*MECHANISM OF VIRAL CARCINOGENESIS BY DNA MAMMALIAN VIRUSES, II. VIRAL-SPECIFIC RNA IN TUMOR CELLS INDUCED BY "WEAKLY" ONCOGENIC HUMAN ADENOVIRUSES**

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Of the 31 human adenoviruses (Ad), 8 are carcinogenic for newborn hamsters: Ad 12,[1] Ad 18,[2] and Ad 31[3] are designated as "highly" oncogenic because they induce tumors in most hamsters within 1–2 months after injection, while Ad 3,[4, 5] Ad 7,[6] Ad 14,[5] Ad 16,[5] and Ad 21[5] are called "weakly" oncogenic because they produce tumors in a small proportion of hamsters 4–18 months after injection. Infectious virus is not present in adenovirus tumor cells,[2, 7, 8] and it is clear that virion replication is not essential for the neoplastic state. We have recently presented evidence for the presence of viral genetic material in Ad 12 tumor and transformed cells: 2–5 per cent of the mRNA fraction in the polyribosomes was found to be complementary to Ad 12 DNA.[8]

In this paper we present evidence that tumor cells induced by "weakly" oncogenic adenoviruses possess related viral-specific RNA's, distinct from that present in Ad 12 tumor and transformed cells. Two different classes of "adenovirus-specific RNA's" exist, one in tumor cells induced by the "highly" oncogenic adenoviruses and the other in tumor cells induced by the "weakly" oncogenic adenoviruses.

Experimental Methods.—Cell culture: Cultures of hamster tumor cells induced by Ad 3 (15520), Ad 7 (C14500), Ad 14 (DeWitt), and Ad 16 (Ch 79) were kindly provided by Drs. R. V. Gilden, R. J. Huebner, and A. Freeman. Cell monolayers were grown in 32-oz prescription bottles using Eagle's minimal essential medium plus 10 per cent calf serum and were subcultured twice a week.

Cell labeling and polyribosome preparation: These methods have been described.[8]

Preparation of cell nuclei and nuclear RNA: The isolation of nuclei and the purification of nuclear RNA were performed by the procedure of Penman[9] with the following additional steps: purified RNA was treated with DNase I (Worthington Biochemicals, electrophoretically purified) for 15 minutes at 37° and $^1/_{10}$ volume of 5 per cent sodium dodecyl sulfate was added. The sample was extracted with equal volumes of cold phenol 3–5 times. the phenol was removed with water-saturated ether, and the solution was dialyzed overnight against 3–5 changes of 0.1 × SSC (SSC = 0.15 *M* NaCl, 0.015 *M* Na_3 citrate).

Viral DNA preparation: The methods of Green and Piña[10, 11] were used for viral cultivation, purification, and isolation of viral DNA. The following strains of adenovirus were used: Ad 2 (38-2), Ad 3 (15520, 19485, and Master seed), Ad 4 (Wyeth, AGMK HEP-2, and 75680), Ad 7 (C14500), Ad 12 (Huie), Ad 14 (DeWitt), Ad 16 (Ch 79), Ad 18 (DC), Ad 21 (AV1645). (Strain designations (in parentheses) are those given by the laboratories providing the virus seeds.) Ad 14, 16, and 21 were obtained from the American Type Culture Collection and the remaining

adenoviruses were provided by Drs. R. J. Huebner, R. M. Chanock, and B. Forsyth.

DNA-RNA hybridization: Viral-specific H^3-RNA's were identified and quantitated by their ability to form RNase-resistant DNA-RNA hybrids with specific adenovirus DNA's by the procedures described previously.[8]

Results and Discussion.—Nuclear versus polyribosomal viral-specific RNA: Experiments with actinomycin D suggested that viral-specific RNA, like cell mRNA, is made on a DNA template in the cell nucleus.[12] The data in Table 1 show that the ratio of labeled viral-specific RNA to total input labeled RNA is about the same in the cell nucleus as in the polyribosomes. (However, this ratio varies from one cell line to another.) Since the nucleus contains 5–8 times as much radioactive RNA as the polyribosomes (after a 180-min labeling period), we used labeled nuclear RNA also for the detection and characterization of viral-specific RNA.

Viral-specific RNA in tumor cells induced by "weakly" oncogenic adenoviruses: Labeled nuclear and polyribosomal RNA from hamster tumor cells induced by Ad 3, 7, 14, and 16 were tested for ability to hybridize with the DNA's of "highly" oncogenic, "weakly" oncogenic, and nononcogenic adenoviruses (Tables 2–5). Viral-specific RNA was readily detected in each of the four different adenovirus tumor cell lines examined. Therefore the synthesis of viral-specific RNA is a common feature of oncogenesis by human adenoviruses. DNA's from three different oncogenic[5] strains of Ad 3 bind labeled RNA to about the same extent (Table 2).

Relationship among viral-specific RNA's induced in tumor cells by "weakly" oncogenic adenoviruses: RNA from tumor cells induced by Ad 3, 7, 14, and 16 hybridizes to about the same extent with the DNA's of the five other "weakly" oncogenic adenoviruses as it does with the DNA's of the homologous virus (Tables 2–5). For example, 5.8×10^{-4} of input labeled RNA from Ad 14 tumor cells binds to Ad 14 DNA, and 4.3, 5.1, 4.5, 4.8, and 5.4×10^{-4} binds to Ad 3, 7, 11,[13] 16, and 21 DNA's, respectively (Table 4). These data strongly suggest that Ad 3, 7, 14, and 16 tumor cells synthesize a common viral-specific RNA. These results are in accord with the very close relationship shown by DNA-DNA homology

TABLE 1

HYBRIDIZATION OF RNA FROM POLYRIBOSOMES AND NUCLEI OF AD 7 TUMOR CELLS WITH VIRAL DNA

RNA[a] from	Input RNA (cpm)	Ad 7 DNA[b] (μg/filter)	Bound RNA (cpm)	Bound (%) (background not subtracted)
Polyribosomes	179,600	3	172	0.096
	"	3	165	0.092
	"	None	24	0.013
	"	None	18	0.010
Polyribosomes	87,500	3	75	0.086
	"	3	77	0.088
	"	None	10	0.011
Nuclei	238,900	3	195	0.082
	"	3	205	0.086
	"	None	19	0.008
	"	None	12	0.005
Nuclei	147,800	3	154	0.104
	"	3	166	0.112
	"	None	10	0.007

[a] RNA from cells labeled for 180 min with H^3-uridine (4 μc/ml).
[b] DNA of Ad 7 (strain C14500).

TABLE 2

VIRAL-SPECIFIC RNA IN AD 3 TUMOR CELLS—RELATEDNESS TO OTHER ADENOVIRUSES

Expt. no.	DNA from	Virus oncogenicity	RNA Bound: Fraction × 10^4[a]	RNA Bound: Per cent relatedness[b]	DNA-DNA % homology with Ad 3 DNA[c]
1[d]	Ad 3 (15520)	Weakly oncogenic	3.9	100	100
	Ad 7	" "	3.9	100	102
	Ad 14	" "	3.9	100	67
	Ad 16	" "	3.7	95	100
	Ad 21	" "	3.9	100	81
	Ad 12	Highly oncogenic	0.4	<15[e]	13
	Ad 18	" "	0.6	<15[e]	20
	Ad 2	Nononcogenic	0.5	<15[e]	27
2[d]	Ad 3 (15520)	Weakly oncogenic	4.7	100	100
	Ad 11	?[f]	2.9	62	80
3[g]	Ad 3 (15520)	Weakly oncogenic	5.3	100	100
	Ad 4 (Wyeth)	Nononcogenic	0.9	17	46
	Ad 4 (AGMK HEP-1)	"	0.9	17	46
4[g]	Ad 3 (15520)	Weakly oncogenic	6.1	100	100
	Ad 3 (19485)	" "	5.3	87	100
5[g]	Ad 3 (19485)	Weakly oncogenic	6.7	100	100
	Ad 3 (master seed)	" "	6.5	97	100
	Ad 12	Highly oncogenic	0.8	<15[e]	13
	Ad 2	Nononcogenic	0.9	<15[e]	27

[a] Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane ($0.7–1.6 \times 10^{-4}$ of input counts).
[b] Binding to Ad 3 DNA normalized to 100%.
[c] Data of Lacy and Green.[14]
[d] Nuclear RNA from Ad 3 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).
[e] The per cent relatedness, if any, can not be estimated since too few counts were bound (less than 15 cpm above that bound to a DNA-free membrane).
[f] Although Ad 11 has not yet produced tumors, base composition[15] and DNA-DNA homology measurements[14] suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.
[g] Polyribosomal RNA from Ad 3 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).

measurements[14] (Tables 2–5); 70–100 per cent of the nucleotide sequences among viral DNA's of this group are held in common.

RNA's from tumor cells induced by the "weakly" oncogenic adenoviruses do not hybridize significantly with the DNA's of highly oncogenic Ad 12 and 18 (Tables 2–5). These data are compatible with the distant relationship between "weakly" oncogenic and "highly" oncogenic adenoviruses indicated by DNA-DNA homology studies[14] (Tables 2–5).

RNA's from Ad 3, 7, 14, and 16 tumor cells do not hybridize significantly with Ad 2 DNA but appear to bind to Ad 4 DNA to a small extent in some cases (10–20%). Since Ad 4 DNA exhibits up to 50 per cent homology with the DNA's of "weakly" oncogenic adenoviruses[14] (Tables 2–5), we conclude that only part of the viral genome is functioning in these tumor cells.

Viral-specific RNA and adenovirus carcinogenesis: Our data demonstrate the presence of viral-specific RNA in tumor cells induced by four "weakly" oncogenic human adenoviruses, Ad 3, 7, 14, and 16. We previously found viral-specific RNA in tumor and transformed cells induced by "highly" oncogenic Ad 12[8] and recently we detected viral-specific RNA in hamster tumor cells induced by "highly" oncogenic Ad 31.[12] It is clear that the formation of viral-specific RNA is a common feature of carcinogenesis by human adenoviruses.

Implications for the etiology of human cancer: These data and techniques suggest means for testing adenoviruses as causative agents of human cancer. It may be feasible to examine fresh human cancer tissue for the presence of adenovirus

TABLE 3

VIRAL-SPECIFIC RNA IN AD 7 TUMOR CELLS—RELATEDNESS TO OTHER ADENOVIRUSES

Expt. no.	DNA from	Virus oncogenicity	RNA bound: Fraction × 10^{4}[a]	RNA bound: Per cent relatedness[b]	DNA-DNA % homology with Ad 7 DNA[c]
1[d]	Ad 7	Weakly oncogenic	8.8	100	100
	Ad 3 (15520)	" "	6.1	69	102
	Ad 14	" "	6.1	69	76
	Ad 16	" "	7.2	82	81
	Ad 21	" "	6.9	78	98
	Ad 12	Highly oncogenic	0.0	<10[e]	15
	Ad 18	" "	0.1	<10[e]	16
	Ad 2	Nononcogenic	0.0	<10[e]	30
2[d]	Ad 7	Weakly oncogenic	10.1	100	100
	Ad 11	?[f]	3.5	35	80
3[d]	Ad 7	Weakly oncogenic	7.7	100	100
	Ad 11	?[f]	1.6	21	80
4[d]	Ad 7	Weakly oncogenic	11.2	100	100
	Ad 4 (AGMK-HEP-1)	Nononcogenic	2.0	18	51
	Ad 4 (75680)	"	2.2	20	51
5[g]	Ad 7	Weakly oncogenic	8.2	100	100
	Ad 12	Highly oncogenic	0.1	<10[e]	15
	Ad 2	Nononcogenic	0.5	<10[e]	30

[a] Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane (0.5–1.9 × 10^{-4} of input counts).
[b] Binding to Ad 7 DNA normalized to 100%.
[c] Data of Lacy and Green.[14]
[d] Nuclear RNA from Ad 7 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).
[e] The per cent relatedness, if any, can not be estimated since too few counts were bound (less than 15 cpm above that bound to a DNA-free membrane).
[f] Although Ad 11 has not yet produced tumors, base composition[15] and DNA-DNA homology measurements[14] suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.
[g] Polyribosomal RNA from Ad 7 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).

TABLE 4

VIRAL-SPECIFIC RNA IN AD 14 TUMOR CELLS—RELATEDNESS TO OTHER ADENOVIRUSES

Expt. no.	DNA from	Virus oncogenicity	RNA Bound: Fraction × 10^{4}[a]	RNA Bound: Per cent relatedness[b]	DNA-DNA % homology with Ad 14 DNA[c]
1[d]	Ad 14	Weakly oncogenic	5.8	100	100
	Ad 3 (15520)	" "	4.3	74	67
	Ad 7	" "	5.1	88	76
	Ad 16	" "	4.8	83	89
	Ad 21	" "	5.4	93	84
	Ad 12	Highly oncogenic	0.2	<10[e]	14
	Ad 18	" "	0.5	<10[e]	13
	Ad 2	Nononcogenic	0.1	<10[e]	22
2[f]	Ad 14	Weakly oncogenic	4.2	100	100
	Ad 11	?[g]	4.5	107	91
3[f]	Ad 14	Weakly oncogenic	12.8	100	100
	Ad 12	Highly oncogenic	0.7	<10[e]	14
	Ad 2	Nononcogenic	0.4	<10[e]	22
4[f]	Ad 14	Weakly oncogenic	5.0	100	100
	Ad 4 (Wyeth)	Nononcogenic	0.7	<15[e]	33
	Ad 4 (AGMK HEP-1)	"	0.7	<15[e]	33
	Ad 2	"	0.5	<15[e]	22

[a] Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane (0.8–1.1 × 10^{-4} of input counts).
[b] Binding to Ad 14 DNA normalized to 100%.
[c] Data of Lacy and Green.[14]
[d] Nuclear RNA from Ad 14 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).
[e] The per cent relatedness, if any, can not be estimated since too few counts were bound (less than 15 cpm above that bound to a DNA-free membrane).
[f] Polyribosomal RNA from Ad 14 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).
[g] Although Ad 11 has not yet produced tumors, base composition[15] and DNA-DNA homology measurements[14] suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.

TABLE 5

VIRAL-SPECIFIC RNA IN AD 16 TUMOR CELLS—RELATEDNESS TO OTHER ADENOVIRUSES

Expt. no.	DNA from	Virus oncogenicity	RNA Bound: Fraction $\times 10^{4a}$	RNA Bound: Per cent relatedness[b]	DNA-DNA % homology with Ad 16 DNA[c]
1[d]	Ad 16	Weakly oncogenic	13.5	100	100
	Ad 3 (15520)	" "	10.2	76	100
	Ad 14	" "	13.5	100	89
	Ad 2	Nononcogenic	0.9	<10[e]	38
2[d]	Ad 16	Weakly oncogenic	24.2	100	100
	Ad 21	" "	23.8	98	92
	Ad 11	?[f]	14.1	58	93
	Ad 18	Highly oncogenic	1.2	<10[e]	7
	Ad 4 (Wyeth)	Nononcogenic	1.3	<10[e]	50
3[g]	Ad 16	Weakly oncogenic	5.7	100	100
	Ad 7	" "	5.1	90	81
	Ad 12	Highly oncogenic	0.2	<10[e]	13
	Ad 2	Nononcogenic	0.2	<10[e]	38
4[g]	Ad 16	Weakly oncogenic	6.8	100	100
	Ad 4 (Wyeth)	Nononcogenic	0.9	13	50
	Ad 4 (AGMK HEP-1)	"	1.2	18	50

[a] Average of duplicate hybridization reactions. Corrected for nonspecific binding to DNA-free membrane (0.7–1.8 $\times 10^{-4}$ of input counts).
[b] Binding to Ad 16 DNA normalized to 100%.
[c] Data of Lacy and Green.[14]
[d] Nuclear RNA from Ad 16 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).
[e] The per cent relatedness, if any, can not be estimated since too few counts were bound (less than 15 cpm above that bound to a DNA-free membrane).
[f] Although Ad 11 has not yet produced tumors, base composition[15] and DNA-DNA homology measurements[14] suggest a close relationship to the weakly oncogenic Ad 3, 7, 14, 16, and 21.
[g] Polyribosomal RNA from Ad 16 tumor cells labeled for 180 min with H^3-uridine (4 μc/ml).

specific RNA molecules by DNA-RNA hybrid formation with adenovirus DNA. Since viral-specific RNA has been detected previously only in tumor cell lines, not in tumors directly, we tested labeled RNA from Ad 12 primary hamster tumor for its ability to hybridize with Ad 12 DNA. As shown in Table 6, Ad 12-specific RNA could be readily detected in fresh tumor tissue. Recently, Benjamin[16] has detected small amounts of polyoma and SV40–specific RNA in transformed cells induced by these viruses. Thus the synthesis of viral-specific RNA appears to be a general feature of carcinogenesis by DNA viruses and the procedure described here provides a means for testing the possible viral etiology of human cancer.

The close relationship of the viral-specific RNA's of "weakly" oncogenic adenoviruses: Labeled RNA from Ad 3, 7, 14, and 16 tumor cells hybridizes with the

TABLE 6

DETECTION OF VIRAL-SPECIFIC RNA IN AD 12 HAMSTER TUMOR TISSUE

Expt. no.	Input RNA[a] (cpm)	DNA (3 μg/filter)	RNA bound (cpm)	RNA bound (%) (background not subtracted)
E004A[b]	185,800	Ad 12	101	0.054
		Ad 12	100	0.054
		None	6	0.003
E004B[c]	138,000	Ad 12	111	0.081
		Ad 12	144	0.104
		None	6	0.004

[a] Whole cell RNA was isolated by the hot phenol-SDS method,[20] followed by a DNase treatment (see *Experimental Methods*), a repeated hot phenol-SDS extraction,[20] and removal of phenol as described in the text.
[b] Minced hamster tumor tissue (1.0 ml wet volume) suspended in 40 ml of media was labeled with H^3-uridine (4 μc/ml; 20 C/mM) for 8 hr.
[c] About 0.25 ml of cells derived by trypsinization of hamster tumor tissue was labeled for 6 hr in 10 ml of media as in footnote (*b*).

DNA's of Ad 3, 7, 11, 14, 16, and 21 to similar degrees in most cases,[17] suggesting that a common viral-specific RNA is synthesized by "weakly" oncogenic adenoviruses. The close relationship among the six "weakly" oncogenic adenoviruses is also suggested by the results of DNA-DNA homology studies[14] and by the presence of a common "tumor" and T antigens.[18, 19] The analogous relationships among tumor antigens and among viral-specific RNA's suggest that part of the viral-specific RNA codes for the synthesis of tumor antigen(s).

The significance of the lack of relationship between "weakly" and "highly" oncogenic adenoviruses: RNA's in tumors induced by the weakly" oncogenic adenoviruses do not hybridize significantly with the DNA of "highly" oncogenic adenoviruses. Although this suggests that a "carcinogenic cistron" common to both of these oncogenic groups does not exist, the sensitivity of hybrid detection is not sufficient to eliminate the sharing of one or two viral cistrons (i.e., 10% relatedness).

How much of the viral genome function in the tumor cell? Although the amount of the viral genome functioning in the tumor cell is not known, an upper limit of about 50 per cent is suggested by the following comparison: Ad 4 DNA hybridizes to a small extent (10–20%) or not at all with RNA from Ad 3, 7, 14, and 16 tumor cells, yet Ad 4 DNA shares about 50 per cent of its nucleotide sequence with Ad 3, 7, 14, and 16 DNA's (Tables 2–5). Whether this upper limit represents an integration of only part of the viral genome or the transcription of part of an integrated genome is not known.

Summary.—Cultures of tumor cells induced by "weakly" oncogenic adenovirus (Ad) types 3, 7, 14, and 16 possess viral-specific RNA's readily detected by the formation of specific DNA-RNA hybrids with viral DNA. Each of these viral-specific RNA's hybridizes with the DNA's of all six members of the "weakly" oncogenic adenovirus group (Ad 3, 7, 11, 14, 16, and 21) but not with the DNA's of "highly" oncogenic Ad 12 and 18. Thus two different classes of adenovirus-specific RNA are synthesized by adenovirus tumor cells, one in tumor cells induced by "highly" oncogenic adenoviruses and the other in tumor cells induced by "weakly" oncogenic adenoviruses. At most one half of the viral genome functions in the synthesis of viral-specific RNA.

Ad 12-specific RNA is readily demonstrated in primary hamster tumors induced by Ad 12.

The presence of viral-specific RNA in cultured tumor cells induced by six different human adenoviruses and the ability to detect viral-specific RNA in tumor tissue directly suggest the feasibility of testing human cancers for viral etiology by the detection of viral-specific RNA.

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