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Effects of Adenovirus Infection on rRNA Synthesis and Maturation in HeLa Cells

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The production of cytoplasmic and nucleolar rRNA species was examined in HeLa cells infected with high multiplicities of adenovirus type 5. Both 28S and 18S rRNA newly synthesized in infected cells ceased to enter the cytoplasm as reported previously (N. Ledinko, Virology **49**:79–89, 1972; H. J. Raskas, D. C. Thomas, and M. Green, Virology **40**:893–902, 1970). However, the effects on 28S cytoplasmic rRNA were observed considerably earlier in the infectious cycle than those on 18S rRNA. The inhibition of cellular protein synthesis and of the appearance in the cytoplasm of labeled cellular mRNA sequences (G. A. Beltz and S. J. Flint, J. Mol. Biol. **131**:353–373, 1979) were also monitored in infected cultures. During the later periods of an infectious cycle, from 18 h after infection, nucleolar rRNA synthesis and processing and exit of 18S rRNA from the nucleus were inhibited, probably reflecting the failure of infected cells to synthesize normal quantities of ribosomal proteins. The earliest responses of cellular RNA metabolism to adenovirus infection were, however, the rapid and apparently coordinate reductions in the levels of newly synthesized 28S rRNA and cellular mRNA sequences entering the cytoplasm.

The value of adenovirus-infected cells as a model system for the study of mechanisms of gene expression and regulation in eucaryotic cells has long been recognized and exploited to construct the detailed picture of the organization and expression of adenoviral genes that we now possess (see references 7 and 42 for reviews). One aspect of the adenovirus-host cell interaction has, however, received much less attention: the extremely efficient usurpation of the cellular biosynthetic machinery, characteristic of the late phase of infection, is a well-established phenomenon, but one whose molecular basis has been little investigated. Inhibition of cellular DNA synthesis by adenoviruses begins with the initiation of viral DNA synthesis (and, by definition, the late phase) some 6 to 8 h after infection by type C adenovirus (adenovirus type 2 [Ad2] or Ad5) and is essentially complete a few hours thereafter (9, 25). Similarly, only adenoviral polypeptides are labeled once the late phase is established (1, 2, 28, 39). Inhibition of cellular protein synthesis is, however, a relatively long-drawn-out affair that, at least to some extent, reflects the failure of type C adenovirus-infected cells to transport mature host mRNA sequences to the cytoplasm (3; S. J. Flint, G. A. Beltz, and D. I. H. Linzer, J. Mol. Biol., in press). The major species of small RNA encoded by the adenoviral genome, VA-RNA$_I$, is required for efficient translation of viral late mRNA species (31), but it is not yet known whether it also mediates selective translation of viral mRNA.

It is clear that adenovirus infection does not inhibit transcription of cellular pre-mRNA sequences (3, 26), including those complementary to specific, nonadenoviral genes (Flint et al., in press; S. M. Berget, personal communication). The molecular mechanism(s) underlying the discrimination between adenoviral and cellular mRNA sequences that must occur during post-transcriptional processing or during transport of sequences destined to function as mRNA remains to be identified. In this respect, it may be significant that adenovirus infection has also been reported to disrupt the normal metabolism of rRNA sequences (15, 27). On the other hand, this phenomenon could be a secondary consequence of the inhibition of synthesis of cellular proteins, including ribosomal proteins. It is well established that inhibition of cellular protein synthesis by the addition of drugs such as cycloheximide (4, 17, 33, 40), by starvation for essential amino acids (18), by incubation in hypertonic medium (22), or after infection by viruses that interfere with cellular gene expression at the level of translation, such as picornaviruses (5, 34), prevents the appearance of 28S and 18S rRNA species in the cytoplasm. Under these circumstances, failure to accumulate mature rRNA species in the cytoplasm is accompanied by a decrease in rates of the synthesis and processing of nucleolar rRNA species.

In an effort to distinguish between these possi-

bilities, the temporal patterns of inhibition of appearance in the cytoplasm of cellular mRNA and rRNA sequences in adenovirus-infected cells were compared with those of inhibition of cellular protein synthesis. The effects of adenovirus infection on synthesis and processing of the 45S rRNA precursor within the nucleolus were also examined.

MATERIALS AND METHODS

Cells and virus. Preparation of viral DNA, labeling of RNA, oligodeoxythymidylate-cellulose chromatography, RNA-DNA hybridization conditions, and the preparation and detection of labeled proteins have been described in detail elsewhere (3).

RNA purification. The procedure used for RNA purification was that reported by Beltz and Flint (3) except that 100 μg of DNase I per ml of nuclear lysate was used to digest chromatin. The DNase I (Worthington Diagnostics) was free of RNase as judged by incubation with [^{3}H]rRNA followed by denaturation and electrophoresis as described below.

RNA gel electrophoresis. RNA was denatured in 50% dimethyl sulfoxide (Fisher Scientific Co.), reacted with glyoxal (Fluka), and separated in 0.75 or 1.0% agarose (BioRad Laboratories; electrophoresis grade) gels cast in 10 mM sodium phosphate (pH 7.0)–1 mM EDTA according to the method of McMaster and Carmichael (20). For fluorography, the gels were soaked in 2.5 volumes of En^{3}Hance (New England Nuclear Corp.) for 8 h, agitated in water for 5 h, dried onto 3MM chromatography paper (Whatman), and exposed to Kodak XRP-1 X-ray film. To quantitate individual RNA species, tracks from dried gels were cut into 1-mm slices and counted. These data were plotted as counts per minute versus distance migrated, and the radioactivity in the peaks was determined. These values were then expressed as a percentage of the total radioactivity recovered in the track to correct for slight variations in this parameter. Fluorograms were also subjected to densitometry with an MkIIIB double-beam recording Joyce-Loebl microdensitometer, and the peaks corresponding to nucleolar or cytoplasmic rRNA species of interest were excised and weighed.

Preparation of ribosomal proteins. [^{3}H]leucine-labeled HeLa cells (6×10^7) were pelleted by centrifugation at 300 × *g* for 3 min, washed once in phosphate-buffered saline, and lysed in hypotonic buffer containing 0.5% Nonidet P-40. Nuclei were removed by centrifugation at 500 × *g* for 5 min, and the microsomal fraction was removed by centrifugation at 27,000 × *g* for 20 min. The supernatant was then layered onto a 30% sucrose cushion and centrifuged at 150,000 × *g* for 16 h to pellet polyribosomes (12). The ribosomal subunits, dissociated in 5 mM EDTA, were separated in 15 to 30% sucrose gradients (23). Fractions containing 60S and 40S subunits were pooled, ethanol precipitated (6), and suspended in water and gel sample buffer (13), and the labeled proteins were examined by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels followed by fluorography. The identity of the 60S and 40S subunits was confirmed by parallel preparation of 60S and 40S subunits labeled with [^{3}H]uridine and analysis of their RNA by electrophoresis in denaturing gels as described in the previous paragraph.

RESULTS

Inhibition of appearance of cellular rRNA species in the cytoplasm of adenovirus-infected cells. The effects of adenovirus infection upon both the appearance of newly synthesized RNA species in the cytoplasm and the synthesis of cellular polypeptides were examined to determine whether the inhibition of cytoplasmic appearance of mature 28S and 18S rRNA is simply a secondary consequence of the well-documented inhibition of cellular protein synthesis observed in adenovirus-infected cells (1–3, 28, 39).

Exponentially growing HeLa cells were infected with high multiplicities of Ad5, 50 to 150 PFU/cell, to ensure that all cells in the culture were infected more or less synchronously. A portion of the culture was removed before infection to serve as a mock-infected control sample. At regular intervals after infection, portions of the culture were removed and labeled for 2-h periods with [^{3}H]uridine or [^{3}H]leucine as described above. Mock-infected samples were labeled in similar fashion, and nuclear and cytoplasmic RNA or proteins were prepared from all samples as described above. Cytoplasmic polyadenylate [poly(A)]-lacking RNA was isolated from each preparation of labeled RNA by two cycles of chromatography on oligodeoxythymidylate-cellulose. To examine the newly synthesized RNA species reaching the cytoplasm after Ad5 infection, poly(A)-lacking RNA fractions were glyoxalated (20), and equal portions were subjected to electrophoresis in 1% agarose gels. A typical fluorogram of a gel containing samples from an infected culture in which complete inhibition of cellular protein synthesis was attained by 18 to 20 h after infection is shown in Fig. 1. As expected on the basis of earlier reports (15, 27), a dramatic inhibition of the appearance of mature 28S and 18S rRNA in the cytoplasm could be seen as the infection progressed. It is also clear, however, that the inhibition of appearance of cytoplasmic 28S rRNA was both greater and more complete, within the period shown in Fig. 1, than that of 18S rRNA.

Despite the poor resolution of low-molecular-weight poly(A)-lacking RNA in 1% agarose gels, a new species could be readily detected by 6 to 8 h after infection (Fig. 1). This species migrated somewhat more slowly than the tRNA band and presumably corresponds to the VA-RNA species (19, 21, 29). It is of interest that little change in the appearance of newly synthesized, cellular, low-molecular-weight RNA species could be discerned within the time span examined. More detailed experiments to investigate the effects of adenovirus infection upon different classes of small RNA species are in progress.

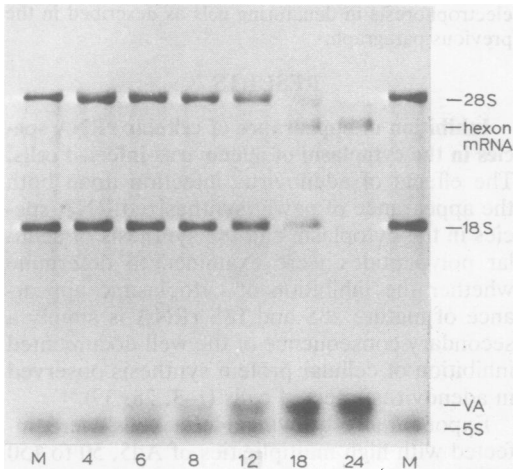

FIG. 1. Appearance of labeled cytoplasmic rRNA in Ad5-infected HeLa cells. Ad5-infected HeLa cells were labeled with [^{3}H]uridine for 2-h periods beginning at 4, 6, 8, 12, 18, and 24 h after infection. Cytoplasmic, non-polyadenylated RNA, prepared as described in the text, was denatured by reaction with 1 M glyoxal (20) and subjected to electrophoresis in 1% agarose gels for 12 h at 40 V. Shown is a fluorogram of such a gel. Equal amounts of RNA labeled at the indicated times were applied to each track, whereas RNA prepared in identical fashion from mock-infected cells was analyzed in the flanking tracks marked M. In this experiment, 1.5×10^4 cpm of infected cell poly(A)-lacking [^{3}H]RNA was loaded onto each track. The percentages of the total RNA recovered that this amount represents were 0.24, 0.21, 0.29, (0.22), 0.26, 0.22, and 0.23% at 4, 6, 8, (10), 12, 18, and 24 h after infection, respectively. As equal numbers of cells were labeled under identical conditions at each time point, each track was therefore loaded, within experimental error (12%; 0.237 ± 0.028% of the RNA), with poly(A)-lacking RNA prepared from the same number of cells.

Quantitation of the amounts of newly synthesized 28S and 18S rRNA entering the cytoplasm of Ad5- or mock-infected HeLa cells was accomplished by measurement of the amount of radioactivity in the 28S and 18S rRNA bands in each lane of the gel shown in Fig. 1 and others like (see Materials and Methods). Within experimental error (less than 12% in the experiment shown in Fig. 1 and 2), the constant amount of labeled RNA applied to each track represents RNA from the same number of HeLa cells, implying that adenovirus infection does not induce gross changes in their total poly(A)-lacking RNA content (that is, rRNA, tRNA, and other small, cytoplasmic RNA species together with VA-RNA). After correction for slight variations in the total amount of RNA recovered from each portion of the infected culture, the values obtained at the various time points after adenovirus infection were expressed as a percentage of mock-infected control values. Such data are presented in Fig. 2. The appearance in the cytoplasm of newly synthesized 28S rRNA was inhibited greater than 90% by 12 h after Ad5 infection and declined to less than 1% of the control value by 18 h. Inhibition of the appearance of 18S rRNA occurred more slowly; indeed, in this and other experiments a slight stimulation in the levels of labeled 18S rRNA reaching the cytoplasm in the first few hours after infection was observed. We do not understand the reasons for this result, which is confined to 18S rRNA. Neither this effect, however, nor the inhibition of 28S rRNA observed early after Ad5 infection appears to result from the manipulations to which cells are subjected during infection, that is, incubation for 1 h at 10 times their normal concentration followed by dilution into fresh medium, because uninfected

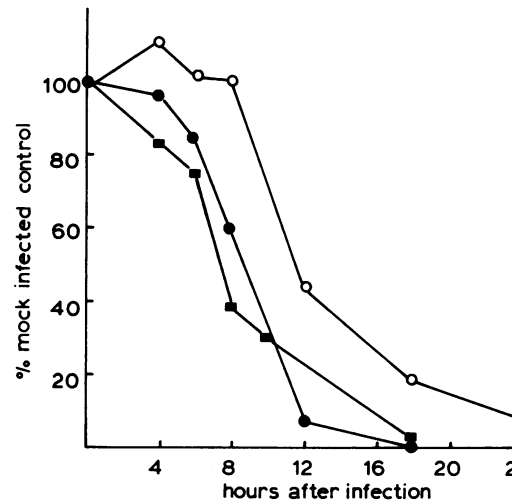

FIG. 2. Time course of inhibition of appearance in the cytoplasm of cellular mRNA and rRNA in Ad5-infected HeLa cells. The amount of newly synthesized 18S and 28S rRNA present in the cytoplasm of Ad5-infected HeLa cells was determined from the distribution of radioactivity in the gel shown in Fig. 1, as explained in the text. The resulting values were corrected as explained in the text and expressed as a percentage of the values obtained with mock-infected control cultures. The inhibition of appearance in the cytoplasm of 28S (●) and 18S (○) rRNA is depicted. Also shown (■) is the inhibition of appearance in the cytoplasm of nonviral mRNA sequences; poly(A)-containing cytoplasmic RNA was prepared from infected cells labeled with [^{3}H]uridine at the indicated times and hybridized to saturation with Ad5 DNA as described in the text. By 18 h after infection, 96% of such RNA hybridized to adenoviral DNA. Labeled RNA sequences that failed to hybridize were assumed to represent cellular RNA sequences.

cells treated in the same fashion yielded identical amounts of 28S and 18S cytoplasmic rRNA whether labeled immediately after mock infection or 4, 6, or 8 h thereafter (data not shown).

The results obtained when equal amounts of [^{3}H]leucine-labeled polypeptides prepared from equal portions of the same infected culture were displayed in sodium dodecyl sulfate-polyacrylamide gels are shown in Fig. 3. Similar experiments, in which two different multiplicities of infection (40 and 100 PFU/cell) were employed and the labeled cytoplasmic proteins recovered from an identical number of mock- and Ad5-infected cells were compared, are shown in Fig. 4. Inhibition of cellular protein synthesis was eventually attained in all of these infected cultures, in agreement with previous reports (1–3, 28, 39). It is, however, clear that the outcome of inhibition is influenced by the multiplicity of infection. Cultures infected with 40 PFU of Ad5 per cell and labeled at the earlier time shown in Fig. 4 not only synthesized smaller quantities of viral late proteins than the sibling culture infected at 100 PFU/cell, but also exhibited a less dramatic decrease in labeling of prominent cellular polypeptides.

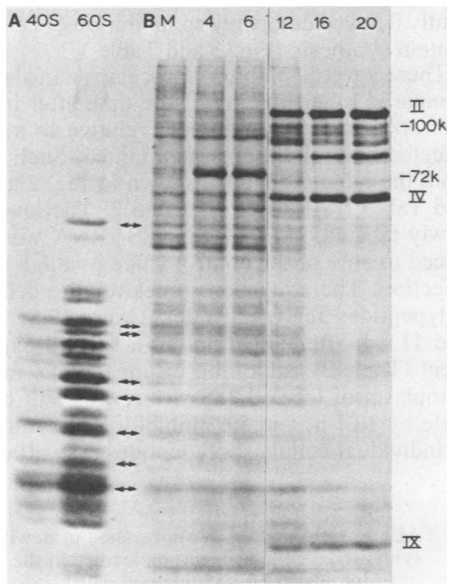

FIG. 3. Synthesis of cytoplasmic proteins in Ad5-infected HeLa cells. Ad5-infected HeLa cells were labeled as described in the text for 2-h periods with [^{3}H]leucine beginning at the time points after infection indicated in part B. Mock-infected HeLa cells were labeled in similar fashion. Samples, each containing the same amount of trichloroacetic acid-precipitable radioactivity, were denatured and subjected to electrophoresis in a 15% polyacrylamide gel for 15 h at 90 V according to the procedure described by Laemmli (13). Part B shows cytoplasmic polypeptides from mock-infected (M) or infected (4, 6, 12, 16, and 20 h) cultures, whereas part A shows polypeptides contained in 40S and 60S ribosomal subunits purified from the mock-infected culture as described in the text. Part B has been deliberately overexposed with respect to viral polypeptides to reveal less strongly labeled polypeptides. Major viral polypeptides are identified to the right of part B.

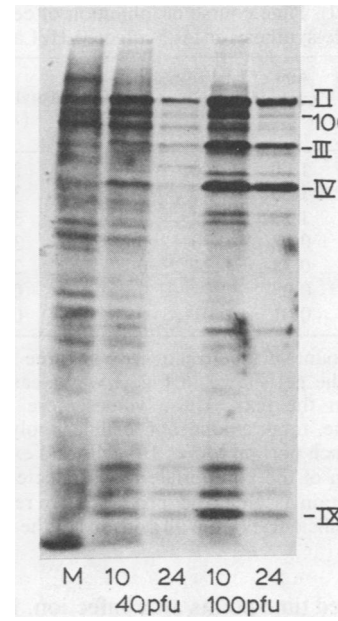

FIG. 4. Synthesis of cytoplasmic protein in HeLa cells infected with different multiplicities of Ad5. HeLa cells infected at 40 or 100 PFU/cell with Ad5 were labeled with [^{3}H]leucine for 2 h after incubation for 10 or 24 h at 37°C. A mock-infected culture (M) was labeled in identical fashion. The cytoplasmic polypeptides recovered from 3×10^5 cells were applied to each track of the gel shown in the fluorogram. Major viral polypeptides are identified on the right.

As illustrated in Table 1, total protein synthesis is not constant during an adenovirus infection; rather, the amount of protein labeled in a constant number of cells can be seen to increase during the early phase of infection and then decrease quite rapidly to a value that is substantially lower than that of mock-infected control cultures. This pattern is typical of that seen in all experiments we have performed. To determine whether the early stimulation merely reflects synthesis of viral proteins and to establish the time course of inhibition of cellular protein synthesis, the amounts of label present in well-resolved cellular polypeptides were measured as described in Materials and Methods. Such data for the experiment shown in Fig. 3 are also listed in Table 1. All values, expressed in Table 1 as fractions of the appropriate mock-infected control value, were calculated as the total amount of the protein of interest labeled in 3×10^6 cells at

TABLE 1. Time course of inhibition of cellular polypeptide synthesis in Ad5-infected HeLa cells[a]

Time (h) after infection	Amt of labeled polypeptide relative to control			Total ^{3}H cpm ($\times 10^6$)
	1	2	3	
0 (mock)	1.00	1.00	1.00	2.99
4	1.38	1.17	1.22	3.06
8	1.39	1.22	1.47	3.80
10	0.98	0.70	0.86	3.70
12	0.38	0.21	0.27	2.64
18	0.08	0.04	0.05	0.86
24	0.01	0.01	0.00	0.58

[a] The amount of label recovered in three selected regions of the gel shown in Fig. 3 was measured as described in the text. These values were used to calculate the total amount of cellular polypeptide labeled in each portion of 3×10^6 cells and expressed as a fraction of the appropriate mock-infected value. This calculation assumes that proteins were recovered with equal efficiencies from all portions of the infected culture.

the indicated time points after infection. In each of the three cases examined, adenovirus infection induced some stimulation in cellular polypeptide synthesis up to 8 h after infection, which was followed by inhibition of synthesis, particularly dramatic between 8 and 12 h after infection. Thus, this quantitative assessment, which takes into account the increasing contribution to the total protein population of adenoviral structural proteins during an infection, confirms conclusions based simply upon inspection of fluorograms like those shown in Fig. 3 and 4.

To investigate whether synthesis of cellular ribosomal proteins was especially sensitive, or resistant, to inhibitory effects of adenovirus infection, [^{3}H]leucine-labeled ribosomal subunits were isolated from uninfected HeLa cells as described in Materials and Methods, and their polypeptides were separated in one dimension in parallel to labeled cytoplasmic polypeptides from uninfected and Ad5-infected HeLa cells. Comparison of the tracks of such a gel (Fig. 3A) containing ribosomal proteins with that to which mock-infected cytoplasmic polypeptides were applied permits tentative identification of the most abundant ribosomal proteins, as indicated by the arrows in Fig. 3. Inspection of these results suggests that inhibition of this group of proteins cannot be distinguished from that of other cellular proteins. Indeed, the majority of [^{3}H]leucine-labeled proteins recovered in 40S and 60S ribosomes fall within a molecular weight range (15,000 to 51,000) which includes no major cytoplasmic viral proteins and in which no labeled polypeptides can be detected once complete shutoff has been attained (see also reference 3).

To assess the relationship between inhibition of synthesis of mature rRNA species and inhibition of protein synthesis in Ad5-infected cells, the temporal relationship of the two inhibitory processes was examined. Were inhibition of rRNA synthesis a secondary consequence of the inhibition of production of ribosomal proteins, the former process could not occur before the latter. The inhibition of entry into the cytoplasm of cellular mRNA sequences and 28S and 18S rRNA as well as inhibition of cellular protein synthesis were therefore monitored in the same infected culture. The results obtained in one such experiment, summarized in Fig. 2 and Table 1, establish that the inhibition of appearance of 28S rRNA in the cytoplasm does not lag significantly behind inhibition of cellular protein synthesis. Rather, the inhibition of cytoplasmic 28S rRNA production occurs at a slightly earlier time than inhibition of cellular protein synthesis. By contrast, the inhibition of release of newly labeled 18S rRNA from the nucleus is significantly delayed compared to inhibition of cellular protein synthesis (Fig. 2 and Table 1).

These several inhibitory processes can also be compared by estimation of the time after infection at which 50% inhibition, relative to mock-infected control cultures, is attained. Such data from the infected culture shown in Fig. 2 and 3 and Table 1 are listed in Table 2. The level of newly labeled, cytoplasmic 28S rRNA was reduced to 50% of the control value by 8.6 h after infection. The equivalent times for the cellular polypeptides analyzed in detail were 10.8, 11.3, and 11.7 h after infection. In a second experiment (Table 2), inhibition of appearance in the cytoplasm of labeled 28S rRNA was half complete by 10.7 h, and 50% inhibition of synthesis of individual cellular polypeptides was attained

TABLE 2. Inhibition of appearance of newly synthesized cellular macromolecules in the cytoplasm of Ad5-infected cells[a]

Expt	Time after infection (h) at which 50% inhibition was attained			
	mRNA	28S rRNA	18S rRNA	Cellular polypeptides
1	7.4	8.6	11.5	10.8, 11.3, 11.6
2	9.6	10.7	18.2	12.5, 11.5, 14.0, 13.2, 13.5

[a] The inhibition of the appearance in the cytoplasm of newly synthesized cellular mRNA and 28S and 18S rRNA and the inhibition of cellular polypeptide synthesis are expressed as the time at which 50% inhibition is reached in infected cultures. Experiment 1 shows the data from the experiment shown in Fig. 1 to 3, and experiment 2 summarizes equivalent data from a second, independently infected culture. Experiments 1 and 2 were performed at multiplicities of infection of 150 and 50 PFU/cell, respectively.

by 11.5 to 14.0 h after infection. A similar pattern of inhibition, that is, an earlier effect on appearance in the cytoplasm of newly made 28S rRNA than on synthesis of cellular polypeptides, was observed in two additional experiments. These data are not included in Table 2 because in one case only about half of the cells in the culture were infected, as judged by the criterion of complete inhibition of cellular protein and mRNA synthesis, and in the other case cellular mRNA sequences were not examined. Inhibition of the appearance in the cytoplasm of labeled 18S rRNA after Ad5 infection is a much slower process; 50% inhibition of 18S rRNA was not observed until 3 to 7 h after the appearance of 28S rRNA was 50% inhibited in both experiments summarized in Table 2.

Some variation in the kinetics of these inhibitory processes has been observed in different infected cultures. Inhibition is, for example, more rapid in experiment 1 (summarized in Table 2) than in experiment 2, although in both cases essentially complete inhibition of both cellular protein synthesis and appearance in the cytoplasm of cellular mRNA species is eventually attained (see Fig. 2 and 3 and Table 1 for experiment 1; data for experiment 2 not shown). As infections 1 and 2 were performed with 150 and 50 PFU/cell, respectively, these results confirm the conclusions made on the basis of the data shown in Fig. 3.

Synthesis of nucleolar RNA in adenovirus-infected cells. The decrease in the amounts of newly synthesized rRNA species entering the cytoplasm of adenovirus-infected cells could reflect any one of a number of virus-induced changes, including inhibition of transcription of rRNA genes, disruption of the normal processing pathways whereby rRNA is matured, or inhibition of transport of mature rRNA species from the nucleolus or the nucleus to the cytoplasm. In an effort to distinguish between such possibilities, nucleolar rRNA species were labeled for 2 h at different periods after Ad5 infection, purified as described in Materials and Methods, and analyzed in 0.75% agarose gels after denaturation. Shown in Fig. 5A is a typical set of results, obtained in this case with the nucleolar RNA samples from the infected culture used in the experiments shown in Fig. 1–3.

The gel system employed provides good resolution of the nucleolar RNA species and permits the ready detection not only of the major nucleolar species 45S and 32S pre-rRNA but also of several minor species. The sizes of these species have been estimated, using as standards the estimates of the lengths of denatured 45S, 32S, and 28S rRNA made by Wellauer and Dawid (37). The size of species h and its comigration with cytoplasmic 18S rRNA (Fig. 5A) identified it as the nucleolar counterpart of the smaller rRNA species. Species g migrated to the position expected for 20S pre-rRNA, the immediate precursor to 18S rRNA (34, 35). Both labeled 20S and 18S RNA were present in the nucleolus in small quantities, even in mock-infected HeLa cells, probably reflecting their rapid exit from the nucleolus (35), as was species i, which was of the size predicted for the second product of cleavage of 20S pre-rRNA. Ribonucleoprotein particles containing 20S or 18S rRNA dissociate from the nucleolus within 2 min of their formation after cleavage of 41S pre-rRNA at site III shown in Fig. 5B, a schematic representation of the 45S pre-rRNA primary transcript and its mode of cleavage (37). Nucleolar RNA species b and f corresponded in size to the initial products of cleavage of 45S pre-rRNA at site I (Fig. 5B). The minor species c, 36S, appeared to be derived by aberrant cleavage of 41S rRNA at site II. In HeLa cells, 36S RNA is present in low concentrations in the nucleolus and does not accumulate after poliovirus infection, as do 41S, 32S, 28S, and 18S rRNA species (34, 35). Thus, it does not appear to be a productive processing intermediate, by contrast with a species of similar size found in mouse L cells (38).

Upon Ad5 infection of HeLa cells, only a small reduction in the amount of nucleolar 28S rRNA labeled in a 2-h period could be discerned before 12 h after infection (Fig. 5A). By 18 h after infection, however, almost no newly made nucleolar 28S rRNA could be detected, and a significant decrease in the amount of 32S rRNA had also taken place (Fig. 5A). A comparable reduction in the amount of 45S pre-rRNA labeled was not observed in the time period investigated in this experiment (Fig. 5A). Thus, it appears to be unlikely that the inhibition of appearance of labeled 28S rRNA in the cytoplasm during the earlier part of the infectious cycle (Fig. 1 and 2) could be a consequence of inhibition of synthesis of the 45S primary transcript, a conclusion consistent with previous failures to find any change in the activity of form I RNA polymerase in infected compared to uninfected HeLa cells (36).

Synthesis of cellular proteins and the appearance of labeled 18S rRNA in the cytoplasm were substantially inhibited by 18 h after infection (Fig. 2 and Table 1). By this time, little newly synthesized 28S rRNA could be detected in the nucleolus, and the levels of labeled 41S, 36S, 24S, 20S, and 18S nucleolar RNA species were also visibly reduced (Fig. 5A). Such inhibition became even more pronounced by 24 h after infection (data not shown). The amounts of the labeled minor species designated a and e in Fig. 5A were also decreased by 18 h after infection, whereas labeled species d appeared at a some-

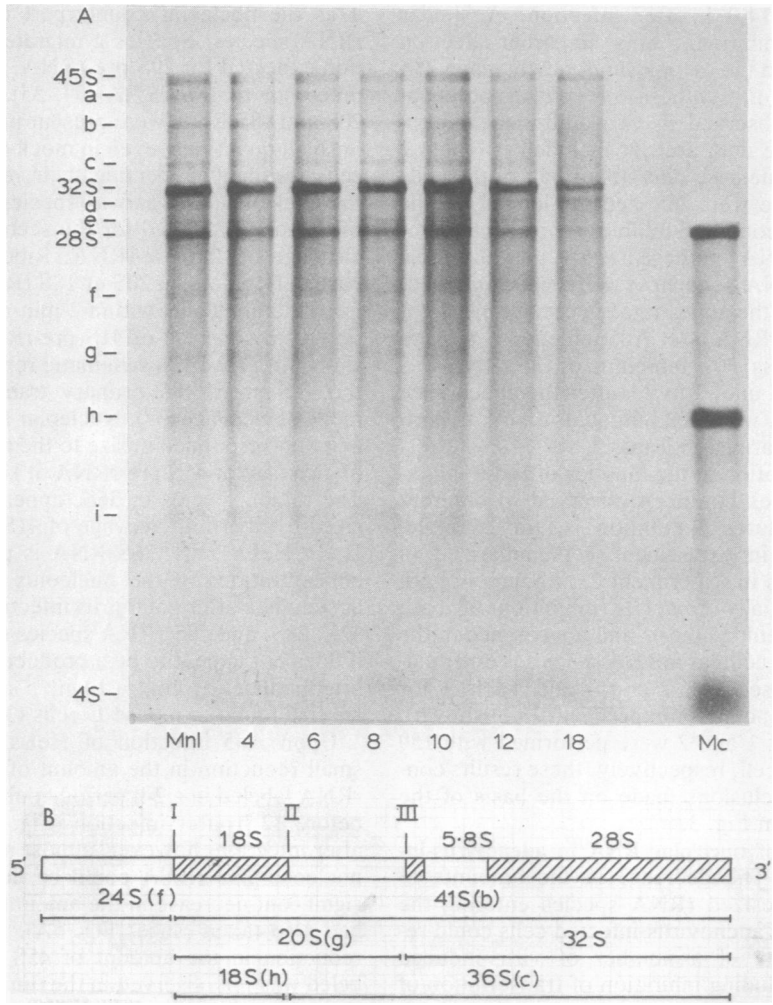

FIG. 5. Labeling of nucleolar RNA species in Ad5-infected HeLa cells. Ad5-infected HeLa cells were labeled with [^{3}H]uridine as described in the text beginning at the times after infection indicated in (A). The RNA was purified, glyoxalated, and fractionated by electrophoresis in 0.75% agarose gels as described in the text. (A) shows a fluorogram of such a gel in which samples from the infected culture are flanked by mock-infected HeLa nucleolar RNA (Mnl) and mock-infected cytoplasmic, poly(A)-lacking RNA (Mc). (B) A schematic representation of the HeLa cell 45S rRNA precursor, including the regions conserved during processing and those discarded (hatched and open areas, respectively), and the sites of endonucleolytic cleavage as discussed in the text.

what higher concentration. RNA species a, d, and e, with approximate lengths of 11.9, 6.5, and 5.5 kilobases, respectively, could not be identified or assigned a role in rRNA processing on the basis of the experiments we performed. It is possible, however, that the increase in concentration of species d at a time when little 28S rRNA was being produced reflected nonproductive processing of 32S rRNA.

A summary of the effects of Ad5 infection on the levels of labeled nucleolar 32S and 28S RNA is given in Fig. 6. The relatively low level of 45S pre-rRNA and its proximity to species a in Fig. 5 precluded reproducible estimations of its concentration. The data presented in Fig. 2 for the inhibition of appearance of labeled 28S rRNA in the cytoplasm are also redrawn in Fig. 6 to permit comparison of the relative levels of nucleolar and cytoplasmic pools of 28S rRNA, newly labeled at different times after Ad5 infection. In this experiment, the amounts of 32S and 28S RNA labeled during a 2-h period and present within the nucleolus declined fairly steadily from about 4 h after infection. From that point onward, the ratio of 32S to 28S was also changed in favor of the former compared with mock-infect-

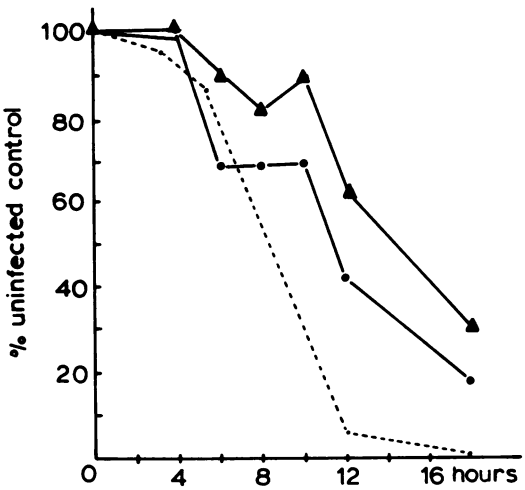

FIG. 6. Time course of inhibition of labeling of nucleolar RNA species in Ad5-infected HeLa cells. The amounts of 28S and 32S nucleolar RNA labeled in 2-h periods at the times after Ad5 infection shown were determined as described in the text. The resulting 32S (▲) and 28S (●) values, corrected for any variations in the amounts of each RNA applied to the gel, are expressed as a percentage of the values obtained with mock-infected control cultures. The dashed line is a replot of the data for cytoplasmic 28S rRNA given in Fig. 2.

ed cells. The most striking result, however, was the very slowness of the inhibition of nucleolar 28S rRNA production compared to the inhibition of appearance of mature 28S rRNA in the cytoplasm. By 12 h after infection, for example, nucleolar and cytoplasmic levels of 28S rRNA were reduced to some 60 and nearly 95% of control values, respectively. Moreover, the amount of nucleolar 28S rRNA made in a 2-h period remained apparently unchanged from 6 to 12 h after infection, a time period during which the cytoplasmic level decreased from about 85 to 7% of the control value.

DISCUSSION

The results presented here of an examination of the temporal patterns of inhibition of cellular protein synthesis and of rRNA and mRNA production in Ad5-infected HeLa cells establish that the effects of the virus are more complicated than might have been anticipated. Although the production of both 28S and 18S cytoplasmic rRNA is eventually completely inhibited in infected cells as reported previously (15, 27), cytoplasmic 28S rRNA is affected much more rapidly than 18S rRNA, suggesting that two distinct inhibitory processes, presumably mediated by different molecular mechanisms, may be at work in adenovirus-infected cells.

Little change in the cytoplasmic levels of newly synthesized 18S rRNA can be discerned at the earliest times after infection, up to 8 h in the experiment shown in Fig. 1 and 2. From this time onward, however, adenovirus infection is characterized by a fairly steady decline in the amount of labeled 18S rRNA reaching the cytoplasm. This occurs in parallel to, but a few hours later than, inhibition of cellular protein synthesis (Fig. 2, Tables 1 and 2). Substantial reductions in the nucleolar levels of newly synthesized 32S and 28S rRNA also become apparent during the later period of infection (Fig. 5 and 6). Although we have not been able to obtain accurate estimates of the amounts of labeled 45S pre-rRNA, inspection of Fig. 5 and the results of other similar experiments suggests that the amount of 45S rRNA labeled in a 2-h period is little changed until even later in the infectious cycle, from 12 to 16 h after infection in different experiments. These later responses in rRNA metabolism observed in Ad5-infected cells therefore appear to resemble those reported when protein synthesis is inhibited by means other than virus infection. The addition of cycloheximide, for example, although slowing the rate of transcription of rRNA genes, exerts a more profound influence on the rate of processing of 45S pre-rRNA. Consequently, the amounts of labeled rRNA species entering the cytoplasm are reduced to very low levels before transcription is inhibited to the same degree (4, 11, 40), a pattern very reminiscent of that seen during the late phase of Ad5 infection (Fig. 2 and 6). We cannot, however, exclude some contribution to the general reduction in rRNA production observed during the later part of an Ad5 infection consequent upon the earlier disruption of the normal metabolism of 28S rRNA.

The earliest effects of Ad5 infection on cellular RNA metabolism in HeLa cells are inhibition of the appearance in the cytoplasm of newly synthesized 28S rRNA and cellular mRNA sequences (Fig. 2, Table 2). These inhibitory processes exhibit quite similar rates after infection and begin several hours before the inhibition of cellular protein synthesis (Fig. 2; Tables 1 and 2). The reproducible precedence of the inhibition of entry of labeled 28S rRNA into the cytoplasm over the inhibition of cellular protein synthesis suggests that the former process cannot be dependent on the latter. Moreover, the well-known disruption of rRNA production that follows inhibition of ribosomal protein synthesis not only affects cytoplasmic 28S and 18S rRNA equally, but also is not substantial until protein synthesis has been 96 to 99% inhibited for at least 30 min (4, 17, 40), conditions that do not pertain to the early phase of Ad5 infection (Fig. 2).

The most dramatic reduction in the amounts of labeled 28S rRNA reaching the cytoplasm in Ad5-infected cells spans a time period (8 to 12 h in the experiment shown in Fig. 1 and 2) in which the effects on production of 45S pre-rRNA in the nucleolus are, by comparison, modest (Fig. 6). Similarly, processing of pre-rRNA does not appear to be substantially deranged at this time, in the sense that normal processing intermediates, such as 41S and 32S pre-rRNA and nucleolar 28S rRNA, continue to be made successfully. Thus, the most rapid phase of the inhibition of entry into the cytoplasm of 28S rRNA occurs in the absence of a comparable reduction in the amounts of labeled 28S rRNA produced in the nucleolus. It therefore appears that adenovirus infection does not induce sufficiently aberrant transcription or processing of 45S rRNA to account for the early failure of newly synthesized 28S rRNA to enter the cytoplasm. It is possible that adenovirus infection preferentially inhibits assembly of the larger ribosomal subunit, either by selective (and early) inhibition of synthesis of a crucial ribosomal protein or by a mechanism distinct from any effects upon synthesis of ribosomal proteins. The attempts described here to identify ribosomal proteins in extracts of Ad5-infected HeLa cells are certainly not of sufficient refinement to address the former possibility. Experiments to isolate and characterize the proteins of nucleolar particles synthesized in infected cells are therefore in progress. We believe, however, that it is unlikely that the specific inhibition of production of cytoplasmic 28S rRNA is mediated at the level of assembly. The processing of pre-rRNA and its assembly into ribonucleoprotein particles are coupled processes, in which addition of proteins to RNA begins very soon after synthesis of 45S pre-rRNA and continues in parallel with rRNA processing in the nucleolus (10, 14, 24). Thus inhibition of any step in the assembly process would be predicted to be reflected in a corresponding alteration in nucleolar rRNA processing, a prediction that is not borne out during the earliest phases of Ad5 infection (Fig. 5 and 6). Alternatively, adenovirus infection might rapidly inhibit the well-characterized methylation of rRNA (8, 16). Inhibition of methylation by starvation of cells for methionine or by addition of ethionine inhibits production of both mature rRNA species, while 32S rRNA accumulates (17, 32, 41). In adenovirus-infected cells, by contrast, 18S rRNA metabolism is quite normal until late times after infection. Indeed, the ready labeling of 45S and 32S RNA with [*methyl*-^{3}H]methionine during the late phase of adenovirus type 2 or type 12 infections has been reported (15, 30). The most likely explanation for the Ad5-induced inhibition of appearance of 28S rRNA in the cytoplasm, therefore, appears to be that the normal transport mechanism must be deranged.

This explanation is also in line with our current understanding of the mechanism(s) whereby adenovirus infection prevents the appearance in the cytoplasm of cellular mRNA sequences (Fig. 2) (3). We have established previously that transcription of cellular genes continues unabated in Ad2- or Ad5-infected cells until well into the late phase (3), an observation that has now been extended to the products of transcription of specific genes (Flint et al., in press; S. J. Flint, G. A. Beltz, J. J. Plunkett, and N. Cowan, unpublished observations). Moreover, neither polyadenylation (3), capping (G. A. Beltz and S. J. Flint, manuscript in preparation), nor splicing (Flint et al., in press) of the transcripts of genes whose mRNA species fail to reach the cytoplasm after adenovirus infection is inhibited. The formation in infected cells of ribonucleoproteins that contain cellular mRNA species and their precursors and transport from the nucleus itself remain to be investigated experimentally. However, the fact that there exists no posttranscriptional processing step that is known to be common to cellular mRNA and rRNA maturation, coupled with the observation described here that transport of these two kinds of cellular RNA species is inhibited with similar kinetics after adenovirus infection (Table 2), leads us to favor the idea that it is transport itself that is the primary target of the adenovirus-induced inhibitory mechanisms. Of course this deduction requires experimental support. Nevertheless, it provides a useful working model with testable predictions. The hypothesis that adenovirus infection inhibits transport from the nucleus to the cytoplasm of mature 28S rRNA and cellular mRNA species implies, for example, either that these two classes of RNA species are normally cotransported, a view for which no experimental support exists, or that there is some common feature to be found in their mode of transport.

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LITERATURE CITED

1. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. J. Virol. 12:241–252.
2. **Bello, L. J., and H. S. Ginsberg.** 1969. Relationship between deoxyribonucleic acid-like ribonucleic acid synthesis and inhibition of host protein synthesis in type 5 adenovirus-infected KB cells. J. Virol. 3:106–113.
3. **Beltz, G. A., and S. J. Flint.** 1979. Inhibition of HeLa cell

protein synthesis during adenovirus infection: restriction of cellular messenger RNA sequences to the nucleus. J. Mol. Biol. 131:353–373.
4. Craig, N. C., and R. P. Perry. 1970. Aberrant intranucleolar maturation of ribosomal precursors in the absence of protein synthesis. J. Cell. Biol. 45:554–564.
5. Darnell, J. E., Jr., M. Girard, D. Baltimore, D. F. Summers, and J. V. Maizel, Jr. 1967. The synthesis and translation of poliovirus RNA, p. 375–401. *In* J. S. Colter and W. Paranchych (ed.), The molecular biology of viruses. Academic Press Inc., New York.
6. Falvey, A. K., and T. Staehlin. 1970. Structure and function of mammalian ribosomes: isolation and characterization of active liver ribosomal subunits. J. Mol. Biol. 53:1–34.
7. Flint, S. J. 1982. Expression of adenoviral genetic information in productively infected cells. Biochim. Biophys. Acta 651:175–208.
8. Greenberg, H., and S. Penman. 1966. Methylation and processing of ribosomal RNA in HeLa cells. J. Mol. Biol. 21:527–535.
9. Ginsberg, H. S., L. J. Bello, and A. J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenovirus, p. 547–572. *In* J. S. Colter and W. Paranchych (ed.), The molecular biology of viruses. Academic Press Inc., New York.
10. Hadjiolov, A. A. 1980. Biogenesis of ribosomes in eukaryotes. Subcell. Biochem. 7:1–80.
11. Higashi, K., T. Matsuhisa, A. Kitao, and Y. Sakamoto. 1968. Selective suppression of nucleolar RNA metabolism in the absence of protein synthesis. Biochim. Biophys. Acta 166:388–392.
12. Horak, I., and D. Schiffman. 1977. Acidic phosphoproteins of the 60S ribosomal subunits from HeLa cells. Eur. J. Biochem. 79:375–380.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
14. Lastick, S. M. 1980. The assembly of ribosomes in HeLa cell nucleoli. Eur. J. Biochem. 111:175–182.
15. Ledinko, N. 1972. Nucleolar ribosomal precursor RNA and protein synthesis in HEK cultures infected with Ad12. Virology 49:79–89.
16. Maden, B. E. H., and M. Salim. 1974. The methylated nucleotide sequences in HeLa cell ribosomal RNA and its precursors. J. Mol. Biol. 88:133–164.
17. Maden, B. E. H., and M. H. Vaughan. 1969. Synthesis of ribosomal proteins in the absence of ribosome maturation in methionine-deficient HeLa cells. J. Mol. Biol. 38:431–435.
18. Maden, B. E. H., M. H. Vaughan, J. R. Warner, and J. E. Darnell. 1969. Effects of valine deprivation on ribosome formation in HeLa cells. J. Mol. Biol. 45:265–275.
19. Mathews, M. B., and U. Pettersson. 1978. The low molecular weight RNAs of adenovirus 2-infected cells. J. Mol. Biol. 119:293–328.
20. McMaster, G. K., and G. C. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. 74:4835–4838.
21. Ohe, K., and S. M. Weissman. 1971. The nucleotide sequence of a low molecular weight RNA from cells infected with adenovirus 2. J. Biol. Chem. 246:6999–7009.
22. Pederson, T., and A. Kumar. 1971. Relationship between protein synthesis and ribosome assembly in HeLa cells. J. Mol. Biol. 61:655–668.
23. Perry, R. P., and D. E. Kelley. 1968. Messenger RNA-protein complexes and newly synthesized ribosomal subunits: analysis of free particles and components of polyribosomes. J. Mol. Biol. 35:37–59.
24. Phillips, W. F., and E. H. McConkey. 1976. Relative stoichiometry of ribosomal proteins in HeLa nucleoli. J. Biol. Chem. 251:2876–2881.
25. Piña, M., and M. Green. 1969. Macromolecule and enzyme synthesis in cells replicating oncogenic and non-oncogenic human adenoviruses. Virology 38:573–586.
26. Price, R., and S. Penman. 1972. Transcription of the adenovirus genome by an α-amanitine-sensitive ribonucleic acid polymerase in HeLa cells. J. Virol. 9:621–626.
27. Raskas, H. J., D. C. Thomas, and M. Green. 1970. Biochemical studies on adenovirus multiplication. XVII. Ribosome synthesis in uninfected and infected KB cells. Virology 40:893–902.
28. Russell, W. C., and J. J. Skehel. 1972. The polypeptides of adenovirus infected cells. J. Gen. Virol. 15:45–57.
29. Söderlund, J., U. Pettersson, B. Vennstromm, L. Philipson, and M. B. Mathews. 1976. A new species of virus coded, low molecular weight RNA from cells infected with adenovirus type 2. Cell 7:585–593.
30. Sommer, S., M. Salditt-Georgieff, S. Bachenheimer, J. E. Darnell, Y. Furuichi, M. Morgan, and A. J. Shatkin. 1976. The methylation of adenovirus-specific nuclear and cytoplasmic RNA. Nucleic Acids Res. 3:749–765.
31. Thimmappaya, R., C. Weinberger, R. Schneider, and T. Shenk. 1982. Adenovirus VA1 RNA is required for efficient translation of viral RNAs at late times after infection. Cell 31:543–551.
32. Vaughan, M. H., R. Soeiro, J. R. Warner, and J. E. Darnell. 1967. The effects of methionine deprivation on ribosome synthesis in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 58:1527–1534.
33. Warner, J. R., M. Girard, H. Latham, and J. E. Darnell. 1966. Ribosome formation in HeLa cells in the absence of protein synthesis. J. Mol. Biol. 19:373–382.
34. Weinberg, R. A., U. Leoning, M. Willems, and S. Penman. 1967. Acrylamide gel electrophoresis of HeLa cell nucleolar RNA. Proc. Natl. Acad. Sci. U.S.A. 58:1088–1095.
35. Weinberg, R. A., and S. Penman. 1970. Processing of 45S nucleolar RNA. J. Mol. Biol. 47:169–178.
36. Weinmann, R., J. A. Jaehning, H. J. Raskas, and R. G. Roeder. 1976. Viral RNA synthesis and levels of DNA-dependent RNA polymerases during replication of adenovirus 2. J. Virol. 17:114–126.
37. Wellauer, P. K., and I. B. Dawid. 1973. Secondary structure maps of RNA: processing of HeLa ribosomal RNA. Proc. Natl. Acad. Sci. U.S.A. 70:2827–2831.
38. Wellauer, P. K., I. B. Dawid, D. E. Kelley, and R. P. Perry. 1974. Secondary structure maps of ribosomal RNA: processing of mouse L-cell ribosomal RNA and variations in the processing pathway. J. Mol. Biol. 89:379–407.
39. White, D. L., M. D. Scharff, and J. V. Maizel. 1969. The polypeptides of adenovirus III. Synthesis in infected cells. Virology 38:395–406.
40. Willems, M., M. Penman, and S. Penman. 1969. The regulation of RNA synthesis and processing in the nucleolus during inhibition of protein synthesis. J. Cell Biol. 41:177–187.
41. Wolf, S. F., and D. Schlessinger. 1977. Nuclear metabolism of ribosomal RNA in growing, methionine-limited and ethionine-treated HeLa cells. Biochemistry 16:2783–2791.
42. Ziff, E. 1980. Transcription and processing by the DNA tumor viruses. Nature (London) 287:491–499.