BACTERIOLOGICAL REVIEWS, Sept., 1968, p. 262-290

Vol. 32, No. 3
Printed in U.S.A.

Ribonucleic Acids from Animal Cells

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INTRODUCTION

One of the important branches of molecular biology at the present time is the study of the synthesis, structure, and function of ribonucleic acid (RNA) molecules; yet only within the past 15 years has it been generally appreciated that cellular RNA comprises not a uniform collection of polymers but many different molecules of defined length. The first distinguishable class of RNA molecules was transfer RNA (tRNA), the carrier of amino acids for protein synthesis (44, 114). Another signal event in the development of successful studies on RNA molecules was the demonstration that the RNA of tobacco mosaic virus (TMV) was infectious by itself (32) and consisted of a very long polymer, about 6,000 nucleotides (30). Furthermore, a damaging chemical or physical event anywhere along the virus RNA chain resulted in inactivation of infectivity of the RNA, implying the necessity for integrity of the whole molecule for viral replication (62).

It was soon found that many RNA-containing animal viruses also possessed infectious RNA molecules comparable in size to TMV (84). These studies on viral RNA were important to the development of RNA biochemistry in general because they provided a yardstick by which other RNA preparations could be judged. Thus, hopefully, extraction procedures which yielded whole viral RNA molecules from infected cells (108) could be used to extract cellular RNA in an undegraded state.

The greatest impetus in recent times to the study of RNA metabolism was provided by the prediction of messenger RNA (mRNA) in 1960–1961 (51), followed shortly by its experimental demonstration in bacteria (9, 38). This hitherto unrecognized RNA species was the direct bearer of genetic information between deoxyribonucleic acid (DNA) and the protein-synthesizing apparatus. These events made the examination of RNA in animal cells a pressing experimental necessity. Cultured animal cells offered a source of virtually unlimited numbers of clonally derived cells which could be easily labeled with radioisotopes. Therefore, experiments were begun which aimed at identifying various classes of animal-cell RNA to compare with those identified in bacteria. From cytochemical and radioautographic data, virtually all (excluding mitochondrial DNA) of

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the cellular DNA was known to be nuclear, and most of the RNA and protein synthesis was known to be cytoplasmic (36, 73, 77, 97). Thus, it might have been anticipated that much of the work of RNA isolation and characterization in animal cells would involve nuclear RNA species and their possible relationship to the cytoplasmic RNA species. This has indeed proved to be the case, and the problems attendant to this question have by no means been completely solved. Enough is now known, however, especially about ribosome biosynthesis, to justify a general summary of RNA synthesis in cultured animal cells. Although most of the experiments to be described were performed in cultured cells, much of the future interest will center on the role of RNA in functioning cells, differentiated cells, and, especially, in differentiating cells. Reference will therefore also be made to specific experiments dealing with differentiated cells. The author takes the prerogative, however, of not attempting to cover comprehensively all points dealing with RNA synthesis in differentiated or, for that matter, cultured cells.

TECHNICAL ASPECTS OF RNA BIOCHEMISTRY

Before dealing with experimental results, it will be helpful to review briefly the major techniques involved in examining RNA molecules. A completely satisfactory comparison of one RNA species with another ultimately demands a knowledge of the linear arrangement of nucleotides in the two species. Recent years have seen remarkable advances along these lines, so that the sequence of a number of tRNA molecules from yeast and *Escherichia coli* (46, 60) and of the 5*S* ribosomal RNA (rRNA) molecules from *E. coli* (15) and KB cells (27), a strain of cultured human carcinoma cells, have been determined. These molecules, however, range between 75 and 121 nucleotides in length. The time when complete sequences of longer polynucleotides will be available is considerably in the future. It is necessary, therefore, to turn to less decisive techniques for comparing the physicochemical properties of various RNA molecules.

Size

Sedimentation velocity is the most common property determined on an isolated RNA sample. Although very accurate conclusions about the molecular weight of various RNA species cannot be obtained by sedimentation velocity experiments (e.g., the sedimentation rates of different types of RNA respond differently to changes in ionic strength of the suspending medium), it is possible by zonal sedimentation to assess the range of molecular weights and the homogeneity of an RNA sample. Sucrose gradient zonal sedimentation has therefore become a standard technique for analyzing and preparing RNA (10). A recent addition to the techniques which separate single-stranded RNA molecules largely on the basis of chain length is acrylamide gel electrophoresis of RNA (59). This technique offers technical advantages of speed and superior resolution between RNA species of very similar sizes, but for preparative purposes the density gradient techniques are still more widely employed.

Composition

After the separation of RNA with a homogeneous sedimentation behavior, the overall composition of the sample with respect to the four common ribonucleotides (base composition) can be determined. The technique usually employed is alkaline hydrolysis, followed by separation of the resulting 2′,3′ ribonucleotides by column chromatography or by paper electrophoresis (18, 83). If a sufficiently large RNA sample is available, the amounts of the four ribonucleotides can be estimated by ultraviolet (UV) absorption. Frequently, however, only a small amount of a radioactive RNA species is available, and it may even contain a large amount of another type of nonradioactive RNA. In this case, if the radioactive species is labeled with ^{32}P, the base composition can be determined by assaying the distribution of ^{32}P among the four ribonucleotides released by alkaline hydrolysis. This latter method, in comparison with UV analysis of large amounts of purified RNA, has proven quite satisfactory for the determination of base composition of long polynucleotides, even after brief periods of label (83, 90).

A simple technique has been developed over the past several years which carries the analysis of RNA molecules one step further than simple average base composition. In place of alkaline hydrolysis, which produces 2′,3′ mononucleotides, the RNA can be subjected to controlled enzymatic digestion with pancreatic ribonuclease or T1 ribonuclease (an enzyme from *Aspergillus oryzae* which preferentially cleaves RNA chains on the 3′ side of guanylic acid residues). This results in a specific pattern of oligonucleotides from each type of RNA molecule. Even if different RNA molecules have identical overall base composition, their oligonucleotide patterns can be distinguished (94).

Methylation

As will be discussed in more detail later, the rRNA and tRNA molecules of animal cells,

just as was first shown to be the case in bacterial cells, have methyl groups transferred to them at the polynucleotide stage from the terminal methyl group of methionine. Other types of cellular RNA lack these methyl groups. The use of methyl-labeled methionine provides a means of differentially labeling the majority species of RNA and other types of cellular RNA (13).

RNA-DNA Hybridization

At the present time, the only reasonable approach to the investigation of sequences of large RNA molecules, which are indistinguishable by the above-mentioned chemical tests, is to compare their ability to hybridize with DNA (41, 93). Especially useful is the so-called "competition" experiment, in which one species of RNA is shown to occupy or fail to occupy the same DNA sites as another species. As will be discussed later, in order to achieve clear-cut results in the comparison of similar species of animal cell RNA by hybridization, care must be taken to measure relatedness of molecules by the most exacting tests possible.

Cell Fractionation

An important aspect in the study of RNA molecules is the determination of their cellular localization. A number of significant advances in cell fractionation techniques have contributed to at least a partial realization of this goal. Not only have these techniques proved very useful in helping to prepare various RNA species of greater purity, but strong evidence also exists that in many cases they provide physiologically valid fractionation of the cell (6, 67, 70). In many experiments, therefore, cell fractionation precedes the release and study of RNA molecules.

One final point in this regard is that all cellular RNA, with the possible exception of tRNA, exists in combination with protein within the cell. In the examination of cell fractions, it is frequently advantageous to examine the types and distribution of ribonucleoproteins of a particular cell fraction before releasing the RNA for further physical or chemical analysis.

The various experimental tactics and maneuvers which will be referred to in the discussion to follow are outlined in Fig. 1.

PATTERNS OF SYNTHESIS OF ANIMAL CELL RNA

"Rapidly Labeled RNA" and Kinetic Analysis Experiments

About 80% of the RNA obtained from any type of cell is made up of the two species of rRNA. Most of the remainder is tRNA. In the simplest of cases, exposure of growing cells to radioactive label would result in these species of RNA becoming labeled in proportion to their amounts within the cell. In fact, if either bacterial cells or growing animal cells are exposed to labeled RNA precursors for a large fraction of their respective generation times, the majority species of RNA do become proportionately labeled. Figure 2 shows that such a result occurs when HeLa cells are labeled for 24 hr with ^{14}C-uridine. If, however, the labeling period is restricted to a small fraction of the generation time (Fig. 3A), it is found that most of the recently incorporated radioactivity (i.e., newly made RNA) is not in the majority species of RNA (85). Figure 3 shows the sedimentation pattern of the total HeLa cell RNA extracted from whole cells after labeling periods of 5, 15, 30, 45, and 60 min. The majority species of RNA (28*S*, 16 to 18*S*, and

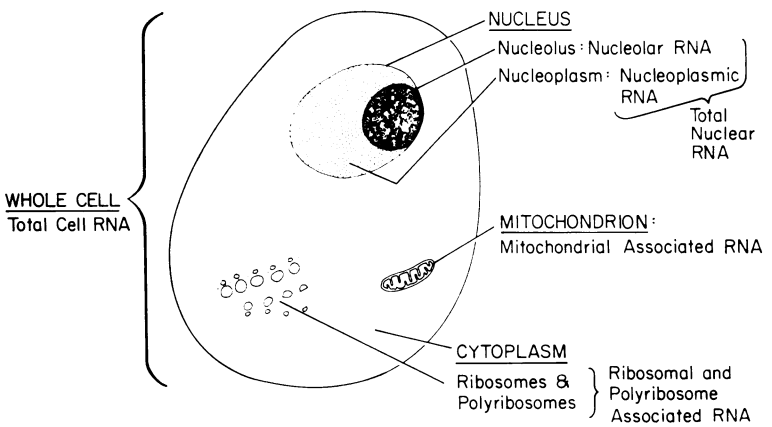

FIG. 1. *Diagram of cellular fractions or structures from which RNA can be extracted*

4S or tRNA) are seen to comprise the majority of the UV-absorbing material. The labeled RNA clearly does not conform to the sedimentation profile of the majority species. Several points are evident. (i) The rapidly labeled RNA is heterogeneous in sedimentation profile with molecules sedimenting from about 20S all the way to 100S. (ii) In the sample taken from cells labeled for 5 min, there appears to be a peak at about 45S which becomes quite distinct after 15 min of labeling. (iii) Between 15 and 30 min, radioactivity appears in a second prominent peak which sediments at 32S, somewhat faster than the 28S or larger rRNA molecule. It is important to point out here a fact which will be dealt with in more detail later. Early experiments on radioautography of animal cells which were briefly labeled with radioactive RNA precursors had established that the nucleus was the initial site of incorporation of the great majority of ^{3}H and ^{14}C purine and pyrimidine nucleosides (36, 73, 77, 97). It was also established in early cell fractionation studies with L cells and HeLa cells that most (about 90%) of the radioactivity incorporated within 30 min of exposure to nucleosides was in the cell nucleus (20, 71). Thus, when the total "rapidly labeled RNA" was extracted from whole cells, as in Fig. 3, it was clear that the majority came from the cell nucleus.

Several possible origins of such "rapidly

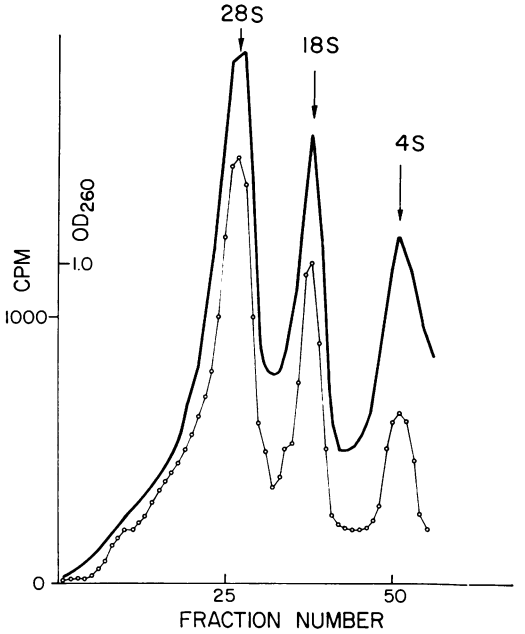

FIG. 2. *Sucrose gradient sedimentation analysis of total cell RNA (HeLa cells) after a 24-hr exposure to ^{14}C-uridine. Fraction 1 is the bottom of the gradient in this and subsequent diagrams; solid line, optical density at 260 nm; O, counts per minute. Redrawn from Scherrer and Darnell (85).*

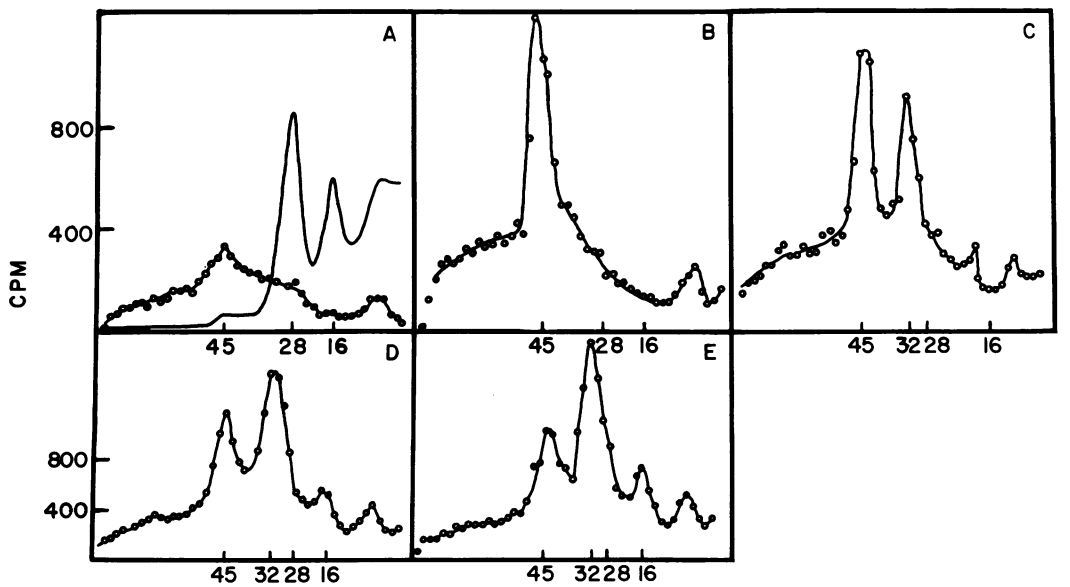

FIG. 3. *Sedimentation analysis of radioactivity in total cell RNA (HeLa cells) after 5, 15, 30, 45, and 60 min (a–e) exposure to ^{3}H-uridine. From Warner et al. (107).*

labeled nuclear RNA" could be immediately suggested. First, the RNA could be "nascent," i.e., not yet completed polynucleotide chains. This possibility was obviously unlikely, at least for the great majority of "rapidly labeled RNA" from animal cells, because the rapidly labeled RNA had physical properties which indicated that it was substantially larger (had a greater chain length) than the majority species of RNA.

For example, samples of RNA isolated from either the 45*S* or 70*S* region of a sucrose gradient again sediment in the same region during a second sedimentation (107). Treatment with deoxyribonuclease, proteolytic enzymes (trypsin and Pronase), ethylenediaminetetraacetate (chelating agent), dimethyl sulfoxide (DMSO; destroys secondary structure of macromolecules), or elevated temperatures failed to alter the sedimentation properties of the rapidly sedimenting molecules (1, 52, 90, 107). Electron micrographs of molecules taken from both the 45*S* and 60*S* regions of the sucrose gradient provide a final bit of evidence on the chain length of the nuclear molecules (88). The 45*S* rRNA is about two to three times as long as the 28*S* rRNA, and the 60*S* molecules are much longer yet (up to 5 to 6 μm in length), indicating molecular weights in excess of 10^7. Thus, it can be safely concluded that the rapidly labeled RNA molecules are not aggregates or artifacts of any kind, but rather are very long polynucleotide chains.

A second explanation of the origin of the rapidly labeled RNA could be that the soluble pools of ribonucleotides from which polynucleotides are built were segregated. In this case, the rapidly labeled material would represent a small amount of the total cell RNA which was made from a pool that became labeled very rapidly in comparison with the pool from which the bulk of the cell RNA might be drawn. This explanation remains virtually impossible to test directly, but, as will become clear from the remainder of the discussion, it is most unlikely to prove accurate.

Strong experimental support has accumulated for a third explanation of the nature of "rapidly labeled" RNA—namely, that these RNA molecules in animal cells represent a mixture of (i) larger molecules which are precursors to ribosomal RNA and (ii) a class of RNA, about 1% of the total cell RNA, which is constantly being synthesized and degraded.

Before further describing experimental results, the importance of a "kinetic analysis" of the flow of radioactivity through and into various RNA species needs emphasis. Precursor-product relationships of molecules within cells are difficult, if not impossible, to establish on kinetic grounds alone (78). This is especially true of RNA molecules, because the introduction of radioactive RNA precursors into the cell pool cannot be followed by an effective chase (90, 107). [Experimentally, this means that incorporation of radioactive RNA precursor cannot be immediately stopped upon addition of unlabeled precursor to the medium. This is in fact true for bacterial as well as animal cells (63).] Thus, without the intervention of drug treatments, it is not possible to label precursor molecules, stop further labeling, and observe the fate of the labeled species. To discover something about the precursor relationship of rapidly labeled species to the major RNA species, another approach has been necessary. First, chemical evidence was obtained of a relationship between rapidly labeled RNA species and a majority species of RNA. Second, it was determined how long it takes to accumulate the maximal label in a rapidly labeled species before the first appearance of label in various chemically related molecules.

Chemical evidence about the rapidly labeled RNA molecules was most easily obtained by labeling cells with $^{32}PO_4^{3-}$, isolating various RNA species by zonal sedimentation, and determining the base composition of RNA molecules with different sedimentation coefficients. Figure 4 shows such an experiment. The total cellular RNA of HeLa cells labeled with $^{32}PO_4^{3-}$ for 35 min was examined. The most important points which come from this experiment are the following. (i) The sedimentation profile of labeled RNA molecules is approximately the same as a 20-min uridine label (compare with Fig. 3) due to the slower entry of PO_4^{3-} into the acid-soluble nucleotide pool. (ii) The guanine plus cytosine (GC) content of the molecules from the 45*S* region is high (>60%). (iii) The larger molecules (those sedimenting from 45 to ~90*S*) have a low GC content. Comparison of these values with the majority species of RNA, rRNA and tRNA, and with HeLa cell DNA (Table 1) reveals that the heterogeneous large RNA has a composition similar to DNA. For convenience, this material has been designated as heterogeneous nuclear RNA, HnRNA (90, 107). (Other authors have referred to this RNA as "giant RNA," "messenger-like" RNA, or dRNA; also, *see* references 1a, 24, 49, 87, 112).

By contrast, the RNA in the 45*S* region was found to have a base composition similar to the rRNA (86, 90, 107). These results, of course, suggested a possible precursor role for the 45*S* RNA in the formation of ribosomes. A decisive experiment to test this hypothesis was made possible by the discovery of the action of actino-

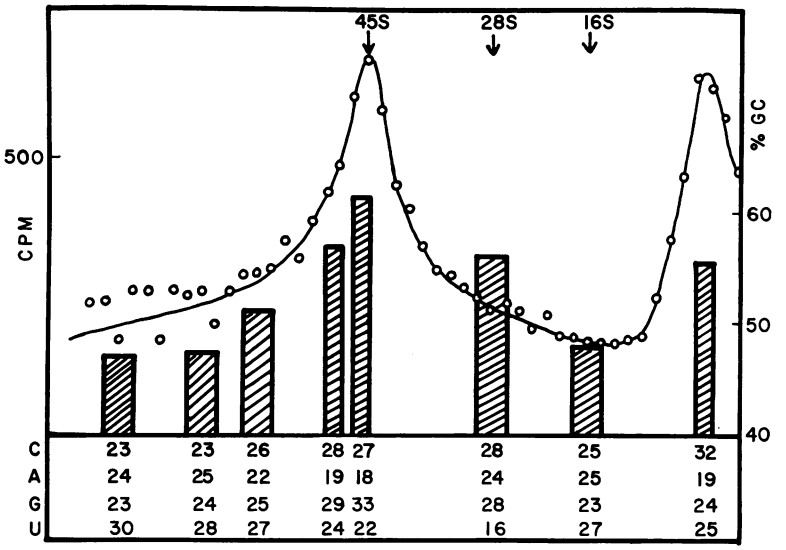

FIG. 4. *Sedimentation and base composition analysis of total "rapidly labeled" HeLa cell RNA after 35-min exposure to ^{32}P-orthophosphate. Samples were taken of various sizes of RNA after the sedimentation analysis and examined for base composition after alkaline hydrolysis. Results are plotted in the bar graphs as percentage guanylic and cytidylic acid (%GC). From Soeiro, Birnboim, and Darnell (90).*

TABLE 1. *Distribution and base composition of various molecules in HeLa cells*[a]

Cell fraction	RNA species	Total cell RNA (%)	Base composition, % as				
			C	A	G	U	GC
Cytoplasma	28S rRNA	53	32	16	36	16	68
	7S rRNA	~1	28	21	28	23	56
	5S rRNA	~1	26	18	34	22	60
	18S rRNA	24	27	21	30	22	57
	tRNA	12	27	22	27	24	54
	"pre-tRNA"	<1					
	mRNA (polysomes)	~3	24	26	21	28	45
	Cytoplasmic heterogeneous[b]	<1					
Total		94.5					
Nucleus	45S r-pre-RNA	1	33	13	37	17	70
	32S r-pre-RNA	3	33	14	37	16	70
	HnRNA		22	26	21	31	43

[a] Data taken from references 52, 90, and 107. The base composition of HeLa cell DNA is C, 21; A, 29; G, 22; T, 28. Abbreviations: pre-tRNA, precursor transfer RNA; r-pre-RNA, ribosomal precursor RNA; HnRNA, heterogeneous nuclear RNA; C, A, G, U, and T represent cytidylic, adenylic, guanylic, uridylic, and thymidylic acids.

[b] Heterogeneous RNA not in polysomes.

mycin D, an antibiotic which binds to DNA, thereby preventing RNA transcription by RNA polymerase (50, 79). Thus, cells which had been labeled long enough so that the 45S peak was very prominent but not long enough for radioactivity to be found in 28 and 18S rRNA were treated with actinomycin to determine the fate of 45S RNA in the absence of further RNA synthesis. It was found (Fig. 5) that the 45S RNA quickly disappeared in actinomycin-treated cells, and coincident with its disappearance radioactivity appeared in 32 and 18S RNA (35, 67, 86). After longer periods in actinomycin, the radioactive RNA in the 32 to 28S region gradually shifted to predominantly 28S (35).

These two experiments—a determination of the base composition of 45S RNA and the "actinomycin chase"—strongly suggested that rRNA in HeLa cells was formed as a long polynucleotide which was subsequently cleaved specifically to yield the 28 and 18S rRNA molecules. Thus, two classes of molecules make up the majority of the rapidly labeled nuclear RNA —the HnRNA, which is DNA-like in composition, and 45S RNA, which is related to rRNA. There are also other types of cytoplasmic RNA which make up only a small proportion of the total rapidly labeled RNA; these will be discussed later.

BIOSYNTHESIS OF RIBOSOMES IN ANIMAL CELLS

Ribosomal Precursor RNA

Since a great deal of morphological and cytogenetic evidence suggested that the nucleolus

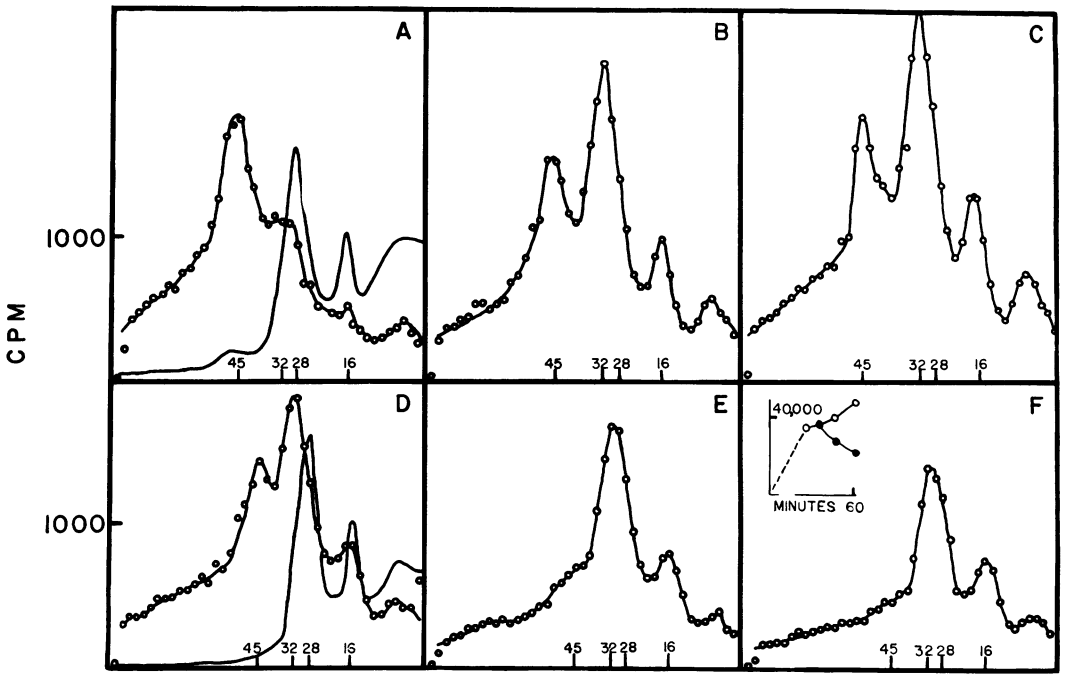

FIG. 5. *Actinomycin chase experiment. HeLa cells were exposed to ^{3}H-uridine for 25 min (A), 45 min (B), or 60 min (C). At 25 min, the culture was divided and a portion was treated with actinomycin (5 μg/ml). Samples of the treated culture were taken after 10 min (D), 20 min (E), or 35 min (F). Total cell RNA was extracted from each sample and analyzed on sucrose gradients.*

was the site of nuclear ribosome accumulation if not also of formation (4, 8, 14, 22, 74), attempts were made in cultured cells to prove that 45*S* rRNA existed in the nucleolus. The first strongly suggestive experiments on this point were performed in L cells using a combination of radioautography, actinomycin D treatment, and examination of extracted RNA from L cells (72). First, it was shown that growing L cells synthesized 45*S* RNA and HnRNA and that radioautography revealed strong incorporation in both the nucleolus and the nucleus at large. Brief treatment of these cells with a low dose of actinomycin (0.05 μg/ml) reduced RNA precursor uptake by about 50%. However, nucleolar RNA synthesis, as observed by radioautography, and 45*S* ribosomal precursor RNA (r-pre-RNA) synthesis, assayed by zonal sedimentation, were almost completely obliterated. It was therefore concluded that 45*S* synthesis occurred in the nucleolus of the cell. Cell fractionation studies have added additional evidence that this is the case. HeLa cell nuclei can be lysed by deoxyribonuclease treatment in the presence of relatively high salt concentrations (0.5 M NaCl, 0.05 M Mg^{++}), leaving the nucleoli intact morphologically so that they can be easily isolated (47, 70). Release and examination of the nucleolar RNA revealed that about 2 to 3% of the total cell RNA could be recovered as 45*S* and 32*S* r-pre-RNA (47, 70; R. Soeiro et al., J. Cell Biol., *in press*). (*See* Fig. 12 and Table 1.) Moreover, no 45*S* or 32*S* r-pre-RNA was found outside the nucleolus (Fig. 6; 47, 70, 90). Thus, a perfect correlation existed between the cytological and cytogenetic evidence and the biochemical evidence that ribosome formation in animal cells was initiated by the formation of 45*S* r-pre-RNA in the cell nucleolus.

Another important chemical event in the manufacture of ribosomes has been shown to occur in the nucleolus. The rRNA isolated from HeLa cells (total cells) had been found to contain methyl groups both attached to nucleic acid bases and to the 2′ OH of the ribose of rRNA (13). The direct precursor of RNA methylation reaction was known to be the terminal methyl group of *S*-adenosyl methionine. Using methyl-labeled methionine as a precursor for these methyl groups, it was shown (Fig. 7) that the first molecules in the pathway to ribosomes which become labeled were the 45*S* r-pre-RNA followed by 32*S* (later

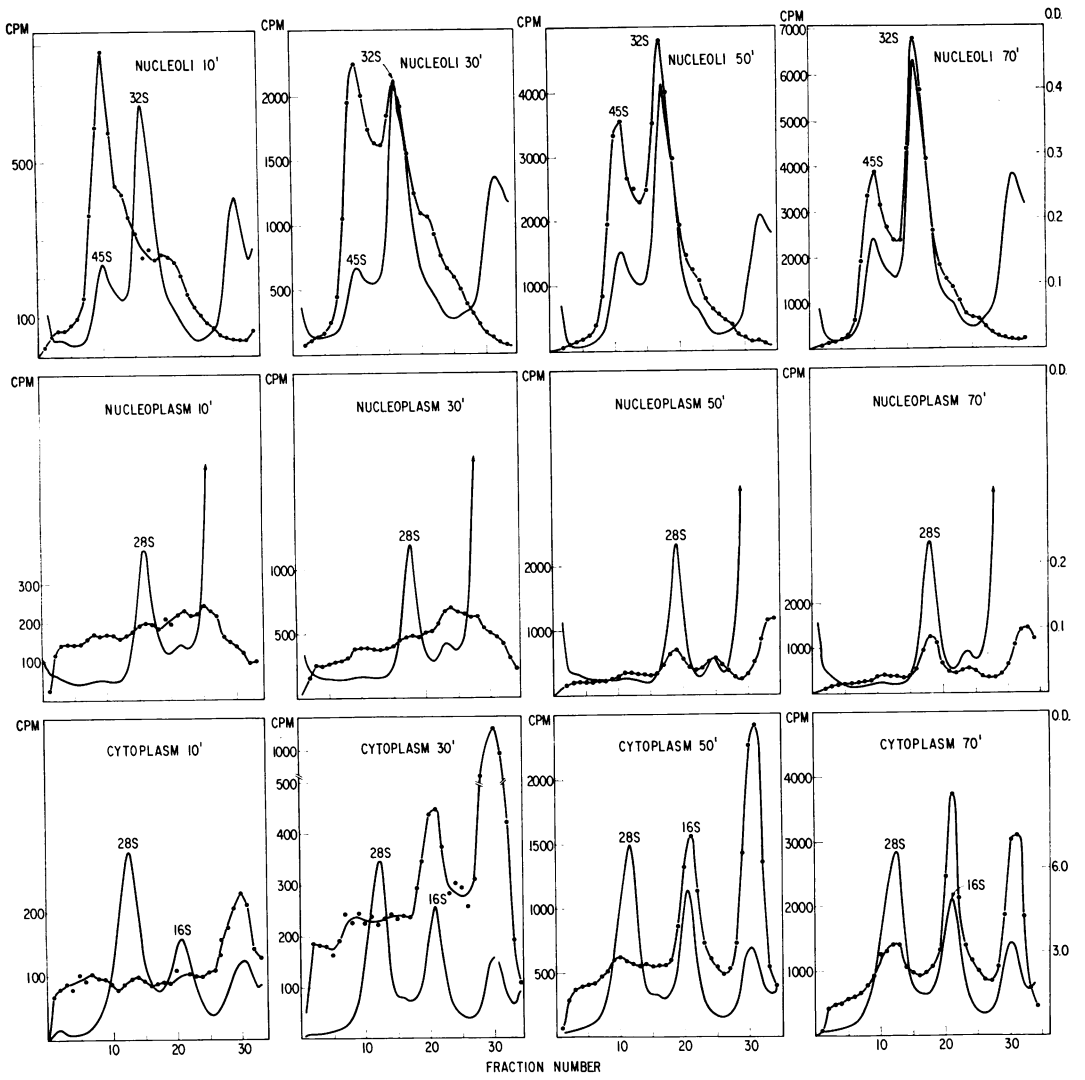

FIG. 6. *Partition of radioactive RNA in fractions of HeLa cells. Cells were exposed to* ^{14}C*-uridine for 10, 30, 50, or 70 min and fractionated; RNA was extracted and analyzed. From Penman, Smith, and Holtzman (70).*

28) and 18*S*, just as was the case when nucleosides were the labeled RNA precursor (37, 115). Thus, the 45*S* ribosomal precursor RNA appeared to be the chief, if not the sole, site of methylation. For example, if 50% of the methylation occurred on rRNA molecules past the 45*S* stage, then methyl incorporation should have proceeded in those species as well as in 45*S*. No evidence of methyl incorporation directly into 32, 28, or 18*S* regions was found. In addition, methylation apparently occurred either very soon after or concomitant with 45*S* synthesis, because actinomycin D treatment abolished within 5 min the ability of the cells to incorporate methyl groups into 45*S* RNA (115) or any other high molecular weight RNA.

Thus far, experiments have been described which indicate that 45*S* ribosomal precursor RNA is made and methylated in the nucleolus. Since 32*S* RNA is also found in the nucleolus, it appears that the cleavage of the 45*S* r-pre-RNA also begins in the nucleolus. Recent experiments, in fact, indicate that a whole series of cleavage steps occur in the nucleolus. These experiments were made possible by the development of the technique of acrylamide gel electrophoresis of RNA molecules (59), which separates single-stranded RNA molecules, presumably on the

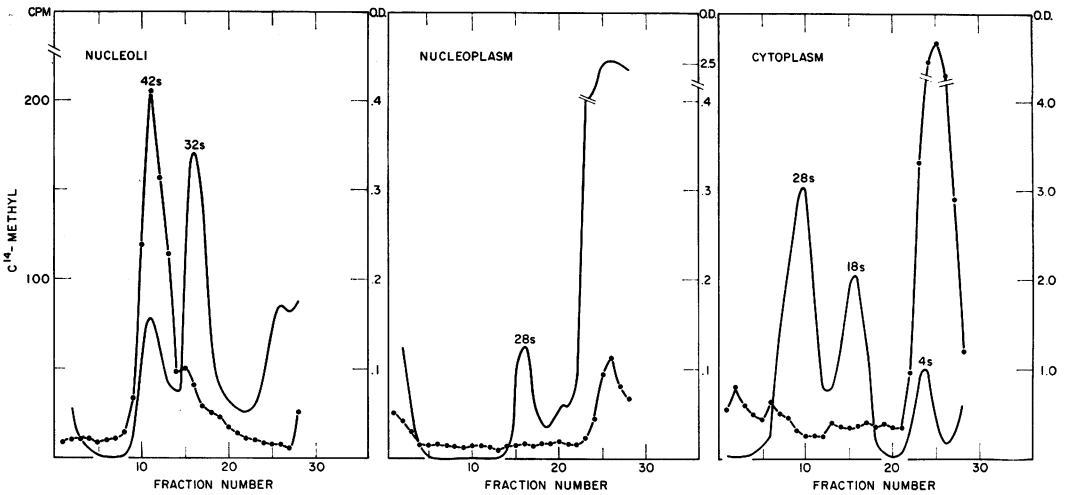

FIG. 7. *Incorporation of methyl groups into 45S ribosomal precursor RNA. HeLa cells were labeled for 10 min with ^{14}C-methyl labeled methionine and RNA was extracted from cell fractions. From Greenberg and Penman (37).*

basis of chain length, but which affords somewhat higher resolution of RNA species than does sucrose gradient zonal sedimentation. When total nucleolar RNA was extracted from cells which had incorporated labeled methionine for 30 min, it was found by gel electrophoresis analysis that, as expected, the 45*S* peak was the major labeled species (109). Moreover, the optical density tracing of the gel showed peaks between 45 and 32*S*, which indicated the possibility of a distinct species intermediate between the two main rRNA precursors (Fig. 8). (For convenience, these intermediate peaks are designated 41*S* and 36*S*, although no careful sedimentation analysis of them has been performed.) When cells labeled for 16 min with methyl-labeled methionine were then exposed to actinomycin, and nucleolar RNA samples were examined 9 and 20 min later, radioactivity was seen to shift through peaks at 41 and 36*S* before appearing at 32*S* (Fig. 9). In addition, a 20*S* molecule was labeled in advance of the 18*S* molecule. One further observation came from this series of experiments: definite evidence was obtained that the earliest labeled 28*S* RNA was located in the nucleolus.

Another set of experiments has demonstrated one additional nucleolar event in the processing of ribosomal precursor RNA. It was found that 28*S* rRNA, regardless of the method of isolation, was not one covalently linked polynucleotide chain. Upon exposure to heat, urea, or DMSO (all procedures which disrupt hydrogen bonding), the "28*S*" RNA gave rise to a small molecule about 150 nucleotides in length which has been termed 7*S* rRNA. It was further found that in

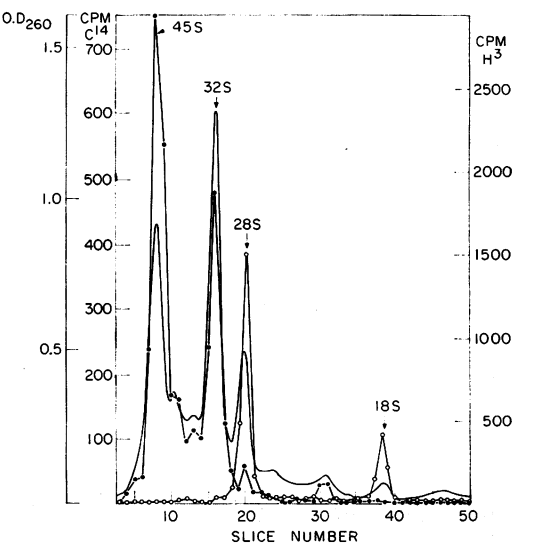

FIG. 8. *Acrylamide gel analysis of nucleolar RNA. Nucleoli from HeLa cells which had been labeled with ^{14}C-methyl methionine for 30 min were prepared, and RNA was extracted and subjected to separation by acrylamide gel electrophoresis. Solid line, optical density at 260 nm; ○, counts per minute. From Weinberg et al. (109).*

nucleolus or RNA after urea treatment an amount of this 7*S* RNA existed which was equivalent in number of molecules to the amount of nucleolar 28*S* RNA (66a).

The present scheme of the nucleolar cleavage of rRNA is summarized in Fig. 10. The exact

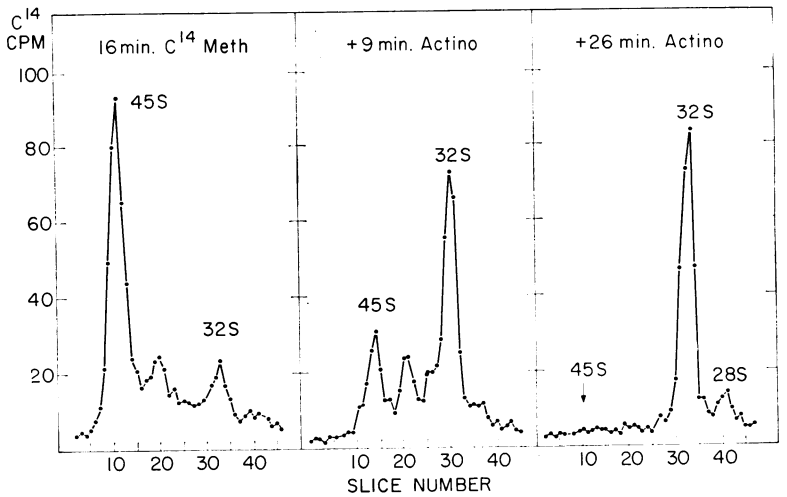

FIG. 9. *"Actinomycin chase" of methyl-labeled RNA. The nucleolar RNA of HeLa cells labeled with ^{14}C-methyl methionine and subsequently chased with actinomycin was analyzed by acrylamide gel electrophoresis. From Weinberg et al. (109).*

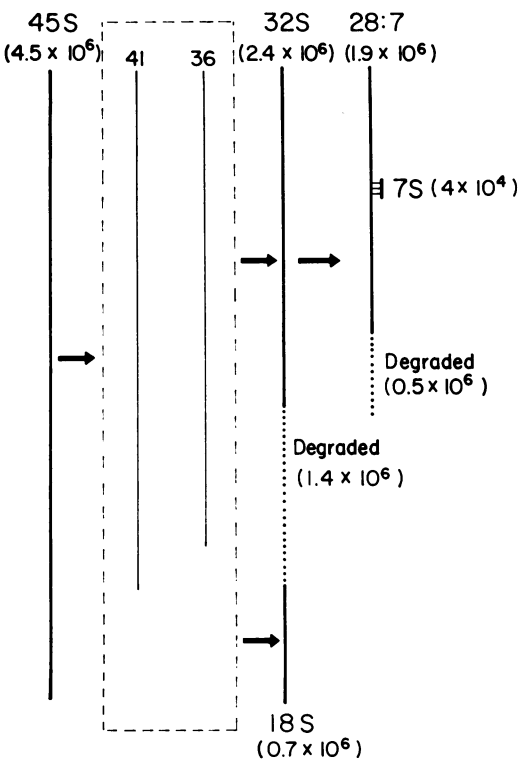

FIG. 10. *Summary of the processing of 45S r-pre-RNA. This diagram indicates the molecular weights and approximate S values of various ribosomal-related RNA molecules as well as the fate of some portions of the RNA which do not survive processing.*

point during the processing at which the 18*S* molecule is released is not clear, but it is present by the time the 32*S* is observed.

These considerations of 45*S* r-pre-RNA cleavage raise a number of important questions. For example, what is the molecular weight of 45*S* r-pre-RNA, and does it contain one 28*S* and one 18*S* molecule? Is the entire 45*S* molecule used in construction of ribosomes? What determines the specificity of the cleavage points in the polynucleotide? Answers to a number of these questions are now at hand.

Since different RNA molecules form random coils of varying degrees of compactness at a given salt concentration (95, 107; J. H. Strauss, Ph.D. Thesis, California Institute of Technology, Pasadena, 1966), it is not possible to obtain reliable molecular weights of RNA species by zonal sedimentation alone. The technique of sedimentation equilibrium determination of molecular weight, however, circumvents this problem, because molecular shape is relatively unimportant in such a determination (113). The availability of nucleolar preparations from which 45*S* and 32*S* r-pre-RNA could be obtained pure in relatively large amounts has allowed a determination of their molecular weights by sedimentation equilibrium. The molecular weight of the main rRNA species, 28 and 18*S*, has also been measured in the same manner, and the data are reported in Fig. 10 (E. D. MacConkey and J. Hopkins, J. Mol. Biol., *in press*). It is clear that only one 32*S* (2.4×10^6) and one 18*S* ($0.7 \times$

10^6) molecule could be drawn from each 45*S* (4.4×10^6) precursor molecule. After subtracting the 32*S* and 18*S* from the 45*S*, there is some RNA (approximately 10^6 daltons) which is left over; likewise, in the 32 → 28*S* conversion a substantial amount ($\sim 5 \times 10^5$ daltons) of RNA is not included in the final rRNA product. Several types of chemical evidence agree with these physical measurements which indicate that only 50 to 60% of the 45*S* is actually conserved during processing (1, 52, 99, 101). (i) A comparison of the oligonucleotide maps obtained after partial enzymatic digestion of 45*S* r-pre-RNA, 32*S* r-pre-RNA, 28*S* rRNA, and 18*S* rRNA reveals that the precursor molecules contain a qualitatively and quantitatively different makeup than the 28*S* plus 18*S* rRNA in about 50% of their length (1, 52). (ii) As previously mentioned, it was shown that the only acceptor of methyl groups was 45*S* r-pre-RNA, and in addition all the incorporated methyl groups in 45*S* r-pre-RNA were conserved during further RNA processing (101). Thus, with RNA samples labeled both with ^{14}C-methyl groups and with ^{3}H-uridine (which would label pyrimidine bases throughout the chain), a test could be made of whether nonmethylated portions of the RNA chains were lost (109). If unmethylated RNA was degraded to acid-soluble material as 45*S* was processed, but all methyl groups were conserved, then the ^{14}C-methyl/^{3}H-pyrimidine ratio would increase during processing. Table 2 shows that this was indeed the case, indicating a loss of about half of the 45*S* RNA during processing. (iii) A third type of evidence, very similar to the second, was obtained by assaying various RNA species labeled with nucleosides for the content of alkali-resistant dinucleotides. This provides a measure of the relative content of methylation of the 2′ OH groups of ribose in various RNA species. The dinucleotide content was found to be 45*S* < 32*S* < 28 and 18*S*. Since $-CH_3$ groups are inserted only into 45*S* RNA, these results also indicate a loss of material lacking methylated ribose as RNA maturation proceeded (99).

TABLE 2. *Extent of methylation of r-pre-RNA compared to rRNA*

RNA species	Uridine/methyl ratio[a]	Dinucleotides[b] (%)
45*S*	1.59	0.86
32*S*	1.16	1.10
28*S*	0.79	2.08
18*S*	0.55	2.74

[a] RNA of various sizes from cells labeled with both ^{14}C-uridine and ^{3}H-methyl methionine shows the increasing methyl content of rRNA compared to precursor RNA (109).

[b] Radioactive RNA of various sizes labeled in uridine and cytidine residues was hydrolyzed by alkali. A measurement of the relative amounts of dinucleotides indicates the frequency of methylation of 2′ OH groups on ribose. From Vaughan et al. (99).

These experiments indicate then that about 50% of the 45*S* r-pre-RNA is made up of nucleotide sequences that are used to make ribosomes, and the other half of the molecule is synthesized and apparently then destroyed because there is no accumulation of any RNA except the 28 and 18*S* resulting from the processing of the 45*S* molecule.

Another question which was repeatedly raised about the 45*S* r-pre-RNA was whether only a single class of precursor molecules exist containing both the 28 and 18*S*. Chemical evidence certainly implies that both 28 and 18*S* are drawn from 45*S* molecules: (i) 45*S* r-pre-RNA, the sole source of high GC RNA in briefly labeled cells, is destroyed in actinomycin with the resulting development of 28 and 18*S* RNA (35, 72, 86, 107); (ii) oligonucleotides of both 28 and 18*S* type are found in 45*S* molecules (1, 52); and (iii) the characteristic distribution of methylated bases for both 28 and 18*S* RNA is represented in 45*S* RNA (101).

A recent experiment performed with nucleic acids from amphibian tissues strongly indicates that the DNA molecule from which 28*S* RNA arises also carries the information for 18*S* RNA. This experiment was performed by isolating hybrids between frog DNA and 28S RNA and demonstrating that the DNA so isolated also has an increased capacity to hybridize 18*S* RNA (12).

It can be concluded that the 45*S* r-pre-RNA is a large polynucleotide of about 4×10^6 daltons which contains one 28*S* and one 16S molecule in addition to some unmethylated stretches of unknown function.

Formation and Distribution of Ribosomal Particles

Thus far, our discussion has been concerned only with experiments in which rRNA has been studied after being freed from combination with protein. Within the cell, rRNA is always found in combination with protein, and we will now move to a discussion of the formation and distribution of ribonucleoprotein particles in cultured cells. Cellular homogenization, either in isotonic sucrose or in hypotonic solutions, has been employed for a number of years to release a large fraction of the cytoplasm of animal cells

(liver cells and thymus cells in particular) without disrupting nuclei (6, 21, 45). When HeLa cells are exposed to hypotonic homogenization and the resulting cytoplasmic extract is examined by zonal sedimentation for its content of RNA-containing (UV-absorbing) particles, a number of structures are observed (Fig. 11; 34, 69). The well-known sedimentation behavior of single ribosomes (75 to 80S) can be used as a guide in describing the other structures (98). The majority (about 75 to 90%) of the UV-absorbing material sediments faster (~100S to 400S) than the single ribosome. These structures have been identified in a variety of ways as polyribosomes, groups of ribosomes which are attached to the same mRNA molecule actively synthesizing protein (31, 64, 104). Approximately 10% of the ribosomes in the extracts of exponentially growing HeLa cells sediment as free single ribosomes which at the time the cell is broken are not engaged in protein synthesis (34). In addition to these classes of "whole ribosomes," there are also observed in the optical density tracing two peaks which sediment more slowly than whole ribosomes. By extracting the RNA from these structures, it was concluded that these were free subunits—a 60S ribosomal subunit, containing 28S rRNA, and a 40 to 45S ribosomal subunit, containing 18S rRNA (34, 53, 98).

After the distribution of cytoplasmic ribosomes was established, experiments were designed to determine in which form newly made ribosomal particles would appear in the cytoplasm. Cells were labeled, fractionated, and examined for the distribution of radioactive RNA in various fractions. It was found that the first appearance of both 18S and 28S RNA was in the form of free subunits (Fig. 12; 34, 53). Within 10 to 15 min of the appearance of new subunits, ribosomes containing newly formed RNA could be found in polyribosomes without radioactivity appearing as a peak in the single ribosome. Thus, it appeared possible that the new ribosomal subunits entered into protein synthesis directly, that is, without first becoming single ribosomes. The obligatory initiation of protein synthesis by

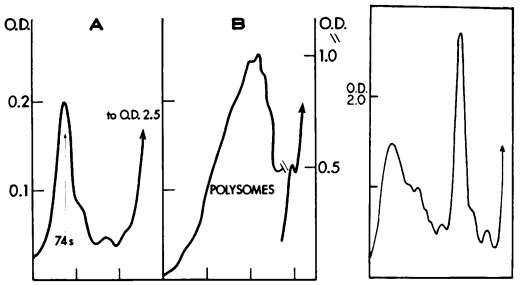

FIG. 11. *Sedimentation of cytoplasmic extracts of HeLa cells. Extracts were prepared by homogenization of cells swollen in hypotonic buffer (69), and three conditions for sedimentation analysis are demonstrated. Left, extract was sedimented for 16 hr at 20,000 rev/min, 4 C, through a 28-ml sucrose gradient (15 to 30%, w/w). Center, extract was sedimented for 90 min at 25,000 rev/min 4 C, through a 28-ml sucrose gradient (15 to 30%, w/w). Right, extract was sedimented for 5 hr at 25,000 rev/min, 4 C, through a 30-ml sucrose gradient (7 to 47%, w/w).*

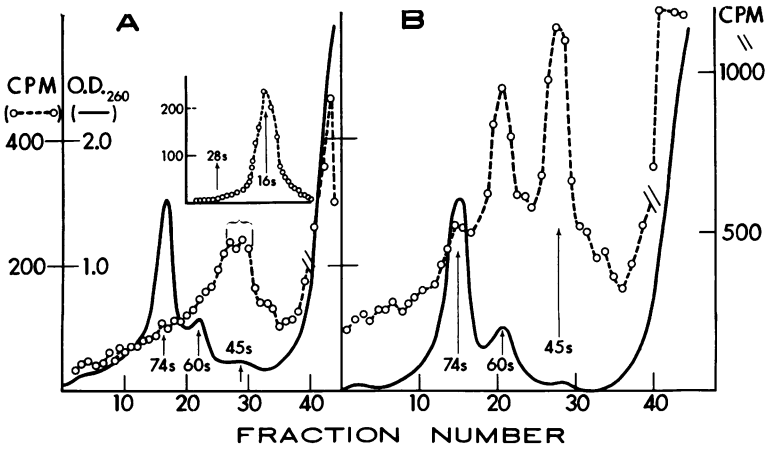

FIG. 12. *Appearance of new ribosomal subunits in HeLa cell cytoplasm. Cytoplasmic extracts of cells labeled with ^{3}H-uridine for 30 (A) or 60 (B) min were sedimented through a sucrose gradient (conditions similar to Fig. 11 left panel) and the gradient contents were assayed for acid-precipitable radioactivity. Inset in part A shows that RNA removed from labeled peak of ribonucleoprotein at 45S is mostly 16S rRNA. From Girard et al. (34).*

subunits has recently been demonstrated in bacteria (65, 89).

The new subunits in mammalian cells have been shown to be distinguishable from the pre-existing free subunits because they have a high protein/RNA content and thus band at a less dense region in CsCl (75). By the time they enter polyribosomes, however, they have the same density as the other ribosomes participating in protein synthesis. The meaning of this change in relative protein content is not known. Eventually, all the cytoplasmic ribosomes of the cell—polysomes, single ribosomes, and subunits —come into equilibrium. This was shown by experiments in which cells were labeled and then grown for a long time in unlabeled medium; the specific activity of the ribosomal RNA in all particles was then the same (100).

One very suggestive result from these earlier experiments on ribosomal particles in cultured cells was the finding that new ribosomes entered the cytoplasm as subunits, not as whole ribosomes. The possibility was raised that nothing but subunits existed within the nucleus.

The simple hypotonic swelling and homogenization technique that had been employed in the earlier experiments with HeLa cells gave variable results in the recovery of rRNA (50 to 90% in the cytoplasmic fraction; 69). Therefore, a technique was sought which would break all the cells, leave the nucleus intact, and leave the smallest amount of contaminating cytoplasm with the nuclear preparation. This latter consideration is of great importance, since many workers had pointed to the large number of ribosomes near or attached to the nuclear membrane (4, 6). Penman, Smith, and Holtzman succeeded in developing a technique for HeLa cells which employed hypotonic swelling and homogenization for the removal of the majority of the cytoplasm, followed by a combined ionic-nonionic detergent treatment of the nuclei. This latter step removed the nuclear rim of cytoplasm and the outer layer of the nuclear membrane without altering the intranuclear architecture (Fig. 13). When nuclei which had been "detergent cleaned" in this manner were isolated from cells which had first been labeled with pyrimidine nucleosides and then grown for several generations in unlabeled medium, they were found to contain almost none (less than 5%) of the total cell 18*S* rRNA and by inference less than 5% of the cellular ribosomes (67). They still contained all of the nucleolar RNA, however, as well as the nuclear DNA. Moreover, the nucleoli looked intact and still had a granular appearance which had been attributed to "nuclear ribosomes" (47, 67, 70).

A search was therefore made in detergent-cleaned nuclei for ribonucleoprotein particles containing rRNA, with the knowledge that there would probably be relatively few such particles. In addition, it was anticipated that any nuclear ribosomes might represent newly synthesized particles which would therefore be the first ribosomal particles labeled in the cell. In experiments on the preparation of nucleoli referred to earlier, it was pointed out that the clean nuclei can be lysed by exposure to high ionic strength (0.5 M NaCl + 0.05 M $MgCl_2$) in the presence of deoxyribonuclease. After removal of the nucleoli by brief centrifugation, these nuclear extracts were examined for ribosomal particles in comparison with cytoplasmic extracts treated in the same manner (100). A reproducible number of particles with the sedimentation characteristics of larger ribosomal subunits (50*S* in high ionic strength) were observed in nuclear extracts. These particles were then shown to contain 28*S* RNA. A final proof that they were truly nuclear particles was obtained by fractionating cells after various periods of labeling to demonstrate that radioactive 28*S* rRNA appeared in these nuclear particles before any could be detected in the cytoplasm (Table 3). A quantitation of the amount of 28*S* rRNA in these nuclear precursor particles indicated that they contained about 1% as much 28*S* rRNA as was present in the total cytoplasm. Evidence was also obtained that 18*S* RNA could be isolated from nuclear subribosomal particles before it was observable in the cytoplasm. However, the speed of exit to the cytoplasm of particles containing 18*S* rRNA was much greater than with the 28*S*-containing particles.

These experiments established the existence of an intranuclear stage of ribosome development when the ribosome is probably largely complete —i.e., has approximately the same sedimentation behavior as a mature subunit. The question naturally arises of whether these nuclear particles are complete with respect to proteins and whether it is possible to isolate other particles which are less completely formed. The topic of ribosomal proteins is too large to be dealt with extensively here, but a few points from this area of research are necessary to further consideration of the assembly of ribosomal structures.

Ribosomal Proteins and Nucleolar Ribosomal Precursors

Ribosomal protein and ribosomal RNA are not held together by covalent bonds. Thus, it is

possible, depending on a variety of conditions (*p*H, ionic strength, Mg^{++} concentration, etc.), to isolate rRNA in combination with variable amounts of protein (66). The first decision in dealing with ribosomal protein, therefore, is to decide what belongs with rRNA, or at least to adopt techniques which allow reproducible isolation of ribosomes containing a reproducible

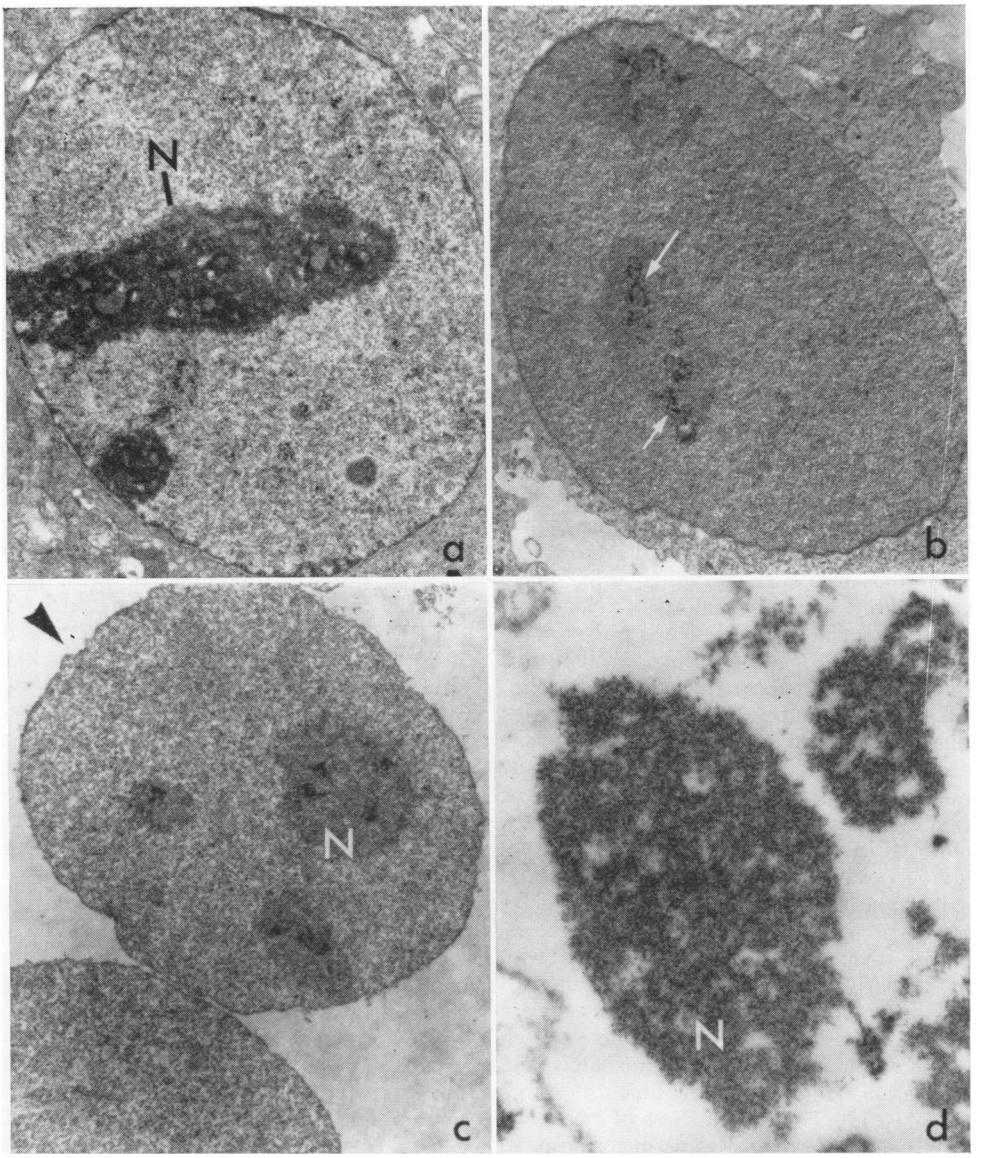

FIG. 13. *Electron micrographs of HeLa cell nucleoli. All preparations were fixed in glutaraldehyde and stained with uranyl acetate and lead citrate. (a) Untreated HeLa cell. The nucleus occupies most of the field. N indicates a nucleolus. × 8,000. (b) Cell swollen in hypotonic buffer. The nucleus occupies most of the field. Its contents appear considerably more homogeneous than in untreated cells. The nucleoli are less prominent except for the intranucleolar strands which stand out sharply (arrows). × 7,000. (c) Nucleus isolated after hypotonic swelling, homogenization, and detergent treatment. The outer nuclear envelope is no longer seen at the surface (arrow). Nucleoli are present. × 7,000. (d) N indicates a nucleolus isolated from a preparation as in part c. The overall form of the nucleolus has been preserved through the isolation, and many small granules, possibly ribonucleoprotein, are present within. × 20,000. Photographs courtesy Eric Holtzman, similar to those published in Holtzman, Smith, and Penman (47).*

TABLE 3. *Content of labeled 28S rRNA in nuclear and cytoplasmic particles*[a]

Label time (min)	Radioactivity (counts/min) in 28S rRNA		ratio, nucleus/ cytoplasm
	Nuclear 50S particles	Cytoplasmic 60S particles	
37	>500	Not detected	—
42	>1,500	<150	>10
90	8,000	8,600	0.91
Steady state	—	—	0.1-0.15

[a] After various exposure times to (^{14}C) uridine the amount of radioactive 28S rRNA in nuclear and in cytoplasmic subribosomal particles was determined (99).

set of proteins. Warner has studied the conditions necessary for producing HeLa cell ribosomes uncontaminated by large amounts of extraneous protein (105). In addition, he has characterized the ribosomal proteins by gel electrophoresis so that a number of different characteristic protein peaks can be identified (102). Two functional classes of HeLa cell proteins are of interest for our discussion. (i) Since ribosomal RNA originates in the nucleolus and after some delay arrives in the cytoplasm in the form of a ribosomal subunit, experiments were designed to determine whether newly formed ribosomal protein also underwent a delay between synthesis and appearance. It was found that a large fraction of the ribosomal protein did in fact behave this way, i.e., appeared in cytoplasmic ribosomes only after a 30- to 120-min delay. This fraction will be referred to as structural ribosomal protein. (ii) A second class of proteins, about 20% of the total ribosomal protein, is regularly found on HeLa cell ribosomes but shows little or no delay between synthesis and appearance in ribosomes. Moreover, the synthesis and association with cytoplasmic ribosomes of this second class of ribosomal proteins continues even after cells are treated with actinomycin, a treatment which stops the synthesis of rRNA immediately and the appearance of new cytoplasmic ribosomes within 1 hr (35). This second class of ribosomal proteins will be termed cytoplasmic ribosomal protein to indicate that they exchange with ribosomal particles in the cytoplasm.

One further important point should be made about the ribosomal protein of HeLa cells. As stated, the structural ribosomal protein does not appear in the cytoplasm for about 30 to 120 min after it is synthesized. This indicates that a pool of structural ribosomal protein exists through which new material must pass before being observed on cytoplasmic ribosomes. An additional experiment which indicates that such a protein pool exists has been reported. If cells are treated with cycloheximide, a drug which completely blocks all new protein synthesis, they are still able to synthesize new rRNA which becomes associated with ribosomal protein to form apparently functional ribosomes (103). Also, at least 80% of the labeled protein which appears in ribosomes after a pulse and cold amino chase will also appear in cytoplasmic ribosomes in the presence of cyclohexamide. Thus, the ribosomal protein pool can be used by rRNA synthesized in absence of ongoing protein synthesis.

Association of Ribosomal Protein with rRNA

We can now address the question of when, in the course of ribosome formation, rRNA and ribosomal protein come together. We previously pointed out that nuclear particles with sedimentation properties similar to cytoplasmic subunits were identified in nuclear extracts after removal of the nucleoli. It is not known whether these particles were part of the nucleolus inside the cell and were dislodged by the high ionic strength deoxyribonuclease treatment. For example, particles of the appropriate size for the larger ribosomal subunit have been seen repeatedly by electron microscopy around the border of the nucleolus (4, 76), and these might easily be lost in purification of the nucleus. At any rate, HeLa cell nucleoli prepared by the high ionic strength deoxyribonuclease method are relatively free from these mature particles containing 28S RNA. Recently, a technique has been worked out for solubilizing the nucleolus, which results in the release of yet another class of ribonucleoprotein particles with sedimentation coefficients of approximately 80S and 55S (Fig. 14; 106). The RNA from these particles has been shown to be ribosomal precursor RNA, not mature rRNA. In addition, gel electrophoretic analysis of the protein of these nucleolar particles shows that their protein corresponds in considerable detail to the structural ribosomal protein of cytoplasmic ribosomes. The ribosomal proteins which are added in the cytoplasm are not found in the nucleolar particles. These experiments demonstrate that the association of ribosomal proteins and rRNA begin immediately after, or even coincident with, the synthesis of the ribosomal precursor RNA. A summary of ribonucleoprotein particle formation in the HeLa cell is given in Fig. 15.

Place of Small RNA Molecules in Ribosomal Maturation

Within the past several years, a number of laboratories have reported the existence of a

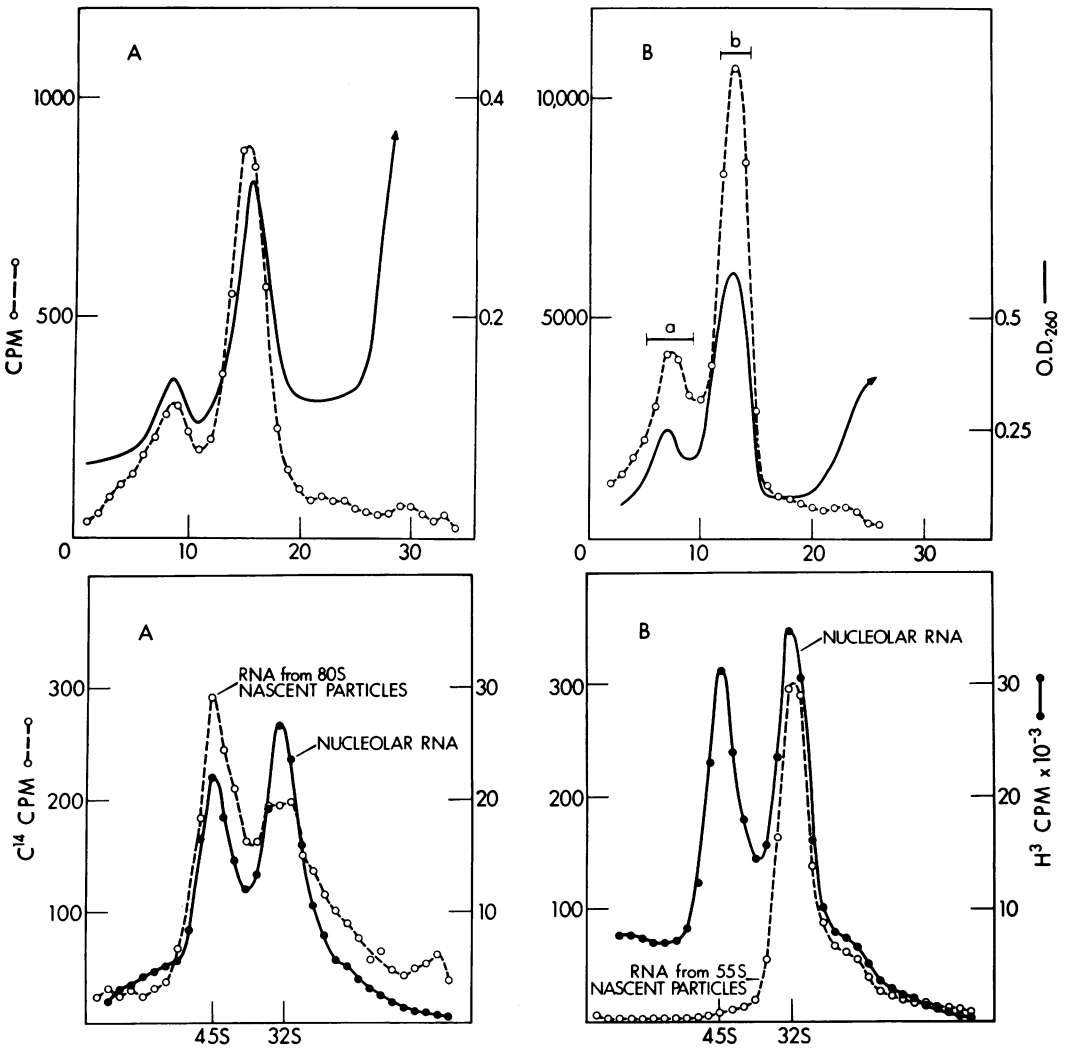

FIG. 14. *Nucleolar ribonucleoprotein particles. Upper panels: HeLa cells were labeled either with* ^{14}*C-leucine (A) or* ^{14}*C-uridine (B) and nucleoli were isolated and disrupted in buffer containing EDTA and dithiothreitol. Ribonucleoprotein particles were displayed by sucrose gradient analysis. The two peaks are approximately 80S and 55S. Lower panels: RNA from 55S (A) and 80S (B) nucleolar particles was released and cosedimented with total nucleolar RNA. From Warner and Soeiro (106).*

small rRNA, termed 5*S* RNA, one molecule of which is found in every larger ribosomal subunit (19, 28, 81). It has been determined that in HeLa cells this 5*S* RNA is contained within all the mature ribosomal particles containing 28*S* rRNA (55). In addition, the 5*S* RNA has also been detected in the newly described nucleolar particles containing ribosomal precursor RNA (106). It is of interest, however, that the 5*S* RNA is apparently not part of the 45*S* r-pre-RNA. The clearest evidence of a separate origin of r-pre-RNA and of 5*S* is that the DNA to which 5*S* RNA hybridizes is separable from that with which rRNA hybridizes (12). In addition, it was shown that the 28*S* molecule in a newly formed HeLa cell ribosome is not accompanied by a new 5*S* RNA molecule. This result indicates a "pool" of 5*S* RNA through which new molecules must pass before entering into ribosome manufacture (55).

Control of Ribosome Formation

The control of the rate at which new ribosomes are formed may be assumed to be important

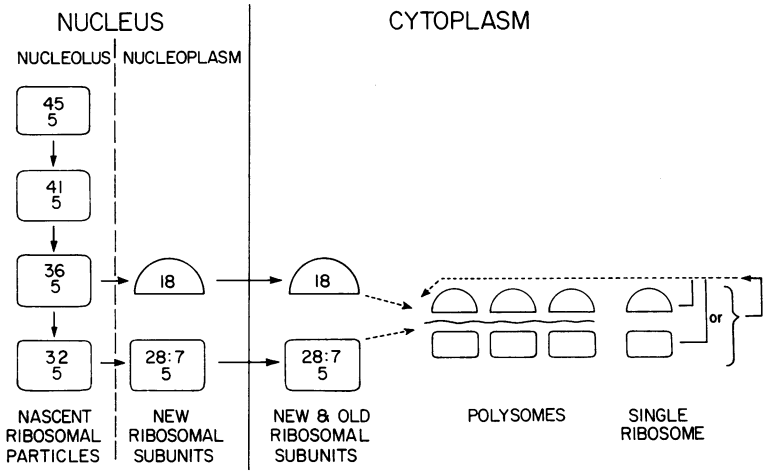

FIG. 15. *Summary of ribosome formation in HeLa cells. Solid shapes indicate ribonucleoprotein structures containing rRNA or ribosomal precursor RNA plus ribosomal protein. Points of detection of 5S and 7S RNA are indicated. Although the ribosomal subunit containing 18S RNA is indicated as arising from the structure bearing 36S RNA, this is not definite and may occur earlier.*

because of the central role occupied by ribosomes in protein synthesis and, therefore, in cellular metabolism. Two recent lines of experimentation have made clear that various means are utilized in animal cells to influence the rate of ribosome formation. Let us first consider a case where vastly accelerated ribosome biosynthesis occurs. In developing oocytes of frogs and salamanders (12, 29), it has been noted morphologically that multiple large nucleoli exist and that rapid incorporation of isotopes into ribosomal precursor RNA and ultimately into ribosomes can be demonstrated. Since one of the components of the nucleolus is probably the DNA from which ribosomal precursor RNA is formed and since the oocyte nucleoli are so prominent, attempts were made to determine whether there existed in these cells increased amounts of DNA complementary to rRNA. It has now been demonstrated in both types of oocytes that the amount of DNA complementary to rRNA has increased perhaps as much as 100-fold compared to DNA from other tissues or whole animals. Thus, in these selected cases, the need for rapid synthesis from ribosomal genes has been met by greatly increasing the number of genes (11, 12).

After oocytes reach the stage of mature eggs, they no longer can be demonstrated to be making ribosomes. Even after fertilization, no new ribosome synthesis occurs. This apparent complete lack of ribosome formation is accompanied by a lack of formation of 45*S* precursor RNA. These findings have been reported not only for frog eggs but also for sea urchin eggs (11, 39). These results appear to indicate a complete dormancy of rRNA genes, the antithesis of the first control situation.

Perhaps a more common need encountered in animal cells from various tissues is not a complete suppression of ribosome formation but rather a fluctuating rate of synthesis. For example, the cell life span in liver and in kidney is very long, yet these cells incorporate isotopes into ribosomal RNA (43, 58, 61). Since the amount of tissue and ribosomal mass in adult liver and kidney is essentially constant, the likely explanation of such findings was that ribosomes were being constantly synthesized and degraded, i.e., "turned over." The half-life of ribosomes in resting liver tissue, in fact, has been estimated at 4 to 6 days (58). If a portion of the liver or a kidney is removed from rats, the remaining tissue—liver or kidney—undergoes very rapid growth, including a sustained synthesis and increase in total ribosomal mass (17, 61). While it is clear in these situations that the rate of accumulation of new ribosomes is greatly enhanced compared to resting tissue, it is not clear at what stage in the complicated chain of events leading to ribosome formation this increase has been effected. For example, it is possible that the rate of 45*S* r-pre-RNA synthesis is unchanged but, in normal liver or kidney, it is not all processed into ribosomes.

The potential focal points of control of ribosome biosynthesis can possibly be studied more effectively in cultured cells where the stages of ribosome production can be more easily ob-

served. Therefore, experiments were undertaken to compare ribosome formation in rapidly growing cultured cells with cells which have had their growth slowed or stopped in a variety of ways.

It was previously mentioned that cycloheximide, a drug which essentially stops polypeptide chain elongation (26, 110), does not immediately stop ribosome production by HeLa cells (103). These results allow two conclusions: (i) there is a pool of ribosomal proteins which can be used to construct ribosomes in the absence of ongoing protein synthesis; (ii) r-pre-RNA has no necessary role in directing protein synthesis in order to be processed into a ribosome. All cells, however, are not equally capable of producing ribosomes after cycloheximide treatment. For example, after cycloheximide treatment, L cells can continue to synthesize r-pre-RNA and process it into 28*S* rRNA which appears in cytoplasmic ribosomal particles, but the smaller or 18*S* rRNA is lost in the process (25).

Treatment of HeLa or L cells with puromycin, another drug which interrupts protein synthesis, has a drastically different effect from cycloheximide on ribosome formation (56, 91). Although r-pre-RNA continues to be made after puromycin treatment, it cannot be effectively incorporated into a ribosome. Thus, a condition is induced where synthesis and degradation proceed in parallel. The speed of degradation of the smaller 18*S* rRNA is especially obvious, there being no sign of labeled 18*S* ever observable in either the nucleus or cytoplasm in cells labeled with ^{3}H-uridine after puromycin treatment. There is no evidence of a defect in the r-pre-RNA synthesized in the presence of puromycin, since RNA which is made in the presence of the drug can be used to make ribosomes when the drug is removed (56, 91).

Studies of ribosome formation under conditions of amino acid deprivation have also been carried out in HeLa cells. The omission of methionine is of special interest, since this amino acid has a direct role to play in ribosomal protein synthesis in addition to providing methyl groups for RNA methylation. During methionine starvation, ribosomal precursor RNA synthesis continues, and accumulation of undermethylated 45*S* occurs (99). Cleavage of r-pre-RNA also continues, thereby producing undermethylated 32*S* RNA. No new ribosomal RNA ever reaches the cytoplasm, however. Also, as was the case with puromycin treatment, the 18*S* RNA is so quickly lost that no radioactive 18*S* RNA is ever observed after uridine labeling of methionine-deprived cells. The undermethylated RNA can be rescued for processing into ribosomes by the restoration of methionine. Furthermore, it has been demonstrated that undermethylated r-pre-RNA can be methylated during this recovery.

In summary, these experiments on ribosome formation after puromycin treatment and during methionine deprivation focus attention on the capacity of the animal cell to degrade ribosomal RNA molecules which cannot be properly made into ribosomes. Whether such a mechanism exists or is ever used by cells in a tissue is at the moment unknown. However, many tissues which make ribosomes only fast enough to replace those lost by turnover are known to make substantial amounts of r-pre-RNA. Therefore, the possibility that the rate of ribosome formation is regulated by degradation rather than by processing of the r-pre-RNA molecule should be borne in mind.

Another recent set of experiments strongly points to the ability of cultured cells to modulate ribosome formation by changing the *rate* of r-pre-RNA synthesis. A comparison of the rate of uridine incorporation into 45*S* RNA in growing HeLa cells and in cells deprived of valine for longer than 4 hr showed that the starved cells form 45*S* less than half as fast as do controls (99; Maden, Vaughan, Warner, and Darnell, *unpublished data*). In addition, the rate of maturation of a 45*S* molecule, once formed, was also slowed down. Thus, the whole machinery for making ribosomes was synchronously slowed down. Starvation for a growth-essential amino acid would be expected to have its effect due to the limitation of some critical protein, perhaps a ribosomal structural protein. It is possible, therefore, that continued work with cultured cells which are making ribosomes at different rates could disclose particular proteins which are critical in controlling the rate of ribosome synthesis.

Such information would not only be very important with regard to control of ribosome synthesis in normal cells but might also ultimately be useful in defining a critical point where control has been lost in cancer cells.

PRECURSORS TO TRNA MOLECULES

The role in protein synthesis and the chemistry of the RNA molecules are far better known and understood for tRNA than that for other cellular RNA species (46, 60, 114). Very little is known, however, about the site of tRNA origin or whether precursor molecules to it exist. Since the major emphasis in this article is on the synthesis of RNA molecules in animal cells, it is appropriate to describe briefly some results in

HeLa cells and Ehrlich ascites cells, from which it appears that a precursor to tRNA has been found. One of the difficulties in the past in investigating molecules which might be related to tRNA was that simple methods for the separation of small RNA molecules of similar size did not exist. The development of acrylamide gel electrophoresis of RNA molecules has been particularly useful in overcoming this difficulty. For example, the small molecules (5*S* and 7*S*) which are part of the larger ribosomal subunit are clearly resolved from 4*S* RNA by electrophoresis (55, 66a).

It has been found that HeLa cells which have been labeled for 30 min or less contain labeled RNA species which migrate on gel electrophoresis between 5*S* and tRNA (16; D. Bernhardt and J. E. Darnell, *in press*). These molecules are labeled before tRNA becomes labeled and have been termed pre-tRNA. If RNA synthesis is stopped by actinomycin in cells where pre-tRNA is the predominantly labeled small RNA species, the pre-tRNA disappears and labeled tRNA appears (Fig. 16). Since the pre-tRNA migrates more slowly than true tRNA, even in formaldehyde (Bernhardt and Darnell, *in press*), a reagent that destroys secondary structure in polynucleotides, it is possible that pre-tRNA is longer than the tRNA derived from it.

Another finding of interest is that both pre-tRNA and tRNA formation proceed more slowly during methionine deprivation. This suggests that there may be an involvement of methylation in the conversion of pre-tRNA to tRNA. If this newly described RNA species is truly precursor to tRNA, it should aid considerably in locating the cellular origin of tRNA.

DNA-LIKE RNA SPECIES

While the ribosome and transfer RNA molecules have roles to play in the synthesis of every protein, the actual dictation of amino acid sequence is accomplished by the mRNA. Many events of interest in animal cells are expressions of changing patterns of protein synthesis (e.g., differentiation, cell division, and regeneration), and these must revolve around the availability of mRNA. Ultimately to understand these processes in molecular terms, it clearly would be desirable to follow mRNA from its synthesis to its utilization and ultimate destruction. Although much effort has been expended in attempting to study mRNA in animal cells, very little concrete knowledge about this RNA species has been uncovered. One of the outgrowths and one of the significant complications of these studies has been the discovery that several classes of cellular RNA exist which share some of the properties expected of mRNA but which may not be mRNA. This section of the present paper will be concerned with this group of RNA species, which we will call generically DNA-like RNA.

Definition and Distribution of DNA-like RNA

Several properties of viral and bacterial mRNA influenced the design of experiments aimed a

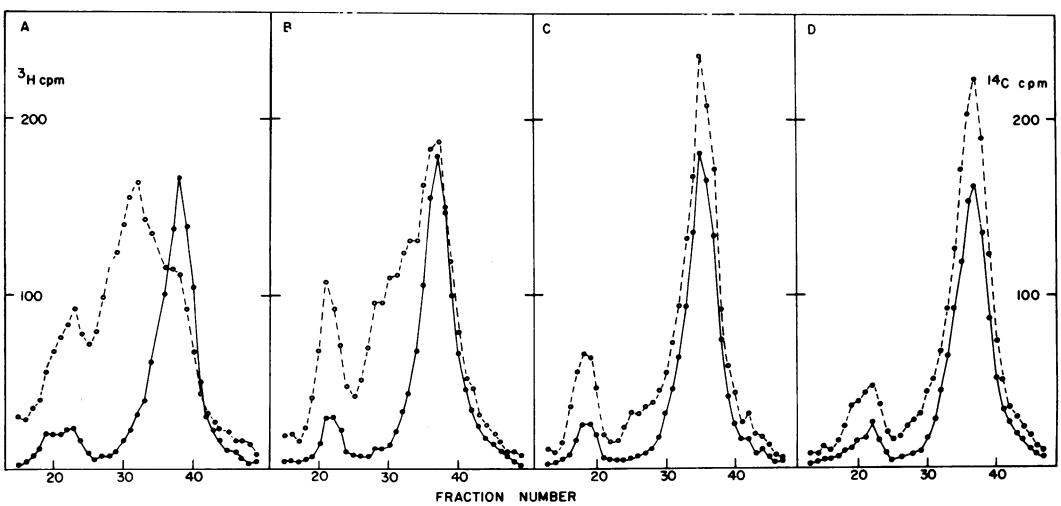

FIG. 16. *An apparent precursor to tRNA. A culture of HeLa cells was labeled with ^{14}C-uridine (solid lines) for one generation and then exposed to ^{3}H-uridine (dashed lines) for 5 min. Actinomycin was added and samples were taken after 2, 10, 35, and 60 min (A, B, C, and D, respectively). RNA was isolated from cytoplasmic extracts of each sample and examined by acrylamide gel electrophoresis for small RNA molecules. The ^{14}C label furnished a marker for the mobility of 4S (tRNA) and 5S RNA (Bernhardt and Darnell, unpublished data).*

locating mRNA in animal cells. First, mRNA in bacteria represents a small proportion of the total cellular RNA, and it is rapidly synthesized and degraded (9, 38, 51). Therefore, if bacterial cells are exposed to labeled RNA precursors for only a brief period, then the amount of radioactivity in mRNA relative to rRNA and tRNA is high enough so that mRNA can be studied as a radioactive RNA species. Second, rRNA represents a distinct species of RNA and does not therefore necessarily reflect the average composition of the total cell DNA; mRNA, on the other hand, comes from many genes and reflects the average DNA composition (92).

One final property of mRNA needs emphasis. Although distinct from rRNA, it must be associated with ribosomes, at least during the time it is directing protein synthesis (9).

It was previously pointed out (Fig. 4) that the total labeled RNA from animal cells exposed to ^{32}P for 30 to 40 min contained a large proportion of labeled molecules which sedimented more rapidly than 45*S* r-pre-RNA. When the base composition of the large molecules was determined, it was found that they resembled DNA, i.e., had a GC content of 43 to 47% (1a, 24, 87, 90, 112). Thus, this material was potentially mRNA. However, as previously mentioned, most of the rapidly labeled RNA of total cells was known to be in the cell nucleus. This large heterogeneously sedimenting DNA-like RNA, termed HnRNA, also has been shown to exist in the nucleus. Recent cell fractionation studies show that the HnRNA is largely, if not completely, unassociated with the nucleolus (107; S. Penman, C. Vesco, and M. Penman, J. Mol. Biol., *in press*). The HnRNA therefore may represent what has been termed by cytologists "chromosomal RNA" (23).

If cytoplasmic extracts of briefly labeled cells are examined, it is found that about 5 to 10% of the total incorporated radioactivity is cytoplasmic rapidly labeled RNA (107). Examination of the distribution of rapidly labeled cytoplasmic RNA shows that, although most of the radioactivity is in the so-called soluble fraction (i.e., nonsedimentable in 1 to 2 hr at 100,000 × *g*), there is labeled RNA associated with structures of all sizes from 40 to 300*S* (Fig. 17; 57). In the polyribosomes of cells labeled with ^{3}H-uridine for 30 min or less, labeled RNA can be recovered free from protein with the sedimentation profile shown in Fig. 17. It will be immediately recognized that this polysome-associated rapidly labeled RNA (i) sediments more slowly than the HnRNA and (ii) does not conform to the sedimentation pattern of rRNA or tRNA. In addition, it has been found that the base composition of this polysomal RNA is very different from tRNA or rRNA and is more similar to cellular DNA (Table 1). This rapidly labeled DNA-like RNA species from polyribosomes has been considered to represent mRNA. Several additional lines of experimentation indicate that this interpretation is correct. (i) The cytoplasmic extracts of cells infected with various animal viruses have been shown to contain virus-specific RNA complexed with host ribosomes in viral polyribosomes (3, 68, 82, 96). Such a result is diagrammed in Fig. 18, where it is shown that *whole* molecules of poliovirus RNA are recoverable from poliovirus polyribosomes. An additional point of importance comes from this experiment —since whole viral RNA molecules are found in virus polyribosomes, it is likely that mRNA molecules, cellular or viral, obtained from polyribosomes of HeLa cells are not degraded. (ii) The chemical stability of polyribosomes suggests a complex between ribosomes and mRNA. For example, the chelating agent ethylenediaminetetraacetate destroys polyribosomes (34) and releases most of the rapidly labeled RNA from the polysome region of a sucrose gradient (Fig. 19). (iii) Puromycin is known to cause the discharge of the nascent polypeptide chain from ribosomes of mRNA, and polyribosomes are thereby degraded by puromycin treatment. If cells which have been briefly labeled are treated with puromycin, very little of the rapidly labeled cytoplasmic RNA is found in the polysome region (Fig. 19).

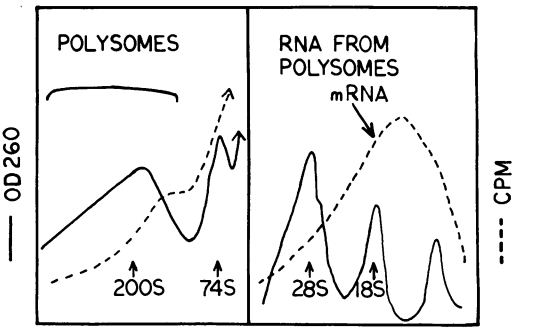

FIG. 17. *Distribution of radioactivity in cytoplasmic extracts of briefly labeled HeLa cells. Left panel: cytoplasmic extracts of HeLa cells labeled with ^{3}H-uridine were sedimented in sucrose gradients (conditions as for center panel, Fig. 11) and assayed for acid-precipitable radioactivity. Right panel: polysomal-associated RNA was further examined (by releasing this RNA with sodium dodecylsulfate and examining it on a sucrose gradient. Redrawn from Latham and Darnell (57).*

In summary, the species of rapidly labeled

cytoplasmic RNA considered to be mRNA is that which is associated with polyribosomes and can be discharged by ethylenediaminetetraacetate or puromycin treatment. An extensive study on the time course of synthesis and appearance of mRNA employing these criteria for mRNA has just been completed (S. Penman, C. Vesco, and M. Penman, J. Mol. Biol., *in press*).

Perhaps the two most important questions which arise from present experiments on DNA-like RNA species are the following. (i) Does the rapidly labeled RNA found in cytoplasmic structures which sediment at 40 to 100*S* before deproteinization represent mRNA on the way to becoming polyribosomes? (ii) What is the relationship, if any, between HnRNA and polysomal mRNA?

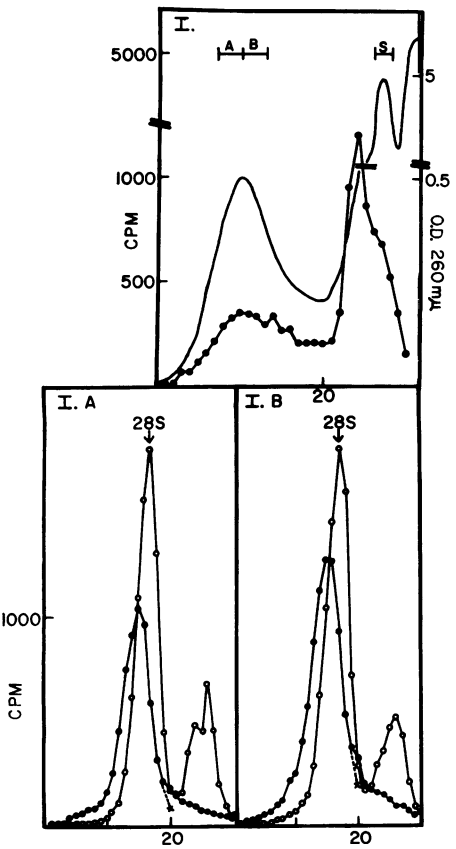

FIG. 18. *RNA from poliovirus polyribosomes. HeLa cells which had been grown in ^{14}C-uridine were infected with poliovirus and treated with actinomycin to stop host-cell RNA synthesis. The culture was labeled with ^{3}H-uridine throughout virus infection. Viral polyribosomes were isolated by sedimentation of cytoplasmic extracts (top panel, A and B are polyribosome fractions; S is single ribosomes; solid line, optical density at 260 nm) and RNA from these was released and further examined by sucrose gradient analysis (lower panel). Viral RNA is ^{3}H-labeled (●) and host cell RNA is ^{14}C-labeled (○). From D. F. Summers, J. V. Maizel, and J. E. Darnell, (Virology 31:427, 1967).*

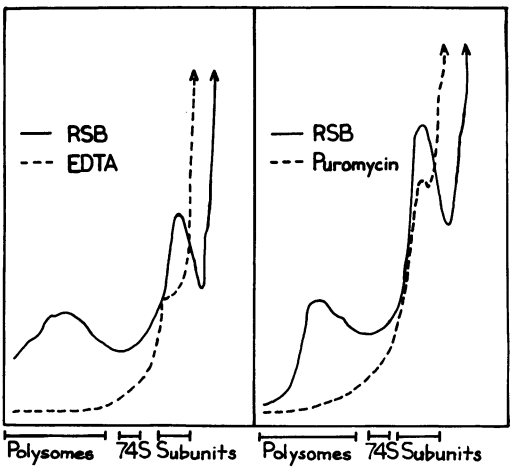

FIG. 19. *Effect of EDTA and puromycin treatment on the radioactivity in polyribosomes. Left panel: HeLa cells were labeled for 25 min with ^{3}H-uridine, and sedimentation of the total acid-precipitable radioactivity was examined in hypotonic buffer containing 0.0015 M $MgCl_2$ (RSB) or EDTA (0.01 M). Conditions of sedimentation were similar to those shown in right-hand panel of Fig. 11 (7 to 47% sucrose gradients). Only radioactivity profiles are shown. Regions of gradients are marked according to OD_{260} of RSB gradient. Right panel: HeLa cells were labeled for 20 min with ^{3}H-uridine and the culture was divided. One half was continued at 37 C for 5 additional min, and the other half was treated with 200 μg/ml of puromycin during the last 5 min of incubation. Cytoplasmic extracts were then made and analyzed as described above (data of C. Birnboim and J. E. Darnell, unpublished).*

Several results argue against the possibility that the rapidly labeled RNA recovered from the 40 to 100*S* structure found in cytoplasmic extracts is new mRNA enroute to polyribosomes. (i) If cells are treated with actinomycin, thus stopping RNA synthesis but leaving protein synthesis unaffected for several hours, the labeled RNA from the 40 to 100*S* region does not "chase" into polysomes (57). (ii) It was found with L cells that the manner in which cytoplasmic extracts were prepared determined how much label was found in the 40 to 100*S* region. Thus, if cellular extracts of cells which were broken in isotonic medium containing a nonionic detergent were compared to extracts prepared by the more common hypotonic swelling and homogenization, it was found that the yield of polysomes was equivalent but the amount of rapidly labeled RNA in the 40 to 100*S* region was decreased by a

factor of 10 in the isotonically prepared extracts (Perry and Kelley, J. Mol. Biol., *in press*). These results suggest that a large fraction of the 40 to 100*S* material might derive from the nucleus. While the exact nature of the labeled RNA in the 40 to 100*S* region is not clear at present, these results suggest that it may bear no relation to mRNA.

In order to adequately discuss the possible relation of mRNA to HnRNA, we must describe experiments on the HnRNA in more detail.

HnRNA: Properties and Problems

The very rapidly sedimenting (20 to 100*S*) HnRNA which we wish to consider in some detail has been found not only in the nuclei of cultured cells but in many differentiated cells as well. Work in one of these systems, nucleated duck erythroblasts, has provided good evidence about the eventual fate of the HnRNA found in those cells. Because the duck erythroblast, a highly differentiated cell making primarily hemoglobin, makes few if any new ribosomes, it was possible to label the cells with an RNA precursor, remove the label, and study what happens to the previously labeled HnRNA as a function of time (1a, 87). Experiments of this type definitely show that the majority of the HnRNA is not transferred to the cytoplasm even hours after its synthesis, and 60 to 70% of the total radioactivity in RNA is gradually lost, presumably back to the acid-soluble pool. If after the labeling of HnRNA, either in duck erythroblasts or cultured cells, the cells are treated with actinomycin D, the great majority (about 90%) of the HnRNA decays to acid-soluble material (48, 107). It was strongly suggested therefore that, at least in the duck erythroblast, the majority of the HnRNA never serves a cytoplasmic function (1, 87).

To investigate this idea more thoroughly in growing cultured cells, an attempt has been made to quantitate the various fractions of the cell involved and to determine whether the amount of label found in various fractions was consistent with a constant nuclear turnover of the HnRNA or whether it was synthesized at a rate consistent with its utilization as cytoplasmic mRNA (Table 1; Soeiro et al., *in press*).

The cytoplasmic polyribosomes of HeLa cells constitute the majority of the cell's ribosomes, and the average size is about six ribosomes per chain of mRNA, the average size of which is about 12 to 16*S* or approximately 6×10^5. Thus, the ratio of rRNA to mRNA in these structures is at least 20:1 [$6 \times (1.7 \times 10^6 + .6 \times 10^6)/6 \times 10^5$]. If both the rRNA and mRNA were stable, then the cell would have to synthesize 20 times as much RNA which was precursor to ribosomes as that which was precursor to mRNA. The mRNA, however, is thought to be an unstable molecule (relative to the rRNA) with a half-life of about 3 to 4 hr at the shortest (69). Taking the minimum ratio of rRNA to mRNA of 20:1, and the shortest possible half-life for mRNA, it was calculated that the cell should still make at least three times as much rRNA as that which was precursor to cytoplasmic mRNA (90, 107). Actually, since we now know that approximately 50% of the r-pre-RNA molecule is itself synthesized and degraded and not used in the final ribosomal product, we would expect six times as much 45*S* r-pre-RNA as any DNA-like RNA which was precursor to cytoplasmic mRNA.

With these thoughts in mind, a careful assessment of the relative amounts of radioactivity in 45*S* r-pre-RNA and HnRNA was made by labeling cells with ^{32}P and analyzing the RNA by zonal sedimentation followed by base analysis. Because the average GC content of HnRNA is low, about 44%, and that of highly purified 45*S* had been determined to be 70% GC (Table 1; 1, 52), it was possible to determine accurately the distribution between these two classes of RNA in the total rapidly labeled nuclear RNA (Soeiro et al., J. Cell Biol., *in press*).

The results shown in Fig. 20 and Table 4 indicate that the 45*S* r-pre-RNA does not constitute the bulk of rapidly labeled material, but on the contrary the HnRNA represents 75% or more of the nuclear radioactivity after 10- or 20-min exposure to ^{32}P. Furthermore, the shorter the label time the higher the proportion of radioactivity in the HnRNA. The flow of radioactivity is much faster into HnRNA than into r-pre-RNA, although they constitute almost the same total amount of RNA. As pointed out previously, any precursor to cytoplasmic mRNA should be synthesized no faster than about one-sixth the rate of synthesis of r-pre-RNA.

The results described above prove that most of the HnRNA turns over in the cell nucleus, an idea first proposed a number of years ago by Harris (42).

While it is clear from the foregoing discussion that most of the HnRNA does not eventually become cytoplasmic mRNA, it is possible that a small portion does. Several explanations might be suggested, all of which involve a scission of the HnRNA with partial use of the products as mRNA. (i) The HnRNA has a much higher molecular weight (electron micrographs indicate

that it may be as long as 10 μm or about 10^7 daltons; 88) than cytoplasmic mRNA, and a small portion of each molecule could serve as mRNA. (ii) Many extra copies of each HnRNA molecule might be made and only a few of each type eventually used to generate mRNA. (iii) Most of the HnRNA might represent types of molecules which are never used even in part, but a small fraction of the total number of HnRNA molecules might be used wholly or in part.

Of course, it should again be pointed out that it is possible that none of these conjectures is correct and that the HnRNA bears no relationship to the cytoplasmic mRNA but is entirely destroyed within the nucleus.

These crucial questions cannot be answered by kinetic experiments alone. Some means of comparing the sequences of HnRNA and the various cytoplasmic DNA-like molecules is necessary. As pointed out in the first section of this paper, at present the only feasible approach to sequence relatedness among RNA molecules is hybridization of RNA to DNA. Because this technique has the potential of answering this question, experiments on the hybridization of DNA-like RNA from cultured cells will be discussed in a separate section.

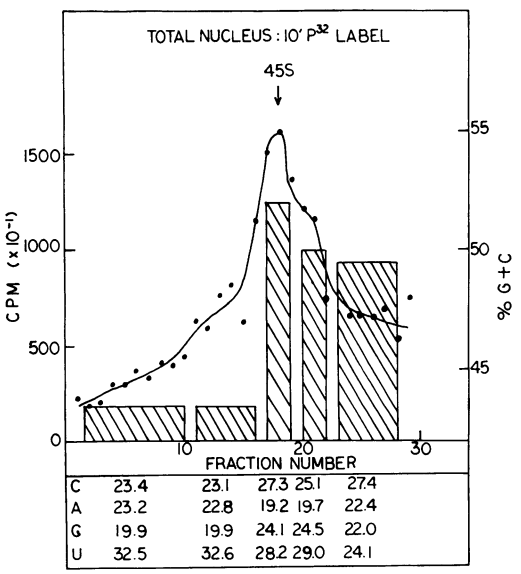

FIG. 20. *Base composition analysis of RNA labeled in 10 min by ^{32}P. Total nuclear RNA was isolated from HeLa cells after a 10-min exposure to ^{32}P and subjected to sucrose gradient separation. Base composition analysis of RNA from various sections of the sucrose gradient was then performed. Bar graph indicates percentage GC obtained. From Soeiro, Vaughan, Warner, and Darnell (in press).*

TABLE 4. *Relative amounts of HnRNA and r-pre-RNA from briefly labeled cells*[a]

Length of label (min)	Sedimentation values of nuclear RNA	Base composition (% GC)	Total count/min	Calculated counts/min as	
				HnRNA	r-Pre-RNA
10	77–115*S*	43	31	31	
	45–77*S*	43	46	46	
	45*S*	51.4	46	31	15
	37–45*S*	50	32	22	10
	10–37*S*	49	40	30	10
	Total			160	35
20	77–115*S*	41	105	105	
	45–77*S*	44	190	190	
	45*S*	54	120	72	48
	40–45*S*	53	91	61	30
	37–40*S*	47	86	74	12
	10–37*S*	54	80	55	25
	Total			557	115

[a] ^{32}P-labeled RNA from experiment shown in Fig. 20 was analyzed for base composition and amount of RNA of each major class (HnRNA, 44% GC; r-pre-RNA, 70% GC) estimated.

Use of RNA-DNA Hybridization in the Study of DNA-like RNA Species

The original demonstration of RNA-DNA hybridization was with mRNA from bacteriophage-infected cells and bacteriophage DNA (41, 93). This was soon extended to bacterial RNA and DNA. We wish to consider how the techniques used in these studies and the results obtained can help design hybridization studies with animal cell DNA-like RNA and the DNA from which it came.

First, it is worthwhile to mention that two basic methods of allowing the RNA to interact (anneal) with the DNA have been employed. (i) The annealing procedure can be carried out by mixing the RNA and DNA in liquid at an elevated temperature and a chosen salt concentration (41, 93). One disadvantage of this technique has been pointed out, namely, the opportunity for DNA-DNA reannealing to proceed and thereby foreclose the opportunity for formation of RNA-DNA hybrids. A second disadvantage, which will be emphasized in later discussions, is that, since the reactants are present in solution, mixtures cannot be easily washed free from one type of RNA in order to re-expose the DNA to a second type of RNA. (ii) Another means of conducting hybridization is to fix the single-stranded DNA to a solid substrate (2, 7, 33) prior to exposure to RNA. Two advantages of such techniques exist. Not only is the fixed DNA

prevented from reannealing, but it also becomes possible to expose the DNA to one RNA sample, wash this away, and introduce a second RNA sample. As will be described in detail, the fixation of DNA to nitrocellulose filters (33) is of particular value in doing competition experiments with different RNA samples.

With bacteriophage mRNA and the various bacterial RNA species, two general types of results have been attained. The first type of result is of qualitative or diagnostic value. For example, the rapidly labeled RNA is isolated from a small culture of bacteria or bacteria infected with T-even bacteriophage and the labeled RNA is exposed to bacterial or phage DNA (41, 93). In the case of labeled RNA from growing cells, a large fraction of the added radioactive RNA will become bound to bacterial DNA but none will bind to phage DNA. The labeled RNA from phage-infected cells conversely will bind to phage and not bacterial DNA. Two important points emerge from experiments of this type. First, the hybridization reaction has specificity and, second, it is implied, especially from the experiments with bacterial RNA, that a broad segment of the genome is represented in the labeled RNA because a large amount (10 to 50%) hybridizes. If the labeled bacterial RNA used in the hybridization reaction is purified rRNA, only a very small fraction of the input (1% or less) is found to hybridize. Thus, readily hybridizable RNA is often equated with RNA drawn from a broad segment of the genome of the cell in question.

The introduction of quantitation into hybridization experiments came with the hybridization of purified bacterial rRNA to bacterial DNA (41, 93). In this type of experiment, increasing amounts of labeled rRNA were mixed with a fixed amount of DNA. Ultimately, a point was reached where continued addition of labeled rRNA did not result in the formation of additional rRNA-DNA hybrid. Since the specific activity (counts per min per μg of rRNA) of the rRNA and the amount of DNA employed in these experiments was known, the amount of rRNA which could bind to a given amount of DNA could be calculated. This *saturation* value of *Escherichia coli* DNA for rRNA has been found to be 0.3%; i.e., 0.3% of the *E. coli* DNA represents sites from which rRNA could be produced. As was pointed out earlier in this review, this "saturation" technique has found great use in ascertaining the percentage of metazoon genomes responsible for rRNA, 5*S* RNA, and tRNA (11, 12).

Another type of hybridization experiment which is based on the fact that the DNA sites can be saturated is the so-called "competition" hybridization experiment. The first example of this type of experiment to be described involved the extraction of unlabeled or labeled RNA from T4 bacteriophage-infected cells early and late during the phage replicative cycle (40). A qualitative difference in the types of mRNA molecules of the "early" and "late" type was inferred from the finding that the hybridization of radioactively labeled "early" or "late" mRNA could be reduced most effectively by the addition to the hybridization mixture of homologous unlabeled mRNA. An important modification of the basic competition technique has recently been described (54). Because the RNA-DNA hybrid once formed at a given temperature and salt concentration is a stable structure under the conditions of its formation, Kasai and Bautz exposed phage DNA (bound to nitrocellulose filters) to unlabeled competing RNA (early or late phage mRNA) and then washed the filters free from any unbound RNA. Upon subsequent challenge of the filters with labeled RNA, it was found that the sites on the DNA which would have accepted labeled RNA had been presaturated by the unlabeled competing RNA. The DNA on the filters was still capable of reacting to heterologous phage mRNA. Obviously, this stable preoccupation of all of a certain type of sites on DNA implies that a saturating amount of unlabeled phage mRNA was employed in these experiments.

Hybridization of DNA-like animal cell RNA to its homologous DNA has been demonstrated in many instances (5, 49, 86, 111). A great many recent attempts to achieve saturation conditions and perform competition hybridization have also been reported. In almost all of these competition experiments, labeled RNA and "competing" unlabeled RNA have been simultaneously exposed to DNA. A decrease in the amount of labeled RNA-DNA hybrid has been interpreted to mean that sites on the DNA which would have been occupied by labeled molecules had been pre-empted by unlabeled molecules. In support of this, it has been pointed out that totally unrelated RNA from bacterial or yeast cells does not prevent the animal cell RNA-DNA hybrids. What has not been demonstrated in any of these experiments is that the decrease in hybrid formed is due to a specific occupation of the DNA sites by the competing unlabeled RNA. In other words, the Kasai-Bautz presaturation technique has not been employed.

From recent experiments involving relatively large amounts of purified HnRNA from HeLa

cells, it appears that true saturation of the DNA by this species of RNA has been achieved and that true competition between unlabeled HnRNA and labeled HnRNA can be demonstrated by first exposing DNA to unlabeled HnRNA, washing the DNA-bearing filter and re-exposing to labeled RNA (Soeiro and Darnell, *unpublished data*). With 1 μg of HeLa cell DNA attached to a Millipore filter, between 0.025 and 0.05 μg of HnRNA can be bound. (Conditions employed were 65 C, 20 hr of incubation in 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% sodium dodecyl sulfate.) Saturation of the DNA on the filter is not achieved, however, with inputs of less than about 20 μg of HnRNA, a 400-fold excess compared to what is bound. In this connection, it is pertinent to point out that a number of reports exist in the literature of studies in which saturating amounts of rRNA and also tRNA and 5S RNA have been determined (61a, 80). In all cases, a ratio of input RNA molecules to available DNA sites of at least 100 was necessary to achieve saturation.

If the sites on HeLa cell DNA can be saturated by HnRNA, then pre-exposure of the 1-μg HeLa DNA filters to unlabeled HnRNA in the range of 20 μg should result in the saturation of the DNA sites for HnRNA by stable hybrid molecules with unlabeled HnRNA. Subsequent reaction of the DNA with labeled HnRNA should be blocked. Such a competition experiment carried out by presaturation of the DNA is shown in Fig. 21 (left panel). Most of the sites on the DNA can obviously be occupied by stable hybrids of unlabeled HnRNA.

Also shown in Fig. 21 is the result of simultaneously exposing HeLa DNA to labeled and unlabeled HnRNA molecules. It can be seen that simultaneous exposure to hot and cold HnRNA causes a depression in the amount of labeled RNA-DNA hybrid formed which is greater than that observed by presaturation. Even at RNA inputs substantially below the saturation level for the 1 μg DNA filters, there is considerable interruption of hybrid formation. This interruption cannot be due to *stable* hybrid formation of unlabeled HnRNA with a DNA site which might otherwise be occupied by a labeled molecule because of the results obtained in the presaturation experiments.

Presaturation of DNA would appear to be the method of choice to demonstrate true competition between unlabeled and labeled samples of RNA. A final point in support of this conclusion can be seen in Fig. 21 (right panel) where it is shown that, although HeLa cell DNA can be presaturated by cold HnRNA, L-cell DNA cannot be so blocked.

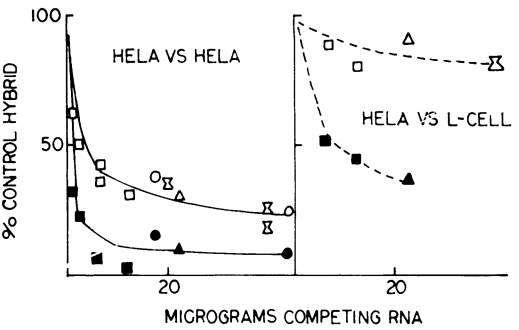

FIG. 21. *Competition hybridization of HnRNA. Left panel: radioactive HnRNA from HeLa cells was hybridized with HeLa cell DNA (1 μg, bound to Millipore filters; 33), and the effect of unlabeled HeLa cell HnRNA on this process was studied. Increasing amounts of unlabeled HnRNA were added during the hybridization of the hot RNA ("simultaneous competition," filled symbols) or the unlabeled HnRNA was added prior to the labeled RNA, and unbound RNA was removed before the DNA was exposed to labeled RNA ("presaturation," open symbols). Different symbols represent different preparations of unlabeled RNA. The level of hybridized labeled RNA without competing unlabeled RNA ranged from 400 to 1,000 counts/min. Right panel: radioactive HnRNA from L cells was purified and hybridized to L-cell DNA. The effect of HeLa-cell HnRNA on the L-cell hybridization was studied by "simultaneous" and "presaturation" competition techniques.*

However, simultaneous addition of unlabeled HeLa HnRNA depresses both HeLa and L-cell RNA-DNA hybrids.

Using the presaturation method, it may be possible to obtain more meaningful information about sequence similarities among molecules which are similar in overall composition but which come from different cellular locations.

CONCLUSIONS

In an active field of research, an article attempting to summarize available knowledge is doomed to obsolescence by press time because of the rapid accumulation of new information. Nevertheless, it seems worthwhile to pause at least momentarily to list the positive statements about animal-cell RNA metabolism which have come from the work of the past few years as well as to focus as clearly as possible on those areas which need a great deal more illumination.

On the positive side, it can be stated that the manufacture of ribosomes in animal cells is begun in the nucleolus by the *transcription* of a high molecular weight precursor to rRNA. This molecule is methylated and becomes associated with ribosomal protein within a brief time after

its synthesis. It is cleaved at specific sites so that two ribosomal subunits, one containing 18*S* rRNA and the other 28*S* rRNA, plus two small RNA chains eventually emerge. A substantial portion of the ribosomal precursor RNA molecule is lost in the maturation process.

To choose one example of the kind of problem which awaits solution in the area of ribosome formation, one can mention the presumably enzymatic scission of the rRNA precursor. Isolation of such enzymes and extensive study of this process should contribute greatly to an understanding of how specific protein-nucleic acid interactions are mediated.

In addition to ribosome biogenesis, this article has dealt with various types of animal cell RNA molecules which resemble DNA in their overall base composition. Here, we have progressed only far enough to distinguish various types of this DNA-like RNA on operational grounds as mRNA, cytoplasmic DNA-like RNA, and HnRNA. As was heavily stressed, a problem of commanding importance is to understand what, if any, relationship these various types of DNA-like RNA molecules have to one another.

Finally, as a prime example of a whole body of new information which is just around the corner (but, fortunately for this reviewer, enough in the future to have just missed this report), one could mention the RNA associated with mitochondria. It is possible that mitochondria not only generate some mRNA from their DNA but also some ribosomes may originate in mitochondria. It is conceivable that some of the knottier problems of animal cell biology such as mRNA generation may not be solved by a study of nuclear function but by dealing with mitochondria.

At any rate, the general subject of gene expression in animal cells as approached by examination of animal cell RNA is progressing rapidly and can undoubtedly be profitably reviewed again soon.

ACKNOWLEDGMENTS

The work described in this paper from the author's laboratory was supported by grants from the National Institutes of Health (CA 07861-04), the National Science Foundation (GB 4565), and the Health Research Council of the City of New York.

The author also thanks Sheldon Penman, Jonathan Warner, and Eric Holtzman for making available various prints, and H. C. Birnboim and Deborah Bernhardt for making available some unpublished data.

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