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Original Article

Characterization of Novikoff hepatoma small RNAs homologous to repetitive DNAs

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Abstract

Three minor small RNA species from Novikoff hepatoma cells, with homology to repetitive DNA sequences, have been identified and characterized. These small RNAs, designated 5.1S, 6S and T3 RNAs, show homology to Alu 1, Alu 2, and Alu 3 sequences, respectively. 6S and T3 RNAs were found both in the nucleus and cytoplasm, whereas 5.1S RNA was not found in the nucleus. Neural tissues were found to contain a 6S-sized BC1 RNA with homology to I.D. sequences [19]; in contrast, the current study shows that Novikoff hepatoma cells contain a 75–80 nucleotide long (T3) RNA, homologous to I.D. sequences. These data suggest that BC1 and T3 small RNAs, homologous to I.D. sequences, are expressed in a tissue-specific manner. These results also show that in addition to the abundant 7SL, 4.5S and 4.5S1 RNAs having homology to repetitive DNA, Novikoff hepatoma cells also contain several minor small RNAs with homology to repetitive sequences.

Introduction

All eukaryotic cells contain several discrete small RNA molecules that are not directly involved in protein synthesis [1–4]. These small RNAs fall broadly into two groups: 1. Capped U-snRNAs which are involved in the processing of precursor RNAs [5–7] and 2. noncapped small RNAs generally involved in translation [8–10].

Many noncapped small RNAs show striking homology to the abundant, dispersed, and reiterated sequences found in the genomes of higher eukaryotes [11–14]. The most abundant types of repeated sequences are referred to as Alu 1, Alu 2 and Alu 3 in the case of the human genome and B1, B2 and B3 (I.D.), respectively, in the case of the rodent genomes. 4.5S RNA and 7SL RNA were previously found to be homologous to Alu 1 sequences [15, 16]. 6S-sized RNAs were found to be homologous to both B2 or I.D. sequences [17–19]. The functions of these 'Alu related' small RNAs are not known.

During our efforts to characterize small nuclear RNA genes in the human genome, we observed several minor RNA species that consistently hybridized to the cloned DNAs. In this study three small RNAs that have not been previously reported in Novikoff hepatoma cells have been characterized. These RNAs, designated 5.1S, 6S and T3, were found to be homologous to Alu 1, Alu 2 and Alu 3 sequences, respectively.

Materials and Methods

Preparation of nuclei and nucleoli

HeLa cell nuclei were prepared by the citric acid procedure [20]. Nucleoli were prepared from HeLa

cells by sonication of nuclei prepared by the NP-40 method [21]. DNA was isolated from nuclei, nucleoli or from bacteriophages as described by Maniatis *et al.* [22].

Preparation of uniformly labelled RNA

Novikoff hepatoma cells were incubated at 37°C with (32P) orthophosphate for 16 h in low phosphate medium as described previously [23]. The 4–8S RNA was prepared by centrifugation of the total nuclear or whole cell RNA on a sucrose density gradient and pooling the fractions corresponding to 4–8S RNAs.

Hybridizations

Dot hybridizations were carried out as described by Kafatos *et al.* [24]. Hybridizations were carried out at 42° in 50% formamide, 4 × SET (1 × SET is 0.15 M NaCl, 1 mM EDTA, 0.03 M Tris-HCl, pH 8.0), 5 × Denhardt's reagent (1× Denhardt's reagent is 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll), 0.1% SDS, and 10 μg/ml yeast tRNA. The nitrocellulose filters were hybridized for 16 h and washed at 42 °C for 20 min each with 3 × SSC, 1.5 × SSC, and 0.5 × SSC containing 0.1% SDS.

Analysis of the RNAs

The hybridized RNAs were eluted with sterile water at 90°C and the RNAs were analyzed on either a 5% or 10% polyacrylamide gels. The gels were dried and subjected to autoradiography. Fingerprinting of the RNAs was carried out as described by Brownlee *et al.* [25].

Results

5.1S RNA was hybrid-selected by four different human genomic clones

While characterizing human U3 snRNA clones isolated from a human genomic library, we observed an RNA which hybridized to each of these human genomic clones. Results obtained with four of the lambda clones are shown in Fig. 1. Lambda phage DNAs obtained from four different human genomic clones were immobilized onto nitrocellulose filters and hybridized with *in vivo* labeled Novikoff hepatoma whole cell 4–8S RNAs. The RNAs which hybridized to the phage DNAs were then eluted and analyzed on polyacrylamide gels. Lane 1 shows the total 4–8S RNAs which were used in the hybridization experiments. Lanes 2 to 5 correspond to four

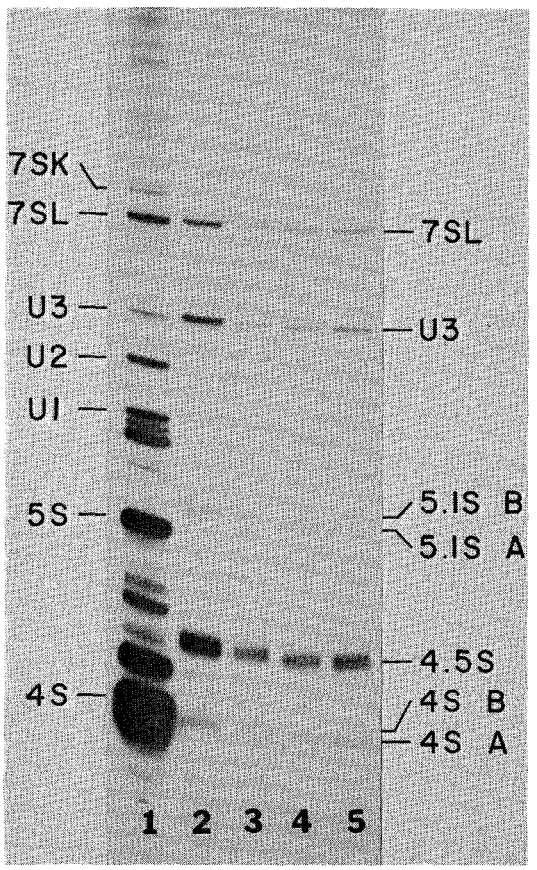

Fig. 1. Analysis of small RNAs hybridized to human genomic clones. *In vivo* labelled Novikoff hepatoma whole cell 4–8S RNA was hybridized to four different human U3 genomic clones immobilized on nitrocellulose filters. The hybridizations and washings were carried out at 42°C as described in Materials and methods. The hybridized RNAs were analyzed on a 5% polyacrylamide/7M urea gel. Lane 1: (32P)-labelled Novikoff hepatoma 4–8S RNA used for hybridizations. Lanes 2 to 5: genomic U3 snRNA clones, 1, 2, 3, and 4, respectively. The bands labelled U3, 4.5S and 7SL RNAs gave expected fingerprints.

different cloned human DNAs. Since these clones were isolated with U3 snRNA as a probe, all these four clones hybridized to U3 snRNA; in addition, the 7SL and 4.5S RNAs also hybridized to all the clones; the 7SL and 4.5S RNAs are previously characterized RNAs which show high degree of homology to Alu 1 sequences [15, 16].

In addition to these abundant and previously characterized small RNAs, an RNA with electrophoretic mobility slightly less than 5S RNA was consistently observed. This RNA, designated 5.1S RNA, always separated into two bands with approximately equal intensity and gave fingerprints identical to each other (results not shown). Therefore, these two RNA bands were designated 5.1S-A and 5.1S-B. In addition to the 5.1S RNA, two RNA bands with the same electrophoretic mobility as the tRNA were also observed (Fig. 1, Lanes 2 to 5). However, these RNA bands did not yield fingerprints consistent with single RNA species. Therefore, these RNAs were not characterized further.

5.1S RNA is also hybrid-selected by total human DNA

Since 5.1S RNA was hybrid-selected by several different clones, it appeared to us that this RNA, in all probability, is homologous to Alu type sequences that are abundant and dispersed in the genomes of higher eukaryotes. If this were the case, one would expect the total genomic DNA to also hybrid select 5.1S RNA. The results obtained when total DNAs from HeLa cells and Novikoff hepatoma cells were used for hybridization are shown in Fig. 2. Lane 1 shows the Novikoff hepatoma whole cell 4-8S RNAs used in the hybridization experiments. Lane 2 shows the RNAs which hybridized to Novikoff hepatoma nuclear DNA and Lane 3 shows the RNAs selected by HeLa nuclear DNA. The 5.1S RNA as well as 7SL RNA were hybrid-selected by human DNA but not by Novikoff hepatoma (rat) DNA (compare lanes 2 and 3, Fig. 2). This result indicates that 5.1S RNA is homologous to a repetitive sequence found in human genome.

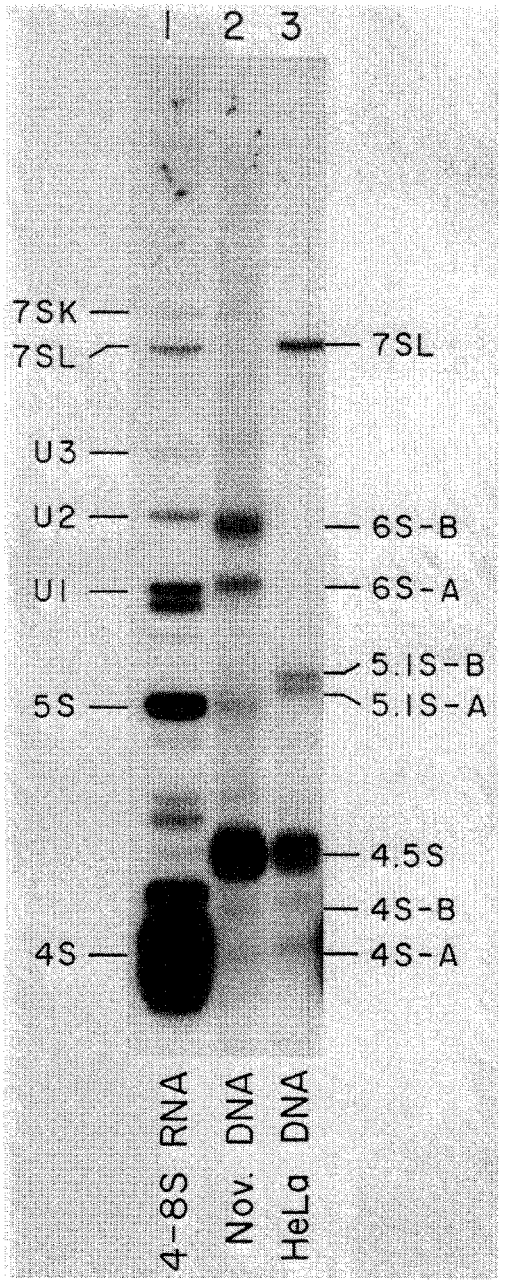

Fig. 2. Analysis of small RNAs hybridized to Novikoff hepatoma and HeLa DNAs. 10 μg of Novikoff hepatoma nuclear or HeLa nuclear DNA was immobilized on nitrocellulose paper and hybridized to uniformly labelled Novikoff hepatoma whole cell 4–8S RNA. The hybridized RNAs were analyzed on a 5% polyacrylamide gel. The conditions for hybridization and washings were as described in Materials and methods. Lane 1: uniformly labelled Novikoff hepatoma 4–8S RNA. Lane 2 (Nov. DNA): Novikoff hepatoma nuclear DNA used for hybridization. Lane 3 (HeLa DNA): HeLa nuclear DNA used for hybridization.

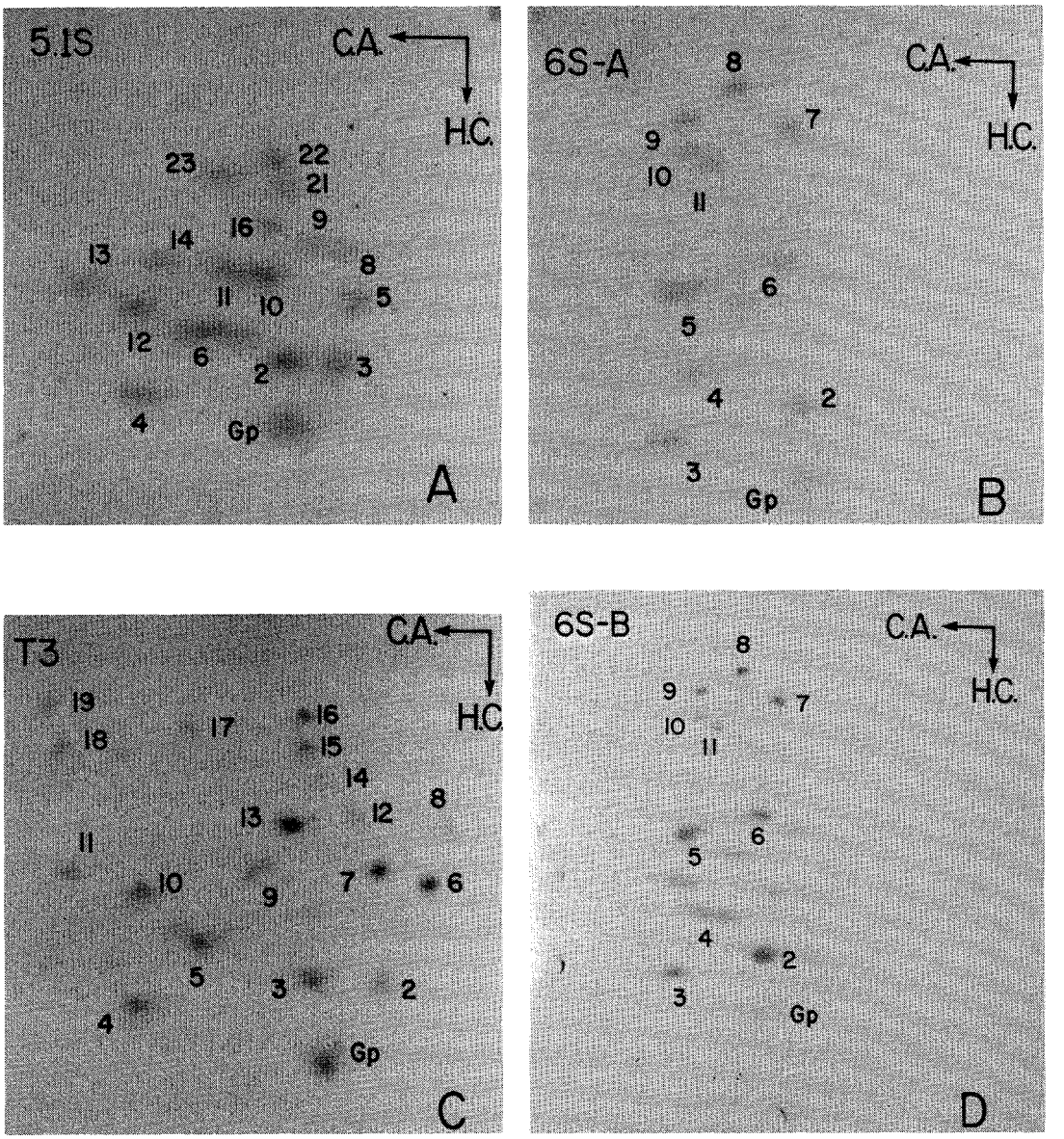

Fig. 3. T1 RNase fingerprints of 5.1S, 6S and T3 small RNAs. The 5.1S RNA from Fig. 2, Lane 3, the 6S-A and 6S-B RNAs from Fig. 2, Lane 2 and the T3 RNA from Fig. 4, Lane 7, were eluted from the acrylamide gels and subjected to fingerprinting after digestion with T1 RNase. The fingerprinting was carried out as described by Brownlee *et al.* (1968). The first dimension was chromatography on cellulose acetate (C.A.) and the second dimension was homochromatography (H.C.). The oligonucleotides in the case of 5.1S RNA were designated as in 7SL RNA [26].

print revealed that all the oligonucleotides present in 5.1S RNA are also present in human 7SL RNA [26, 27]. In addition, the 5.1S RNA fingerprint pattern corresponded to the 5′-end 120 nucleotides of the 7SL RNA. The T_1-RNase oligonucleotides were numbered as in 7SL RNA [26] based on their position in the fingerprint. This observation is not very surprising since the 5′-portion of 7SL RNA was shown to be homologous to the Alu 1 sequences [26, 27]. From these data, it is clear that 5.1S RNA is homologous to the Alu 1 sequences.

Fingerprint of 5.1S RNA corresponds to the 5'-end of 7SL RNA

The 5.1S RNA was digested with T_1-RNase and fingerprinted. The fingerprint of 5.1S-A RNA is shown in Fig. 3, Panel A. Careful analysis of this finger-

6S RNAs are homologous to Alu 2 (B2) repetitive DNA

The DNA from Novikoff hepatoma cells hybridized to 4.5S RNA and to 6S-sized RNAs (Fig. 2, Lane 2). The 6S RNAs separated into at least two and sometimes three bands. These bands were designated 6S-A RNA and 6S-B RNA.

The Fig. 3, Panel B shows the fingerprint of 6S-A RNA and Panel D shows the fingerprint of 6S-B RNA. The fingerprints of 6S-A and 6S-B RNAs were very similar to each other and all major oligonucleotides had same mobilities. These data indicate that 6S-A and 6S-B RNAs are structurally related. In addition these 6S RNAs are hybrid-selected by clone B2 DNA sequences (see Fig. 4, Lane 6). A 6S-sized RNA from hamster and mouse tissues, homologous to B2 repetitive sequences, has previously been described by several groups [17–19]. Our present data show that Novikoff hepatoma cells, like other rodent cell types, synthesize 6S-sized RNAs homologous to B2 sequences. Instead of a single discrete RNA band observed in the case of cultured hamster ovary cells [17], the Novikoff hepatoma cells contain at least two distinct B2 (6S) RNA populations differing in their electrophoretic mobilities.

T3 RNA is the only Novikoff hepatoma small RNA homologous to I.D. (Alu 3) sequences

To identify other small RNAs of Novikoff hepatoma cells, previously characterized and cloned repetitive DNAs were used for hybridization. The results obtained with Alu 2 (B2) and Alu 3 (I.D.) clones are shown in Fig. 4. The B2 clone [28] hybridized to 6S-A and 6S-B (Fig. 4, Lane 6). In addition to 6S RNAs, two other RNA bands with the apparent

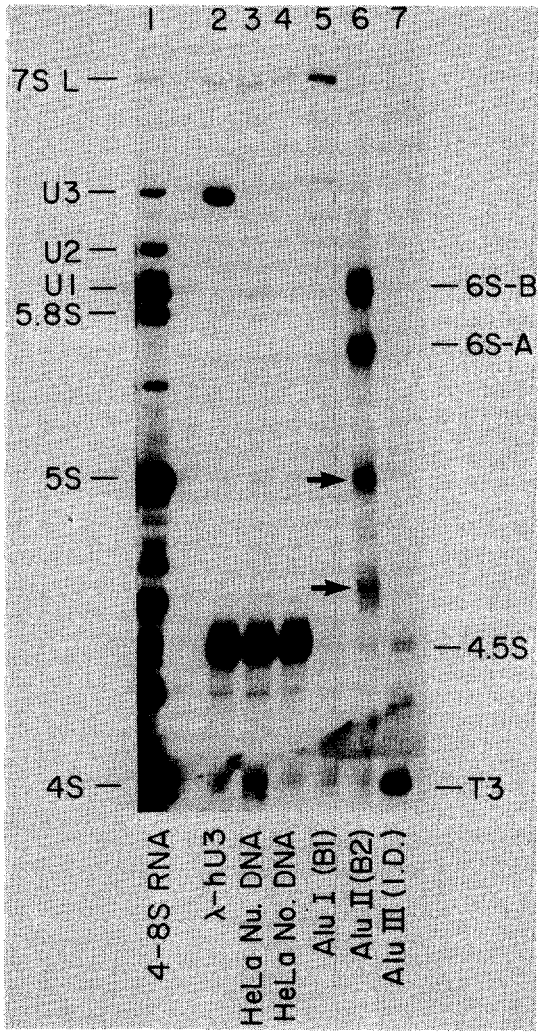

Fig. 4. Analysis of small RNAs hyrid-selected by cloned repetitive DNAs. The cloned DNAs containing the Alu 1 sequence [36], B2 sequences [28] and I.D. sequences [19] were immobilized on nitrocellulose and hybridized to Novikoff hepatoma nuclear 4–8S RNA. The hybridization conditions and washing conditions were as described in Materials and Methods. The RNAs were fractionated on a 5% polyacrylamide/7M urea gel. In addition, HeLa nuclear, HeLa nucleolar, and cloned U3-1 DNA used in Fig. 1, Lane 2 were also used as controls.

nucleotide chain lenghts of 100 and 120 nucleotides were also found (Fig. 4, Lane 6; marked by arrows). These RNAs were found only with B2 DNA but not with Alu 1 or Alu 3 sequences (compare Lanes 6 with Lanes 5 and 7, Fig. 4). These results indicate that Novikoff hepatoma cells contain several B2 RNA populations.

The most interesting result was obtained with I.D.

sequences [19]. A single RNA was hybrid-selected with clone I.D. sequences (Fig. 4, Lane 7). This RNA was analyzed by fingerprinting (Fig. 3, Panel C) and the number of T_1 RNase oligonucleotides observed was consistent with T3 RNA being a homogeneous RNA species. Note that there was no detectable 6S-size (BC1) RNA when I.D. sequences were used for hybridization (Fig. 4, Lane 7). This result is consistent with the observation made by Sutcliffe and associates that BC1 RNA is present in only tissues of neural origin and not in liver [19].

The hybridization experiments presented in Fig. 4 were carried out with 4–8S RNA prepared from purified nuclei. The presence of 6S RNAs and T3 RNA in these purified nuclear preparations suggests that these RNAs are, at least in part, nuclear in localization. The absence of 5.1S RNA in these nuclear preparations (Fig. 4, Lane 5) indicates that 5.1S RNA is primarily cytoplasmic in localization. When the Novikoff hepatoma total nuclear or whole cell 4–8S RNAs were fractionated on polyacrylamide gels and stained with methylene blue, stained bands corresponding to 5.1S RNA or 6S RNAs were not detected (Results not shown). Since RNAs present at a concentration of 10000 copies/cell or more are detectable by this staining procedure, the absence of stainable amounts of 5.1S and 6S RNAs indicates that 5.1S RNA and 6S RNAs were minor RNA species with fewer than 10000 copies/cell in the Novikoff hepatoma. Since the T3 RNA comigrates with abundant tRNAs, no conclusions can be drawn regarding the copy number of this RNA.

Discussion

We have identified three RNAs in Novikoff hepatoma cells which are homologous to the highly repetitive sequences found dispersed in the genomes of higher eukaryotes. Several questions arise from the observations made in this study. 1. Are these RNAs distinct and new small RNAs? 2. Are these RNAs found only in the Novikoff hepatoma cells or are RNAs corresponding to these RNAs present in other species also?, and 3. What are the function(s) of these small RNAs?

Several lines of evidence suggest that these RNAs are distinct small RNAs and not degradation products. The 5.1S RNA was observed consistently in many experiments. This RNA always gave two closely spaced RNA bands. Many other distinct small RNAs like 7SL, 7SK, 5S, 4.5S and 4.5SI RNAs contain two major populations differing in length by one nucleotide [29]. The two bands observed in the case of 5.1S RNA fit this pattern. The cloned DNA used in Fig. 1 also contained U3 snRNA genes. If there was degradation of RNA during isolation of RNA one would expect degradation of U3 RNA also. In this case the degradation products of U3 RNA would have been hybrid selected. Since no extra bands were observed (Fig. 1, Lanes 2 to 5), it is unlikely that the 5.1S RNA is a degradation product. Although the labeled RNAs used in these experiments were from Novikoff hepatoma, it is interesting that 5.1S RNA was hybrid-selected by HeLa DNA (Fig. 2, Lane 3) and not by Novikoff DNA (Fig. 2, Lane 2). One possible explanation for this observation is that 5.1S RNA is transcribed in Novikoff hepatoma from genes that are few in number. When whole Novikoff DNA is used for hybrid selection (Fig. 2, Lane 2), DNA corresponding to these genes is not sufficient to hybrid-select 5.1S RNA. However, because 5.1S RNA is highly homologous to Alu 1 sequences that are abundant in HeLa DNA, total HeLa DNA may be able to hybrid-select 5.1S RNA (Fig. 2, Lane 3).

However, the identity of the fingerprint of 5.1S RNA to the 5′-portion of the abundant cytoplasmic 7SL RNA raises the possibility that 5.1S RNA is simply a degradation product of 7SL RNA. It is theoretically possible that 7SL RNA was degraded to yield discrete fragments corresponding to 5.1S RNA. The secondary structures proposed by several investigators show a high-yield cleavage site around the nucleotide 120 in isolated 7SL RNA as well as in signal recognition particles [26, 30, 31]. It is also possible that the abundant cytoplasmic 7SL RNA has a natural physiological turnover, and the formation of 5.1S RNA is a step in this pathway. Further work is needed to unequivocally establish that 5.1S RNA is a distinct small RNA.

The 6S RNAs homologous to B2 sequences and T3 RNA homologous to I.D. sequences have been observed by other investigators in other species [15,

16, 32]. Therefore, it is likely that these RNAs are distinct small RNA species. Although the 6S RNA, homologous to B2 sequences, has been described previously [15], the Novikoff hepatoma cells appear to contain at least three major and two minor classes differing in size (Fig. 4, Lane 6).

The T3 RNA homologous to I.D. sequences has recently been observed by Sutcliffe and his associates [32]. Our data supports the observations made by these workers. In addition, our results show that T3 RNA is the only Novikoff cell RNA with homology to I.D. sequences. This is in contrast to the synthesis of only BC1 RNA in neural tissues [19] and BC1, BC2 and T3 RNAs in many other tissues [32].

One model proposed for the origin of I.D. sequences is that they arose from the phenylalanine tRNA [33, 34]. Therefore, it was possible that the T3 RNA hybrid-selected by I.D. sequences was phenylalanine tRNA. However, the T_1-RNase fingerprint obtained for T3 RNA (Fig. 3, Panel C), was not consistent with the rat phenylalanine tRNA sequence [35]. Therefore, T3 RNA is distinct from phenylalanine tRNA.

The functions of these small RNAs are not understood. Functions related to gene expression [19, 37], and transport [8] have been previously suggested. Further work is needed to confirm hypotheses that these RNAs may play a role in gene expression [19, 37] and transport [8].

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