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The Mechanism of Viral Carcinogenesis by DNA Mammalian Viruses: RNA Transcripts Containing Viral and Highly Reiterated Cellular Base Sequences in Adenovirus-Transformed Cells*

(DNA–RNA hybridization/viral–cell mRNA)

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ABSTRACT **Virus-specific RNA was isolated from cells transformed by human adenoviruses 2 and 7 by multiple hybridizations with and elutions from homologous viral DNA; RNA molecules purified by this selection procedure hybridized efficiently with both viral DNA (24–50%) and DNA from untransformed cells (12–27%). Virus-specific RNA isolated in the same manner from cells productively infected with adenoviruses did not hybridize significantly with cellular DNA. These findings suggest that RNA molecules containing covalently-linked viral and cellular sequences are transcribed in cells transformed by human adenoviruses. The high efficiency of hybridization with DNA from untransformed cells implies that viral DNA is integrated adjacent to highly reiterated cellular DNA sequences.**

We previously reported findings suggesting that RNA molecules containing both cell- and virus-specific RNA sequences are synthesized in cells transformed by human adenoviruses (1–4). The possible existence of such RNA molecules was indicated by the following observations. Virus-specific RNA is distributed heterogeneously in adenovirus 2-transformed cells in molecules that sediment at 10–30 S in the cytoplasm and 10–40 S in the nucleus (1, 4), yet the fraction of the viral genome transcribed in adenovirus 2-transformed cells (4–10%) is too small to account for RNA molecules larger than 23 S (5). These large molecules containing virus-specific RNA could be transcribed from multiple viral DNA sequences integrated in tandem or from integrated viral DNA together with contiguous cellular DNA. We present in this paper the results of experiments that support the second alternative. Virus-specific RNA from adenovirus 2-transformed rat cells and adenovirus 7-transformed hamster cells, purified by hybridization to viral DNA hybridizes with high efficiency both with viral DNA and DNA from untransformed cells.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Adenovirus 2-transformed rat embryo cells (8617), adenovirus 7-induced hamster tumor cells (5728), normal rat cells (9258), and normal hamster cells (Nil 2E) were grown in monolayer culture as described (6). Suspension cultures of KB cells at a density of 2–3 × 10^5 cells/ml were infected with 50–100 plaque-forming units of adenovirus 2 or 7 per cell (7).

DNA. Adenovirus DNA was isolated from purified virus (8, 9). DNA from untransformed cells was prepared by either of two procedures (10, 11).

RNA. KB cells infected with adenovirus 2 and 7 were labeled with [^{3}H]uridine (20 μCi/ml, 20 Ci/mmol) from 2 to 18 hr after infection. Adenovirus 2- and 7-transformed cells were labeled with [^{3}H]uridine for 8 hr. RNA was extracted from cell pellets by the hot phenol-sodium dodecyl sulfate (SDS) method (12) with DNase treatment.

DNA–RNA Hybridization and Elution Procedures. Hybridization in the presence of 7.5 M urea at 37°C was used throughout this work (13). Denatured viral DNA and cellular DNA was immobilized on 25-mm membranes (14, 15); 6.5-mm circles were made with a paper punch. Filters were placed in 10 × 65-mm test tubes containing [^{3}H]RNA, 7.5 M urea (Mann Research Lab), 0.01 M N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) (Calbiochem Co.), 2 × SSC (0.30 M NaCl–0.030 M trisodium citrate), and 0.05% SDS in a final volume of 150 μl. Annealing was performed at 37°C for 2 or 4 days. Filters were then washed five times with 1 ml of 2 × SSC; RNA was eluted with 8.0 M urea, 0.01 M TES, 0.1 × SSC, and 0.05% SDS at 60°C for 10 min. 60–70% of bound [^{3}H]RNA was usually recovered. RNase treatment, when necessary, was performed with 0.5 ml of pancreatic RNase (20 μg/ml) in 2 × SSC at room temperature for 1 hr.

RESULTS

Purification of virus-specific RNA by hybrid formation with and elution from viral DNA

The purification of viral RNA from adenovirus 2- and 7-transformed cells and adenovirus 2-infected cells by repeated hybrid formation with and elution from homologous viral DNA is illustrated by the data in Table 1. The first hybridization step involved 30 to 50 reaction vessels, each containing 1–2 × 10^7 cpm of input RNA and 5 μg of immobilized viral

Abbreviations: SSC, standard saline citrate (0.15 M NaCl–0.015 M trisodium citrate); SDS, sodium dodecyl sulfate.

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TABLE 1. *Purification of viral RNA by hybridization with viral DNA using the urea procedure*

Source of RNA	Hybridization step	RNA input (cpm)	DNA (5 μg/filter)	Bound radioactivity (cpm)*	%†
Adenovirus 2-transformed rat cells	1st	1.85×10^7	Adenovirus 2	21,650	0.05
			None	12,300	
	2nd	29,050	Adenovirus 2	1,020	3
			None	140	
	3rd‡	500	Adenovirus 2	178	32
			None	17	
Adenovirus 7-induced hamster tumor cells	1st	1.5×10^7	Adenovirus 7	27,650	0.1
			None	12,200	
	2nd	1.5×10^5	Adenovirus 7	5,960	3.7
			None	340	
	3rd	786	Adenovirus 7	229	26.5
			None	24	
	4th‡	1330	Adenovirus 7	532	39
			None	10	
Adenovirus 2-infected KB cells	1st	2.7×10^5	Adenovirus 2	5,760	2.2
			None	290	
	2nd‡	1950	Adenovirus 2	670	34
			None	7	

* Average of duplicate hybridization reactions.
† cpm bound to empty filter was subtracted.
‡ RNase treatment was performed in the final hybridization step only.

DNA. Omission of RNase treatment caused large amounts of nonspecific binding in the first hybridization reaction. Viral RNA was eluted from the DNA on the filters, pooled, and further incubated with fresh viral DNA on filters. Highly purified viral RNA was obtained after two cycles of hybridization and elution, as indicated by the high binding efficiency with homologous viral DNA.

TABLE 2. *Hybridization of virus-specific RNA from adenovirus 2-transformed rat cells with viral DNA and DNA from untransformed cells*

Exp. no.	RNA input* (cpm)	DNA Source	DNA μg/filter	Bound radioactivity cpm†	Bound radioactivity %
1	2170	Adenovirus 2	5	523	24
		Rat cell	50	254	11.7
		E. coli	50	83	3.8
		None	0	37	1.7
2	1920	Adenovirus 2	5	569	30
		Adenovirus 2	0.01	11	0.6
		Rat cell	50	508	26.5
		E. coli	50	36	1.9
		None	0	4	0.3
3	1550	Adenovirus 2	5	619	40
		Adenovirus 2	0.01	10	0.7
		Rat cell	50	381	24.5
		E. coli	50	38	2.5
		None	0	3	0.2

* [^{3}H]RNA was purified by two cycles of hybridization with and elution from adenovirus 2 DNA without treatment with RNase.
† Average of duplicate hybridization reactions performed by the urea procedure with RNase treatment.

Hybridization of virus-specific RNA from adenovirus 2- and adenovirus 7-transformed cells with viral DNA and DNA from untransformed cells

Virus-specific [^{3}H]RNA from adenovirus 2-transformed rat cells, purified by two cycles of hybridization with and elution from viral DNA, hybridized not only with viral DNA but also with DNA from untransformed rat cells (Table 2). The efficiency of hybridization of three different [^{3}H]RNA preparations was 24, 30, and 40% with viral DNA and 12, 27, and 25% with cellular DNA. [^{3}H]RNA did not bind significantly to empty filters or to filters containing 50 μg of *Escherichia coli* DNA.

Similar results were obtained with virus-specific RNA isolated from adenovirus 7-induced hamster tumor cells (Table 3). Three separate preparations of [^{3}H]RNA hybridized both with adenovirus 7 DNA (36–50%) and with DNA from untransformed hamster cells (16–19%).

Lack of hybridization of virus-specific RNA from cells productively infected with adenovirus 2 and adenovirus 7 with DNA from untransformed cells

Virus-specific [^{3}H]RNA from KB cells productively infected with adenovirus 2 or 7 was purified by one cycle of hybridization with and elution from viral DNA, and annealed with viral or cellular DNA. RNA derived in this manner from cells productively infected with adenovirus 2 and 7 hybridized to homologous viral DNA with 26–41% efficiency but not to DNA of untransformed KB, rat, hamster, or *E. coli* cells (Table 4). Hybridization to heterologous viral DNA occurred with 10–28% efficiency, as expected from the relationship between adenoviruses 2 and 7 established by DNA–DNA

homology measurements (16), and from the complete transcription of the viral genome late after infection.

DISCUSSION

We show here that virus-specific RNA isolated from cells transformed by members of two groups of human adenoviruses hybridizes efficiently with both viral DNA and DNA of untransformed cells. The high efficiency with which RNA selected by hybridization to viral DNA hybridizes to cellular DNA cannot be due to the annealing of virus-specific RNA sequences to cellular DNA, since RNA from productively infected cells that contain sequences derived from the entire viral genome does not hybridize to cellular DNA, nor does viral DNA hybridize with cellular DNA (6, 15). The most likely explanation is that virus-specific RNA transcripts contain cellular RNA sequences derived from highly reiterated cellular DNA sequences (17). Recently, Simian Virus 40 (SV40)-transformed cells were reported to contain RNA molecules possessing both viral and cellular base sequences (18). The relative proportion of cellular and viral sequences in these RNA molecules cannot be estimated accurately, since (*a*) virus-specific RNA is present in a heterogeneous population of molecules, (*b*) degradation of RNA may occur during purification, and (*c*) the hybridization efficiency of cellular RNA to cellular DNA is unknown.

The synthesis of polycistronic RNA molecules containing both viral and cellular base sequences in adenovirus-transformed cells is additional evidence for the integration of viral DNA into the host cell genome. As we have suggested previously, these RNA molecules may be formed by the cell RNA polymerase transcribing both viral and cellular DNA sequences without interruption. Whether transcription initiates

TABLE 3. *Hybridization of virus-specific RNA from adenovirus 7-induced hamster tumor cells with viral and cellular DNA*

Exp. no.	RNA input* (cpm)	DNA Source	DNA μg/filter	Bound radioactivity cpm.†	Bound radioactivity %
1	1180	Adenovirus 7	5	587	50
		Hamster cell	50	182	15.5
		E. coli	50	28	2.4
		None	0	12	1
2	1340	Adenovirus 7	5	532	40
		Hamster cell	50	213	16
		None	0	10	0.7
3	1600	Adenovirus 7	5	574	36
		Adenovirus 7	0.01	10	0.6
		Hamster cell	50	305	19
		E. coli	50	34	2.1
		None	0	7	0.4

* [^{3}H]RNA was purified by two cycles of hybridization with and elution from adenovirus 7 DNA without treatment with RNase in Exps. 1 and 3, and 3 cycles in Exp. 2.

† Average of duplicate hybridization reactions performed by the urea procedure with RNase treatment.

TABLE 4. *Hybridization of virus-specific RNA from adenovirus 2 and adenovirus 7-infected KB cells with viral and cell DNA*

Exp. no.	RNA* Source	RNA* input (cpm)	DNA Source	DNA μg/filter	Bound radioactivity cpm†	Bound radioactivity %
1	Adenovirus 2-infected cells	2,440	Adenovirus 2	5	832	35
			KB	50	21	0.8
			KB	6	11	0.4
			Rat cell	50	22	0.9
			E. coli	50	9	0.4
			Adenovirus 7	5	83	3.5
			None	0	12	0.5
2	Adenovirus 2-infected cells	1,950	Adenovirus 2	5	670	34
			Rat cell	50	13	0.7
			E. coli	50	13	0.7
			Adenovirus 7	5	105	5.3
			None	0	7	0.4
3	Adenovirus 7-infected cells	22,900	Adenovirus 7	5	9360	41
			KB	50	46	0.2
			Hamster cell	50	21	0.07
			Adenovirus 2	5	2620	11
			E. coli	5	38	0.2
			None	0	38	0.2
4	Adenovirus 7-infected cells	1,270	Adenovirus 7	5	325	25.6
			KB	50	3	0.3
			Adenovirus 2	5	148	11.7
			Hamster cell	50	50	3.9
			None	0	12	1

* [^{3}H]RNA was purified by hybridization with and elution from adenovirus 2 DNA (Exp. 1 and 2) or adenovirus 7 DNA (Exp. 3 and 4) without treatment with RNase.

† Average of duplicate hybridization reactions performed by the urea procedure at 37°C with RNase treatment.

at a cellular or viral DNA site is not known. It is possible that cellular RNA sequences covalently linked to virus-specific RNA may play a role in the regulation of the growth of the transformed cell.

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