An Examination of the Nuclear RNA of Adenovirus-transformed Cells

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Soon after a molecule of bacterial RNA polymerase initiates the transcription of an operon in bacterial cells, the nascent mRNA chain is engaged by ribosomes—*the transcript is the message* (Miller et al. 1970). It appears that little or no post-transcriptional modification is generally required before bacterial mRNA can function, and any modification that might occur would be at or close to the 5′ end, the initiation site for a new RNA chain (Maitra and Hurwitz 1967). In addition, because bacterial mRNA has a fairly constant and rapid half-life (Levinthal et al. 1962; Salser et al. 1968), measurements of how much of a specific mRNA has accumulated basically reflect different rates of transcription of the gene in question (Hayashi et al. 1963).

In the past several years, work with mammalian cells (in fact, eukaryotic cells in general) has led to the conclusion that mRNA is derived after transcription is completed by a series of nuclear events near to the 3′ end of newly formed HnRNA (heterogenous nuclear RNA) (Kates 1970; Edmonds et al. 1971; Lee et al. 1971; Mendecki et al. 1972; Darnell et al. 1971a,b; Jelinek et al. 1973a,b). Thus in mammalian cells, the transcript itself is not the mRNA; only after proper processing does an mRNA participate in protein synthesis (see Darnell et al. 1973).

Furthermore, in mammalian cells there is no assurance that measurements of accumulated mRNA reflect the transcription rate, since a potential differential efficiency of post-transcriptional processing of mRNA as well as widely differing mRNA lifetimes may occur (Stewart and Papaconstantinou 1967; Perry and Kelley 1973; Singer and Penman 1973). A decision between the relative importance of transcriptional and this hypothetical post-transcriptional regulation can only be made if it is possible to measure the just completed HnRNA, i.e., the unprocessed transcript. The problem may be phrased: If protein X is made in accelerated amounts from an increased supply of mRNA, has more HnRNA containing mRNA X been synthesized or has a more efficient processing of the same amount of HnRNA occurred? The critical quantity is the rate of synthesis of the initial HnRNA transcript containing mRNA X.

Our laboratory has recently been concerned with the general features of organization of sequences within HnRNA in the hopes of establishing guidelines for the measurement of *new transcripts* which will give rise to mRNA, as opposed to processed nuclear molecules which may accumulate.

We have recently reported on the distribution within these large nuclear RNA molecules of various types of sequences that do not exit to the cytoplasm as part of mRNA (Jelinek et al. 1973a; Molloy et al. 1972). Certain repeated sequences, e.g., a uridylate-rich oligoribonucleotide, have a preferential location at a considerable distance (greater than 10,000 nucleotides) from the poly(A) which lies at the 3′ end of HnRNA. Other repeated sequences occur interspersed with nonrepeated sequences throughout the length of HnRNA, beginning about 3000 or 4000 nucleotides from the 3′ end and extending toward the 5′ end (Molloy et al. 1974; Jelinek et al. 1973a,b). From the relatively slower hybridization rate of the 3′-most portion of HnRNA, it was concluded that this region contained mRNA, but no decision was possible about whether other regions of the HnRNA might represent potential mRNA molecules. In the present report, we describe the location within HnRNA from virus-transformed cells of virus-specific sequences apparently destined to function as mRNA in the cytoplasm. In addition, we briefly review additional substantiating evidence that HnRNA in general is a precursor to mRNA.

Evidence for Some HnRNA As Precursor to mRNA

Among the most convincing evidence that mRNA derives from HnRNA is that some molecules of HnRNA have added, after transcription, a 200 nucleotide-long polyadenylic acid [poly(A)] segment at their 3′ termini (Philipson et al. 1971; Darnell et al. 1971b; Mendecki et al. 1972; Molloy and Darnell 1973) and this segment is also found at the 3′ end of cytoplasmic mRNA. Prevention of addition of poly(A) is associated with failure of appearance of newly made mRNA (Penman et al. 1970; Darnell et al. 1971b; Adesnik et al. 1972). Experiments with very brief labeling times (1–5 min) showed all the newly labeled poly(A) to be in the nucleus, attached to HnRNA, arguing for a nuclear origin of the poly(A) (Darnell et al. 1971b; Jelinek et al. 1973a,b). An important consideration is whether every HnRNA to which

poly(A) is added can contribute mRNA to the cytoplasm. Two types of experiments were originally performed to probe this question.

First, cells were briefly labeled with adenosine, and any additional labeled poly(A) synthesis was prevented by treatment with 3′-dA (3′-deoxyadenosine, "cordycepin"). Approximately 30–40% of the labeled nuclear poly(A) then appeared in the cytoplasm within about 30 minutes. During further incubation, the nuclear poly(A) decreased greatly, while the cytoplasmic poly(A) remained approximately constant for 60 minutes and then also declined (Jelinek et al. 1973a,b). Thus it was only possible to conclude that 30–40% of the poly(A) (and presumably associated mRNA) appeared in the cytoplasm. The remaining poly(A) could have either been transferred to the cytoplasm where decay occurred or decayed in the nucleus. Experiments involving addition of both 3′-dA and actinomycin did not result in the observable 30–40% transfer that the chase with 3′-dA alone revealed (LaTorre and Perry 1973; Sheiness and Darnell, unpubl.). It was, however, consistently possible to observe the 30–40% transfer with 3′-dA alone.

Second, a study was made of the kinetics of accumulation of total radioactivity and of labeled poly(A) in HnRNA and mRNA (Jelinek et al. 1973a,b). Whereas total radioactivity in HnRNA remained considerably higher than in mRNA for many hours (an old finding indicating nuclear turnover of a large fraction of HnRNA: Harris 1962; Soeiro et al. 1968), the cytoplasmic poly(A) exceeded the nuclear poly(A) during the first hour after exposure to [^{3}H]adenosine and was fourfold greater by three hours. These results demonstrated a much greater conservation of poly(A) than of total HnRNA in conversion to mRNA.

These conclusions have been challenged recently by a kinetic analysis of poly(A) accumulation in L cells labeled with a high concentration of adenosine (Perry et al. 1974). Under these labeling conditions, nuclear poly(A) continued to rise past the point where the maximal rate of accumulation of cytoplasmic poly(A) occurred, suggesting nuclear poly(A) turnover. Furthermore, it was argued that the rapid accumulation of cytoplasmic poly(A) was not consistent with a nuclear origin of the poly(A) because mRNA turnover was slow (Perry and Kelley 1973; Singer and Penman 1973) and there is an initial lag in mRNA appearance due to processing.

A reexamination of poly(A) labeling kinetics in HeLa cells has explained the discordance in results and substantiates the original conclusion that most, if not all, nuclear poly(A) may exit to the cytoplasm (Puckett et al., unpubl.). This conclusion is based on two new findings: (1) If the labeled nuclear poly(A) content was compared to relative radioactivity in the acid-soluble pool, the nuclear poly(A) only continued to rise so long as the acid-soluble pool continued to rise. (2) When cells grown in adenine were briefly labeled with [^{3}H]guanosine, followed by removal of the label, over 50% of the labeled mRNA that entered the cytoplasm disappeared with a half-life of 1 hour or less. This rapidly "turning-over" fraction of mRNA appears to be the basis of the apparent overly rapid accumulation of cytoplasmic poly(A). Kinetic analysis of such a complicated multistep process [adenosine or adenine → ATP → nuclear poly(A) → cytoplasmic poly(A) → (?) AMP] cannot, we believe, prove either total exit or fractional turnover of nuclear poly(A). The kinetics in these recent studies, however, are consistent with possible total transfer of nuclear poly(A).

This is an important issue to the further study of the relationship of HnRNA to mRNA because techniques for the selection of nuclear HnRNA terminated by poly(A) exist (Molloy et al. 1974). If poly(A) transfer to the cytoplasm is conservative, such selected HnRNA molecules are the direct precursors to mRNA; if poly(A) were turned over in the nucleus, then the homopolymer might not mark potential mRNA. The new kinetic results give renewed confidence that analysis of poly(A)-terminated HnRNA does provide information about mRNA precursor.

HnRNA from Virus-transformed Cells

The earliest strongly suggestive evidence that HnRNA was a precursor to mRNA was that HnRNA from cells transformed by the integration of SV40 DNA (Sambrook et al. 1968) synthesized high molecular weight HnRNA containing virus-specific sequences, whereas the polysomal virus-specific presumed mRNA was smaller in size (Lindberg and Darnell 1970; Tonegawa et al. 1970). Similar findings have also been described for HnRNA from cells transformed by adenovirus type 2 (Ad2) (Wall et al. 1973), where it was also shown that virus-specific nuclear and cytoplasmic RNA contain largely overlapping sequence distributions (Shimada et al. 1972) and that the cytoplasmic virus-specific mRNA consists mainly of one 20S RNA species with perhaps a lesser amount of a lower molecular weight component (Wall et al. 1973).

We have analyzed the distribution of virus-specific RNA in various sizes of nuclear RNA samples and determined the intramolecular location of the Ad2-specific regions within the largest available HnRNA molecules. The aim of these investigations was to determine, if possible, some properties of the initial transcript of the region of the genome containing virus DNA and the nature of any accumulated virus-specific molecules in the nucleus of the transformed cells.

In previous experiments, a large fraction (≈70%) of the Ad2-specific polysomal RNA had been shown to bind to poly(U)-Sepharose (Wall et al. 1973), indicative of a poly(A) terminus. In the present ex-

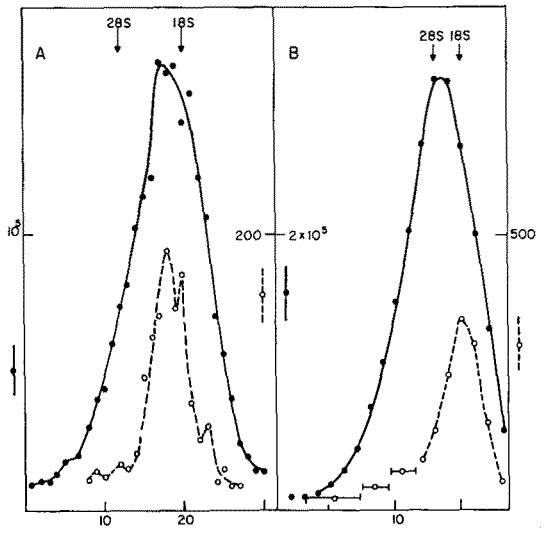

Figure 1. Sedimentation profile of Ad2-specific RNA in Ad2-transformed rat cells. Poly(A)-terminated cytoplasmic *(A)* and HnRNA *(B)* were collected, precipitated and sedimented through sucrose gradients in SDS buffer (see text) so that 28S and 18S markers in separate tubes migrated as shown by arrows. Total cpm (●) and virus-specific RNA (○) are plotted.

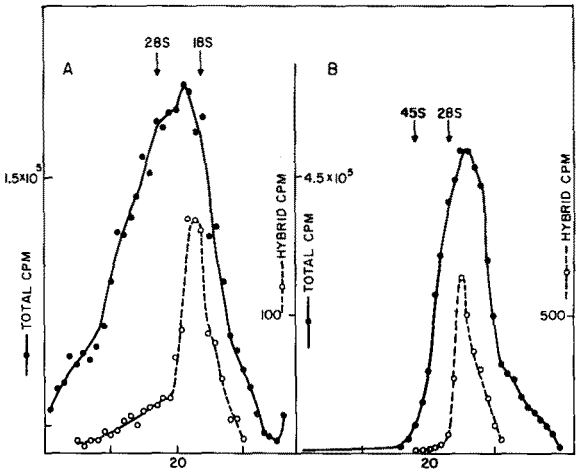

Figure 2. Ad2-specific RNA from nucleus of Ad2-transformed rat cells. [^{32}P]HnRNA was prepared, sedimented and molecules from 15–32S collected. After selection of poly(A)-terminated RNA, the sample was divided into two parts (not equal), precipitated, and one portion sedimented through sucrose-SDS buffer (see Fig. 1) so that the 32S marker had migrated as shown *(A)*. The second portion was redissolved and sedimented through a 5–20% DMSO gradient *(B)* as described in Experimental Procedures. The markers shown were sedimented at the same time in a parallel DMSO gradient.

periments, the total poly(A)-terminated cytoplasmic and nuclear RNA was collected and subjected to zonal sedimentation analysis through standard sucrose gradients (Fig. 1). The major species of Ad2-specific RNA in both the nuclear and cytoplasmic fractions was the previously observed "20S" Ad2 species. In order to better characterize this peak, poly(U)-selected nuclear samples from another preparation of HnRNA, originally sedimenting less than 32S, were resedimented through either a standard sucrose gradient or through sucrose gradients in DMSO and the sedimentation of virus-specific RNA again assayed. Again the major virus-specific poly(A)-terminated RNA sedimented faster than the 18S rRNA, but considerably slower than the 28S rRNA, i.e., ≈20S (Fig. 2). Based on these comparative sedimentations in the two solvents, the Ad2 RNA is about 2500 nucleotides, assuming the 18S to contain 2100 and the 28S, 5100 nucleotides (Strauss et al. 1968). It appears, therefore, that there is in the nucleus of transformed cells a poly(A)-terminated virus-specific molecule the same size as the major cytoplasmic virus-specific mRNA. In addition, it is clear in Figures 1 and 2 that the overall profile of poly(A)-terminated RNA from the 15–32S region resembles mRNA in its general sedimentation pattern.

Since the data in Figure 1, as well as earlier experiments, indicated virus-specific sequences in molecules larger than 28S, we wished next to determine if virus-specific RNA was contained in poly(A)-terminated molecules longer than the 20S molecule. Highly labeled ^{3}H- or ^{32}P-nuclear RNA was prepared and separated by conventional sucrose gradient sedimentation into > 40S and 15–40S fractions (Fig. 3A). Poly(U) selection of poly(A)-terminated molecules was carried out by a modification of the technique previously demonstrated to yield authentically poly(A)-terminated large molecules (Molloy et al. 1974). When the poly(A)-terminated 40S fraction was resedimented, almost all the radioactive RNA resedimented more rapidly than 28S rRNA (Fig. 3C). Assay for Ad2-specific RNA showed that all sedimentation classes of RNA contained Ad2 RNA with three-fourths sedimenting faster than 20S (ranges 60–80% in various experiments). The majority of the virus-specific RNA in the poly(A)-terminated fraction from the RNA originally obtained from the 15–40S sedimentation zone was found to sediment like the majority of the 20S Ad2 RNA fraction (Fig. 3D).

A summary of a number of experiments performed with transformed cell HnRNA preparations is given in Tables 1–3. Several features of the results are noteworthy. In the >40S HnRNA, well over half of the virus-specific RNA was contained in molecules terminated by poly(A)-containing molecules. Experiments 2 and 3 in Table 1 emphasize that not all poly(A)-containing large molecules can be regularly bound to poly(U). Repeated passage through the filter columns (Exp. 2) or extremely slow flow (Exp. 1 sample required 90 min for 1 ml to flow through column) result in better yields of poly(A)-containing large HnRNA. Second, the poly(A) content of the larger molecules decreased as the sedimentation increased, indicating that the

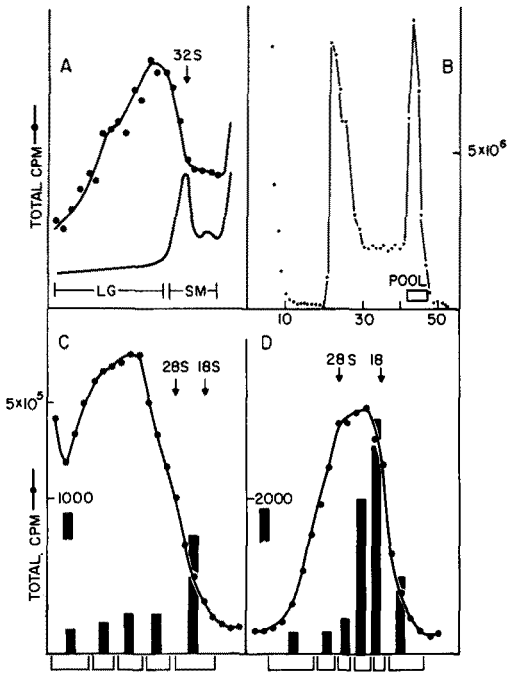

Figure 3. Demonstration of virus-specific sequences in poly(A)-terminated HnRNA of Ad2-transformed rat cells. *(A)* [^{32}P]HnRNA was prepared (50 mCi ^{32}P, 4×10^8 cells in 200 ml; 4.5-hr label) and sedimented through sucrose-SDS gradients and aliquots of gradient fractions assayed for radioactivity (●) and absorbance at 260 nanometers (———). The gradient was divided as indicated into a "large" (40–70S) and "small" (15–32S) portion and poly(A)-terminated molecules selected from each sample. *(B)* Elution pattern of large HnRNA from the poly(U) filter column. Fractions 43–48 constituted the poly(A)-terminated fraction and represented about 10% of the large HnRNA. The poly(A)-terminated molecules were again sedimented through sucrose-SDS gradients and Ad2 sequences measured in fractions indicated by brackets. ("large" HnRNA, *C*; "small" HnRNA, *D*). Total cpm is one-third of actual total and the black bars (———) represent Ad2-specific cpm.

faster sedimenting molecules were truly longer (data not shown; see Molloy et al. 1974). To relate the virus-specific RNA content of various poly(A)-terminated samples to the number of *molecules* of RNA, the percentage of virus-specific RNA was divided by the percentage of poly(A), providing an estimate of the content of Ad2 sequences *per molecule* in the various-sized poly(A)-terminated sedimentation classes (Table 2). The longer molecules (>10–15,000 nucleotides) tended to have a greater Ad2 content per molecule than intermediate-sized molecules, but slightly less Ad2 sequences than samples including the 20S size class. The concentration of Ad2-specific sequences was similar in the <30S nuclear RNA and in the cytoplasmic RNA. Thus the concentration of virus-specific sequences in the largest poly(A)-containing molecules and poly(A)-containing molecules of 10–30S and cytoplasmic mRNA are all about the same. The location of the virus-specific regions within the large poly(A)-terminated molecules was examined by exposing such molecules briefly to alkali and reselecting the poly(A)-containing fragments resulting from this treatment (Table 3). The poly(A) content of such fragments was 3 to 4-fold higher than the original sample, indicating an average of 3–4 breaks per large molecule to yield the smaller fragments. In several such experiments, about 60–70% of the Ad2 sequences remained associated with the poly(A)-containing fragments, indicating a preferential location next to poly(A) of the virus-specific sequences. These results are consistent with, but certainly do not prove, the original transcription of a large Ad2-containing HnRNA molecule to which poly(A) is added followed by nuclear and eventual cytoplasmic accumulation of a processed 20S product from such molecules (Wall et al. 1973). Kinetic experiments indicating that label enters the large molecules first would be necessary to conclude that

Table 1. Ad2-specific RNA in Poly(A)-terminated HnRNA

	RNA prep.	Poly(U) reaction	Content of fraction	
			cpm, Ad2 hybrid	% total poly(A)
Exp. 1	large (>40S)	bound	3460	>95
		wash	2080	< 5
	small (15–32S)	bound	8420	64
		wash	12,800	36
Exp. 2	large (>40S)	bound 1st	523	52
		2nd	297	36
		wash	327	12
	small (15–32S)	bound 1st	2370	68
		2nd	280	11
		wash	1250	21
Exp. 3	large (>45S)	bound	4900	27
		wash	13,800	73

Labeled HnRNA from Ad2-transformed rat cells was obtained after zonal centrifugation in SDS-sucrose gradients and subjected to chromatography on poly(U) filters and the material specifically eluting in about 30–40% formamide collected as "bound" (see Figs. 3 and 4). The RNA in this flow-through fraction was collected as "wash." The amount of Ad2-specific RNA (Wall et al. 1973) and poly(A) was measured (Molloy et al. 1974) in each fraction. In Exp. 2, the wash from the first chromatography was precipitated, redissolved in ETS (0.01 M EDTA, 0.01 M Tris pH 7.4, 0.2% SDS), heated for 1 min to 60°C and chromatographed a second time.

Table 2. Relationship of Size of Poly(A)-terminated RNA to Content of Ad2-specific RNA

S value	Fraction Ad2 hybrid/fraction in poly(A)			
	Exp. 1 (large)	Exp. 2 (large)	Exp. 2 (small)	Exp. 3
>60	.022	.020		
50–60	.028	.02		
40–50	.015	.019		
35–40	.019	.016	.0116	
30–35	.012		.0097	
25–30			.0079	
20–25			.027	
15–30	.02	.047		
18–20			.041	
6–18			.014	
Total from				
45S				.018
15–32S				.03
Cytoplasmic				.03

HnRNA of large size (>40S) or small size (15–32S) or total cytoplasmic RNA was subjected to poly(U) filter chromatography to select poly(A)-terminated molecules. The selected preparations were then sedimented through SDS-sucrose gradients and fractions of the stated approximate "S" values collected. Each sample was then analyzed for Ad2-specific RNA and poly(A). The fraction of each sample as Ad2 sequences was divided by the fraction in poly(A) to provide an estimate of average Ad2 content per molecule.

the large molecules were precursors. The small relative amount of virus-specific RNA makes experiments employing labeling times equal to the synthesis time of such molecules virtually impossible.

The rapidly sedimenting virus-specific sequences might represent (1) the most newly made molecules which are intact but partially processed, i.e., poly(A) addition only, or (2) incompletely processed molecules, i.e., poly(A) added and the molecules nicked but not completely cleaved or (3) artifacts due to nonspecific aggregation of virus RNA with larger cellular sequences. In an attempt to investigate these possibilities the experiments depicted in Figures 4–6 were carried out.

Transformed cells were labeled with [^{3}H]uridine and nuclear RNA sedimenting between 15–32S was collected (Fig. 4A). A second culture was labeled with ^{32}P and the nuclei isolated. The 15–32S [^{3}H]RNA was added to the ^{32}P-labeled nuclei which were then lysed, and the total labeled RNA was extracted and sedimented through an SDS-sucrose gradient. Figure 4 shows very rapidly sedimenting ^{32}P-labeled HnRNA (40–70S) and that the ^{3}H-labeled RNA did not aggregate with the ^{32}P-labeled RNA but resedimented in the 15–32S region. Poly(U) chromatography was then performed on "large" (40–70S) and "small" (15–40S) HnRNA and the poly(A)-terminated molecules collected. In the poly(A)-terminated molecules from the "large" sample, the ^{32}P/^{3}H ratio was 9/1, whereas in the poly(A)-terminated molecules from the "small" sample, the ratio was about 2/1. Likewise, when virus-specific RNA was assayed in each sample, there was about 9-fold more ^{32}P than ^{3}H in the Ad2 RNA from the large sample, and about 1.8 times as much in the small sample. It appeared, therefore, that the large *virus-specific molecules* could not have been an artifact created by an aggregation of the virus-specific 20S RNA with larger RNA during the phenol extraction or subsequent sample preparation.

Both the "large" and "small" samples of poly(A)-terminated HnRNA were then sedimented through

Table 3. Localization of Ad2-specific Sequences within Poly(A)-terminated HnRNA

RNA prep.			Ad2 hybrid (cpm)	Ad2 hybrid (%)	Chain length calculated from poly(A) content[a]
Exp. 1	large (>40S) alkali, 2 sec	bound	358	69	6500
		wash	161	31	
Exp. 2	large (>40S) alkali, 2 sec	bound	561	71	5000
		wash	225	29	

Poly(A)-terminated [^{32}P]HnRNA selected by poly(U) filter chromatography (see Figs. 3 and 4) was ethanol-precipitated and dissolved in 0.1–0.2 ml NETS (0.1 M NaCl plus ETS, see Experimental Procedures and Table 1). One-fifth vol. of 1 N NaOH was added at 4°C and 2 sec later neutralized with 0.3 vol. of 1 M HEPES. This was diluted to 1–2 ml with 0.2 NETS and again subjected to poly(U) chromatography. "Bound" and "wash" fractions were assayed as in Table 1.

[a] 200/chain length = % of ^{32}P as poly(A).

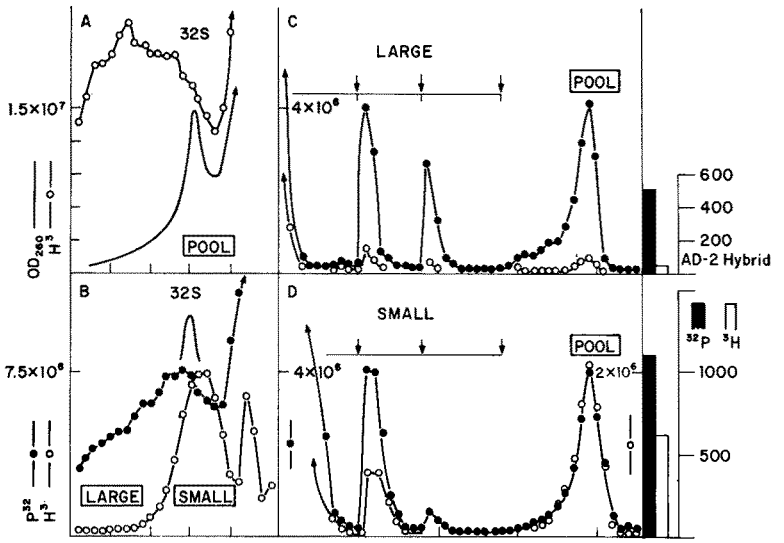

Figure 4. Origin of large HnRNA containing Ad2 sequences. [^{3}H]Uridine-labeled HnRNA (40 mCi[^{3}H]uridine, 20 mCi/mmole; 4×10^8 cells in 200 ml) was prepared and sedimented through sucrose-SDS gradients. The RNA from the 15–32S region was collected and added to the isolated nuclei from 4×10^8 cells labeled with ^{32}P as in Fig. 3. The total RNA was then extracted and sedimented through sucrose-SDS gradients. This preparation was divided into large and small samples as indicated and poly(A)-terminated HnRNA was selected from each sample *(C, D)*. The arrows on the elution diagrams represent first, wash with 10 ml of ETS; second, 10 ml of 10% formamide–90% ETS; third, 10–50% exponential formamide gradient in ETS. A portion of these selected molecules was hybridized to Ad2 DNA as shown on far right.

sucrose gradients in DMSO, a procedure that should reveal any breaks in the molecules (Fig. 5). The total radioactivity in the small samples sedimented with an average size of about 5000 nucleotides (<32S but greater than 18S), and the virus-specific RNA was almost exclusively (more than 90%) in the 20S region, with a ^{32}P/^{3}H ratio of about 2. The "large" sample had an average sedimentation for the total ^{32}P in excess of 8000 nucleotides (>32S). The Ad2-specific RNA, however, sedimented mainly (~80%) in the 20S region, while only about 20% of the total sedimented faster than the 20S virus-specific peak. Thus it appeared as if the large molecules contained "nicks" which did not disrupt the molecule until after denaturation. The ^{32}P/^{3}H ratio in this "derived" 20S peak was 7/1 in contrast to about 1.8/1 in the 20S RNA present in the "small" HnRNA, evidence against aggregation being responsible for the presence of "DMSO-derived" 20S Ad2 RNA from the large molecules. Presumably the large amount of internal base-pairing known to exist in HnRNA could be responsible for such nicked RNA molecules which remain associated (Jelinek et al. 1974). The breaks in the large molecules did not appear to be random, however, because the "derived" smaller Ad2 molecules sedimented predominantly in the 20S region, while the remaining cellular molecules were mainly larger than the 32S marker.

Finally, the extent to which such internal nicking occurs in long molecules was tested by putting a sample of total HnRNA containing all sizes of [^{32}P]HnRNA (but only the 15–32S [^{3}H]HnRNA) directly onto DMSO gradients without previous selection on columns in order to minimize the handling of the sample (Fig. 6). The sedimentation profile of this material showed that about 30% of the virus-specific [^{32}P]RNA sedimented faster than 20S and that the average size of the total [^{32}P]HnRNA was considerably larger than the selected poly(A)-terminated class of HnRNA. In addition, the total [^{3}H]HnRNA sedimented mainly

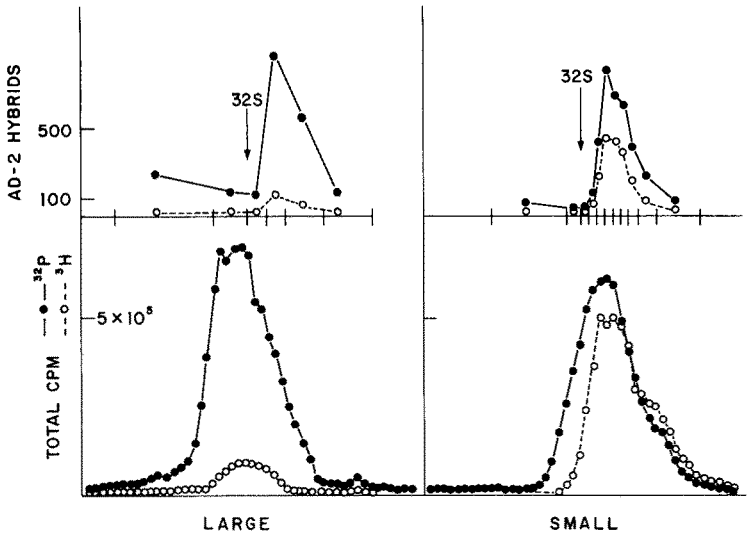

Figure 5. Nature of large HnRNA containing Ad2 sequences. The poly(A)-terminated HnRNA from the experiment of Fig. 4 was precipitated, dissolved and sedimented through DMSO gradients (see Fig. 2 and text). Total cpm and Ad2-specific RNA were then measured in the designated fractions of the separated RNA.

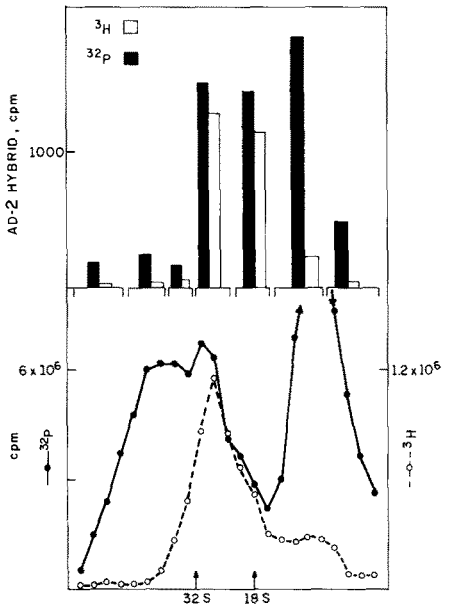

Figure 6. Sedimentation of total HnRNA and "small" HnRNA in DMSO. Total [^{32}P]HnRNA and a sample of 15–32S ^{3}H-labeled HnRNA were sedimented through DMSO and total radioactivity and Ad2-specific RNA measured.

from 15–32S, and the ^{3}H-labeled virus-specific RNA at about 20S, i.e., no shift in position compared to the SDS-sucrose gradient. Perhaps, therefore, in the total HnRNA compared to the poly(A)-terminated fraction there are slightly more large molecules containing virus-specific sequences.

DISCUSSION

In searching for nuclear RNA that is precursor to cytoplasmic mRNA, the use of poly(A) as a marker appears still to be valid. Evidence is summarized indicating that the kinetics of poly(A) accumulation in the nucleus and cytoplasm is consistent with complete transfer from the nucleus to the cytoplasm, although it is recognized that kinetic experiments of this sort cannot prove 100% transport.

Large nuclear poly(A)-terminated molecules containing virus-specific (Ad2) RNA were then identified. However, it was found that the great majority of poly(A)-terminated nuclear Ad2 sequences were in fact in a 20S species that sedimented similarly to the major 20S cytoplasmic Ad2 RNA. Moreover, even in the largest poly(A)-terminated Ad2-containing molecules, there were internal "nicks" in the molecules which upon denaturation appeared to yield specifically the 20S species. Evidence was also obtained that in the poly(A)-containing large molecules, the Ad2 region was preferentially next to poly(A) (Table 3). However, since the large molecules had internal nicks, it is not possible to tell whether the 3′ terminus to which poly(A) was added was actually a 3′ terminus due to RNA polymerase termination or an internal 3′ terminus created by endonucleolytic action.

Thus it is clear that if the pathway of biosynthesis of the 20S Ad2 RNA is first as part of a long molecule which is then processed (including both cleavage and poly(A) addition in unknown order), then a very small part—perhaps 5% or less (see Figs. 3 and 5)—of the *total* poly(A)-containing nuclear RNA represents unprocessed HnRNA transcripts. In order to characterize and measure the initial RNA polymerase transcription product from integrated viral genes or probably any other types of genes, it will be necessary to restrict labeling to very short times, interrupt processing somehow, or go to partial cell-free synthesis (isolated nuclei perhaps) or a combination of all these approaches. Ultimately these steps will be necessary to study regulation in the production of *transcripts* from mammalian genes.

Experimental Procedures

Cell growth of Ad2-transformed rat cells (line 8617, obtained from Dr. Aaron Freeman) as well as RNA labeling and preparation procedures of both mRNA and HnRNA have been described (Wall et al. 1973). All labeling was carried out after cells were treated for 25 min with 0.025 μg/ml actinomycin to suppress rRNA synthesis. Poly(U)-Sepharose chromatography for selection of poly(A)-terminated molecules has been previously described. In the present studies, columns prepared in Pasteur pipettes from glass fiber filters (3 1-inch GFC filters) impregnated with poly(U) as described by Sheldon et al. (1972) were used instead of poly(U) Sepharose. Best results for selection of poly(A)-terminated molecules were obtained by applying HnRNA (in 0.2 M NaCl, 0.01 M Tris pH 7.4; 0.01 M EDTA, 0.2% SDS = 0.2 NETS) and washing the columns with 10 ml of the 0.2 NETS buffer, followed by 10 ml of the same buffer lacking the NaCl (ETS), then 10 ml of the ETS plus 10% formamide, and finally, the column was eluted with 30 ml of a 10–50% formamide-ETS gradient. RNA-DNA hybridization procedures were performed as previously described (Wall et al. 1973).

Sedimentation in sucrose-NETS (0.1 M NaCl, EDTA, Tris, SDS as described above) was carried out by layering RNA samples dissolved in 1 ml of 0.1 NETS onto 12 ml of 15–30% w/w sucrose sedimenting at 40,000 rpm, 28°C, for sufficient time so that marker rRNA or precursor rRNA had migrated a predetermined, desired distance. All results show position of marker RNA.

Sedimentation in DMSO was achieved by dissolving RNA (after ethanol precipitation) in 0.05 ml of ETS; 0.05 ml of dimethyl formamide was then added and the sample incubated at 37°C for 5 min followed by the addition of 0.45 ml of DMSO. After a few minutes of further incubation at 37°C, the sample was layered on 5–20% sucrose gradient prepared in DMSO. Sedimentation conditions were chosen then which sedimented rRNA or pre-rRNA markers on desired distance (e.g., 40,000 rpm for 26 hr sedimented 45S RNA about 50% of the distance down a 12.5-ml gradient in an SW40 Spinco tube).

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