Mechanism of Viral Carcinogenesis by Deoxyribonucleic Acid Mammalian Viruses

IV. Related Virus-specific Ribonucleic Acids in Tumor Cells Induced by "Highly" Oncogenic Adenovirus Types 12, 18, and 31

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Formation of hybrids between viral deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was used to detect virus-specific RNA in the nuclei and polyribosomes of transformed and tumor cells induced by "highly" oncogenic human adenovirus (Ad) types 12, 18, and 31. The presence of virus-specific RNA in the cell nucleus, and the inhibitory effect of actinomycin D on its synthesis, suggest that adenovirus-specific RNA is transcribed from a DNA template in the nucleus. Ad 12, 18, and 31 virus-specific RNA did not hybridize significantly with the DNA of the "weakly" oncogenic adenovirus group (Ad 3, 7, 11, 14, 16, and 21) or with that of nononcogenic Ad 2 and 4. Labeled RNA from Ad 12, 18, and 31 tumor cells hybridized with heterologous Ad 12, 18, and 31 DNA 30 to 60% as efficiently as with homologous DNA. Thus, common viral genes are transcribed in tumor cells induced by Ad 12, 18, and 31.

Eight human adenovirus (Ad) types have been shown to be oncogenic (3, 6, 7, 9, 12; Green, Piña, Tochstein, and Thornton, *unpublished data*). Of these, Ad 12, 18, and 31 are "highly" oncogenic and produce tumors in a large percentage of newborn hamsters within a few months after inoculation (7, 9, 12). Infectious virus has not been detected in these tumor cells (1, 7), indicating that virion replication is not necessary for the neoplastic state.

We previously reported (1) that a large proportion of the messenger ribonucleic acid (mRNA) fraction in the polyribosomes of Ad 12 tumor and transformed cells is virus-specific. In this paper, we show that virus-specific ribonucleic acid (RNA) is present also in Ad 18 and 31 tumor cells, both in the polyribosomes and in the nuclei. The extent of hybridization of Ad 12, 18, and 31 virus-specific RNA with different Ad deoxyribonucleic acid (DNA) preparations was determined.

MATERIALS AND METHODS

Cells and RNA. Seed cultures of Ad 18 and Ad 31 hamster tumor cells were kindly provided by K. Schell, R. V. Gilden, and R. J. Huebner. The origin of other cell lines was described previously (1). Monolayers were grown in Eagle's minimal essential medium containing 10% calf serum. Cells were labeled with

³H-uridine, polyribosomes and nuclei were isolated, and RNA was purified as described previously (1). Purified polyribosomal RNA contained ³H-RNA plus carrier RNA. Purified ³H-nuclear RNA contained no carrier. Specific activities were determined in many preparations, and ranged from 15,000 to 30,000 counts per min per μ g of nuclear RNA and from 900 to 1,300 for polyribosomal RNA preparations. However, it should be noted that these values are overall specific activities of total RNA. Labeled RNA which hybridizes with viral DNA represents virus-specific RNA whose specific activity is not readily measured.

Viral DNA. Viral DNA was isolated by the methods of Green and Piña (4, 5). The following Ad strains were employed: Ad 2 (38-2), Ad 3 (15520), Ad 4 (Wyeth, 75680), Ad 7 (C14500), Ad 12 [Huie, B, C, D (5), 01516, and 568], Ad 14 (DeWitt), Ad 16 (Ch 79), Ad 18 (DC), Ad 21 (AV1645), and Ad 31. Ad 14, 16, and 21 were obtained from the American Type Culture Collection, and the remaining adenovirus strains were obtained from R. J. Huebner, R. M. Chanock, and B. Forsyth.

Detection and quantitation of virus-specific RNA. Virus-specific RNA was measured as ribonuclease-resistant DNA-RNA hybrids by the procedures of Gillespie and Spiegelman (2). Alkali-denatured viral DNA was immobilized on a nitrocellulose membrane (1) and incubated with labeled RNA isolated from Ad tumor and transformed cells (1). Conditions described previously for quantitating Ad 12-specific

TABLE 1. Hybridization of RNA from polyribosomes and nuclei of Ad 12-transformed cells with viral DNA

RNA ^a from	Input RNA	Ad 12 (Huie) DNA (μg/filter)	Labeled RNA bound	Bound (back-ground ^b not subtracted)
	counts/min		counts/min	%
Polyribosomes	52,440	3	146	0.28
	52,440	3	134	0.26
	52,440	None	2	0.004
Nuclei	214,000	3	438	0.20
	214,000	3	423	0.20
	214,000	None	26	0.01
	42,450	3	97	0.23
	42,450	3	114	0.27
	42,450	None	12	0.03
Whole cells	283,100	3	356	0.13
	283,100	3	406	0.14
	283,100	None	60	0.02
	59,600	3	113	0.19
	59,600	3	103	0.17
	59,600	None	14	0.02

^a Labeled with ³H-uridine (4 μc/ml) for 180 min.^b Counts bound to a DNA-free membrane.TABLE 2. Distribution of labeled RNA in cell fractions^a

Fraction	Total labeled RNA		Total labeled virus-specific RNA	
	counts/min ^b	% (of total)	counts/min ^c	% (of total)
Nuclei ^d	7.8×10^6	69	17,300	65
Cytoplasm.....	3.5×10^6	31	9,200	35
Polyribosome	1.0×10^6	9	2,800	11

^a Ad 12-transformed cells (3×10^6 /ml, 100 ml) were labeled with ³H-uridine (4 μc/ml, 20 c/mmole) for 180 min.^b Radioactivity of acid-precipitable material.^c Radioactivity of RNA complementary to Ad 12 (Huie) DNA = total radioactivity in fraction $\times$ proportion of radioactivity which hybridized with viral DNA.^d The radioactivity of the nuclear fraction was not determined directly because of sampling difficulties, but instead was obtained by subtracting cytoplasmic radioactivity from whole-cell radioactivity.

RNA from polyribosomes (1) were optimal for nuclear RNA as well, namely, incubation of membrane and labeled RNA at 66 C for 14 to 18 hr, and the use of 3 μg of DNA per membrane, an amount sufficient to bind a constant proportion of labeled virus-specific RNA (180-min labeling periods) at input levels of RNA up to 300,000 counts per min.

RESULTS

Virus-specific RNA in nuclei and the effect of actinomycin D. In previous studies (1), we looked for virus-specific RNA in the cytoplasmic polyribosomes, the major site of protein synthesis in the cells, since brief exposures to ³H-uridine labeled mainly the mRNA fraction. In the experiments reported here, we used 180-min labeling periods to prepare larger amounts of radioactive RNA (1). Virus-specific RNA was present not only in polyribosomes but in nuclei as well (Table 1). From 0.20 to 0.28% of the input nuclear and polyribosomal RNA hybridized with Ad 12 DNA; for example, with an input of 214,000 counts (per min) of nuclear RNA, 438 and 423 counts were bound in two separate hybridization experiments, whereas only 26 counts were bound to an empty membrane.

The incorporation of ³H-uridine into total RNA

TABLE 3. Absence of RNA complementary to Ad 12 DNA in the nucleus of "normal," polyoma, and SV40 tumor and transformed cells

RNA ^a from	Input	Ad 12 (Huie) DNA (μg/filter)	Labeled RNA bound	Bound (back-ground not subtracted)
	counts/min		counts/min	%
Ad 12 transformed hamster embryo cells	105,800	3	174	0.164
		3	205	0.194
		None	10	0.009
Ad 12 hamster tumor cells	167,100	3	59	0.035
		3	56	0.034
		None	12	0.007
KB ^b cells	170,000	3	32	0.019
		3	34	0.020
		None	30	0.018
BHK-21 ^c cells	172,100	3	39	0.023
		3	32	0.019
		None	30	0.017
Polyoma hamster tumor cell	158,300	3	28	0.018
		3	19	0.012
		None	27	0.017
SV-Py-3T3 ^d	209,400	3	25	0.012
		3	31	0.015
		None	23	0.011

^a Nuclear RNA labeled with ³H-uridine (4 μc/ml) for 180 min.^b Human cell line.^c Baby hamster kidney cell line.^d Mouse cell line transformed by SV40 and polyoma virus.

and virus-specific RNA in nuclei, cytoplasm, and polyribosomes is presented in Table 2. After a 180-min labeling period, nuclei contained about eight times as much total labeled RNA and six times as much labeled virus-specific RNA as did polyribosomes.

RNA complementary to Ad 12 DNA is absent from polyribosomes of "normal" human and hamster cell lines, and simian virus 40 (SV40) and polyoma virus-induced tumor and transformed cells (1). Likewise, nuclear RNA from human KB cells, hamster BHK-21 cells, polyoma virus-induced tumor cells, and SV40- and polyoma virus-transformed mouse cells did not hybridize significantly with Ad 12 DNA (Table 3).

Actinomycin D (1 and 5 $\mu\text{g}/\text{ml}$) inhibited incorporation of ^3H -uridine into both total RNA and virus-specific RNA in the nucleus and polyribosomes by 95 to 99% (Fig. 1). These data suggest that virus-specific RNA and cell RNA are synthesized by similar mechanisms on a DNA template.

Virus-specific RNA in Ad 12-transformed cells—relationship to DNA of other Ad types. We measured the extent of hybridization of labeled nuclear

FIG. 1. Ad 12-transformed hamster embryo cells ($3 \times 10^6/\text{ml}$) were labeled with ^3H -uridine (4 $\mu\text{C}/\text{ml}$, 20 c/mmole) for 180 min in the presence and absence of actinomycin D (1 and 5 $\mu\text{g}/\text{ml}$), and ^3H -RNA was isolated from the polyribosomes and nuclei. The radioactivity of the RNA of each fraction was determined by acid precipitation and collection on membrane filters. The total incorporation into virus-specific RNA was determined by hybridization reactions between Ad 12 (Huie) DNA and purified RNA from polyribosomes and nuclei.

RNA from Ad 12-transformed cells with the DNA of "highly" oncogenic Ad 18, "weakly" oncogenic Ad 3, 7, 11, 14, 16, and 21, and non-oncogenic Ad 2 and 4. [Ad 3 (6), Ad 7 (3), Ad 14, Ad 16, and Ad 21 (Green, Piña, Tochstein, and Thornton, *unpublished data*) have produced tumors in a small percentage of newborn hamsters inoculated. Although Ad 11 has not produced tumors, base composition (10) and DNA-DNA homology measurements suggest that Ad 11 belongs to this group of viruses.] In Table 4 is presented the "per cent relatedness" of Ad 12-specific RNA (i.e., virus-specific RNA from Ad 12-transformed cells) with each viral DNA, calculated by comparison with the homologous reaction; Ad 12-specific RNA $\times$ Ad 12 DNA is normalized to 100%. However, the "per cent relatedness" does not necessarily reflect the number of transcribed genes, since the frequency of transcription of individual genes is not known. The last column gives the per cent relatedness (between each viral DNA pair), established by DNA-DNA homology measurements (Lacy and Green, *J. Gen. Virol.*, *in press*).

RNA from Ad 12-transformed cells hybridized 34% as efficiently with Ad 18 DNA as it did with Ad 12 DNA. Thus, only part of the Ad 12-specific RNA is complementary to the Ad 18 DNA.

Ad 12-specific RNA did not hybridize significantly with the DNA of nononcogenic Ad 2 or 4 or "weakly" oncogenic Ad 3, 7, 11, 14, 16, or 21 (Table 4); the absence of detectable binding is consistent with the distant relationship found by DNA-DNA homology studies (8; Lacy and Green, *in press*).

Virus-specific RNA in Ad 18 tumor cells—relationship to DNA of other Ad types. RNA from the nucleus and the polyribosomes of Ad 18 tumor cells hybridized not only with Ad 18 DNA (Table 5) but also with Ad 12 and 31 DNA; however, Ad 12 and 31 DNA bound only 30% as much RNA as did Ad 18 DNA. No hybridization was detected between Ad 18 virus-specific RNA and the DNA of the "weakly" oncogenic and nononcogenic adenoviruses, or between Ad 18 and *Pseudomonas aeruginosa* DNA.

Virus-specific RNA in Ad 31 tumor cells—relationship to DNA of other Ad types. Ad 31-specific RNA is readily demonstrable in Ad 31 tumor cells (Table 6). RNA from Ad 31 tumor cells hybridized also with Ad 12 and 18 DNA (62% as efficiently as it did with Ad 31 DNA). No hybridization was detected with the DNA of the "weakly" oncogenic and nononcogenic adenoviruses. Thus, the RNA present in Ad 12, 18, and 31 tumor cells hybridizes with the homologous

TABLE 4. Hybridization of nuclear RNA from Ad 12-transformed cells with Ad DNA

Expt	DNA from	Virus oncogenicity	Labeled RNA bound			DNA-DNA homology with Ad 12 DNA ^c
			Counts/min	Fraction (× 10 ^{4d})	Per cent relatedness ^b	
1 ^d	Ad 12 (Huie)	High	113 120	18.6	100	% 100
	Ad 18	High	51 49	6.3	34	79
	Ad 7	Weak	23 25	1.5	<10 ^e	15
	Ad 14	Weak	20 14	0.5	<10 ^e	14
	Ad 16	Weak	25 21	1.3	<10 ^e	13
	Ad 21	Weak	17 25	0.9	<10 ^e	14
	Ad 2	Nononcogenic	25 21	1.3	<10 ^e	20
	Ad 4 (Wyeth)	Nononcogenic	27 18	1.2	<10 ^e	18
	<i>P. aeruginosa</i>	—	14 15	0.0	<10 ^e	—
	None	—	12 20	—	—	—
2 ^d	Ad 12 (Huie)	High	144 152	15.2	100	100
	Ad 3	Weak	17 22	0.3	<10 ^e	13
	Ad 11	? ^f	12 16	0.0	<10 ^e	11
	None	—	14 20	—	—	—

^a Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane.

^b Binding to Ad 12 DNA normalized to 100%.

^c Data of Lacy and Green (8; J. Gen. Virol., *in press*).

^d Nuclear RNA from Ad 12-transformed hamster embryo cells labeled for 180 min with ³H-uridine (4 μc/ml). In experiment 1, the input was 53,900 counts/min; in experiment 2, 97,500 counts/min.

^e The relatedness, if any, cannot be estimated, since too few counts were bound (less than 15 counts/min above that bound to a DNA-free membrane).

^f Although Ad 11 has not yet produced tumors, base composition (10) and DNA-DNA homology measurements suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.

and heterologous DNA of "highly" oncogenic Ad 12, 18, and 31, but not with the DNA of the "weakly" oncogenic and nononcogenic adenoviruses.

Hybridization of Ad 12-specific RNA with DNA from different Ad 12 strains. Virus-specific RNA from transformed cells induced by the prototype Huie strain of Ad 12 was tested for its ability to hybridize with six different strains of Ad 12 (Table 7). Only DNA from Ad 12, strain D, bound as much labeled RNA as did the DNA of the prototype strain. DNA from the five other strains bound only from 30 to 50% as much

labeled RNA as did the DNA of prototype Ad 12. Thus, these strains of Ad 12 lack some viral genes that are transcribed in Ad 12 (Huie)-transformed cells.

DISCUSSION

The human adenoviruses provide a series of molecular tools for analyzing the mechanism of viral oncogenesis. Highly purified DNA of "highly" oncogenic, "weakly" oncogenic, and nononcogenic adenoviruses (5, 10) were used here as reagents to analyze the presence of, and rela-

TABLE 5. *Virus-specific RNA in Ad 18 tumor cells—relatedness to other adenoviruses*

Expt	DNA from	Virus oncogenicity	Labeled RNA bound			DNA-DNA homology with Ad 18 DNA ^c
			Counts/min	Fraction (× 10 ^{4a})	Per cent relatedness ^b	
1 ^d	Ad 18	High	171	7.3	100	%
			173			100
	Ad 12 (Huie)	High	57	2.1	29	79
			59			
	Ad 31	High	64	2.2	30	Not done
			57			
	Ad 3	Weak	15	0.2	<5 ^e	20
			21			
	Ad 7	Weak	18	0.3	<5 ^e	16
			20			
	Ad 11	? ^f	15	0.1	<5 ^e	13
			17			
	Ad 14	Weak	11	0.2	<5 ^e	19
			22			
	Ad 16	Weak	20	0.2	<5 ^e	13
			14			
	Ad 21	Weak	16	0.1	<5 ^e	23
2 ^g	Ad 2	Nononcogenic	15	0.1	<5 ^e	23
			17			
	Ad 4 (75680)	Nononcogenic	14	0.1	<5 ^e	26
			16			
	<i>P. aeruginosa</i>	—	20	0.2	<5 ^e	—
	None	—	10	—	—	—
			17			
	Ad 18	High	106	5.2	100	100
			121			
	Ad 3	Weak	28	0.2	<5 ^e	20
			25	—	—	—
	None		24			

^a Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane.

^b Binding to Ad 18 DNA normalized to 100%.

^c Data of Lacy and Green (8; J. Gen. Virol., *in press*).

^d Nuclear RNA from Ad 18 tumor cells labeled for 180 min with ³H-uridine (4 μc/ml). In experiment 1, the input was 216,700 counts/min; in experiment 2, 172,100 counts/min.

^e The relatedness, if any, cannot be estimated, since too few counts were bound (less than 15 counts/min above that bound to a DNA-free membrane).

^f Although Ad 11 has not yet produced tumors, base composition (10) and DNA-DNA homology measurements suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.

^g Polyribosomal RNA from Ad 18 tumor cells labeled for 180 min with ³H-uridine (4 μg/ml).

tionships among, virus-specific RNA in Ad tumor and transformed cells. Tumor cells induced by Ad 12, 18, and 31 synthesize related virus-specific RNA. The presence of virus-specific RNA in the nuclear fraction and the inhibition of its synthesis by actinomycin D suggest that virus-specific RNA is transcribed from viral DNA in the nucleus by a DNA-dependent RNA polymerase.

RNA from Ad 12 and 18 tumor cells hybridizes with heterologous Ad 18 and 12 DNA only about one-third as efficiently as with homologous DNA,

yet Ad 12 and 18 DNA share four-fifths of their nucleotide sequences as determined by DNA-DNA homology measurements (8). If we assume that different viral mRNA molecules are present in equal amounts, then a simple calculation suggests that a maximum of 30% of the Ad 12 and 18 genomes are transcribed in the tumor cell. However, these assumptions are untested. The frequency of expression of individual viral genes and the rate of synthesis and degradation of different viral mRNA molecules are unknown. Possibly,

TABLE 6. *Viral-specific RNA in Ad 31 tumor cells—relatedness to other adenoviruses*

Expt	DNA from	Virus oncogenicity	Labeled RNA bound		
			counts/min	Fraction ($\times 10^{4a}$)	Per cent relatedness ^b
1 ^c	Ad 31	High	88	4.0	100
	Ad 12 (Huie)	High	85	2.5	62
			62		
	Ad 18	High	61	2.5	62
			64		
	Ad 7	Weak	20	0.1	<10 ^d
			27		
	Ad 2	Nononcogenic	22	0.0	<10 ^d
None	—	22	—	—	
2 ^c	Ad 31	High	89	6.7	100
	Ad 3	Weak	26	0.8	<15 ^d
			22		
	Ad 14	Weak	16	0.0	<10 ^d
			14		
	None	—	15	—	—
3 ^c	Ad 31	High	73	4.7	100
	Ad 3	Weak	84	0.4	<10 ^d
			26		
			27		
			21		
	Ad 11	? ^e	27	0.3	<10 ^d
			21		
	Ad 16	Weak	21	0.0	<10 ^d
			17		
	Ad 21	Weak	23	0.4	<10 ^d
27					
<i>P. aeruginosa</i>	—	25	0.1	<10 ^d	
None	—	20	—	—	
4 ^f	Ad 31	High	112	6.0	100
	Ad 4 (Wyeth)	Nononcogenic	118	0.3	<10 ^d
			34		
			24		
			25		
	None	—	25	—	—

^a Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane.

^b Binding to Ad 31 DNA normalized to 100%.

^c Nuclear RNA from Ad 31 tumor cells labeled for 180 min with ³H-uridine (4 μc/ml). In experiment 1, the input was 160,200 counts/min; experiment 2, 109,700 counts/min; experiment 3, 123,100 counts/min; experiment 4, 150,700 counts/min.

^d The relatedness, if any, cannot be estimated, since too few counts were bound (less than 15 counts/min above that bound to a DNA-free membrane).

^e Although Ad 11 has not yet produced tumors, base composition (10) and DNA-DNA homology measurements suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.

^f Polyribosomal RNA from Ad 31 tumor cells labeled for 180 min with ³H-uridine (4 μc/ml).

most of the mRNA molecules are coded for by a small proportion of the transcribed viral genes. Until we know more about viral gene expression in transformed cells, it is difficult to extrapolate from data on abundance of viral mRNA molecules to numbers of expressed viral genes.

Virus-specific RNA in tumor cells induced by “highly” oncogenic Ad 12, 18, and 31 does not hybridize with the DNA of the six “weakly” oncogenic adenoviruses. Likewise, virus-specific RNA from tumor cells induced by “weakly” oncogenic Ad 3, 7, 14, and 16 does not hybridize with Ad 12,

TABLE 7. Hybridization of nuclear RNA^a from Ad 12 (Huie)-transformed cells with Ad DNA

DNA ^b from	Labeled RNA bound ^c	Bound ^c (avg)	Per cent relatedness ^d
	counts/min	%	
Ad 12 (Huie)	361 374	0.156	100
Ad 12 (B)	149 155	0.065	42
Ad 12 (C)	119 118	0.050	32
Ad 12 (D)	351 341	0.148	95
Ad 12-01516	143 159	0.065	42
Ad 12-565	187 167	0.075	48
Ad 12-568	166 159	0.070	45

^a Nuclear RNA from Ad 12 (Huie)-transformed hamster embryo cells labeled for 180 min with ³H-uridine (4 μ C/ml). RNA input, 235,500 counts/min.

^b Contained 3 μ g of DNA per filter.

^c Corrected for nonspecific binding to DNA-free membrane (0.6×10^{-4} of input counts).

^d Binding to Ad 12 (Huie) DNA normalized to 100%.

18, or 31 DNA (Fujinaga and Green, Proc. Natl. Acad. Sci. U.S., *in press*). Thus, tumor cells induced by "highly" and "weakly" oncogenic adenoviruses synthesize different virus-specific RNA molecules. However, possession of one or two viral genes in common is not excluded by these data.

Strains of Ad 12 differ in degree of neutralization by heterologous Ad 12 antisera (11). This observation, and the differences observed here in ability to bind Ad 12-specific RNA, suggest that Ad 12 strains differ considerably in genetic composition.

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