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A COMPARISON OF THE HETEROGENEOUS NUCLEAR RNA OF HELA CELLS IN DIFFERENT PERIODS OF THE CELL GROWTH CYCLE

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ABSTRACT

HeLa cells synthesize heterogeneous nuclear RNA (HnRNA) in the G_1, S, and G_2 portions of the cell cycle. HnRNA prepared from these various periods was compared by RNA-DNA hybridization experiments. The results indicated that some of the HnRNA molecules were equivalent at all times in the cell cycle, but limitations in the sensitivity of the hydridization reactions, as well as in the spectrum of hybridizing molecules, restrict the conclusions that can be drawn from these comparisons.

INTRODUCTION

One of the outstanding unanswered problems in cell biology is how eucaryotic cells, all of which from a given metazoan organism are generally presumed to have the same DNA complement (1, 2), regulate the production of specific proteins at specific times. The growth cycle in animal cells has been demonstrated to be clearly divisible into a period of DNA synthesis (S phase) separated from mitosis (3). Much recent work has been concentrated on delineating the biochemical events that occur during different phases of the cell cycle, and it has been established in cultured cells that a number of proteins are made discontinuously throughout the cell cycle (4–6). Thus, a synchronized growing cell culture offers an excellent model in which to study the regulation of protein synthesis at the molecular level. Since the synthesis of specific proteins requires the translation of specific mRNA molecules, an examination of the RNA molecules present in cultured cells during the growth cycle seems in order. We have concentrated on two types of RNA, the HnRNA, a metabolically active nuclear RNA species of various-sized molecules with a base composition resembling DNA (7–9), and cytoplasmic mRNA from polyribosomes (10). The question of the relationship between these two RNA species is unsettled at the moment. While it is quite clear that the majority of the HnRNA does not leave the nucleus and enter the cytoplasm (7, 9, 11), recent experiments comparing HeLa cell HnRNA with polysomal RNA from randomly grown cells indicate a considerable overlap in the sequences of some of the RNA molecules. These experiments are compatible with the possibility that a small selected fraction of the HnRNA is used to make mRNA. It therefore becomes of interest to compare these RNA species in synchronized cells.

MATERIALS AND METHODS

The growth of HeLa cells in suspension culture, cell fractionation, RNA extraction from nuclei and

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Published March 1, 1970

polyribosomes, DNA preparation from purified nuclei, and hybridization procedures have been described previously (12, 13).

Cell Synchronization

Since large numbers of cells (up to 5×10^9 cells) were needed for preparations of RNA, the method used for cell synchronization was the two-cycle thymidine blockade of DNA synthesis (14). Growing cultures were exposed to 2 mM thymidine for 16 hr and removed from thymidine-containing medium by centrifugation and resuspension in growth medium (15) for 10 hr. A second cycle of thymidine treatment of 16 hr then allowed capture of all the cells in late G_1 (just prior to S phase, the DNA synthesis period). Centrifugation and resuspension of the cells (at 2×10^6/ml) was followed within 1 hr by the beginning of DNA synthesis, which was monitored by the addition of 0.5 μCi of thymidine-^{14}C (10 μCi/μmole to 5×10^5 cells in 1 ml) and by the measurement 15 min later of incorporated acid-precipitable radioactivity (Fig. 1). DNA synthesis reached a maximum which was three times the incorporation of asynchronously grown cells by 4–5 hr after removal from excess thymidine. Since the S phase is one-third of the cell cycle (16), the threefold increase over asynchronous cells indicates that all or nearly all of the cells were in S phase at this time. Cells were taken at this point for preparation of unlabeled S phase RNA from either polysomes or purified nuclei (Fig. 1). For the preparation of labeled S phase RNA, synchronized cells were exposed to uridine-^{3}H (20 μCi/μmole, 200–400 μCi/ml) between 3 and 4 hr after removal from excess thymidine, and then RNA was extracted.

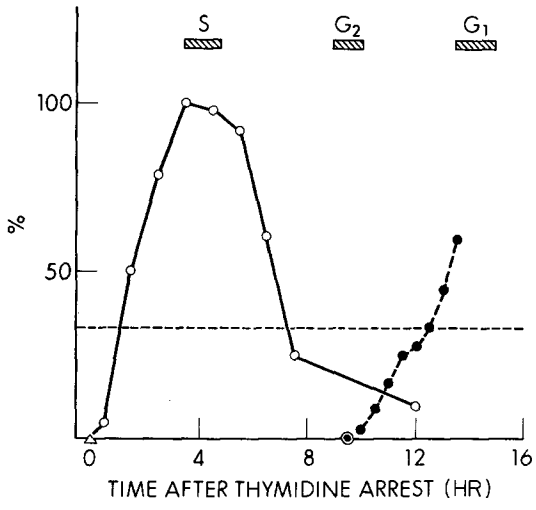

FIGURE 1 Cell synchronization. DNA synthesis was monitored in synchronized cells by thymidine-^{14}C incorporation after the removal of an inhibitory dose of thymidine (△, zero time). The percentage of the maximum thymidine-^{14}C incorporation is plotted on the ordinate (see Materials and Methods, —○—, maximum incorporation during S phase was 1500 cpm). The relative incorporation in the same number of cells from a logarithmically growing control culture is shown by the dashed line (----). Colcemid (0.03 μg/ml) was added (◉) to a portion of a synchronized culture 9½ hr after release from the second thymidine block, and cells arrested in metaphase were scored at various times thereafter [—●—%, cells in metaphase after Colcemid). Shaded segments indicate the times after release from thymidine inhibition at which cultures were harvested for preparation of labeled and unlabeled RNA of S, G_1, or G_2 cells.

For obtaining cells in G_2, the rate of DNA synthesis was monitored in thymidine-synchronized cells and, 2–3 hr after the rate had declined to less than 20% of the maximum, cells were harvested as G_2 cells. The mitotic index was less than 5% at this time, and an additional test indicated that the cells had not passed through mitosis. Portions of the synchronized cultures were treated with Colcemid at 9.5 hr after reversal from thymidine blockade. It was found that more than 60% (as much as 80%) of the cells were arrested in metaphase within the next 3 hr (Fig. 1). Thus the great majority of cells that were considered to be in G_2 were in fact in this phase of the cell cycle. Labeled RNA from cells in G_2 was prepared as described above.

For obtaining cells in G_1, thymidine-synchronized cells in S phase were carefully counted in a hemacytometer, and the culture was followed by thymidine incorporation and subsequent cell counts. By 12 hr after the peak of DNA synthesis, the cell number had doubled and DNA synthesis was less than 10% that of the maximum of S phase. Cells were considered to be in G_1 at this time, and labeled RNA was prepared after 60–75 min of exposure to uridine-^{3}H in growth medium or to ^{32}P in phosphate-free Eagle's medium (15) containing dialyzed horse serum.

RESULTS

Formation of HnRNA Throughout the Cell Cycle

Previous studies on synchronized cells in culture have indicated that it is likely that both HnRNA and r-pre-RNA (ribosomal precursor RNA) are formed throughout the cell cycle (16–18). These studies, however, either were performed before the clear recognition of the two nuclear RNA species or were designed specifically to investigate the rate of rRNA synthesis and not the relative amounts of each type of nuclear RNA being formed.

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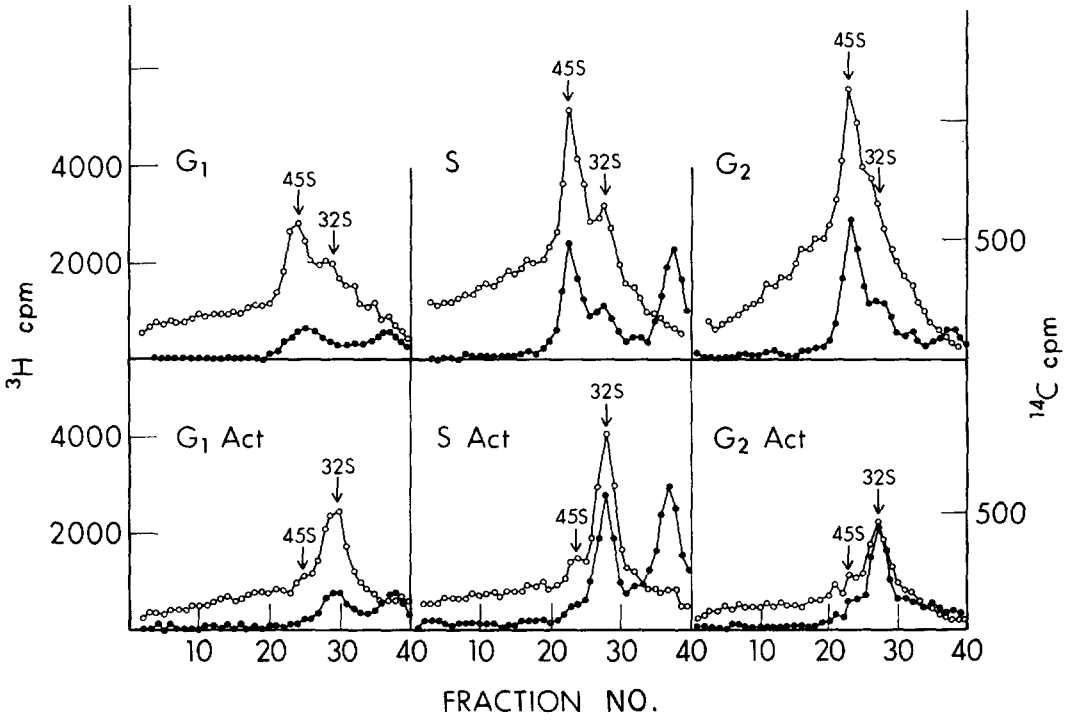

FIGURE 2 Ribosomal precursor RNA and HnRNA synthesis in synchronized HeLa cells. Synchronized HeLa cells were released from the second thymidine block and resuspended in normal medium supplemented with 10^{-4} M adenine. At 4, 9, and 14 hr after release, samples of 10^8 cells were concentrated 10-fold by centrifugation and resuspended in 20 ml of fresh medium lacking methionine and containing 7% dialyzed serum, 4 μCi uridine-^{3}H, 10 μCi methionine[methyl-^{14}C], and 10^{-4} M adenine. After 15 min of incubation one-half of the culture was removed and actinomycin D, 5 μg/ml, was added to the remainder for an additional 15 min. RNA was isolated by phenol extraction of detergent-cleaned nuclei (13, 32), followed by sedimentation analysis of radioactive RNA in sucrose gradients (37 ml, 15–30% sucrose in 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.01 M EDTA, and 0.2% sodium dodecyl sulfate; Spinco SW 27 rotor, 16,000 rpm, 15 hr, 25°C). Open symbols, cpm, uridine-^{3}H; closed symbols, cpm, methyl-^{14}C.

Therefore the experiment shown in Fig. 1 was performed to determine whether the HnRNA was formed rapidly in all phases of the cell cycle. Cells in G_1, S, and G_2 were labeled simultaneously with uridine-^{3}H and methionine[methyl-^{14}C], one-half of the culture was removed after 15 min and actinomycin was added to the remainder for an additional 15 min. Examination of the total nuclear RNA from each sample revealed that both HnRNA and r-pre-RNA were indeed being formed throughout the cell cycle (Fig. 2). The 45S r-pre-RNA is recognized most clearly in these diagrams because it is labeled by methyl-^{14}C groups from methyl-labeled methionine (19–21). The HnRNA is marked most easily as material not labeled by methyl groups and sedimenting in advance of the 45S r-pre-RNA (8).

Several points are obvious from the diagrams.

(*a*) As has been previously reported (16–18), there seems to be a generally more rapid incorporation of uridine into all types of RNA in going from G_1 to S to G_2.

(*b*) In the actinomycin-treated cultures the 45S RNA disappears while the 32S RNA appears within 15 min, indicating that processing of the 45S r-pre-RNA is approximately the same throughout the cell cycle (22).

(*c*) By comparing the total nuclear uridine-^{3}H incorporation and methyl-^{14}C incorporation (Table I), one can see that the amount of methyl incorporation into r-pre-RNA compared to HnRNA synthesis rises as cells progress from G_1 to S to G_2. Data of this type are in accord with the idea of increased ribosome formation throughout

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TABLE I

Comparison of Uridine-^3H and Methyl-^{14}C Incorporation into Total Nuclear RNA

Phase of cell cycle	Before chase			After actinomycin chase		
	^3H	^{14}C	^3H:^{14}C	^3H	^{14}C	^3H:^{14}C
	cpm			*cpm*		
G_1	38,600	1,100	35.0	28,219	1,113	25.5
S	65,462	2,594	26.3	34,183	2,437	14.1
G_2	66,570	3,095	22.2	23,934	2,207	11.0

Total ^3H and ^{14}C counts were summed from the first 31 fractions of the gradients of Fig. 2 so as not to include methyl-^{14}C incorporation not in RNA in the fractions at the top of the gradients.

interphase (16, 17), but could conceivably be due to more rapid or effective entry of methyl groups into the S-adenosyl methionine (donor of methyl groups) pool in $G_2 > S > G_1$. This matter can only be settled by direct measurements.

(*d*) There is a greater loss of radioactivity incident to actinomycin treatment in the G_2 cells than in the G_1 or S cells. This is most easily observed in the loss of ^3H relative to loss of ^{14}C. Loss of nuclear radioactivity in these cells during actinomycin treatment can come from three sources. (i) About 30% of the ^3H counts in r-pre-RNA would be destroyed to acid-soluble radioactivity in going from 45S to 32S, and about 15% would be lost from the nucleus (23–26) in the form of 18S rRNA in smaller ribosomal subunits that very rapidly leave the nucleus to enter the cytoplasm (22). (ii) Due to the more extensive methylation of 18S rRNA (0.6×10^6) compared to 28S (1.6×10^6) (19, 24), about 40% of the methyl-^{14}C groups in r-pre-RNA will be lost from the nucleus. The only methyl counts are in these two RNA species. (iii) The turnover of HnRNA will lead to a loss of ^3H but not methyl-^{14}C groups (11). Thus the relative amount of ^3H and ^{14}C lost from 45S r-pre-RNA as it is processed to 32S RNA should be approximately equal, although due to losses of different portions of the molecule. The observed greater loss in the G_2 cells of ^3H counts relative to methyl-^{14}C counts therefore indicates a more extensive loss (a more rapid turnover) of HnRNA in the G_2 cells. This fact may be related to the apparently more rapid rate of synthesis of G_2 cells when brief label times are used to monitor synthesis.

Although definite differences in the rates of rRNA synthesis and HnRNA turnover during the cell cycle are therefore apparent, the main conclusion from this type of experiment, for the purposes of this paper, is simply that HnRNA is being formed at a rapid and similar rate (within a factor of 2) throughout the cell cycle.

Comparison by Presaturation Competition of HnRNA from G_1, S, and G_2

For the comparison of HnRNA of G_1, S, and G_2 cells by competition hybridization, large amounts (1–1.5 mg) of unlabeled and labeled RNA from the nuclei of cells in each part of the cell cycle were prepared.[1] The highly labeled RNA from each part of the cell cycle was found to hybridize equally to filters containing 1 μg of HeLa cell DNA. Pairwise comparison in various combinations was then made by presaturation competition hybridization experiments that involve, as previously described (12, 13), exposure to unlabeled RNA of DNA bound on a nitrocellulose filter, thorough washing of the filter at 65°C in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate), subsequent exposure to labeled RNA molecules, and finally an assay of the labeled RNA bound as RNase-resistant hybrid.

The general result in the present experiments was that a majority of the hybridization sites for HnRNA that had been labeled during any time of the cell cycle could be occupied by unlabeled molecules from another portion of the cycle (Fig. 3). This was true when either S phase or G_1 phase RNA was used as the competitor.

In the hybridization experiments in this work, precautions were taken to increase the validity of comparisons between RNA samples. For example, in Fig. 3, Panel A, the presaturation was carried out with RNA from S phase cells, and then the filters were exposed simultaneously to HnRNA-^3H from S and HnRNA-^{32}P from G_1. Differences in specific activities between two samples of RNA having the same distribution of molecules are cancelled out, for the following reason. For example, let us suppose that the proportions, as well as the types of molecules in the two samples, are the same but that their specific activities are very

[1] HnRNA preparations used were taken from sucrose gradients. The labeled preparations were collected in the region of the gradient in front of the 45S peak; as were most of the unlabeled preparations. Some of the unlabeled preparations included also the region of 32S of the gradient.

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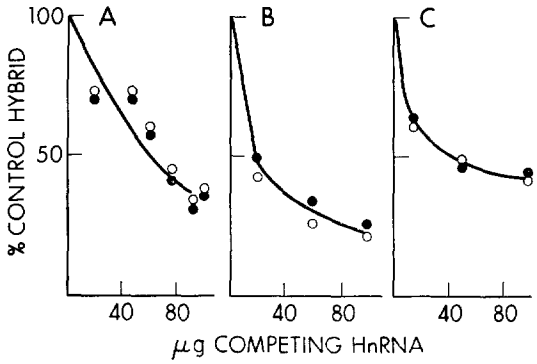

FIGURE 3 Competition hybridization with HnRNA from G_1, S, and G_2 phases of the cell growth cycle. Panel A: 1 μg HeLa DNA filters were subjected to "presaturation" with various amounts of S phase HnRNA for 18 hr at 65°C in 2X SSC (sodium chloride, 0.3 M and sodium citrate, 0.03 M), followed by exposure to ^{3}H-labeled S phase (closed symbols) and ^{32}P-labeled G_1 phase HnRNA (open symbols). Nuclease-resistant hybrid molecules were scored as described (12, 13). Each point represents two or three filters. Panel B: The effect of G_1 phase unlabeled HnRNA as competitor against ^{3}H-labeled HnRNA from S and ^{32}P-labeled HnRNA from G_1. Panel C: The effect of G_1 phase unlabeled HnRNA as competitor against G_2 phase ^{3}H-labeled HnRNA (open symbols) and G_1 phase ^{32}P-labeled HnRNA (closed symbols). All the labeled RNA in these experiments were of high specific activity (derived from 5×10^7 cells with at least 2×10^7 cpm in nuclear RNA), and the uncompeted DNA filters contained 200–400 cpm ^{3}H and 200–500 cpm ^{32}P from inputs of 50,000–100,000 cpm. Blank filters containing no DNA contained no more than 5% of the cpm on the HeLa cell filters.

different. In order technically to perform the experiment, i.e., to achieve a sufficient input of radioactivity to monitor the hybrid, we would need different amounts of RNA from the two samples; and different molecules might therefore be represented in the hybrid structures from the two samples, although the two samples contained the same distribution of molecules. This type of difficulty is avoided by the use of ^{3}H and ^{32}P mixtures.

On the other hand, it is possible that the ^{3}H S phase and ^{32}P G_1 phase samples contained the same types of molecules but in different proportions, such that certain types accounted for a larger fraction of the observed hybrid with the ^{3}H S phase, while another type accounted for the larger fraction with the ^{32}P G_1 phase. This should be reflected by better competition of unlabeled S phase against ^{3}H than against ^{32}P, even though the types of hybridizing molecules were similar. All the comparisons given in Fig. 3 were made by presaturation with this double-label technique.

In addition, the fact that competition occurred on the same filter for the two types of labeled RNA economizes on the amount of competitor RNA needed and increases the accuracy of comparison. Finally, since the competition was carried out by prehybridization, any differences observed would be expected to have a greater degree of specificity than if simultaneous addition of labeled and unlabeled molecules had been employed (12).

Other variations of the hybridization technique were carried out to determine whether differences between RNA samples could be observed. In the experiment of Fig. 4, filters with larger amounts of DNA were used in an effort to hybridize a broader spectrum of RNA molecules. Competition by simultaneous addition of labeled and unlabeled RNA molecules failed to reveal any differences.

The experiments described thus far have examined hybridization and competition against the total population of large HnRNA molecules. From the previously determined kinetics of hybridization of these molecules, it appears that the hybridization reaction is fastest at first and constantly decreases in rate and may still be slightly increas-

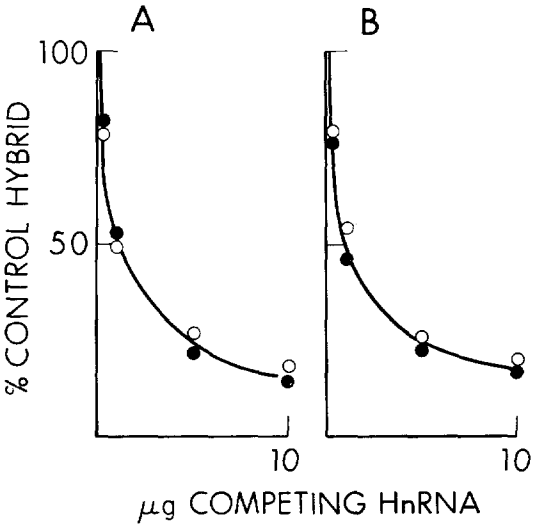

FIGURE 4 The effect of simultaneous addition of labeled and unlabeled HnRNA to filters containing 5 μg HeLa DNA. Various amounts of cold S phase HnRNA (left panel) and G_1 phase HnRNA (right panel) on the hybridization of ^{32}P-labeled G_1 phase (open symbols) and ^{3}H-labeled S phase HnRNA (closed symbols) were studied with 5 μg DNA filters.

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ing even after 48 hr (27, 28). One suggestion to explain these kinetics is that molecules that hybridize faster than other types of molecules may be gradually depleted from the population as time goes on. Therefore an experiment designed to test "more rapidly hybridizing" molecules compared to those remaining after removal of some fraction of these rapidly hybridizing molecules was performed. The main purpose of the experiment was to magnify any small differences in the hybridizing behavior of the later hybridizing RNA molecules from G_1 or S phase, which would be masked by the preponderant hybridization of the rapidly hybridizing molecules. In this experiment, a larger amount of DNA (20 μg) was used on the filters. A first set of filters was added to the various RNA samples, followed after 6 hr by a second set added to the same vials and 16 hr later by a third set. Each successive set of filters hybridized smaller amounts of radioactive RNA. If there were a depletion of certain types of RNA by attachment of the most readily hybridizable RNA to the DNA on the first filter sets, the three sets of filters should be hybridizing different types of molecules. That this was true was indicated first by the fact that the third set of filters contained only about one-third the amount of hybrid as the first set, and second by the different behavior of the RNA species hybridized to the third filter set with regard to inhibition by unlabeled RNA.

In this latter type of test, it was observed that a considerable and increasing inhibition was obtained with increasing amounts of unlabeled RNA for the first two filter sets. However, in the third filter set, only a small inhibition was observed.

A likely interpretation of these results is that the earliest molecules that hybridize belong in the class that are in the related "families" (in the terminology of Britten and Kohne [29]) with the largest number of members so that they cross-react and reanneal fastest in the hybridization test. Because of this cross-reaction, these molecules may be easier to block from hybridization than those that remain to hybridize to the third filter set. The molecules in this third set are therefore of a different class, as they are not inhibited to the same degree by the unlabeled RNA. They may represent RNA that comes from DNA regions not included in large families or from families that are transcribed into RNA less frequently and therefore are more difficult to compete.

Even though the experiment in Fig. 5 examines apparently different types of molecules, it can be seen that the S phase and G_1 phase behaved in parallel, indicating that, with yet another test, differences among these two RNA samples were not observable.

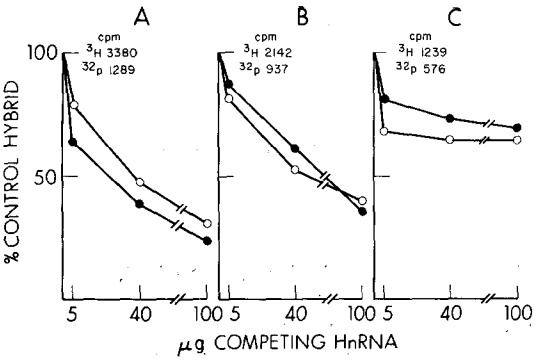

FIGURE 5 Stepwise hybridization of HnRNA and effect of simultaneous addition of cold HnRNA to 20-μg HeLa DNA filters. Three sets of 20-μg DNA filters were added to the same vials containing labeled RNA alone or labeled plus the designated amount of unlabeled S phase HnRNA. The first filter set was added at 0 time (*A*) followed 6 hr later by a second set (*B*), and after 16 hr by a third set (*C*). The hybridization was stopped and assayed after a further 22 hr. Each vial contained labeled HnRNA of high specific activity from S (uridine-^{3}H, closed symbols) and from G_1 (^{32}P, open symbols).

Comparison of Polysomal mRNA from G_1, S, and G_2

It is known that certain proteins are manufactured at discrete times during the cell growth cycle; this presumably means that at least some differences in the distribution of mRNA molecules would exist in polysomes during the cell cycle. One such difference in specialized mRNA molecules has been detected in association with histone synthesis in HeLa cells (30).

Polysomal RNA, both labeled and unlabeled, was prepared from various times in the cell cycle, and competition by both presaturation and simultaneous addition of labeled and unlabeled RNA was performed just as described in the accompanying paper (13). It was not possible to obtain extensive presaturation of DNA filters with polysomal RNA, although as much as 3 mg of polysomal RNA was exposed to a single filter bearing 1 μg of DNA. This result was also found when unsynchronized cell RNA was tested (13). Simultaneous addition of hot and cold RNA, a considerably less specific technique for demonstrating competition

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between RNA samples than presaturation (12, 13), was performed with the RNA from S, G_1, and G_2; although almost complete depression of hybridization was achieved, to the extent of 70–90%, no consistent or large differences were observed between the samples.

DISCUSSION

These experiments indicate, first of all, that the synthesis of HnRNA is not confined to any particular period of the cell cycle.

The interpretation of comparison by hybridization techniques between the HnRNA or polysomal mRNA molecules from the different times in the cell cycle presumes a clear understanding of the hybridization technique for animal cell RNA and DNA. If the hybridization were perfect, i.e. if RNA molecules *only* hybridized to DNA sites of the type from which they originated, then we could conclude that no significant variations in HnRNA existed throughout the cell cycle in HeLa cells because of the cross-competition results.

Unfortunately, no such unambiguous interpretation is possible. Various laboratories have pointed out in recent years that DNA from animal sources contains repeated sequences that are similar but probably not exact (29, 31). When animal cell nucleic acids are allowed to hybridize, these sequences are the first to participate in the hybridization (27). To the extent that a molecule from any of these "repeated" sites hybridizes to a similar nonidentical site, the specificity of the hybridization and competition reactions is decreased.

In addition to the competition experiments involving the total HnRNA from various phases of the cell cycle, another type of experiment (Fig. 5) was performed in which different classes within the total HnRNA have been examined for differences between G_1 RNA and S RNA without detecting distinct differences. Since the experiment of Fig. 5 indicated that different classes of HnRNA were, in fact, distinguishable, further experiments in which the HnRNA is fractionated into rapidly and slowly hybridizing molecules deserve further work.

All the approaches used in this paper to compare RNA from G_1, G_2, and S phase increase the level of specificity with which we are comparing RNA samples from the different portions of the cell cycle, but it is still likely that the majority of the hybrid molecules examined come from DNA sites that are similar but not identical. It should also be pointed out that in comparison by hybridization, one can only compare those molecules that hybridize and not those that do not, and only about 1% of the input molecules hybridize in the usual experiment. Thus, the final results are compatible with the interpretation that some of the HnRNA from all three phases of the cell cycle is the same, but further work with the hybridization technique in the direction of hybridizing a broader sample of the RNA molecules will be necessary to determine the real degree of similarity.

We should point out, however, in conjunction with the findings of the previous paper, the possibility that large segments of the HeLa cell DNA are transcribed at all times, usually to be destroyed but perhaps sometimes to be used to generate mRNA. Such a mechanism would then not demand a changing profile of HnRNA as the cell goes throught the cell cycle, in order for different mRNA to be selected for use in the manufacture of proteins.

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