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Virus-Specific Ribonucleic Acid in the Nucleus and Cytoplasm of Rat Embryo Cells Transformed by Adenovirus Type 2

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Nuclei were isolated from rat embryo cells transformed by adenovirus type 2. Nuclear and cytoplasmic virus-specific ribonucleic acids (RNA) were characterized and quantitated by deoxyribonucleic acid (DNA)-RNA hybrid formation with adenovirus DNA. The results indicate that most, if not all, virus-specific RNA molecules are synthesized in the cell nucleus and subsequently transported into cytoplasm where they degrade with a half-life of 1 to 2 hr. No difference in base sequences between nuclear and cytoplasmic virus-specific RNA species can be detected by hybridization competition experiment with viral DNA.

Virus-specific ribonucleic acid (RNA) is transcribed in cells transformed by adenoviruses which synthesize no infectious virus (4-6, 9). Adenovirus type 2 (Ad 2) has not induced tumors in newborn rodents, but Freeman et al. (3) have shown that Ad 2 (a group C adenovirus) transform rat embryo cells morphologically in vitro. Hybridization competition studies show that 4 to 10% of the viral genome (one to five viral genes) are transcribed in rat embryo cells transformed by Ad 2 (8).

It was of interest to investigate further the properties of nuclear and cytoplasmic virus- and cell-specific RNA in Ad 2-transformed cells. We report here studies on nuclear and cytoplasmic virus-specific RNA species, the synthetic pattern, transportation from nucleus to cytoplasm, half-life, and nucleotide sequence homology.

MATERIALS AND METHODS

Cells. The growth of Ad 2-transformed rat embryo cells and human KB cells has been described (8). Cells labeled with a radioactive precursor were harvested by centrifugation at 1,200 rev/min for 10 min and washed with cold phosphate-buffered saline.

Separation of nuclear and cytoplasmic fractions. The procedures described by Borun et al. (1) and Penman (15) have been modified. The cell pellet was resuspended in RSB buffer [1×10^{-2} M NaCl, 1×10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 1.5×10^{-3} M $MgCl_2$, *p*H 7.4] plus 0.2% Nonidet P-40 (NP-40) (Shell Chemical Co.) and incubated at 0 to 4 C for 15 min.

Nuclei were centrifuged at 2,200 rev/min for 10 min and washed with RSB containing NP-40, and the supernatant fractions were pooled as cytoplasmic fraction. Nuclei were free of cytoplasmic contaminants by phase microscopy.

Isolation of RNA from nuclei. The nuclear pellet was suspended in HS buffer (0.5 M NaCl, 5×10^{-2} M $MgCl_2$, 1×10^{-2} M Tris-hydrochloride, *p*H 7.4) (15) and treated with deoxyribonuclease (50 μg/ml, electrophoretically purified, Worthington Biochemical Co.) for 30 min at room temperature. Nuclear RNA was extracted as described previously (5, 18). RNA was precipitated with ethanol, treated again with deoxyribonuclease and phenol, and dialyzed against $0.1 \times$ SSC (SSC is 0.15 M NaCl, 1.5×10^{-2} M sodium citrate).

Cytoplasmic RNA. Cytoplasmic RNA was purified by the hot phenol-sodium dodecyl sulfate (SDS) method (15).

DNA preparations. Viral deoxyribonucleic acid (DNA) was isolated from purified virus by the method of Green and Piña (11, 12) using adenovirus types described previously (8). Cellular DNA was purified by Marmur's procedure (11).

DNA-RNA hybridization. DNA-RNA hybridization was performed by the procedure of Gillespie and Spiegelman (10) with minor modifications (7).

Hybridization competition. Membrane filters containing immobilized DNA were incubated with unlabeled RNA in 1 ml of $2 \times$ SSC-0.1% SDS for 20 hr at 66 C. ^{3}H-RNA from the Ad 2-transformed

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rat embryo cells in 0.4 ml of 2 × SSC-0.1% SDS was added, and the filters were incubated further at 66 C for 20 hr. The filters were processed (7).

RESULTS

Separation of nucleus from cytoplasm. The separation of nuclei and cytoplasm of Ad 2-transformed rat embryo or KB cells was evaluated by labeling cells with ^{3}H-thymidine. Nuclear and cytoplasmic fractions were prepared with 0.1, 0.2, or 0.5% NP-40. As shown in Table 1, less than 4% of the acid-insoluble radioactivity was present in the cytoplasmic fraction when 0.1 to 0.5% NP-40 was used. Further experiments used 0.2% NP-40. Nuclei were free of cytoplasmic contaminants by phase microscopy.

Incorporation of ^{3}H-uridine into virus-specific RNA of nuclear and cytoplasmic fractions. Ad 2-transformed rat embryo cells were labeled with ^{3}H-uridine, and the virus-specific RNA content of the nuclear and cytoplasmic RNA fractions was determined. Viral RNA was detected in the nucleus after 30 min of incorporation and in the cytoplasm at later times (Fig. 1a). The amount of viral RNA in both fractions increased in proportion to the labeling time, but the ratio of viral RNA in the nucleus to that in the cytoplasm decreased with increasing labeling time (Fig. 1b).

Stability of viral RNA. After labeling Ad 2-transformed rat embryo cells with ^{3}H-uridine for 12 hr at 37 C, the medium was replaced with medium containing unlabeled uridine and actinomycin D and the cells were incubated further at 37 C. Samples were removed after the indicated periods of time. As shown in Fig. 2, nuclear viral ^{3}H-RNA decreased 41% at 1 hr, 79% at 2 hr, and 95% at 4 to 24 hr. The faster decrease of nuclear viral RNA than cytoplasmic viral RNA was reproducible in several experiments.

Relationship between nuclear and cytoplasmic viral RNA. Hybridization competition analysis was performed with RNA isolated from the nucleus and cytoplasm of Ad 2-transformed rat embryo cells. Unlabeled nuclear and cytoplasmic

TABLE 1. *Acid-insoluble radioactivity in cytoplasmic fraction from ^{3}H-thymidine-labeled cells*[a]

Cell	Nonidet P-40 (%)	Whole cell: nucleus + cytoplasm (×10^2 counts per min per 100 ml of culture)	Cytoplasm (×10^2 counts per min per 100 ml of culture)	Radioactivity in cytoplasm
Ad 2-transformed rat embryo	<0.1	8,636	248	2.9
	0.2	7,385	285	3.9
	0.5	8,210	234	2.8
KB	0.1	7,685	169	2.2
	0.2	8,096	154	1.9
	0.5	7,302	196	2.7

[a] Cells in suspension (2 × 10^5 to 5 × 10^5/ml) were labeled with ^{3}H-thymidine (0.5 μCi/ml, 15.5 Ci/mmole, thymidine-*methyl*-^{3}*H*, Schwarz BioResearch) for 16 to 18 hr at 37 C. Nuclear and cytoplasmic fractions were prepared, and acid-insoluble radioactivity of whole cells and cytoplasmic fractions was measured after precipitation with 2.5% perchloric acid.

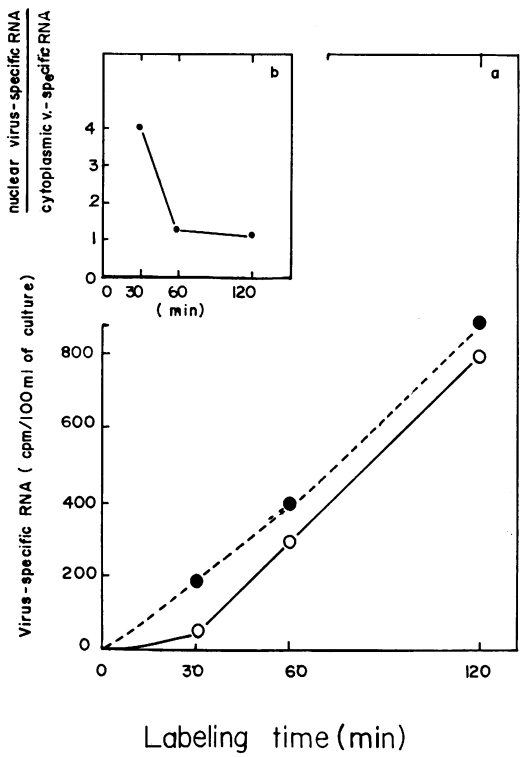

FIG. 1. *Incorporation of ^{3}H-uridine into nuclear and cytoplasmic virus-specific RNA. Ad 2-transformed rat embryo cells (10^6/ml) were labeled with 8 μCi of ^{3}H-uridine per ml (uridine-5-T, Amersham, 22.7 Ci/mmole) at 37 C. Samples were harvested at indicated intervals, and nuclear and cytoplasmic RNA was prepared as described in the text. The amounts of labeled viral RNA were estimated by hybridization with excess viral DNA immobilized on nitrocellulose filter (Fig. 1a). In Fig. 1b, the ratio of labeled viral RNA in the nucleus to that in the cytoplasm was plotted against the time of labeling. Nuclear ^{3}H-RNA (●-----●); cytoplasmic viral ^{3}H-RNA (○——○).*

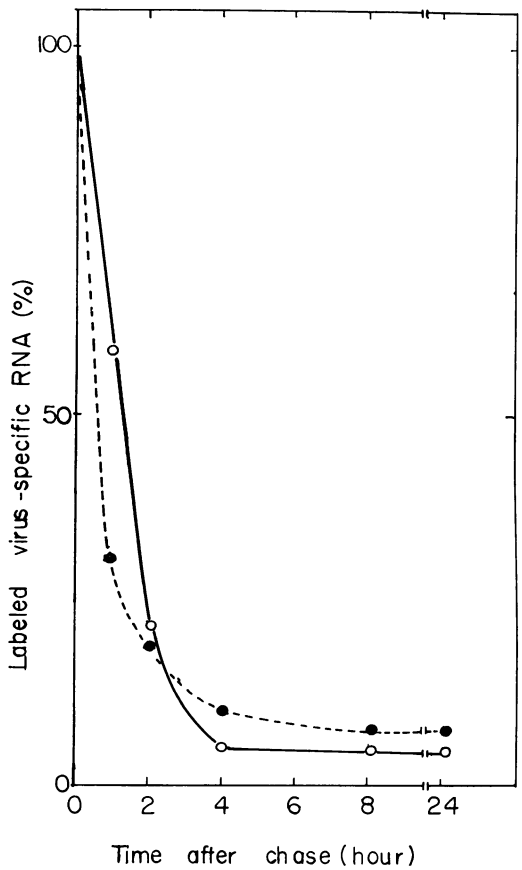

FIG. 2. *Stability of labeled viral RNA. Ad 2-transformed rat embryo cells (10^6/ml) were labeled with 8 μCi of ^{3}H-uridine per ml at 37 C for 12 hr. The medium was replaced and 1 μg of actinomycin D per ml and 5×10^{-4} M unlabeled uridine were added. Cells were incubated further at 37 C, and samples were removed after 0, 1, 2, 4, 8, and 24 hr. The amount of labeled viral RNA in the nuclear and cytoplasmic fractions was estimated by hybridization with excess viral DNA. Nuclear ^{3}H-RNA (●-----●); cytoplasmic viral ^{3}H-RNA* (○——○).

RNA inhibited hybrid formation between labeled cytoplasmic ^{3}H-RNA and viral DNA over 80% (Table 2). Similarly, unlabeled cytoplasmic and nuclear RNA inhibited hybrid formation between labeled nuclear RNA and Ad 2 DNA over 90%, and the same nuclear and cytoplasmic RNA preparations of uninfected KB cells did not inhibit hybrid formation significantly (Table 2). Unlabeled nuclear and cytoplasmic RNA inhibited equally well hybrid formation between labeled cytoplasmic ^{3}H-RNA and cellular DNA (Fig. 3). On the contrary, the hybrid formation between labeled nuclear ^{3}H-RNA and cellular DNA was inhibited to 90% by unlabeled nuclear RNA, but only to 30% by unlabeled cytoplasmic RNA (Fig. 4), indicating that most of the nuclear RNA synthesized is restricted to the nucleus. In both cases, KB cell RNA did not inhibit hybrid formation. These results strongly suggest that most, if not all, of the nucleotide sequences of nuclear and cytoplasmic virus-specific RNA species are identical.

DISCUSSION

Labeled viral RNA was at first detected in the nucleus by the incorporation of ^{3}H-uridine. The ratio of labeled viral RNA in the nucleus to that in the cytoplasm decreased with increasing labeling time. Chased with unlabeled Ur and actinomycin D, labeled viral RNA started to decrease in the nucleus earlier than in the cytoplasm. These results strongly suggest that virus-specific RNA is synthesized in the nucleus and transported into the cytoplasm. Hybridization competition experiments between nuclear and cytoplasmic viral RNA species show that they share, if not all, most of the nucleotide sequences. However, difference in minor components cannot be excluded, for the condition of DNA excess is not perfectly attained in hybridization competition. From the above results, we conclude that most of the virus-specific RNA species synthesized in the nucleus are transported into the cytoplasm. Therefore, virus-specific RNA differs from most cellular nuclear RNA, of which over 80% is retained in the nucleus of the cells. Existence of such nuclear-specific RNA species has been previously reported in L cells (16).

Heterogeneous nuclear RNA has been established as an entity having the character of specific localization in the nucleus, rapid turnover rate, heterogeneous high molecular weight, and high hybridizability to cellular DNA (17). Recently, nuclear viral RNA has been demonstrated in the cells transformed by SV-40, which has a molecular size larger than corresponding to the whole viral genome (13). Similar viral RNA has been observed in the nucleus of Ad 2-transformed rat embryo cells (Sekikawa et al., *unpublished data*). A hybrid molecule of viral RNA covalently linked to cellular RNA seems to be synthesized in the nucleus of the same transformed cells (2). These results suggest that viral RNA is transcribed as a hybrid RNA molecule bound covalently to cellular RNA, and at least a portion including viral RNA seems to be transported into the cytoplasm.

It will therefore be interesting to investigate in detail the transport mechanism of viral RNA from nucleus to cytoplasm in relation to that of cellular RNA.

TABLE 2. *Nucleotide sequence homology between virus-specific RNA species present in nucleus and cytoplasm: hybridization competition experiment*[a]

Labeled RNA (input counts/min)	Cold RNA (μg/filter)		Ad 2 DNA (μg/filter)	Counts/min bound[b]	Percent of control[c]
Ad 2 cytoplasmic[d] (5.10×10^6)	Ad 2 cytoplasmic	1,500	0.2	98	29
		1,200	0.2	106	32
		800	0.2	174	52
	Ad 2 nuclear[e]	600	0.2	63	19
		400	0.2	123	37
		60	0.2	287	86
	KB cytoplasmic[f]	1,500	0.2	324	96
	KB nuclear[g]	600	0.2	332	99
	2 × SSC-0.1% SDS		0.2	336	100
Ad 2 nuclear (9.86×10^5)	Ad 2 cytoplasmic	1,500	0.4	12	6
		1,200	0.4	23	12
		800	0.4	21	11
	Ad 2 nuclear	600	0.4	14	7
		400	0.4	33	17
		60	0.4	40	21
	KB cytoplasmic	1,500	0.4	152	78
	KB nuclear	600	0.4	164	84
	2 × SSC-0.1% SDS		0.4	196	100

[a] Labeled RNA was isolated from the nucleus and cytoplasm of Ad 2-transformed rat cells (10^6/ml) labeled for 6 hr with ^{3}H-uridine (8 μCi/ml, 22.7 Ci/mmole). Ad 2 DNA was hybridized first with different amounts of unlabeled RNA (cold RNA) for 20 hr at 66 C. The DNA filter was incubated further for 20 hr at 66 C with labeled RNA in the presence of cold RNA. Hybrid formation of labeled RNA with viral DNA was measured as described in the text.

[b] Background (counts per minute bound to an empty filter) subtracted.

[c] Radioactivity bound to DNA filter preincubated in 2 × SSC-0.1% SDS was normalized to 100%.

[d] Cytoplasmic RNA from adenovirus type 2-transformed rat embryo cells.

[e] Nuclear RNA from adenovirus type 2-transformed rat embryo cells.

[f] Cytoplasmic RNA from KB cells.

[g] Nuclear RNA from KB cells.

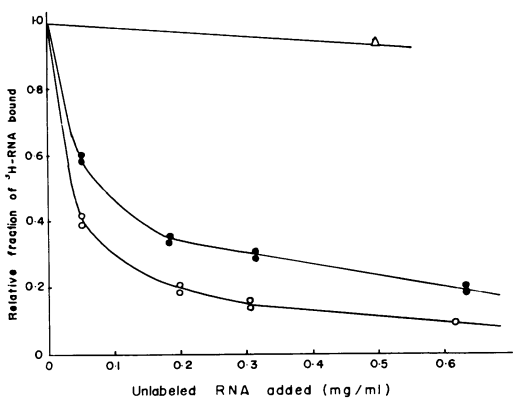

FIG. 3. *Hybridization competition of cellular RNA: cytoplasmic RNA with nuclear or cytoplasmic RNA. Hybridization competition was performed as described in Methods and Materials. Cellular DNA (0.4 μg/filter) of Ad 2-transformed rat cells was incubated with the indicated amount of cold RNA for 20 hr at 66 C. After adding cytoplasmic ^{3}H-RNA (133 μg, 6×10^6 counts per min per filter) of the same cells, incubation was performed for another 20 hr at 66 C. The filters were washed, treated with ribonuclease, washed, and counted. Counts bound to DNA filters incubated with 2 × SSC-0.1% SDS instead of unlabeled RNA were normalized to 1.0. Unlabeled RNA added: KB cell RNA (▲); cytoplasmic RNA of Ad 2-transformed cells (●); and nuclear RNA of Ad 2-transformed cells (○).*

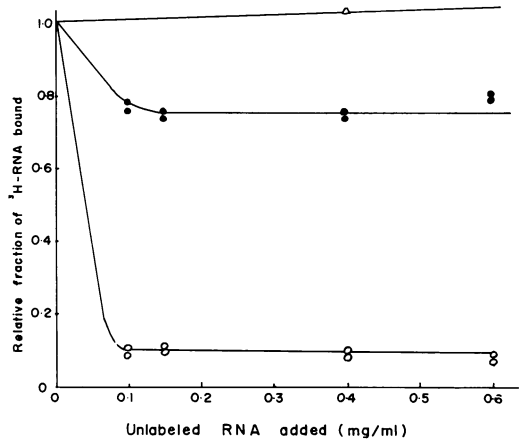

FIG. 4. *Hybridization competition of cellular RNA: nuclear RNA with nuclear or cytoplasmic RNA. DNA (0.1 μg/filter) of Ad 2-transformed rat cells was incubated for 20 hr at 66 C with different amounts of unlabeled RNA. After adding nuclear ^{3}H-RNA (46.7 μg, 4.1×10^6 counts per min per filter) of the same cells, incubation was continued for another 20 hr at 66 C. The filters were washed, treated with ribonuclease, washed, and counted. Counts bound to DNA filters incubated with 2 × SSC-0.1% SDS instead of unlabeled RNA were normalized to 1.0. Unlabeled RNA added: nuclear RNA of Ad 2-transformed rat cells (○); cytoplasmic RNA of the same cells (●); and KB cell RNA (▲).*

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