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Further Evidence That the Majority of Primary Nuclear RNA Transcripts in Mammalian Cells Do Not Contribute to mRNA

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Nuclear RNA from Chinese hamster ovary cells was effectively separated into polyadenylic acid [poly(A)]-containing [poly $(A)^+$] and non-poly(A)-containing [poly$(A)^-$] fractions so that ~90% of the poly(A) was present in the $(A)^+$ fraction. Only 25% of the 5'-terminal caps of the large nuclear molecules were present in the $(A)^+$ class, but about 70% of the specific mRNA sequences (assayed with cDNA clones) were in the $(A)^+$ class. It appears that many long capped heterogeneous nuclear RNA molecules are of a different sequence category from those molecules that are successfully processed into mRNA.

The rapid turnover (30) of a large part of nonribosomal nuclear RNA—the heterogeneous nuclear RNA or hnRNA—has posed a long-standing question in eucaryotic molecular biology: Are nuclear RNA transcripts made and not processed into mRNA in one cell under one physiological condition, but successfully processed in another cell or under another condition (6, 7)? That at least part of every transcript, or at least some molecules of each type of transcript, may be used in mRNA manufacture has always remained an important possibility. A partial resolution to "wasted" nuclear RNA came with the discovery of noncontiguous coding of mRNA in the nuclear RNA precursors to mRNA (2, 5, 16). Intramolecular loss of nuclear sequences clearly occurs in many, perhaps most, mammalian cell primary transcripts (8, 11, 19). But many studies still suggested more nuclear RNA loss than could be reasonably accounted for by loss of intervening sequences alone (14, 15, 17). For example, we recently analyzed the synthesis of 5'-methylated caps and the 3'-terminal polyadenylic acid [poly(A)] segments in high-molecular-weight hnRNA and the appearance of caps and poly(A) in the mRNA of Chinese hamster ovary (CHO) cells in culture (25). There are four caps formed for every poly(A) segment in the high-molecular-weight hnRNA, but the great majority (>90%) of the newly synthesized capped mRNA molecules in polyribosomes, even after brief methyl labels, contained poly(A). This result infers that over half of the individual primary nuclear RNA transcripts did not contribute a stable capped mRNA to the cytoplasm. These previous experiments, however, did not settle the question of whether the sequences of molecules in the nonproductive, non-polyadenylated [poly$(A)^-$] nuclear RNA group were the same as in the poly(A)-containing [poly$(A)^+$] group. We have now used available recombinant DNA clones complementary to a series of different CHO mRNAs (12, 13) to examine the distribution of specific sequences within the nuclear RNA fractions that contain or lack poly(A) [the poly$(A)^+$ or poly$(A)^-$ fractions]. The sequence composition of the long capped hnRNA molecules that lack poly(A) is different from that of the group of molecules that contain poly(A). It appears that as much as 50 to 75% of the nuclear RNA may be primary transcripts whose sequences are not found (or perhaps only very rarely found) in stable poly$(A)^+$ cytoplasmic mRNA.

MATERIALS AND METHODS

Cell growth and labeling procedures. CHO cells in suspension culture were grown as described (12) and labeled at 2×10^6 to 4×10^6 cells per ml for times up to several hours with [^{3}H]uridine (250 μCi/ml; 25 to 30 mCi/μmol) or [^{3}H]adenosine (100 to 200 μCi/ml, 25 to 30 mCi/μmol) or mixtures of the two labels.

cDNA clones and RNA:DNA hybridization. The recombinant CHO cDNA clones used in this work have been described (12), as has preparation of the DNA for filter hybridization. Ribosomal DNA clones from mouse cells were kindly made available by Norman Arnheim. The clone containing 28S rRNA was complementary to ~4,800 bases, and that containing 18S RNA was complementary to ~1,800 bases. RNase-resistant hybrids were scored as described (12).

RNA preparation. RNA was extracted from nuclei or cytoplasm as described (for recent protocol see reference 24). Fractionation of nuclear and cytoplasmic RNA by affinity to oligodeoxythymidylic acid [oligo(dT)-cellulose or polyribouridylic acid [poly(rU)], usually linked to Sepharose, has been widely used for a number of years to prepare poly$(A)^+$ and poly$(A)^-$ RNA fractions (1, 4, 20, 21). In our

experiment we have used commercial poly(U) (Miles) attached to Sepharose by the procedure of Wagner et al. (32). Although this absorbent selects the majority of the poly(A)$^+$ cytoplasmic RNA, we (20) have never been able to achieve 100% binding of cultured cell nuclear poly(A)$^+$ RNA to poly(U)-Sepharose. However, Edmonds and her co-workers (21, 31) described quantitative selection of the nuclear poly(A)$^+$ RNA by oligo(dT)-cellulose prepared in their laboratory. Since we needed as complete a separation as possible of the poly(A)$^+$ and poly(A)$^-$ of nuclear RNA, we made comparisons of the two selecton procedures (Table 1). The total radioactivity in poly(A)$^+$ RNA was assayed, and in the nuclear sample the labeled RNase-resistant ~200-nucleotide poly(A) segment was determined by gel electroporesis (20). Poly(U)-Sepharose consistently selected ~20% more labeled poly(A)$^+$ RNA than did oligo(dT) from labeled cytoplasmic RNA. We believe the reason for this is that as the poly(A) segments become shorter with age (29) the binding of the mRNA to poly(U)-Sepharose remains greater than that to oligo(dT). In contrast, in the nuclear RNA samples where poly(A) is uniformly ~235 ± 35 bases (27), the oligo(dT) proved more effective than the poly(U)-Sepharose. About two times as much labeled nuclear RNA bound to oligo(dT) as to poly(U)-Sepharose. A possible explanation for the difference between the two adsorbents is the exclusion of larger molecules from contact with the poly(U) attached to Sepharose. About 30 to 40% of the RNase-resistant poly(A) present within hnRNA failed to bind to poly(U)-Sepharose. In using a number of batches of oligo (dT) (obtained from P. L. Biochemicals and from Collaborative Research), different amounts of nuclear poly(A) in hnRNA were retained by oligo(dT). Consistent results of >90% binding have been obtained recently by using the P-L Biochemicals product (see Table 2).

TABLE 1. Binding of cytoplasmic nuclear RNA to oligo(dT)-cellulose and poly(U)-Sepharose[a]

Determination	cpm	
	Oligo(dT)	Poly(U)-Sepharose
RNA bound		
Cytoplasmic, total (input, 16×10^6 cpm)	4.1×10^5	5.6×10^5
Nuclear, untreated (input, 13.3×10^6 cpm)	2.1×10^6	0.9×10^6
DMSO treated (input, 13.3×10^6 cpm)	2.0×10^6	0.8×10^6
Poly(A) present in nuclear samples[b]		
Originally bound	30,400	21,000
Originally unbound	5,400	20,000

[a] After a 30-min label with both [^{3}H]uridine and [^{3}H]adenosine, RNA was extracted from nuclear and cytoplasmic samples as described (12, 24), and equal samples were subjected to chromatography on poly-(U)-Sepharose or oligo(dT) as outlined in the text.

[b] Poly(A) was assayed as RNase-resistant ~200-nucleotide fragment (20) after first selecting samples on oligo(dT) or poly(U)-Sepharose.

The standard conditions for binding that have been adopted are as follows.

(i) Cytoplasmic RNA bound to poly(U)-Sepharose. All operations were at room temperature. Dissolve RNA in ETS (EDTA, 0.01 M; Tris, 0.01 M, pH 7.4; sodium dodecyl sulfate, 0.2%). Add NaCl to 0.2 M final concentration and apply at room temperature. Use a column bed of 1 ml for the RNA from 10^8 cells. Wash with binding buffer followed by ETS; elute with ETS-formamide (1:1). Preparation of poly(U)-Sepharose is according to Wagner et al. (32), and 50 mg of poly(U) is used with 100 ml of Sepharose slurry.

(ii) Nuclear RNA bound to oligo(dT). Dissolve RNA in elution buffer (0.01 M Tris, pH 7.4; 0.001 M EDTA; 0.05% sodium dodecyl sulfate). Add two volumes of 100% dimethyl sulfoxide (DMSO) and heat to 65°C for 10 min. Adjust the buffer concentration for binding (final concentrations: 0.01 M Tris, pH 7.4; 0.002 M EDTA; 0.01% sodium dodecyl sulfate; 0.5 M NaCl), and apply the sample at room temperature to an oligo(dT) column at 0.5 ml/min. Wash the column with 10 volumes of binding buffer followed by binding buffer with 0.1 M NaCl. Elute the bound RNA with the elution buffer described above.

RESULTS

Characterization of poly(A)$^+$ nuclear RNA selected by oligo(dT). With an adequate separation of nuclear RNA into (A)+ and (A)$^-$ fractions, we wished to examine the size of nuclear RNA in the two classes and the division of rRNA-related molecules and poly(A) between the two fractions (Table 2). Both the bound and the unbound nuclear RNA fractions were subjected to size analysis on DMSO-containing sucrose gradients (Fig. 1). The majority of the total RNA, as judged by optical density at 260 nm, was in the wash fractions. The intermediate salt wash (0.1 M NaCl) contained a significant fraction of the largest rRNA precursors. The rRNA was also assayed by hybridization of labeled samples to an excess of rDNA (cloned 18S and 28S regions) attached to nitrocellulose filters; over 85% of the rRNA was in the flow-through. There was some contamination of the bound poly(A)$^+$ fraction with rRNA.

One interesting sidelight provided by the analysis of rRNA content of these samples was a direct measurement of the rate of labeling of rRNA versus hnRNA. This is, of course, a reflection of polymerase I versus polymerase II activity in the nucleus (22). We and others have attempted to measure this ratio indirectly in the past (3, 30). From the four experiments in Table 1, it can be concluded that rRNA synthesis makes up about 1/4 of the total very briefly labeled nuclear RNA. Thus the size comparison of the two nuclear fractions (Fig. 1) is mainly a comparison of hnRNA with or without poly(A). These two fractions are similar in average size. Even if the 20% of the (A)$^-$ fraction that is rRNA were removed from consideration, the

TABLE 2. Preparation and characterization of labeled nuclear RNA[a]

Expt	Time/Label	Determination	cpm		rRNA as % of total	% Poly(A) retained	% of rRNA retained
			$(A)^+$	$(A)^-$			
1	10 min/A + U	Total cpm	41×10^6	148×10^6	14		
		rRNA, total	5×10^6	22×10^6			18
		Poly(A)	1.97×10^5	2.7×10^4		88	
	30 min/A + U	Total cpm	61×10^6	263×10^6	31		
		rRNA, total	5.5×10^6	97×10^6			5.3
		Poly(A)	4.3×10^5	2.4×10^4		92	
2	20 min/A + U	Total cpm	57×10^6	65×10^6	16		
		rRNA, total	4.4×10^6	17×10^6			20
		Poly(A)	2.8×10^5	5.7×10^4		84	
3	30 min/A + U	Total cpm	18×10^6	80×10^6	19		
		rRNA, total	1.2×10^6	17.5×10^6			6.4
		Poly(A), total	1.95×10^6	4×10^4		98	

[a] Growing cells were labeled, and RNA was extracted and separated into $(A)^+$ and $(A)^-$ fractions by affinity chromatography on oligo(dT)-cellulose as described in the text. rRNA was estimated by hybridization to p28S or p18S (pBR plasmids with 4,800 or 1,800 bases complementary to 28S or 18S rRNA, respectively). To obtain total ribosomal precursor RNA contribution, cpm hybridized to excess p28S + p18S was multiplied by 2.15 because the total pre-rRNA is 14,000 bases long. This corrected figure is entered as "rRNA, total." Poly(A) was assayed as the T_1 RNase-digested fraction that bound specifically to poly(U)-Sepharose (20, 24) as the 235 ± 35 base-long nuclear poly(A) segment (24).

average size of $(A)^+$ and $(A)^-$ nuclear RNA would be similar.

Cap content. We reported several years ago that 5'-methylated cap structures are present in both poly$(A)^+$ and poly$(A)^-$ nuclear fractions, with the bulk of capped material failing to bind to poly(U)-Sepharose (26). We repeated this determination with adenosine-labeled RNA in which both poly(A) segments and caps could be assayed with the same label. (About 40% of the CHO caps have A as the first nucleotide of the RNA chain [25].) The assay was performed after separating the nuclear RNA on DMSO gradients, and only molecules larger than 750 bases were included (see references 12 and 25 for illustrations of separation on DMSO gradients). Although 84% of the poly(A) was retained on the oligo(dT) column, only 24% of the caps were retained. The percentages of total labeled $(A)^+$ and $(A)^-$ hnRNA that were recovered as caps were similar (see legend to Fig. 2), indicating that the two classes of RNA are about the same length. This extends and quantitates the earlier conclusions that most capped large hnRNA molecules do not contain a poly(A) (26); other studies mark the time of nuclear poly(A) addition very soon after chain synthesis (24). Therefore, we conclude that the poly$(A)^-$ long capped molecules will not ever acquire a poly(A) segment.

Sequence composition of $(A)^+$ and $(A)^-$ nuclear RNA. We next wished to determine whether hnRNA molecules were distributed differently between the $(A)^+$ and $(A)^-$ hnRNA fractions. For this purpose specific cloned DNA segments complementary to eight individual CHO mRNA molecules were available (12). The total radioactive RNA complementary to each of these cloned DNA samples was determined in a series of experiments. Results are presented of several experiments in which the total hybridizable RNA was measured and the percentage of the $(A)^+$ and $(A)^-$ nuclear RNA fractions complementary to each clone was calculated (Table 3). In calculating the hnRNA input the rRNA contribution to both the $(A)^+$ and $(A)^-$ fractions was measured and subtracted. About 70% of the total labeled RNA complementary to each sequence was in the $(A)^+$ fraction, and the percentage of each sample represented by each specific RNA sequence was from 5- to 10-fold greater in the $(A)^+$ fraction. This division was about the same for the label times between 15 and 30 min. Since the distribution of molecules in the $(A)^+$ and $(A)^-$ fractions was similar in size (Fig. 1), it appears that on a molar basis the sequences in the $(A)^-$ fraction contain many sequences not present in the $(A)^+$ fraction.

DISCUSSION

Adherence to the protocol of Edmonds and co-workers (21, 31) has allowed us to fractionate

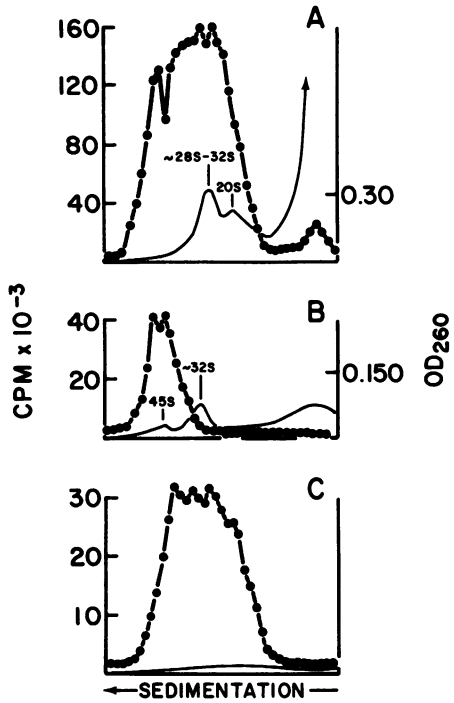

FIG. 1. Sedimentation analysis of nuclear RNA fractions. Nuclear RNA was extracted from 2×10^8 CHO cells after 30 min of incorporation of [^{3}H]uridine and [^{3}H]adenosine (see the text). The nuclear RNA was chromatographed in oligo(dT) as described in the text. Three fractions were collected: (A) unbound or poly(A)$^-$ fraction; (B) 0.1 M NaCl wash; and (C) bound poly(A)$^+$ fraction. The plotted (●) radioactivity represents 1/100 of total label. The optical density at 260 nm is given by the solid line, and the S values are nominal for ribosomal species.

high-molecular-weight nuclear RNA from CHO cells into poly(A)$^+$ and poly(A)$^-$ fractions. As it should, the majority (~85 to 95%) of the rRNA partitions with the poly(A)$^-$ fraction. Only about 25% of the caps in the high-molecular-weight nuclear RNA are in poly(A)$^+$ molecules. If the poly(A)$^-$ hnRNA represented simply an oversupply of the same type of molecules as found in the poly(A)$^+$ fraction, then the specific mRNA sequences characteristic of (A)$^+$ mRNA should be found in equal concentration in the poly(A)$^+$ and poly(A)$^-$ hnRNA fractions. We did not find this to be so. While a small fraction of the (A)$^-$ nuclear RNA appears to be transcripts from the same DNA regions as are the poly(A)$^+$ sequences, at least half of the long capped nuclear RNA appears to be transcribed from other regions of DNA that are not the same as for the (A)$^+$ group.

Based on several possible, but we believe unlikely, arguments this conclusion could be wrong. For example, suppose all (or most) of the poly(A)$^-$ hnRNA fraction were nascent (unfinished) RNA or prematurely terminated hnRNA chains that on average had not been elongated to include the 3'-most portion of a transcriptional unit. Since our cDNA clones to mRNA were made by the conventional reverse transcription techniques (9, 12, 18), their sequence representation may be weighted to the 3' mRNA sequences. Thus if poly(A)$^-$ hnRNA were all or mainly nascent or prematurely terminated we would find an underrepresentation of 3' sequences. However, the size profile of the [^{3}H]uridine- or [^{3}H]adenosine-labeled (A)$^-$ hnRNA fraction (Fig. 1) does not resemble the distribution expected for nascent chains or prematurely terminated molecules. Neither does the steady-state distribution of *methyl*-^{3}H-labeled cap structures appear to be equivalent to a group of short transcripts (23). Moreover, for the majority of labeled nuclear RNA to be nascent after a 30-min label time would imply that turnover (or nuclear exit) was faster than chain synthesis. Chain synthesis is estimated to be 50 nucleotides per s (28) for an average synthesis time of 2 to 3 min for an hnRNA molecule (8, 28), whereas turnover is estimated to be ~15 to 45 min (13, 17). Thus the majority of hnRNA is not likely to be nascent RNA. Finally, although definite evidence exists for premature termination of adenovirus transcription units in infected HeLa cells (10), and we also presented

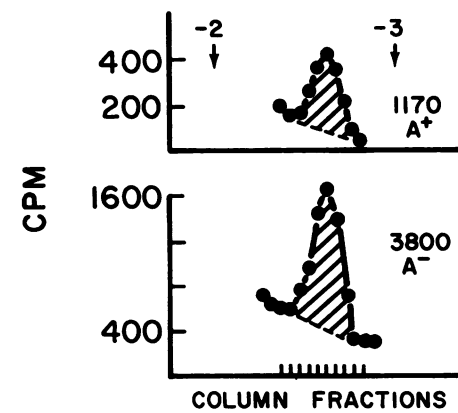

FIG. 2. Cap content of nuclear poly(A)$^+$ and poly(A)$^-$ fractions. Adenosine-labeled nuclear RNA was prepared as described for Table 1 and Fig. 1. RNA larger than ~750 bases (equivalent to fractions 1 to 28 in Fig. 1) was collected and digested with T_2 RNase and P1 exonuclease, followed by DEAE chromatography (see reference 23 for details). The labeled nuclease-resistant structures with a charge of −2.5 have been characterized as 5'm^{7}GpppN. Approximately 24% of the caps were in the poly(A)$^+$ fraction (1,170/4,970).

TABLE 3. Specific sequence content of $(A)^+$ and $(A)^-$ hnRNA[a]

Expt	Label time (min)	hnRNA input (cpm × 10^6)	cpm in hybrid							
			A	B	C	D	E	F	G	I
1	10 $(A)^+$	36	5,730	3,270	1,250	740	780	560	1,130	450
	$(A)^-$	126	2,960	2,330	1,110	260	450	950	250	450
	30 $(A)^+$	55	6,100	3,150	1,310	1,550	2,750	1,980	1,420	760
	$(A)^-$	167	3,600	2,300	1,250	430	850	1,500	320	650
2	20 $(A)^+$	52.5	6,250	3,610	1,560	840	1,380	900	890	710
	$(A)^-$	48	1,790	1,090	590	160	270	250	140	110
3	$(A)^+$	16.8	1,880	2,150	970					
	$(A)^-$	62.5	890	920	230					
Ratio (avg) A^+/A^-			68/32	66/34	64/36	78/22	76/24	57/43	82/18	62/38
Avg % hnRNA										
as A^+			1.2×10^{-4}	8.3×10^{-5}	3.8×10^{-5}	2.2×10^{-5}	3.3×10^{-5}	2.3×10^{-5}	2.5×10^{-5}	1.2×10^{-5}
as A^-			$2{,}4 \times 10^{-5}$	1.7×10^{-5}	8.0×10^{-6}	2.6×10^{-6}	4.8×10^{-6}	7.2×10^{-6}	2.4×10^{-6}	3.3×10^{-6}

[a] The hnRNA fractions prepared as described in Table 2 were used in RNA:DNA hybridization experiments with excess plasmid DNA containing CHO mRNA sequences (12, 13). Entered in the table are cpm hybridized. A background on pBR plasmid without CHO DNA insert, which ranged from 50 to 150 cpm, was subtracted. In the last three lines of the table, the calculated average from all the experiments is given to show the ratio of specific RNA between the $(A)^+$ and $(A)^-$ fractions and the percentage of each specific sequence in the $(A)^+$ and the $(A)^-$ fractions. The total hnRNA input was corrected after subtracting rRNA as in Table 2.

results of *methyl*-^{3}H-labeled CHO nuclear RNA that suggested premature termination in CHO cells, the prematurely terminated chains are quite short (23), extending only a few hundred bases from the initiation site. Again, the poly(A)$^-$ hnRNA examined in this paper has an average size of ~5 kilobases (kb) (8, 25; Fig. 1). Thus premature termination of the (A)$^-$ hnRNA seems unlikely to be the reason for the underrepresentation of the specific CHO mRNA sequences found in the nuclear fraction.

A third possibility that could lead to the erroneous conclusion of two nonoverlapping populations of large hnRNA molecules would be breakage of hnRNA, either in the cell before isolation or outside the cell during RNA extraction. For example, if the average hnRNA were really all ~20 kb, if all molecules were polyadenylated, and if these molecules suffered an average of four breaks, we could end with molecules that sedimented with an average size of ~5 kb. If the cloned sequences were mainly in the 3′ portion, then most caps would not bind to oligo(dT), but most specific sequences might. If this were the true distribution for hnRNA primary transcripts, then nascent chain analysis after the briefest label times should reveal very few nascent primary transcripts of less than 5 kb. However, the nascent chain analyses of both HeLa and CHO cells suggest a weighted average transcript size of 5 kb, with an equal number of primary transcripts shorter and longer than 5 kb (8, 23). Moreover, the same extraction procedures yield intact primary adenovirus transcripts greater than 20 kb in length (7). Thus extensive breakage of long hnRNA does not seem a likely explanation for the present results.

A final possibiltiy is that all primary transcripts are in fact used as (A)$^+$ molecules some of the time, but the frequency is 50 to 75% for the group of mRNAs for which we have cDNAs and much less frequent, say 10% or less, for most other primary transcripts. This could lead to a distribution of mRNA-related sequences similar to what was found. However, this requires the ad hoc hypothesis that the CHO clones that were selected randomly from a cDNA cytoplasmic library all happened to be examples with high nuclear utilization, whereas the majority of primary transcripts are in fact infrequently polyadenylated. We, of course, cannot rule out this possibility, especially if very scarce mRNA molecules fall into this category.

However, we are driven to suggest that many (>50%) of nuclear RNA molecules in CHO cells belong to an unusual group of transcripts. These molecules do not become polyadenylated and therefore do not contribute a stable mRNA to the cytoplasm, because, as earlier discussed, over 90% of the newly arrived capped polysomal molecules have poly(A) (25). Some hnRNA molecules of this class have been described, those containing oligo(A)s that are not found in poly(A)$^+$ nuclear or cytoplasmic RNA (21, 31). If primary transcripts that do not yield mRNA can be positively identified, what may be the explanation for their existence? As already mentioned, such transcripts may not be processed in CHO cells but processed in other cells, although no evidence exists at present for such regulatory decisions. Another possibility is that these transcripts emanate from "pseudogenes" (9, 18) that cannot yield mRNAs. "Leaky" transcription for many regions in the DNA could also be the rule. The final proof or refutation of these or other possibilities will have to come by identifying, with specific DNA clones, individual primary nuclear RNA transcripts that do not yield any mRNA. We have begun to search for such primary transcripts. M. Nemer (personal communication) has performed similar experiments with sea urchin cytoplasmic polysomal RNA and finds no individual sequence present in the (A)$^-$ fraction that is not present in the (A)$^+$. From the experiments described in this paper, we should expect one-half of the genomic clones that contain sequences complementary to nonrepetitive hnRNA sequences to be part of transcription units that do not produce an mRNA. If, on the other hand, all primary transcripts are used to make poly(A)$^+$ mRNA but with very different efficiencies, we should recover only genomic clones at least some of whose transcripts are in the (A)$^+$ hnRNA fraction.

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