A COMPARISON BETWEEN HETEROGENEOUS NUCLEAR RNA AND POLYSOMAL MESSENGER RNA IN HEla CELLS BY RNA-DNA HYBRIDIZATION

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ABSTRACT

Heterogeneous nuclear RNA (HnRNA) and mRNA from cytoplasmic polyribosomes of HeLa cells have been compared by RNA-DNA hybridization tests. 1 µg of HeLa cell DNA binds 0.05-0.10 µg of either HnRNA or mRNA. In addition, HeLa DNA that is preexposed to unlabeled HnRNA was found to have a reduced capacity to bind either HnRNA or mRNA. The results are compatible with considerable sequence similarity in the two types of RNA but, as is discussed, firm conclusions are precluded by imperfections of the hybridization reaction as presently employed.

The possible precursor relationship in animal cells of the high molecular weight, rapidly labeled nuclear heterogeneous RNA (HnRNA) to polysomal messenger RNA (mRNA) has been the focus of considerable interest in the past several years. Present evidence indicates that all nucleated animal cells synthesize HnRNA (1-6), and it has been demonstrated by analysis of the kinetics of HnRNA synthesis that a majority of this RNA never leaves the cell nucleus (1-8). This finding does not, however, preclude the possibility that a small portion of the HnRNA (4-10 × 10⁶ daltons) is excised in some manner to serve as mRNA (~2-4 × 10⁵ daltons) in the cytoplasmic polyribosomes.

In this paper, we report experiments utilizing RNA-DNA hybridization to investigate the sequence similarity of polysomal mRNA and HnRNA. These experiments have been based on the previous finding (7, 13) that sufficiently large amounts of purified unlabeled HnRNA from HeLa cells can react with HeLa DNA bound to a nitrocellulose filter, so that when the filter is washed and exposed to labeled HnRNA the majority of the DNA sites to which HnRNA can hybridize remain blocked. This "presaturation" technique, first described by Kasai and Bautz (9), has been found to show a higher degree of specificity than the more common technique utilized for comparison of two mammalian cell RNA samples, that is, by simultaneous exposure of DNA to labeled and unlabeled molecules (10).

The present results, which indicate an extensive similarity in sequence between some polysomal mRNA molecules and HnRNA, are compatible with, but do not prove the hypothesis that mRNA is derived by specific cleavage of large HnRNA molecules.

METHODS

Cells and Labeling Procedures

HeLa cells grown in suspension as previously described (11) were concentrated to 5 × 10⁶/ml and
labeled either with uridine-\(^{3}\text{H}\) (500 \(\mu\text{Ci/ml; 20 mCi/\mu mole}\)) or with \(^{32}\text{P}\) (1mCi/ml in PO4-free Eagle's medium [1959]) for periods up to 1 hr. Cells were also grown in medium containing uridine-\(^{3}\text{H}\) for 24 hr (0.125 \(\mu\text{Ci/ml; 0.025 mm}\), under which conditions the cells doubled.

**RNA**

RNA, labeled and unlabeled, was prepared as described (12, 13) with the use of DNase lysis of purified nuclei (14). In most cases HnRNA sedimenting faster than 4S was used; however, in a few specified cases total nuclear RNA sedimenting faster than 32S was used.

Polysomal RNA was prepared from cytoplasmic extracts made from cells swollen and homogenized in hypotonic buffer (Reticulocyte Standard Buffer [RSB] = 0.01 M Tris pH 7.4, 0.01 M NaCl, 0.0015 M MgCl\(_2\); up to \(10^8\) cells/ml of buffer) (15). The extracts were treated with deoxycholate (final concentration 0.5%) and the nonionic detergent, Brij 58 (final concentration 0.5% Atlas Chemical Industries, Inc., Wilmington, Del.), and sedimented for 4 hr through 7–47% (w/w dissolved in RSB sucrose gradients) to separate polysomes. As shown in Fig. 1, the polysomes were well separated from ribosomes and ribosomal subunits. The RNA from the polysome region was released by sodium dodecyl sulfate (SDS) treatment and precipitated with NaCl (0.1 M) and ethanol, followed by phenol plus CHCl\(_3\) (16) extraction in acetate buffer (14). In agreement with previous results (17) approximately 50% of the labeled RNA in polysomes from cells labeled for 60 min sedimented in sucrose gradients like mRNA. If the polysomal RNA came from cultures of cells greater than \(5 \times 10^7\), it was further purified by phase partition according to Ralph and Bellamy (18) to eliminate anthrone-positive material (presumably glycogen).

**Hybridization and Presaturation**

Hybridization and presaturation procedures were used as previously described (13), including scoring

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**Figure 1** Panel A: \(1.5 \times 10^4\) HeLa cells, which had been incubated at 37°C for 60 min in 30 ml of Eagle's medium containing 10 mCi of uridine-\(^{3}\text{H}\) (90 mCi/\mu mole), were swollen and homogenized and the cytoplasmic extract was fractionated on a 7–47% sucrose gradient (4 hr, 27,000 rpm, 4°C, SW 27 rotor, Spinco ultracentrifuge; for further details see Methods and [18]). OD\(_{260}\) was monitored spectrophotometrically in a flow cell and the cpn in each fraction of about 3 ml was determined by assaying a small aliquot for acid precipitable radioactivity. Panel B: The portion of the gradient from Panel A that contained the polysomes was treated with EDTA (0.01 M), resedimented through four separate sucrose gradients containing EDTA (15–30% in 0.01 M Tris, pH 7.4; 0.01 M NaCl; 0.01 M EDTA; 16 hr, 23,000 rpm, SW 27, 4°C). All the EDTA gradients were identical, and one profile is given with the scales multiplied \(\times 4\). RNA for hybridization studies was extracted from particles between fractions 10 and 28.
for hybrids after washing at 65°C, and digestion of unpaired RNA at 37°C with both RNase and T1 ribonuclease. The reasons for the preference of the presaturation technique are detailed in another paper (13) in which it was shown e.g., that unlabeled HeLa cell HnRNA as a competing species distinguished between HeLa HnRNA and L cell (mouse) HnRNA by the presaturation technique but not by the simultaneous addition technique.

RESULTS

Demonstration of Hybridization by Polysomal mRNA

It was first necessary to determine the hybridizing capacity of newly formed cytoplasmic RNA to filters containing 1 µg DNA, since this amount of DNA was to be used in the presaturation experiments. One other point was kept in mind in these initial comparisons. Small amounts of very highly labeled RNA were employed so that the DNA would be in excess. (That this was true was indicated by the fact that the amount of hybrid formed was linear with input over a considerable range.) The purpose was to test whether under conditions of DNA excess approximately equal or very different proportions of the input HnRNA or polysomal RNA would form hybrid with 1-µg filters. It was found that under these conditions approximately 0.3% of the radioactive RNA from polysomes of cells labeled for 60 min formed hybrids. A comparable input of radioactive, purified HnRNA formed about 0.6% hybrids under the same conditions (Table I). Although about 50% of the radioactivity in labeled polysomal RNA was ribosomal in type, these molecules would not contribute to the hybrids formed here. In all cases (Table I and Fig. 3) a saturating level of rRNA was present. Since only 0.0004 µg of rRNA would bind to a 1 µg HeLa DNA filter at saturation (21) and the input polysomal RNA was about 25 µg of RNA, mostly rRNA, it is clear that any contribution from rRNA hybrids would be negligible. However, RNA taken from cytoplasmic structures that sediment between 40S and 100S also forms hybrids at about 0.3% of the input. These structures are not engaged in protein synthesis, and no evidence exists that nonribosomal RNA in these structures is a precursor to polysomal mRNA (19, 20).

In addition, it has been found that some rapidly labeled cytoplasmic RNA is associated with nonpolysomal structures even larger than the 40–100S structures (19, 20). These structures may be clearly distinguished from functioning polysomes because they do not dissociate when treated with EDTA as do polysomes (19, 20, 26).

The following experiment was carried out to

| Type of labeled RNA | Hybridized with | % |
|--------------------|----------------|---|
| HnRNA >44S ‡       | 0.1-0.2        |   |
| Nuclear RNA >32S ‡ | 0.1-0.2        |   |
| Polysomal RNA §     | 0.2-0.6        |   |
| Cytoplasmic RNA from 40-100S structures § | 0.2-0.6 |   |
| RNA from EDTA-treated polysomes § | 0.3-0.6 |   |

‡ Nuclear RNA was either purified HnRNA, which sedimented faster than 44S ribosomal precursor RNA, or total nuclear RNA that included all molecules as large as or larger than 32S ribosomal precursor RNA. § Cytoplasmic RNA was extracted from polysomes, from cytoplasmic structures that sedimented slower than polysomes (40–100S), or from EDTA-treated polysomes that had been resedimented to display the 50S and 30S ribosomal subunits. Fig. 1 shows the separations of the various fractions. Most of the total RNA added from any cytoplasmic fraction was rRNA.

Table I

Hybridization Efficiency of Various Types of Rapidly Labeled HeLa Cell RNA*
make certain in our experiments that the RNA from the polysome region that was found to hybridize to 1 µg HeLa DNA filters was mRNA. A cytoplasmic extract was subjected to sucrose gradient sedimentation to isolate polysomes, which were then treated with EDTA and resedimented (Fig. 1). The rapidly labeled RNA was now found to sediment in structures from 20 to 60S in EDTA containing buffer as has been reported for mRNA (19, 20). The rapidly labeled RNA released from polysomes by EDTA was still found to hybridize to 1-µg DNA filters to the extent of 0.3–0.5%.

One further comparison between HnRNA and polysomal mRNA hybridization was made involving the kinetics of hybridization of each type of RNA. Since HnRNA hybridizes to DNA at a rate faster than would be predicted, if the molecules came from DNA sites that occurred only once per genome, it was important to determine the kinetics of the polysomal mRNA reaction. It can be seen in Fig. 2 that, over a 48 hr period, the rates of reaction with DNA of these two species are quite similar.

In summary, these experiments indicate that rapidly labeled RNA, which behaves as functioning mRNA (i.e., is in polysomes and can be released by EDTA), does hybridize detectably to small quantities of DNA and that the reaction of this polysomal mRNA is similar both in extent and in rate to that of HnRNA.

**Figure 2** Time course of hybridization of polysomal and HnRNA. Polysomal RNA labeled with uridine-3H and HnRNA labeled with 32P were mixed and hybridized at 65°C in 2 × Standard Saline Culture containing 0.1% SDS to filters containing 1 µg HeLa DNA for the periods indicated. Each point represents an average of three filters. Input: polysomal RNA, 5 × 10⁶ cpm; HnRNA, 10⁶ cpm.

**Figure 3** “Saturation” of DNA with HnRNA. Purified HnRNA labeled with uridine-3H at the indicated inputs was hybridized to filters containing three different levels of DNA that were labeled with ¹⁴C to monitor retention of DNA during the hybridization. From the specific activities of the two nucleic acid preparations, the µg RNA hybridized/µg DNA on the filters could be determined. The amounts of DNA on the filters were 0.55 µg (○); 1.1 µg (●); 1.6 µg (○).

**Presaturation of mRNA Sites by HnRNA**

We have earlier reported the apparent saturation of small amounts of HeLa DNA with purified (>45S) HnRNA (13). This is illustrated again in Fig. 3 where it is shown, in addition, that if approximately 0.5, 1, or 1.5 µg of DNA are bound to a nitrocellulose filter, there is a corresponding threefold change in the amount of RNA hybridized at saturation. Based on the amounts of HnRNA required to attain the apparent saturation of 1 µg of DNA, a number of experiments were performed in which unlabeled nuclear RNA was used as competitor for both labeled nuclear and labeled polysomal RNA. A summary of several experiments is given in Table II. In most of these experiments the presaturation was carried out and then filters were challenged with a mixture of both polysomal RNA-3H and HnRNA-32P. In this way the competition by the unlabeled HnRNA could be simultaneously scored against itself and polysomal RNA. (Additional advantages of this double label technique are discussed in the accompanying paper [22]). The results clearly show that the very high molecular weight nuclear HnRNA (>45S) can presaturate HeLa DNA as efficiently for sites that bind polysomal RNA as for sites that bind HnRNA. Thus, by this test, there is similarity in base sequence between large nuclear RNA molecules and smaller cytoplasmic RNA.
Since techniques for the complete separation of mRNA from the other species of RNA in polysomes (rRNA, 5S, tRNA) were not available, the specific activity of mRNA in these polysomal preparations could not be directly determined. The following indirect estimate of the mRNA specific activity was made so that the amount of mRNA bound by 1 µg of HeLa DNA could be calculated. On the basis that the average polysome contains 6–8 ribosomes (15) (2.4 × 10^6 daltons RNA in each) (23) and that a strand of mRNA represents about 4.5 × 10^5 daltons (24), the relative amount of mRNA in polysomes in HeLa cells has been estimated to be about 3% (7). The 4 and 5S RNA are quantitatively a small part of polysomal RNA. In polysomes from cells labeled for 60 min, about one-half the radioactivity is in mRNA (17). Thus, it is possible to estimate the specific activity of rapidly labeled polysomal mRNA from the specific activity of the total RNA in polysomes. Two separate experiments show (Fig. 4, Panel A) that 1 pg of DNA binds between 0.05 and 0.10 pg of rapidly labeled polysomal mRNA.

A second type of experiment (Fig. 4, Panel B) was also performed in which RNA from the polysomes of a large quantity of cells labeled for 1 generation was isolated and used in saturation experiments. Here again, in order to calculate the specific activity of the mRNA, it was assumed that 31/3 of the polysomal RNA was mRNA, that in 1 generation the rRNA became 50% labeled, and that the mRNA was totally labeled (thus 6% of the radioactivity and 3% of the mass of polysomal RNA was mRNA). As with the rapidly labeled mRNA, it was found that more than 0.05 pg RNA bound/µg DNA.

In addition, RNA from the nuclei of cells labeled for 1 generation was also used to perform a "saturation" experiment. Again, no absolute plateau was found and as much as 0.07 RNA µg bound/µg of DNA (Fig. 4, Panel C).

### Failure of Presaturation with Polysomal RNA

In addition to the previous studies on competition hybridization utilizing presaturation with HnRNA (13), it has also been shown that significant inhibition of hybridization of labeled RNA to filters containing 10–50 µg of DNA could be brought about by the presence of cytoplasmic RNA (25). But, if the unlabeled RNA was added first, and the DNA-filter was washed and then exposed to labeled RNA, no inhibition was observed.
Figure 4. Saturation of 1-µg DNA filters. Panel A: Polysomal RNA-3H was prepared from cells labeled for 60 min. Various amounts were hybridized to filters containing 1 µg of DNA. Different symbols indicate two different RNA preparations. Specific activity of total polysomal RNA: — O — 200 cpm/µg; — ● — 600 cpm/µg. For calculation of specific activity of mRNA see text. Each point is an average of three filters. Blank filters containing no DNA had less than 10% of the cpm attached to HeLa filters. Panel B: Polysomal RNA-3H was prepared from cells labeled for 22 hr (see Methods). Various amounts were hybridized to filters containing 1 µg of DNA. Specific activity of total polysomal RNA was 1500 cpm/µg in both experiments. Number of filters was the same as in Panel A. Experiment 1, — O — ; experiment 2, — ● — . Panel C: The total nuclear RNA (>32S) from long-labeled cells in Panel B was prepared, and various amounts were exposed to 1-µg DNA filters. Specific activity of total nuclear RNA was 3000 cpm/µg. Each point represents a single DNA filter in this panel.

This kind of study has been repeated with large quantities of purified polysomal RNA and filters containing 1 µg of DNA, the results of which are shown in Table III. Again, a very great depression of hybridization of both polysomal RNA and HnRNA is apparent, the amounts of unlabeled polysomal RNA being less than 1 mg when both are present simultaneously. However, even as much as 2.1 mg of unlabeled material fails to block more than 20–30% of the DNA sites when presaturation is the method of competition. It should be stressed here, as has previously been shown to be true with HnRNA (13), that the polysomal RNA used for presaturation could be demonstrated to remain bound to the filter during the course of the second incubation (Table IV).

| RNA preparation | Unlabeled polysomal RNA used | Competition by presaturation | Competition by simultaneous addition |
|-----------------|------------------------------|-----------------------------|-------------------------------------|
| Polysomal mRNA | Polysomal | HnRNA | Polysomal | HnRNA |
| I               | 0.50 | 75 | 74 |
|                 | 2.10 | 67 | 79 |
| II              | 0.19 | 100 | 100 | 50 | 91 |
|                 | 0.30 | 25 |
|                 | 0.76 | 90 | 85 | 10 | 55 |
|                 | 1.52 | 72 | 100 | 6 |
TABLE IV

Stability of Polysomal RNA Hybrids

| Input | cpm | µg RNA | cpm retained in hybrid at 65°C |
|-------|-----|--------|-----------------------------|
|       |     |        | after 18 hr at 65°C as described in Methods |
| 1.0 × 10^5 | 25 | 386 | |
| 1.0 × 10^5 | 25 | 361 | |
| 1.35 × 10^5 | 1800 | 145 | |
| 1.35 × 10^5 | 1800 | 130 | |

Each sample represents average of four 1 µg HeLa DNA filters.
* Assayed after 18 hr at 65°C as described in Methods.
‡ After 18 hr at 65°C, filters were washed and incubated an additional 20 hr before hybrids were assayed.

These results again emphasize the differences between the two competition techniques and imply that much of the reduction in hybridization seen with simultaneous addition of labeled and unlabeled molecules (especially with total cell RNA) is due to interference during hybridization and not due to firm binding of unlabeled RNA molecules to sites where labeled molecules might bind.

DISCUSSION

The problem to which this work is addressed is the origin of mRNA molecules in animal cells. As is clear from experiments with avian erythroblasts (1, 2) and HeLa cells (7), the vast majority of the HnRNA does not function as mRNA in polyribosomes, but the possibility exists that a minority of these large nuclear molecules are cleaved in some specific way to generate mRNA. A precedent for the specific cleavage of larger nuclear molecules exists, e.g. ribosomal RNA is made in mammalian cells by specific cleavage of a precursor (26). Ultimately a firm answer to this question demands a comparison of sequences in mRNA and HnRNA to determine whether identical sequences the size of functional mRNA occur within HnRNA molecules. Since unique species of both mRNA or HnRNA are not available at present, direct sequence comparison is impossible, and we are forced to settle for the only available technique based on detailed sequence comparisons, that is, RNA-DNA molecular hybridization.

For present purposes, the defect in RNA-DNA hybridization is that molecules that have related but not identical sequences (molecules from the same “families,” in the terminology of Britten and Kohne [27]) may give equivalent reactions (28). We are presumably eliminating some of this cross-reaction by performing our competition hybridizations by presaturation, which demands that the competing unlabeled molecules be bound sufficiently well so that they do not detach in a subsequent exposure to 65°C. In addition, only molecules that remain bound after 65°C salt washes plus 37°C digestion with both pancreatic and T1 ribonuclease are scored as hybrid. Nevertheless, because of the rapidity of hybridization of both polysomal mRNA and HnRNA (about 50% maximum in 8–10 hr with 1 µg DNA filters), it is likely that most, if not all, of the hybrid molecules we observed are bound to the DNA regions that make up families with many members (29). Thus our results, which clearly show that high molecular weight HnRNA molecules can block the hybridization of smaller polysomal mRNA molecules, are compatible with the assumption, but do not prove it, that some mRNA molecules are drawn from HnRNA.

The present experiments do help to settle another question that has arisen since the description of the repeated similar sequences in mammalian DNA (11). That is, do these repeated DNA sequences represent genes for proteins with related structure, or do they represent DNA with another function, e.g. for initiation, termination, or replication signals, for structural organization of chromosomes, or for some other unknown non-genetic function? It was recently shown (30) that the most highly reiterated DNA sequences (the satellite DNA) are not transcribed by L cells. In addition, there is very little satellite DNA in human cells (31). Moreover, in our experiments the DNA saturation level for polysomal mRNA is at least as high as that of the HnRNA (between 5 and 10% of the total DNA); therefore, as discussed above, it appears likely that most of our RNA hybrids are bound to reiterated regions of the DNA. It therefore seems likely that the repeated sequences other than the satellite DNA represent families of related structural genes (sequences), since the RNA involved appears in functioning polysomes.

What is obviously needed to solve the questions raised in this work is the ability to follow individual, clearly characterized species of mRNA from a possible nuclear appearance into a subsequent
cytoplasmic functional role. Pending the outcome of such decisive experiments, we must continue to entertain as a possibility that cytoplasmic mRNA arises from cleavage of nuclear molecules (15, 32) and also be aware that this pathway would allow the possibility of control mechanisms in protein synthesis quite different from those believed to operate in bacteria (33).

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