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Distribution of Repetitive and Nonrepetitive Sequence Transcripts in HeLa mRNA

(polyadenylated mRNA/hydroxyapatite/RNA·DNA hybridization)

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ABSTRACT **Polyadenylated messenger RNA extracted from HeLa cells was hybridized with a mass excess of HeLa DNA. The kinetics of the hybridization reaction demonstrated that most of the messenger RNA is transcribed from nonrepetitive DNA. The amount of messenger RNA hybridized to DNA was measured both with and without prior RNase treatment. Comparison of the results indicates that within the limits of detection, HeLa messenger RNA does not contain repetitive sequence elements covalently linked to nonrepetitive sequence transcripts. However, a small fraction of the HeLa messenger RNA preparation is transcribed entirely from repetitive DNA sequences. This fraction represents about 6% of the total polyadenylated messenger RNA preparation.**

Recent studies indicate that most eucaryotic mRNA sequences are transcribed from DNA sequences which occur only once per genome (1–9). Thus, in a variety of systems, ranging from insect to sea urchin and rodent, most of the structural genes are in the class of nonrepetitive DNA sequences. A notable exception is the set of histone structural genes (10, 11), and other exceptions may well exist. In order to avoid cumbersome phrases we will occasionally use the terms repetitive and nonrepetitive transcript to describe RNA sequences that have been transcribed from repetitive and nonrepetitive DNA sequences. The demonstration that mRNAs are complementary to nonrepetitive DNA sequences has generally involved measurements of the kinetics of hybrid formation between trace quantities of labeled mRNA and excess cellular DNA. Assay of hybrid formation in such experiments customarily requires the use of RNase to destroy nonhybridized RNA. Experiments utilizing labeled cDNA (DNA transcribed from mRNA using an RNA-dependent DNA polymerase) and excess cellular DNA have involved treatment with S_1 nuclease for analysis of hybrids. Such measurements, however, preclude investigation of the possibility that a large fraction of mRNA molecules contains repetitive sequence "tags" covalently associated with the nonrepetitive coding sequences. Repetitive tags could be relatively short and, though present on a large fraction of mRNA molecules, might include only a small fraction of the total RNA nucleotides. On the basis of filter hybridization measurements, Dina *et al.* (12) claimed that the repetitive "tag" model applies to a major fraction of the mRNA of *Xenopus* embryos.

To investigate this question further, we recently developed a procedure for the assay of RNA·DNA hybrids on hydroxyapatite (HAP) columns in which ribonuclease is not utilized. This procedure depends on the observation that 8 M urea prevents the binding of nonhybridized RNA to HAP but permits the binding of molecules containing hybrid regions (2, 13). Using this method, we recently showed (2) that total sea urchin gastrular mRNA is transcribed almost exclusively from single copy DNA sequences and that no appreciable portion of the mRNA molecules contains repetitive sequence "tags." Here we describe essentially similar observations on the mRNA of a human cell type, HeLa. We find that most poly(A)-containing mRNA of HeLa cells is transcribed from unique DNA sequences. However, a small class of RNAs transcribed from repetitive sequences can also be detected. Furthermore, we show that within the limits of detection none of the mRNA molecules transcribed from single copy DNA has repetitive sequence "tags" covalently linked to them.

Abbreviations: HAP, hydroxyapatite; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PB, phosphate buffer (pH 6.8); poly(A)mRNA, polyadenylated mRNA.

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MATERIALS AND METHODS

Cell Culture and Labeling of mRNA. HeLa cells (clonal strain S_3) were grown in suspension culture in modified Eagle's medium (14) containing 5% calf serum. The cultures were free of any detectable contamination with PPLO (Mycoplasma). The mRNA was labeled to approximately constant specific activity by growing the cells (1×10^5/ml) in the presence of [5-^{3}H]uridine (26 Ci/mmol) for 48 hr. Isotope was added at zero time to a concentration of 1.25 μCi/ml, and additional label was added at 25 hr (1.25 μCi/ml) and 35.5 hr (0.9 μCi/ml) in order to ensure approximately uniform incorporation of the precursor into the mRNA over the entire 48-hr period. Under these conditions the supply of exogenous radioactive precursor varies less than 2-fold (15). During the last 12 hr of the labeling period the concentration of radioactive precursor in the medium dropped to about 60% of its value at 36 hr. Therefore, the specific activity of any mRNA species which turns over more rapidly than the bulk of the mRNA could be as much as 1.7-times lower than that of the bulk mRNA (16).

Purification of HeLa mRNA. Free HeLa cell polysomes were isolated in essentially pure form, as described earlier (16). The polysomes displayed greater than 99% sensitivity to ethylenediaminetetraacetic acid (EDTA). Polysomal RNA was extracted and the mRNA was purified by poly(T)-cellulose chromatography (16). In the present experiments the mRNA was passed two times through 0.5 × 5.0-cm columns of poly(T)-cellulose.

HeLa DNA Isolation. DNA was isolated from HeLa cells by standard procedures. The DNA was bound to a HAP

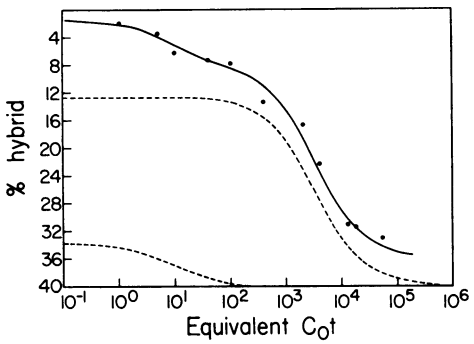

FIG. 1. Hybridization kinetics of HeLa mRNA. Reactions contained 2500 cpm of HeLa mRNA (50,000 cpm/μg) and 250 μg of HeLa DNA. The reactions were incubated in 0.12 M PB or 0.48 M PB at 60° for appropriate times. Reaction volumes were 0.1 ml. Following incubation, the hybrids were analyzed by the urea-phosphate HAP procedure. For details of incubation conditions and hybrid analysis see *Materials and Methods*. The *solid line* represents a least squares fit to the data. The root mean square error for the computer fit is 2.7%. The *broken lines* represent the resolved kinetic components. The fast component reacts with a rate constant of 1.1×10^{-1} M^{-1} sec^{-1} and the slow component reacts with a rate constant of 3.0×10^{-4} M^{-1} sec^{-1}.

column in the presence of 8 M urea, which we found to be of aid in removing RNase contamination. After elution, the DNA was sheared in a Virtis blender to about 300 nucleotides (17).

mRNA·DNA Hybridization. mRNA and DNA were mixed and denatured by immersion in a boiling water bath for 5 min. The samples were incubated at 60° in Kontes microflex vials in 0.12 M phosphate buffer (PB) or 0.48 M PB. Equivalent C_0ts were calculated in the latter medium by correcting for the increase in reassociation rate compared to the rate in 0.12 M PB due to monovalent cation concentration (17). The fraction of RNA molecules containing hybrid regions was measured by binding to HAP (Biorad DNA grade HTP lot, no. 9404) in the presence of 0.2 M PB, 8 M urea, 1% sodium dodecyl sulfate (SDS) at 40° ("urea-phosphate HAP" assay system). The incubation mixtures destined for the urea phosphate HAP analysis usually contained 0.1% SDS. In the other half of the procedure the fraction of RNA nucleotides actually residing in hybrid regions was measured by treating the sample with Worthington RNase A (10 μg/ml in 0.24 M PB), followed by passage over a Sephadex G-200 column equilibrated in 0.12 M PB.

TABLE 1. *Hybridization of HeLa mRNA with HeLa DNA*

DNA	C_0t	% mRNA obtained as hybrid: Urea-phosphate HAP	% mRNA obtained as hybrid: RNase-Sephadex
None	—	0.6, 1.7	—
Sea urchin	40	2.4	—
HeLa	40	5.6, 7.0, 7.2	7.6, 7.1
HeLa + 12.5 μg of rRNA*	40	7.1	—
HeLa (50° incubation)	40	8.1 (30° HAP)	5.8
HeLa	13,000	31.0	24.2

Hybridization reactions and analyses were carried out as described in *Materials and Methods*. The reaction mixtures each contained 2500 cpm of HeLa mRNA (50,000 cpm/μg) and 250 μg of HeLa DNA or sea urchin DNA sheared to 300 nucleotides in length. At C_0t 40 about 1% of the single-copy DNA fraction has reacted.

* rRNA was isolated from human liver. The rRNA mass was 100 times in excess of the rDNA present in the reaction mixture.

RESULTS

Assay of Hybrids Without Nucleases. Since many of the observations reported below are obtained by the urea-phosphate HAP method of hybrid analysis, it is useful to review briefly the evidence that this is a valid procedure for assay of RNA molecules containing regions of DNA·RNA duplex. The results of prior studies (2, 13) have shown the following: (*a*) Binding of nonhybridized RNA to HAP is almost completely suppressed in 8 M urea–0.2 M PB-1% SDS. This has been found to be true for sea urchin mRNA and hnRNA, and for HeLa mRNA (Table 1). (*b*) Optical measurements show that 8 M urea lowers the T_m of DNA by about 20°. DNA duplexes are also thermally eluted from hydroxyapatite in 0.2 M PB–8 M urea at about 20° lower than in 0.12 M PB in the absence of urea. (*c*) Measurements of DNA reassociation kinetics on HAP in the urea-PB at 40° yield essentially the same results as standard measurements at 60°, 0.12 M PB. A small discrimination against lower stability repetitive duplexes is the only difference noted [6% less of the total DNA is bound in urea after incubation to C_0t 40 (i.e., 40%) than is bound under standard conditions (46%)] (13). (*d*) The evidence most directly relevant to the studies reported here comes from observations on sea urchin nuclear RNA (13). Hybridization of this RNA at low C_0t yields structures consisting of 1000 to 2000 nucleotide-long RNA fragments paired on the average over about one-third of their length with DNA, while two-thirds of their length remain single-stranded. These molecules, which are about the same size as the HeLa mRNAs, are bound efficiently to HAP in the urea-PB, due to their duplex regions. The amount of RNA bound in this assay system is about the same as the amount estimated to contain hybridized sequences by isopycnic centrifugation in Cs_2SO_4 (13) or CsCl gradients (unpublished data). These experiments show that the urea-phosphate method adequately recognizes RNA molecules which contain even a minor portion of their length as RNA·DNA duplex. These are the structures which would be formed by low C_0t hybridization if the repetitive "tag" hypothesis of mRNA structure is correct. The minimum length of duplex required for binding has not been determined but it is probably similar to the minimum length of DNA·DNA duplex recognized by HAP under standard conditions, which is less than 20 nucleotides (21).

We emphasize that the urea-phosphate HAP system measures the fraction of RNA in molecules which contain hybrid regions. In contrast, the procedures using RNase measure the fraction of the RNA nucleotides which are actually in duplex regions.

Kinetics of Hybrid Formation Between HeLa mRNA and DNA. The polyadenylated mRNA [poly(A)mRNA] was hybridized with a 5000-fold excess of DNA to various DNA C_0ts, and the hybrid content assayed by the urea-phosphate HAP procedure. Fig. 1 shows the kinetics of hybrid formation as a function of DNA C_0t. By least squares analysis the reaction is best fit assuming two components. The slow component (about 30% of the input RNA) reacts with a rate constant of 3.0×10^{-4} M^{-1} sec^{-1}, which is close to the calculated rate constant for the single copy fraction of human DNA. The faster component (about 6% of the input RNA) reacts with

a rate constant for which the best estimate is 1.1×10^{-1} M^{-1} sec^{-1}. Though a rate constant for this component cannot be evaluated with any great accuracy, it is evident that the faster hybridizing RNA component is transcribed from a class of sequences present about 10^2–10^3 times per genome.

Repetitive Sequences are Confined to a Small Fraction of Entirely Repetitive Transcripts. The next experiments are aimed at determining whether individual molecules in the mRNA preparation contain sequences transcribed from repetitive as well as single-copy DNA. Alternatively repetitive and nonrepetitive sequences could be represented on separate sets of mRNA molecules. In the former case, mRNA molecules, after hybridization to low C_0t, would contain many single-strand "tails" of unhybridized RNA. Table 1 shows that RNase treatment of hybrids yields the same results as the urea-phosphate HAP procedure. About 6–7% of the RNA molecules appear to contain repetitive regions but this is also the fraction of mRNA nucleotides which are present in repetitive regions. It follows that the repetitive sequences in the mRNA preparation must be confined to molecules which consist entirely of repetitive sequence transcript, since these molecules lack RNase-sensitive single-stranded tails after low C_0t hybridization. For this measurement the size of the RNA fragments is important. If the fragments have been reduced in size, a smaller fraction of any possible nonrepetitive sequences would remain linked to the repetitive sequences. To test this possibility, RNA·DNA hybrids formed at C_0t 40 were bound to HAP in urea phosphate, the RNA and DNA were eluted and denatured, and the RNA size was measured on formaldehyde sucrose gradients. Fig. 2 reveals no significant difference in the sedimentation profile between the hybridized and the input RNA. That is, essentially no degradation of the RNA molecules has taken place. The unbound RNA (RNA transcribed from nonrepetitive DNA sequences) sediments slightly more slowly than do the other fractions, but the extent of degradation is negligible.

Table 1 also demonstrates that little if any additional hybrid is formed at low C_0ts when HeLa DNA is reacted with the HeLa mRNA under less stringent conditions. Lowering the temperature of incubation from 60° to 50° and the temperature of assay in urea-phosphate from 40° to 30° causes little change in the amount of RNA which binds to the HAP column. This shows that after low C_0t hybridization there is not a large population of mRNA·DNA hybrids which are only marginally stable under the usual hybridization conditions.

We conclude from the experiments so far presented that HeLa mRNA molecules in general lack any recognizable tags of repetitive sequence transcript covalently linked to nonrepetitive sequence transcript. Most of the hybridizing molecules thus contain only nonrepetitive sequence transcript. However, it is apparent that a small class of RNA molecules transcribed entirely or almost entirely from repetitive DNA sequence is also present in the preparation. This RNA does not appear to be rRNA, since the binding to HAP is not reduced by the presence of large amounts of unlabeled human rRNA (Table 1).

Size and Amount of the Repetitive Component of HeLa Poly(A)mRNA. Analysis of the hybridization kinetics presented in Fig. 1 indicates that about 6% of the mRNA is transcribed from repetitive sequences. However, this interpretation requires that sufficient excess of DNA was present in the experiments of Fig. 1 to hybridize all of the repetitive sequence transcripts. A limit estimate of the DNA:RNA ratio needed

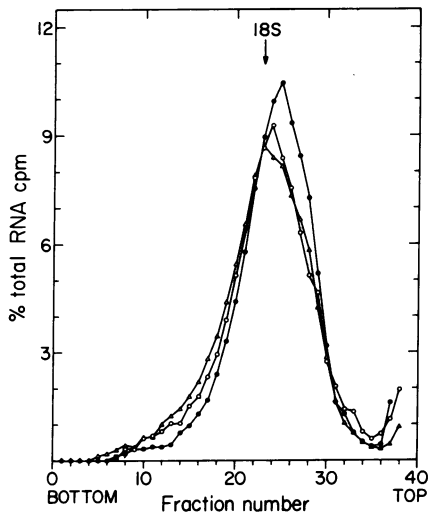

FIG. 2. Effect of annealing and HAP chromatography on the size of HeLa mRNA. 50,000 cpm of HeLa mRNA (1 μg) was incubated with 500 μg of HeLa DNA. The reaction was carried out for 3.5 hr (to C_0t 40) following which the hybrids were bound to HAP in urea-PB (see *Materials and Methods*). The hybrids were eluted from the HAP columns with 0.5 M PB. Both the bound RNA and the unbound RNA were dialyzed against water, sodium acetate was added to 0.3 M, and the RNA was precipitated in ethanol. The mRNA was then denatured by incubation at 63° for 15 min in 0.1 M sodium phosphate buffer (pH 7.7) containing 3% neutralized formaldehyde (22) and then centrifuged through a 5–20% sucrose gradient in formaldehyde-PB [0.1 M NaCl, 0.02 M potassium phosphate buffer (pH 7.4), 1% formaldehyde] for 24 hr at 26,000 rpm, 2°, in a SW 25.3 Spinco rotor. RNA which was bound to HAP (O—O); RNA which was not bound to HAP (●——●); control RNA, no incubation or HAP chromatography, (△——△).

to hybridize all the RNA transcribed from sequences present about 10^2–10^3 times per genome (Fig. 1) can be obtained by assuming the lowest reasonable complexity for the RNA. For this calculation we can assume that there is only one species of such RNA present, the complexity of which is equal only to the average transcript length, i.e., about 2000 nucleotides (Fig. 2) The DNA:RNA ratio needed to hybridize the repetitive RNA is given by the expression

$$\frac{\text{DNA}}{\text{RNA}} = \frac{\text{genome size}}{\text{reiteration frequency} \times \text{complexity}}$$

and even for this extreme case, such a calculation indicates that a total DNA:RNA ratio of about 4 to 40×10^3 would suffice. In accordance with this calculation, Table 2 shows that no change in the amount of hybridization at low C_0t is observed over a range of two orders of magnitude in DNA:RNA ratio. Even at a DNA:RNA ratio of 5×10^4, no more than 6% (background subtracted) of the RNA reacts at C_0t 40. We conclude that no more than 6% of the HeLa poly(A)mRNA preparation is actually transcribed from repeated regions on the HeLa DNA, since this value has not been underestimated due to insufficient DNA:RNA ratio.

Table 3 presents an experiment in which the HeLa poly(A)-mRNA was divided into four size fractions on the basis of sedimentation in sucrose gradients. Each fraction was then reacted with excess DNA to low C_0ts and the amount of hybrid measured. The data suggest a slight enrichment for repeti-

tive transcripts in the heavier classes of RNA. Clearly some molecules >35S in size belong to the repetitive sequence class.

Single Copy Sequence in HeLa mRNA. According to the hybridization kinetics in Fig. 1, about 30% of the input RNA reacts with single copy DNA at DNA:RNA = 5000. The amount of hybrid observed after a high C_0t incubation is shown in Table 1. The difference between the values obtained with the urea-phosphate HAP assay method as compared to the RNase-Sephadex assay method (31% *versus* 24%) is probably due to the fact that some regions of the relatively long RNA are not completely covered by DNA at this DNA:RNA ratio and therefore more RNA is bound to HAP in the urea-phosphate system. Earlier studies (13) showed that as the DNA:RNA ratio is increased, average coverage of an RNA molecule also increases, and the difference between RNase and urea-phosphate value decreases.

At the highest DNA:RNA ratio used in these studies, about 41% of the mRNA molecules contain hybridized regions when the reaction has terminated kinetically (Table 2). Since, as shown above, the 59% of the RNA which remains unhybridized cannot represent repetitive transcripts, this RNA must also be derived from nonrepeated DNA sequences. The question then remains why all the RNA did not hybridize at high C_0t. From calculations of the number of copies of each nonrepetitive DNA sequence in the reaction mixtures and the yield of poly(A)mRNA per cell‡, it is apparent that at the highest DNA:RNA ratio used (5×10^4), mRNAs transcribed from single copy sequences and occurring less than 5×10^3 times per cell should have hybridized. This suggests that the 59% unhybridized RNA represents transcripts present in large numbers of copies, i.e., $>5 \times 10^3$ per cell. Experiments utilizing higher ratios would have required higher specific activity mRNA than was available.

An alternative explanation for lack of complete hybridization is that some form of artefact interferes with hybridization and is responsible for the unhybridized RNA. We have carried out many control experiments in an effort to demonstrate such an artefact (Smith and Davidson, unpublished). In these experiments we have utilized mRNA from sea urchin embryos, which we have studied by methods similar to those used here (2). As with HeLa mRNA, the fraction of the sea urchin mRNA which can be hybridized with nonrepetitive DNA sequences increases as the DNA:RNA ratio is increased, but nearly half (45%) of the mRNA remains unhybridized at the highest DNA:RNA ratios attempted. The RNA was not significantly degraded during the hybridization reaction. Nor was the rate of the hybridization reaction found to be slower at very high DNA:RNA ratios (e.g., 300,000/1) compared to more moderate DNA:RNA ratios (3,000/1). When the unhybridized fractions of the mRNA were re-reacted with fresh DNA there was no significant increase in the amount of hybrid formed. That is, all the RNA which can hybridize appears to do so on first exposure to DNA. Furthermore, using the same techniques, up to 80% of sea urchin hnRNA molecules can be recovered as hybrid-containing molecules at very high DNA:RNA ratios. This shows that in themselves these methods neither inhibit hybridization nor cause substantial losses in the amounts of hybrid measured. These data suggest that the correct explanation for the failure of all the mRNA to hybridize lies in the high frequency with which some of the sequences are represented.

‡ 3.8 pg is the mass of the haploid HeLa genome. At a DNA:RNA ratio of 5×10^4, a reaction mixture contains 2500 μg of DNA and 0.05 μg of RNA. The number of copies of each nonrepetitive sequence per μg of RNA is $2500/0.05 \times 1/3.8 \times 10^{-6} = 1.3 \times 10^{10}$. The amount of mRNA/cell is 4×10^{-7} μg (based on direct measurements of mRNA yield) and $1.3 \times 10^{10} \times 4 \times 10^{-7} = 5.2 \times 10^3$ copies of a given mRNA sequence per cell which should be hybridized at the above DNA:RNA ratio.

TABLE 2. *Hybridization of HeLa mRNA with DNA at various DNA:RNA ratios*

C_0t	DNA:RNA	% Hybrid
40	500	6.1
40	5,000	5.6, 7.0, 7.2
40	50,000	7.3
13,000	5,000	31.0 (35.2)*
13,000	50,000	35.7 (40.6)*

Hybridization reactions contained 2500 cpm of HeLa mRNA and appropriate amounts of HeLa DNA. The hybrids were analyzed by binding to HAP in urea-PB as described in *Materials and Methods.*

* Corrected for kinetic completion of DNA reassociation. At C_0t 13,000, 81% of the nonrepetitive DNA has reacted. As estimated from the DNA reassociation (data not shown), 57% of the DNA fragments react as single copy sequence, 38% react as repetitive DNA, and 5% are unreacted. Thus the correction factor for kinetic completeness is $[(0.81)(0.57) + 0.38]/0.95 = 0.88$.

DISCUSSION

The experiments described in this report lead to three main conclusions regarding the nature of the sequences present in the poly(A)mRNA isolated from free cytoplasmic polyribosomes of HeLa cells. First, most of the mRNA molecules appear to be transcribed from nonrepetitive DNA sequences. This conclusion is based mainly on measurement of the rate of mRNA·DNA hybrid formation, which is consistent with that expected for single-copy DNA sequence transcripts. However, our data are not sufficiently extensive to preclude the (unlikely) alternatives that the sequences from which these mRNAs are transcribed are present an average of 0.5 or 2 times per genome. Nor can we say whether the rate of hybrid formation is less than a factor of two different from the rate of DNA·DNA duplex formation (18, 19). Using RNase treatment for assay of the hybrids, Penman and Bishop also reached the conclusion that HeLa mRNA is mainly nonrepetitive sequence transcript (unpublished data). Thus in human as well as other animal cell types (1–9), most of the structural genes are single-copy DNA sequences.

The second conclusion is that a small but nonnegligible component of the HeLa mRNA preparation (about 6%) [or slightly more if this RNA fraction turns over rapidly compared to the bulk of the mRNA (16)] is transcribed entirely from repetitive sequence elements. Assuming that this RNA is indeed mRNA, there must exist an as yet unidentified class of repetitive structural genes. Histone mRNA is excluded as a possibility, since the mRNA was selected on the basis of its poly(A) content. Other workers have also noticed some repetitive sequence transcript present in total tissue culture cell mRNA preparations (e.g., ref. 7; Penman and Bishop, unpublished data), but it has previously been impossible to distinguish between a small class of RNAs totally transcribed from repetitive sequence or a larger class of RNAs transcribed mainly from single-copy DNA but containing a fraction of repetitive sequence. Only the former

TABLE 3. *Size of RNA hybridizing at C_0t 40*

mRNA fraction	% of total cpm	% of HeLa mRNA bound +HeLa DNA	−HeLa DNA	Δ
Unfractionated	100.0	7.2, 7.0, 5.6	1.7	3.9–5.5
>35S	8.4	10.2	0.6	9.6
25–35S	25.4	9.6	0.6	9.0
15–25S	49.3	4.9	0.7	4.2
<15S	16.9	2.8	0.6	2.2

The mRNA was centrifuged on a 15–30% sucrose gradient (prepared over a 1 ml cushion of 64% sucrose) in SDS buffer for 16 hr, 26,000 rpm, 20°, in an SW 25.3 Spinco rotor. The mRNA was pooled into the four size classes indicated. 10 μg of *Escherichia coli* rRNA was added as carrier, and the RNA precipitated with ethanol. Hybridization reactions contained 2500 cpm (0.05 μg) of the indicated RNA fraction and, where indicated, 250 μg of HeLa DNA. Reactions were incubated to C_0t 40 and the hybrids were analyzed by the urea-phosphate HAP method.

case requires the postulation of repetitive structural genes. This argument, however, depends on the certainty with which the hybridizing radioactivity can be ascribed to mRNA. The following data are relevant for the HeLa mRNA preparation used here. (*a*) After two passages over poly(T)-cellulose, less than 1% of the radioactivity can be attributed to rRNA, according to both formaldehyde and SDS-sucrose gradient analysis, and to the yield of RNA recovered from the column after long-term labeling (2% of the total polysomal radioactivity). (*b*) The size distribution of the RNA species hybridizing at low C_0t (Table 3) includes species larger than rRNA, i.e., >35S. (*c*) Addition of a large excess of unlabeled human rRNA to the hybridization reactions results in no decrease in the amount of labeled RNA bound (Table 1). (*d*) The structures from which the [^{3}H]mRNA is extracted are >99.7% sensitive to EDTA disaggregation. This is of course a diagnostic characteristic of polysomes, and contrasts to the behavior expected of structures which contain hnRNA. Furthermore, the extracted RNA displays the turnover rates and labeling characteristics of polysomal rather than hnRNA (16). (*e*) The hybridization behavior of the RNA is distinct from that of hnRNA. Both in HeLa (20) and in sea urchin cells (13), hnRNA contains interspersed repetitive sequence elements, and the fraction of nucleotides present in repetitive regions is far smaller than the fraction of molecules containing them. In contrast, our measurements yield identical values for the fraction of RNA nucleotides present in the repetitive sequences and the fraction of HeLa mRNA molecules containing these sequences. This is a strong argument that the poly(A)-containing repetitive transcripts actually represent mRNA rather than an hnRNA contaminant.

Our experiments argue strongly against the existence of recognizable repetitive sequence "tags" on a large portion of the mRNA molecules. Prior studies (13) have shown the urea-phosphate HAP assay system on which we rely for this conclusion to be capable of binding with high efficiency RNA molecules which are 1 to 2 × 10^3 nucleotides long, but contain less than one-third their length as RNA·DNA duplex. The present data show that such structures are not detectable in HeLa mRNA. This result was reported earlier for total mRNA of sea urchin gastrulae (2). Our conclusions are thus inconsistent with Dina *et al.* (12) who reported that 80% of *Xenopus* embryo mRNAs contain short repetitive sequence elements recognizable in hybridization experiments carried out at much the same annealing criterion that we have used. Possibly phylogenetic differences account for this discrepancy, but it seems more likely to be technical in origin.

It remains of course possible that mRNAs contain repetitive sequence regions shorter than the recognizable limit. For hydroxyapatite columns operated under standard conditions in 0.12 M PB this limit is known to be very small (Wilson and Thomas report 85% binding of 17 nucleotide long duplexes under our conditions; ref. 21 and unpublished data). Though we see no reason why there should be a substantial difference, we do not known exactly what this limit is for hydroxyapatite columns operated in 8 M urea–0.2 M PB–1% SDS. However, the recovery of short DNA·DNA duplexes in the urea-phosphate medium leads to the conclusion that duplex sequences 100 nucleotides or longer would be bound quantitatively under these conditions. Nontranslated sequences now known on several specific mRNAs appear to exceed this short length. Thus we provisionally conclude that at least part of the nontranslated mRNA must also be transcribed from single-copy DNA.

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1. Davidson, E. H. & Britten, R. J. (1973) *Quart. Rev. Biol.*, **48**, 565–613.
2. Goldberg, R. B., Galau, G. A., Britten, R. J. & Davidson, E. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3516–3520.
3. Bishop, J. O., Pemberton, R. & Baglioni, C. (1972) *Nature New Biol.* **235**, 231–234.
4. Bishop, J. O. & Rosbash, M. (1973) *Nature New Biol.* **241**, 204–207.
5. Harrison, P. R., Hell, A., Birnie, G. D. & Paul, J. (1972) *Nature* **239**, 219–221.
6. Suzuki, Y., Gage, L. P. & Brown, D. D. (1972) *J. Mol. Biol.* **70**, 637–649.
7. Greenberg, J. R. & Perry, R. P. (1971) *J. Cell Biol.* **50**, 774–786.
8. Firtel, R. A., Jacobson, A. & Lodish, H. F. (1972) *Nature New Biol.* **239**, 225–228.
9. Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A. J., Kiley, M. L., Summers, N. M., Bishop, J. M. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 7530–7539.
10. Kedes, L. H. & Birnstiel, M. L. (1971) *Nature New Biol.* **230**, 165–169.
11. Weinberg, E. S., Birnstiel, M. L., Purdom, D. R. & Williamson, R. (1972) *Nature* **240**, 225–228.
12. Dina, D., Crippa, M. & Beccari, E. (1973) *Nature New Biol.* **242**, 101–105.
13. Smith, M. J., Hough, B. R., Chamberlin, M. E. & Davidson, E. H. (1974) *J. Mol. Biol.* **84**, in press.
14. Levintow, L. & Darnell, J. E. (1960) *J. Biol. Chem.* **235**, 70–73.
15. Aloni, Y. & Attardi, G. (1971) *J. Mol. Biol.* **55**, 251–270.
16. Murphy, W. & Attardi, G. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 115–119.
17. Britten, R. J., Neufeld, B. R. & Graham, D. E. (1974) *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. XXIX, part E, pp. 363–418.
18. Hutton, J. R. & Wetmur, J. G. (1973) *J. Mol. Biol.* **77**, 495–500.
19. Melli, M., Whitfield, C., Rao, K. V., Richardson, M. & Bishop, J. O. (1971) *Nature New Biol.* **231**, 8–12.
20. Darnell, J. E. & Balint, R. (1970) *J. Cell Physiol.* **76**, 349–356.
21. Wilson, D. A. & Thomas, C. A. (1973) *Biochim. Biophys. Acta* **331**, 333–340.
22. Boedtker, H. (1968) *J. Mol. Biol.* **35**, 61–70.