments the sixfold increase in radioactivity in cytoplasmic rRNA observed after actinomycin treatment was derived by smaller (~4S) RNA coming together into larger molecules. If this were so, it would mean that small RNA was built into 28 and 16S RNA in the cytoplasm, while large (45 and 35S) RNA was being reduced to the same 28 and 16S size in the nucleus.

The soundest interpretation of the results with the HeLa cell is that the nucleus is the site of ribosome synthesis. Ribosomal RNA is made of large molecules which are divided during ribosome formation to 28 and 16S size. The 50S portion of a ribosome takes longer to complete than the 30S, leading to the appearance of 16S RNA in the cytoplasm in advance of 28S RNA. The interruption in formation of ribosomes by actinomycin, even though 28 and 16S RNA are available, implies that ribosomal protein synthesis is affected by actinomycin to a much greater extent than general cell protein synthesis. For example, it was previously shown that three hr of actinomycin treatment reduced the number of functioning cytoplasmic polysomes by only 50 per cent in HeLa cells.[15] Two possible explanations can be suggested: (1) the completion of a ribosome requires either a structural protein or some unstable functional protein which has as its template an mRNA with a

shorter half-life than is seen in the cell cytoplasm. (2) A system exists for the suppression of formation of ribosomal proteins, which is normally repressed by an unstable RNA. Further analysis of this situation should prove very useful in gaining understanding of the necessary elements in ribosome formation.

Summary.—Kinetic studies on the entry of ribosomal RNA into the HeLa cell cytoplasm after treatment with actinomycin are presented. The major conclusions are: (1) there is demonstrable transfer of nuclear RNA to the cytoplasm; and (2) actinomycin very quickly interrupts the formation of whole ribosomes in the nucleus before the supply of available rRNA has all been made into ribosomes.

It is a great pleasure to acknowledge the capable technical assistance of Misses Ruth Liberfarb, Sara Long, and Mary Jane Madden.

* This work was supported by grant C-5789 from the U.S. Public Health Service and grant GB-513 from the National Science Foundation.

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