

SV40-Specific RNA in the Nucleus and Polyribosomes of Transformed Cells*

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Abstract. Cells transformed by the oncogenic virus SV40 are known to contain viral DNA integrated into cellular DNA and to produce virus-specific RNA. It has been shown that nuclear molecules containing virus-specific sequences are considerably longer than presumed virus-specific mRNA molecules from cytoplasmic polyribosomes. This finding suggests the possibility that cytoplasmic mRNA is derived by the specific cleavage of larger nuclear RNA.

Introduction. Since the demonstration of messenger RNA (mRNA) in bacteria,¹ the nuclear origin of mRNA in eukaryotic cells has been widely assumed. Studies of presumed nucleo-cytoplasmic transport of mRNA in mammalian cells have been obscured by a type of very high molecular weight nuclear RNA (heterogeneous nuclear RNA) which shares some chemical characteristics of mRNA. Unlike mRNA, however, heterogeneous nuclear RNA turns over rapidly within the cell nucleus without apparently participating in protein synthesis.²⁻⁶ In HeLa cells over 90 per cent of this RNA never leaves the cell nucleus, and its average half life is less than five minutes.⁶

We have been examining the hypothesis that, while most of the heterogeneous nuclear RNA is made and destroyed within the nucleus, some small portion of it might serve as a source of mRNA. Recent experiments comparing RNA-DNA hybridization of mRNA from cytoplasmic polyribosomes and heterogeneous nuclear RNA indicated a sequence resemblance in the two types of RNA that is compatible with, but not proof of, the origin of mRNA from heterogeneous nuclear RNA.^{7, 8}

In this paper we report the analysis of heterogeneous nuclear RNA and polysomal RNA of cells transformed by the oncogenic virus SV40, which are known to contain the equivalent of 20 viral genomes included in cellular DNA molecules at least 10^8 in molecular weight.^{9, 10} It would appear, therefore, that these viral genes have become part of the genome of the host cell. RNA transcribed from such integrated viral DNA can be demonstrated in the total RNA from transformed cells. It has now been found that both the very high molecular weight heterogeneous nuclear RNA and polysomal mRNA, which is of lower molecular weight, contain viral specific RNA. This result again suggests the possibility that some portion of the large nuclear molecules can give rise to cytoplasmic mRNA.

Methods and Materials. Labeling procedures of cells and cell fractionation: 3T3 cells¹³ and 3T3 cells transformed by SV40 virus (SV3T3-47 clone)¹⁰ were provided by Dr. Howard Green. The cells were grown as monolayers in Eagle's medium containing 10% fetal calf serum and nonessential amino acids.¹⁴ Labeling of the cells was accomplished by draining all but approximately 0.5 to 1 ml of medium from a culture of 1410 cm² and overlaying for 3 hr with 5 ml of medium containing 5–10 mCi of ³H-uridine. The bottles were then buried in ice and the chilled cells washed with phosphate-buffered saline (PBS)¹⁵ and harvested with a rubber policeman or by trypsin-EDTA treatment (0.05% Difco trypsin, 0.01 M EDTA in PBS). After washing twice in medium lacking serum, the cells were suspended at 10⁸ cells/ml in an isotonic buffer, iso-Hi-pH (0.14 M NaCl; 0.01 M Tris, pH 8.4; 0.0015 M MgCl₂). In order to protect polyribosomes in the extracts of the transformed cells, a high-speed supernatant (150,000 g × 45 min) of a cytoplasmic extract of HeLa cells (extract of 10⁸ HeLa cells/10⁸ 3T3 cells) prepared without deoxycholate according to the method of Penman *et al.*¹⁶ was added to the suspended SV3T3-47 cells. Cell lysis was accomplished by the addition of 1/10 vol of 5% NP 40 (a nonionic detergent from Shell Chemical Co.) followed by Dounce homogenization (four strokes) to ensure rupture of all the cells. Nuclei were sedimented at 1000 rpm for 5 min and washed once with isotonic buffer containing high-speed extract of HeLa cells and NP 40 as above. The cytoplasmic fraction was layered on sucrose gradients (7–47% sucrose w/w in iso-Hi-pH buffer) for separation of the polyribosomes by centrifugation (SW 27 rotor; 210 min, 26,000 rpm, 4°C). RNA from polyribosomes was isolated as previously described and recovered after sucrose gradient sedimentation (23,000 rpm, 16 hr, 25° through 15 to 30% sucrose in SDS buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.01 M EDTA, 0.2% SDS).⁸ The washed nuclei were resuspended and digested with DNAase in high salt buffer^{7, 17} and the RNA recovered after extraction with hot phenol and sucrose gradient sedimentation (15–30% sucrose in SDS buffer, 16 hr, 16,000 rpm, 25°).⁷

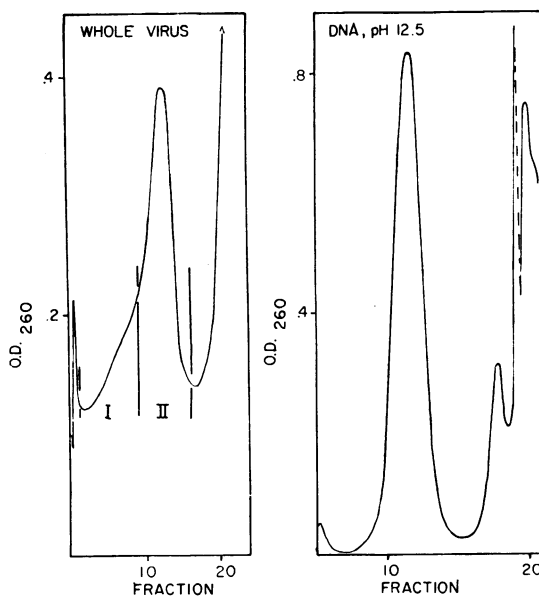
RNA samples to be treated with DMSO (dimethyl sulfoxide, Fisher, analytical) were precipitated with 2 vol of ethanol in the presence of 0.5 M LiCl and redissolved in DMSO containing 0.01 M LiCl.¹⁸ The treated samples were then diluted 1 to 3 with SDS buffer and centrifuged through a 15 to 30% (w/w) sucrose gradient as described above for RNA not treated with DMSO. Before being used in hybridization experiments the RNA preparations were precipitated in 2 vol of ethanol, redissolved in 0.1 × SSC (standard saline citrate = 0.15 M NaCl + 0.015 M NaCitrate),¹⁹ boiled for 10 min, and made equal to 2 × SSC and filtered through both nitrocellulose columns²⁰ and through a Millipore filter.

Virus: Preliminary studies on ³H-thymidine-labeled SV40 virus were done with the advice of Dr. E. Winocour. The following technique was worked out for purification of virus and viral DNA from a commercially obtained SV40 virus stock (Microbiological Associates) grown in BSC-1 cells.²¹ The yield was approximately 50% of closed circular viral DNA/100 ml of stock suspension.

Frozen virus stock containing the cell debris (stored at –20°C) was thawed and centrifuged at 60,000 rpm for 90 min. The gelatinous pellet was resuspended in a glass homogenizer in 1/100 vol of 0.05 M Tris buffer containing 0.01 M EDTA and 1% deoxycholate. The suspension was then sonicated at 4° in a well-tuned MSE ultrasonic device for 45 sec. The virus suspension was layered on a sucrose gradient (5–20% w/w sucrose in a buffer containing 0.01 M Tris, pH 8.8, 0.01 M NaCl, 0.01 M EDTA) and centrifuged at 27°C for 80 min to yield patterns similar to that shown in Figure 1A. (Both sections of the gradient marked I and II contained viral DNA and they were usually pooled.) The material in the OD₂₆₀ peak (Fig. 1A) which was shown to correspond to authentic ³H-thymidine-labeled SV40 virus, was isolated and collected by centrifugation (60,000 rpm; 90 min; +40°C). The pellet of purified virus was resuspended in 0.01 M Tris buffer (pH 7.4) containing 0.01 M EDTA, and DNA was released either by phenol extraction followed by dialysis against the same buffer or by dissolving the virus pellet in buffer containing 0.5% sarcosyl (Geigy). The solution was then made alkaline (pH

FIG. 1.—*Left panel:* Profile of absorption (OD_{260}) in a sucrose gradient during zonal sedimentation step in purification of SV40 virus (see *Materials and Methods* for details).

Right panel: SV40 DNA sedimentation in alkaline sucrose gradient. Peak near top of gradient is presumably Form II and linear DNA.²⁹



12.5) by the addition of 6 *M* NaOH and the DNA sedimented through alkaline sucrose (10–20% sucrose in 0.1 *M* NaCl containing 0.001 *M* EDTA, pH 12.5 for 20 hr at 22°K (Fig. 1B)). The position of the leading peak was confirmed as >40S by comparison with RNA sedimented in a buffer of the same ionic strength. Moreover, the leading peak was shown to sediment much faster above pH 12.5 than at pH 10 or below indicating it was closed circular viral.^{10, 21} The peak fractions were pooled and dialyzed against 0.01 *M* Tris, pH 7.4, 0.01 *M* EDTA.

For hybridization, virus DNA was denatured by boiling for 15 min²¹ and attached to filters (1γ/filter) in 5 × SSC according to Gillespie and Spiegelman.²² Retention of DNA by the filters under hybridization conditions was measured using ³H-thymidine-labeled DNA and found to be greater than 85%. The hybridization reaction was performed at 65° in 5% SSC containing 0.5% SDS in a volume of 1 ml.

Results. Figure 2 shows sedimentation patterns of radioactive nuclear RNA and polysomal RNA from SV40-transformed cells which were labeled for three hours. The predominant nuclear RNA peaks, which correspond to small shoulders in the OD_{260} profile at 45 and 32S (determined by sedimenting HeLa cell nuclear RNA in a parallel gradient), contain large amounts of radioactivity and these regions of the gradient are presumed to contain the ribosomal precursor RNA molecules.²³ In the polyribosomes, the 28S and 18S ribosomal RNA (rRNA) were both labeled, although there is not a constant specific activity of RNA over the entire gradient, indicating a mixture of labeled mRNA with rRNA. The nuclear RNA accounted for about 60 to 65 per cent of the label in these cells after a three-hour exposure to ³H-uridine. The lack of radioactive 28S and 18S rRNA in the nucleus as well as the lack of 45S and 32S ribosomal precursor RNA in the cytoplasm argue for the effective separation of these cells into nuclear and cytoplasmic fractions by the technique used.²⁴

Nuclear RNA molecules sedimenting faster and slower than the 45S ribosomal precursor RNA and polysomal RNA sedimenting slower than 28S RNA were collected after sedimentation analysis, and exposed to filters containing 1γ of

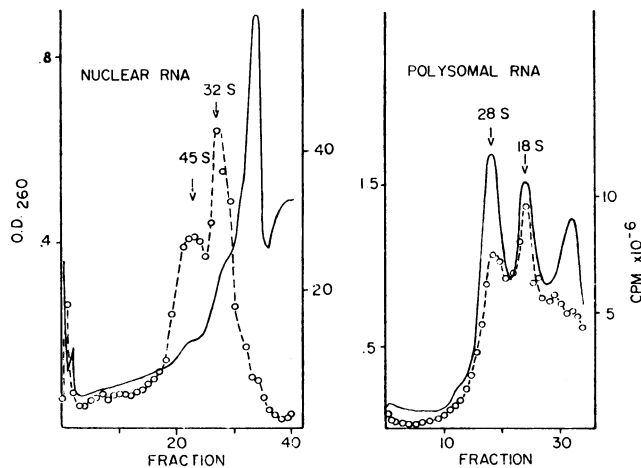


FIG. 2.—*Left panel:* Zonal sedimentation profile of radioactive nuclear RNA from 3T3-47 cells. Location of 32S and 45S were taken from HeLa cell RNA in another tube. Counts per minute (cpm) represent total recovered from about 4.5×10^8 cells labeled with 15 mCi of ^3H -uridine. Only about 0.1% of the total was actually counted. (OD₂₆₀, solid line; cpm, ○).

Right panel: Sedimentation profile of radioactive RNA from polyribosomes. Counts per minute (cpm) represent total recovered from about 3×10^8 cells labeled with 10 mCi of ^3H uridine of which about 1% was counted.

SV40 DNA or no DNA. A summary of several experiments is contained in Tables 1 and 2.

Although the fraction of the input RNA which hybridized was very small, from 0.002 to 0.03 per cent, all of the RNA classes tested from SV3T3-47 cells contained some RNA which hybridized to SV40 DNA. Previous work had shown that the total rapidly labeled RNA from polyoma-transformed and SV40-transformed cells was approximately 0.01 per cent viral specific.^{11, 12} The small amount of RNA hybridized was not due to limiting amounts of DNA on the filters, since larger amounts of RNA in the hybridization reaction resulted in a proportionate increase in the amount of RNA bound (Table 1). In various experiments the amount of SV-40-specific RNA from the polyribosomes was fairly constant (from 0.012 to 0.04% of the input) while the amount of nuclear RNA in both the larger than 45S and smaller than 45S regions seemed to vary somewhat more. Whether these variations result from uncontrolled factors in the cell cultures or in preparation of the RNA, or represent real changes in the output of viral specific RNA as the culture continues to grow remains to be seen.

The amount of radioactivity bound to filters containing no DNA was from 5 to 30 per cent that specifically bound. RNA from nontransformed 3T3 cells bound to SV40 DNA only 5 to 10 per cent as well as transformed cell RNA, and only small differences were observed between SV40 DNA filters and blank filters. These results indicate that SV40-specific RNA is contained in both nuclear and cytoplasmic RNA from transformed cells. Moreover, some of the viral-specific nuclear RNA seems to have a considerably higher molecular weight than the viral-specific RNA from the polyribosomes. In order to assure that the rapidly

sedimenting molecules were not aggregates of any sort, ^3H nuclear RNA from transformed cells was sedimented after treatment with DMSO (Expts. 3 and 4, Table 1; Fig. 3), a solvent known to denature double-stranded nucleic acids.¹⁸ The sedimentation of the RNA after DMSO treatment was quite similar to untreated samples examined in the same buffer, indicating that the majority of molecules which sedimented faster than 45S were not aggregates.

TABLE 1. *Hybridization of various RNA samples to SV40 DNA.*

Cell	Type of RNA	Expt.*	Input cpm $\times 10^{-6}$	Cpm bound to 1γ SV40 DNA†	Blank	Filters‡	% Input bound		
3T3-47 (transformed)	Nuclear, >45S	1	0.48	110	48	3	0.013		
			0.96	160	45	3	0.012		
			1.4	200	50	3	0.011		
		2	0.32	71	56	2	0.005		
			0.64	109	30	2	0.009		
			0.5	86	30	3	0.011		
		3	1.0	145	33	3	0.011		
			1.5	195	28	3	0.011		
			2.2	81	40	3	0.002		
		4	6.6	192	52	2	0.002		
			Nuclear RNA, 30-45S	1	0.5	120	50	3	0.017
					1.0	200	30	3	0.017
	1.5	298			50	3	0.018		
	2	0.5		128	49	3	0.016		
		1.0		197	49	3	0.013		
		1.3		262	3	3	0.016		
	3	0.5	123	30	3	0.019			
		1.0	180	30	3	0.015			
		1.5	290	3	3	0.017			
	4	3.1	241	4	4	0.0068			
		6.2	459	37	4	0.0068			
		12.4	861	44	3	0.0066			
	3T3	Polysomal RNA, 6-30S	1	0.125	78		2	0.026	
				0.250	107	45	2	0.025	
0.300				122	47	2	0.025		
2			0.350	186	50	2	0.039		
			0.1	42		3	0.032		
			0.2	55	10	3	0.022		
3			0.3	72	10	3	0.021		
			0.45	131	42	2	0.021		
			0.86	308	50	2	0.030		
Nuclear, >45S				1.7	576	31	2	0.032	
				1.9	35	19	3	0.0008	
				3.0	23	23	3	0.0000	
				6.0	34	21	3	0.0002	
				<45S	2.0	36	40	3	0.0000
					4.0	57	52	2	0.0001
8.0	44	63	2		0.0000				

* Experiment nos. 1 and 2 were performed with RNA not treated with DMSO; Expt. nos. 3 and 4 were with DMSO treated RNA.

† Counts per minute (cpm) represent averages of number of filters shown in next column.

‡ Refers to number of filters containing SV40 DNA, usually only one or two blank filters were used.

TABLE 2. *Distribution of SV40-specific RNA in 3T3-47 cells.*

Expt.*	Type of RNA	Total cpm in fraction†	Total hybridizable cpm in fraction‡
1	Nuclear > 45S	6.7×10	605
	30-45S	16.3	2670
2	Polysomes		
	6-30S	7.1	1920
	Nuclear > 45S	93	2100
	32-45S	137	5500
3	20-32S	64.0	5150
	Polysomes		
	6-30S	36	9000
	Nuclear > 45S	193	3900
	32-45S	207	14100
	30-32S	50	8500
	Polysomes		
	6-30S	120	37000

* Experiment 1 was performed with RNA not treated with DMSO; Expts. 2 and three were with DMSO treated RNA.

† Represents cpm recovered in purified samples.

‡ Calculated from average hybridization of a series of samples of 0.5 to 4×10^6 cpm bound on a series of filters (see Table 1).

In addition, approximately the same degree of hybridization was found with the DMSO-treated RNA as with the large nuclear RNA which had not been subjected to DMSO treatment (Fig. 3, Table 2). This indicates that the large hybridizing molecules from the nucleus were not aggregates between small viral-specific and large cellular molecules.

These experiments do not allow a precise assessment of the size of the molecules containing SV40-specific sequences, but they do allow certain conclusions. Since the buffer in which the preparative sedimentation was performed had an ionic strength greater than 0.2, all types of RNA molecules (both heterogeneous nuclear and ribosomal precursor RNA) would be quite compact. Size comparisons of RNA by sedimentation are much more reliable under these conditions than in low salt concentrations. For example, both poliovirus RNA molecules and the RNA from MS-2 bacteriophages sediment slower in low salt than the larger ribosomal RNA molecule from their respective host cells. In high salt concentration (0.1 M) however, both virus molecules sediment approximately in proportion to their known molecular weights, using the equation of Spirin.²⁷ The nuclear molecules in the present experiments were taken from the region of a sucrose gradient from 30 to 45S or from the 45S and more rapidly sedimenting region. The 32S ribosomal precursor is known to be 2.4×10^6 daltons and the 45S $4.1-4.5 \times 10^5$ daltons.²⁸ Thus the majority of nuclear molecules with SV40 sequences are larger than approximately 2×10^6 daltons, and some must be at least 4×10^6 daltons. Since the double-stranded SV40 virus DNA molecule is 3×10^6 , these nuclear RNA molecules are considerably larger than a single strand of SV40 DNA.

It is also clear that the majority of the nuclear molecules are larger than the polysomal molecules (6-30S) if the polysomes were intact after isolation. The technique of polyribosome preparation used in the present work was based on the experiments of Scharff and Uhr³⁰ who used HeLa cell extract to counteract the

endogenous nuclease action of lymphocytes in order to study the size of polyribosomes on which gamma globulins are synthesized. Figure 4 shows that, in the presence of HeLa cell extract, polyribosomes from SV40-transformed cells containing labeled nascent protein could be observed. No (or very little) radioactive protein was seen on single ribosomes and there are relatively few dimers and trimers in the polysome preparation. These tests indicate that the polysomes are very well preserved by the isolation procedure and that the viral specific RNA from polyribosomes was probably not degraded.

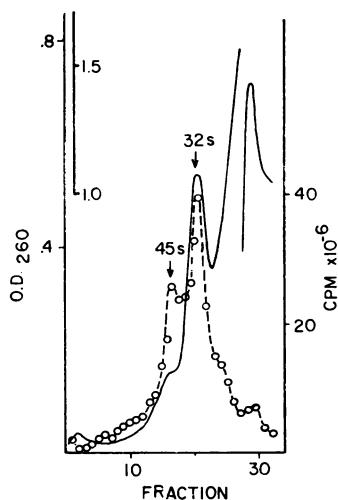


FIG. 3.—Sedimentation of nuclear RNA from 3T3-47 cells after DMSO treatment (see *Materials and Methods* and Fig. 2 for details).

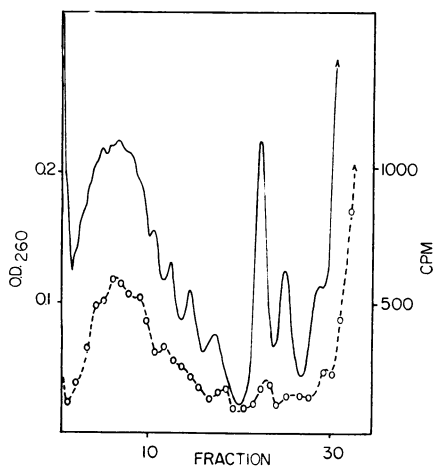


FIG. 4.—Polyribosome profile from 3T3-47 cells. About 10^8 cells were exposed to $30 \mu\text{Ci}$ algal protein hydrolysate for 1 min at 37° in Eagle's medium with $1/50$ the normal amino acid concentration. The cells were broken in presence of HeLa cell extract and analyzed by sedimentation (see *Materials and Methods*).

Discussion. It has been known for several years that nonvirus-producing cells transformed by polyoma virus or by SV40 virus contained RNA which would hybridize specifically to the homologous viral DNA.^{11, 12, 21} Furthermore, cells transformed by adenoviruses had been shown to contain adenovirus-specific RNA in polyribosomes as well as in nuclear RNA.³¹ The aim of the present experiments was to use this system to attempt to study the nuclear transcription products of "cellular genes" (integrated viral genes) when it could be shown that these same genes were contributing mRNA to the cytoplasm. The results indicate that there are nuclear molecules larger than 4×10^6 and polysomal molecules less than 1.7×10^6 which contain RNA sequences complementary to SV40 DNA. No proof is offered in this paper that the large molecules are precursors to the smaller ones but the findings make this hypothesis a definite possibility. Experiments designed to investigate a possible flow of molecules from large heterogeneous nuclear RNA to polysomes are clearly worth attempting.

Since the largest nuclear molecules containing SV40-specific sequences are longer than one SV40 DNA molecule they must either contain cellular RNA co-

valently linked to viral specific RNA or the SV40 DNA molecules must be integrated at least several at a time. Selection by hybridization of very high molecular weight RNA molecules containing SV40 sequences should permit an investigation of the nature of any covalently attached host cell sequences. If nuclear molecules were found to be "hybrid" in this sense, it would be of interest to determine if cytoplasmic SV40-specific molecules also contained cellular sequences.

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