*THE MECHANISM OF VIRAL CARCINOGENESIS BY DNA MAMMALIAN VIRUSES, VI. A NEW CLASS OF VIRUS-SPECIFIC RNA MOLECULES IN CELLS TRANSFORMED BY GROUP C HUMAN ADENOVIRUSES**

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Abstract.—A new class of virus-specific RNA molecules was found in cells transformed by group C human adenovirus types 2, 5, and 6. RNA isolated from virus-free rat embryo cells transformed by adenovirus 2, 5, and 6 hybridized with all group C adenovirus DNA's (adenovirus 1, 2, 5, and 6) equally well, but not appreciably with group A and B adenovirus DNA's. Most likely no viral genes common to group A, B, and C adenoviruses are transcribed in adenovirus-transformed cells.

Group C adenoviruses are closely related since they share 83 to 93 per cent of their base sequences as shown by DNA-DNA homology measurements. Group C DNA's share only 10 to 26 per cent of their base sequences with group A and B DNA's. Moreover, the shared sequences are not transcribed detectably in adenovirus transformed cells.

Virus-specific RNA isolated from group C transformed cells contains 49 to 51 per cent G + C, but viral DNA's possess a 7 to 9 per cent higher G + C content. These differences suggest that only a portion of the viral genome with an average G + C content of 49 to 51 per cent is transcribed in group C adenovirus transformed cells.

Tumor and transformed cells induced by human adenoviruses (Ad) contain no infectious virus but possess functioning viral genes as revealed by the presence of virus-specific T antigens[1] and virus-specific RNA.[2–4] Virus-specific RNA molecules transcribed in cells transformed by Ad 12, 18, and 31,[4] members of "highly oncogenic" group A, differ in nucleotide sequence from those induced by Ad 3, 7, 14, and 16,[3] members of "weakly oncogenic" group B.

Recently Freeman *et al.*[5, 6] and McAllister *et al.*[7] transformed rat embryo cells with members of the "non-oncogenic"[7a] group C of adenoviruses which includes Ad 1, 2, 5, and 6.[7b] These cells were transformed morphologically but did not induce tumors in rats. Group C transformed cells do not contain infectious virus but synthesize virus-specific RNA and virus-specific T antigens.[5] We describe here some properties of the virus-specific RNA species transcribed in group C transformed cells and the relationship between group C, A, and B adenoviruses as determined by DNA-DNA homology measurements.

Experimental Methods.—Cell culture and cell labeling: Seed cultures of Ad 2, 5, and 6 transformed rat embryo cells were generously provided by Drs. A. E. Freeman and R. J. Huebner. Cell culture and cell-labeling procedures have been described.[2, 3]

Purification of RNA: Cellular RNA was isolated and purified by the hot phenol-sodium dodecyl sulfate (SDS) method followed by treatment with DNase.[8, 9] [^{3}H]RNA

had specific activities of 3000 to 7000 cpm/μg and [^{32}P]RNA 30,000 to 80,000 cpm/μg.

Viral DNA: Viral DNA was prepared from purified virus by the methods of Green and Piña.[10, 11] The following strains of adenoviruses were used: Ad 1 (646), Ad 2 (38-2), Ad 4 (Wyeth), Ad 5 (20057), Ad 6 (Tonsil 99), Ad 7 (C14500), Ad 8 (Trim), Ad 12 (Huie), and Ad 26 (BP-2). Seed virus cultures were obtained from Drs. R. J. Huebner, R. M. Chanock, and B. Forsyth. [^{3}H]DNA was prepared from virus grown in cells in the presence of [^{3}H] thymidine (0.1 μc/ml, 5–15 c/mmole).[10, 11]

DNA-RNA hybridization, purification of virus-specific RNA, and base analysis of [^{32}*P*]*-RNA:* DNA-RNA hybridization was performed by the procedure of Gillespie and Spiegelman with minor modifications.[5, 12] Sufficient immobilized viral DNA was used to bind available virus-specific RNA. Virus-specific RNA was purified by elution from DNA-RNA hybrids.[8] Base analysis of purified virus-specific [^{32}P]RNA was performed by alkaline hydrolysis of RNA to [^{32}P] ribonucleotides which were separated by paper electrophoresis and their radioactivity counted in a liquid scintillation counter.[8, 13]

DNA-DNA hybridization: The procedure of Warnaar and Cohen[14] described for bacteriophage DNA was modified: DNA was immobilized on Schleicher and Schuell B6 membrane filters and hybridization was carried out in 2 × SSC containing 0.1% SDS.[9, 15] The use of SDS was necessary to reduce the background levels of bound radioactivity to a low level.

Viral DNA was immobilized on membrane filters as previously described.[8] Viral [^{3}H]DNA in 2 to 5 ml of 0.1 × SSC was sonicated in 13 × 100 mm nitrocellulose tubes for 10 min at full power in a Raytheon DF-101 sonic oscillator to produce DNA fragments of molecular weight 6×10^5 daltons. DNA fragments were denatured by heating for 10 min at 100° and immersed rapidly in an ice bath. DNA filters were incubated with 1.0 ml of denatured [^{3}H]DNA fragments in 2 × SSC containing 0.1% SDS at 60° for 24 hr. Filters were rinsed through two beakers containing 300 ml of 3 mM Tris buffer, pH 9.2, and washed on each side with 100 ml of buffer by suction filtration. Filters were dried and radioactivity determined in a scintillation spectrometer. A 7:1 or greater ratio of immobilized DNA to DNA in solution gave a maximum hybridization efficiency of 80 to 85 per cent.

Results.—Virus-specific RNA in adenovirus-transformed cells and group specificity: Cells transformed by Ad 2, 5, and 6 were labeled with 4 μc/ml of [^{3}H] uridine for 180 minutes. [^{3}H]RNA was isolated and annealed with various DNA's. Virus-specific RNA was detected readily in Ad 2, 5, and 6 transformed rat embryo cells. [^{3}H]RNA from Ad 2 transformed cells hybridized equally well with Ad 2 DNA and the DNA's of the other group C members, Ad 1, 5, and 6 (Table 1). Significant hybridization (greater than 10 to 20% of homologous reactions) did not occur with the DNA's of Ad 12 (group A), Ad 7 (group B), Ad 4, 8, and 26, oncogenic SV40, and *Ps. aeruginosa* (Table 1). Similarly, [^{3}H]-RNA from Ad 5 transformed cells hybridized with Ad 1, 2, 5, and 6 DNA's with equal efficiency (Table 2) but not significantly with the DNA's of Ad 12, 7, 4, 26, and *Ps. aeruginosa.* [^{3}H]RNA from Ad 6 transformed cells hybridized with group C DNA's but not with the DNA's of Ad 4, 7, 12, and *Ps. aeruginosa* (Table 3).

Thermal elution of virus-specific RNA from DNA-RNA hybrids. The thermal dissociation profiles of hybrids formed between purified Ad 2, 5, and 6 specific [^{3}H]RNA and homologous viral DNA are shown in Figure 1. The midpoint of the thermal elution curve (T_e) was 67°, 71°, and 69° for Ad 2, 5, and 6 DNA-RNA hybrids. Thermal elution profiles of hybrids formed between Ad 2 specific RNA's and Ad 1, 5, and 6 DNA's were identical to that of the Ad 2 specific RNA-Ad 2 DNA hybrid; T_e values of 67° were found for each hybrid.

TABLE 1. *Hybridization of [^3H]RNA from Ad 2 transformed cells with different DNA's.*

RNA	DNA from*	Virus oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
Expt. 1	Ad 1	Nononcogenic[7]	211	0.070	102
[^{3}H]RNA from Ad 2	Ad 2	"	207	0.069	100
transformed cells§	Ad 5	"	197	0.065	94
	Ad 6	"	207	0.069	100
	Ad 12	Highly oncogenic	17	0.006	<10
	Ad 7	Weakly oncogenic	19	0.006	<10
	Ad 4	Nononcogenic[7]	12	0.004	<10
	Ad 8	"	19	0.006	<10
	Ad 26	"	9	0.003	<10
	SV40	Highly oncogenic	6	0.002	<10
	Ps. aeruginosa	—	3	0.001	<10
Expt. 2	SV40	Highly oncogenic	72	3.41	—
[^{3}H]RNA from SV40 infected BSC-1 cells**	*Ps. aeruginosa*	—	0	0	—

* 3 μg/filter used throughout.
† Average of duplicate hybridization reactions; corrected for nonspecific binding to DNA-free membrane filters (7×10^{-5} of input counts for Expt. 1, 2×10^{-5} of input counts for Expt. 2).
‡ Binding to Ad 2 DNA normalized to 100%.
§ RNA from whole cells labeled for 180 min with [^{3}H]uridine (1 μc/ml; 20 c/mmole); input 302,500 cpm/filter.
** RNA from SV40 infected cells labeled with [^{3}H]uridine (1 μc/ml; 20 c/mmole) from 48 to 49 hr after infection kindly supplied by Dr. Hiroshi Sakaoka, input 2112 cpm/filter.

TABLE 2. *Hybridization of [^3H]RNA from Ad 5 transformed cells with different DNA's.*

RNA	DNA from*	Virus oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
[^{3}H]RNA from Ad 5	Ad 1	Nononcogenic[7]	471	0.186	105
transformed cells§	Ad 2	"	427	0.169	95
	Ad 4	"	23	0.009	<10
	Ad 5	"	450	0.178	100
	Ad 6	"	467	0.185	104
	Ad 7	Weakly oncogenic	25	0.010	<10
	Ad 12	Highly oncogenic	20	0.008	<10
	Ad 26	Nononcogenic[7]	5	0.002	<10
	Ps. aeruginosa	—	3	0.001	<10

* 3 μg/filter used throughout.
† Average of duplicate hybridization reactions corrected for nonspecific binding to DNA-free membrane filters (1.1×10^{-4} of input counts).
‡ Binding to Ad 5 DNA normalized to 100%.
§ RNA from whole cells labeled for 180 min with [^{3}H]uridine (4 μc/ml, 20 c/mmole); input 253,200 cpm/filter.

Hybridization of purified virus-specific RNA with viral DNA. Virus-specific [^{3}H]RNA isolated from Ad 2, 5, and 6 transformed cells was purified by DNA-RNA hybrid formation and thermal elution of virus-specific RNA as described above. Ad 2, 5, and 6 virus-specific RNA purified in this manner hybridized with Ad 1, 2, 5, and 6 DNA's with efficiencies of 56 to 77 per cent (Tables 4, 5, and 6). No hybrid formation (less than 10%) was detected between purified Ad 2, 5, and 6 specific RNA's and group A and B viral DNA's.

Base composition of purified virus-specific RNA: Group C transformed cells were labeled with [^{32}P]-orthophosphate (50 μc/ml) for 180 minutes. Virus-specific [^{32}P]-RNA was isolated and purified by two cycles of hybrid formation

TABLE 3. *Hybridization of [^{3}H]RNA from Ad 6 transformed cells with different DNA's.*

RNA	DNA from*	Virus oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
[^{3}H]RNA from Ad 6 transformed cells§	Ad 1	Nononcogenic[7]	386	0.226	110
	Ad 2	"	369	0.216	105
	Ad 4	"	58	0.034	<20
	Ad 5	"	355	0.208	101
	Ad 6	"	351	0.206	100
	Ad 7	Weakly oncogenic	55	0.032	<20
	Ad 12	Highly oncogenic	50	0.029	<20
	Ps. aeruginosa	—	12	0.007	<20

* 3 μg/filter used throughout.

† Average of duplicate hybridization reactions corrected for nonspecific binding to DNA-free filter (8×10^{-5} of input cpm).

‡ Binding to Ad 6 DNA normalized to 100%.

§ RNA from whole cells labeled for 180 min with [^{3}H]uridine (4 μc/ml, 20 c/mmole), input 170,600 cpm.

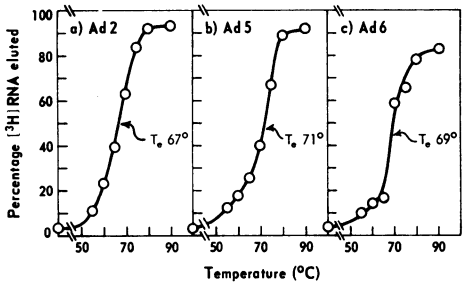

FIG. 1.—Thermal elution of [^{3}H]RNA from membrane filters containing the following DNA-RNA hybrids: (*a*) Ad 2 DNA and Ad 2 specific [^{3}H]RNA (608 cpm), (*b*) Ad 5 DNA and Ad 5 specific [^{3}H]RNA (755 cpm), (*c*) Ad 6 DNA and Ad 6 specific [^{3}H]RNA (2216 cpm). Virus-specific RNA used for hybrid formation was purified previously by one cycle of hybrid formation and elution.

with viral DNA and elution of the RNA from the hybrid. The base composition of purified Ad 2, 5, and 6 specific [^{32}P]RNA's was determined by alkaline hydrolysis and paper electrophoresis as described under *Experimental Methods*. As shown in Table 7, the guanine plus cytosine (G + C) content of Ad 2, 5, and 6 virus-specific RNA's is 49, 51, and 50 per cent respectively. These values are 7 to 9 per cent lower than the G + C content of the respective viral DNA's:

TABLE 4. *Hybridization of purified Ad 2 specific RNA with different DNA's.*

RNA	DNA from*	Virus oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
Purified Ad 2 specific [^{3}H]RNA§	Ad 1	Nononcogenic[7]	218	68	93
	Ad 2	"	235	73	100
	Ad 4	"	5	<3	<5
	Ad 5	"	212	66	90
	Ad 6	"	248	77	106
	Ad 7	Weakly oncogenic	6	<3	<5
	Ad 8	Nononcogenic[7]	7	<3	<5
	Ad 12	Highly oncogenic	5	<3	<5
	Ps. aeruginosa	—	2	<3	<5

* 3 μg/filter used throughout.

† Average of duplicate hybridization reactions corrected for nonspecific binding to DNA-free filter (5×10^{-2} of input count).

‡ Binding to Ad 2 DNA normalized to 100%.

§ [^{3}H]RNA from Ad 2 transformed cells labeled for 180 min with [^{3}H]uridine (4 μc/ml, 20 c/mmole) purified by hybrid formation with Ad 2 DNA; input, 321 cpm/filter.

TABLE 5. *Hybridization of purified Ad 5 specific RNA with different DNA's.*

RNA	DNA from*	Oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
Purified Ad 5 specific [^{3}H]RNA§	Ad 1	Nononcogenic[7]	218	63	104
	Ad 2	"	192	56	92
	Ad 4	"	16	<5	<10
	Ad 5	"	209	61	100
	Ad 6	"	212	62	101
	Ad 7	Weakly oncogenic	12	<5	<10
	Ad 12	Highly oncogenic	7	<5	<10
	Ad 26	Nononcogenic[7]	11	<5	<10
	Ps. aeruginosa	—	1	<5	<10

* 3 μg/filter used throughout.
† Average of duplicate hybridization reactions, corrected for nonspecific binding to DNA-free filters (7×10^{-2} of input counts).
‡ Binding to Ad 5 DNA normalized to 100%.
§ [^{3}H]RNA from Ad 5 transformed cells labeled for 180 min with [^{3}H]uridine (4 μc/ml, 20 c/mmole), purified by hybrid formation with Ad 5 DNA; input 344 cpm/filter.

Ad 2 DNA, 57 per cent G + C; Ad 5 DNA, 58 per cent G + C; and Ad 6 DNA, 59 per cent G + C.[16] These data suggest that viral DNA regions with low G + C contents are transcribed preferentially in adenovirus transformed cells.

The base compositions of Ad 5 and 6 specific RNA's are very similar and differ from that of Ad 2. Adenovirus-specific RNA's isolated from group A, B, and C tumor and transformed cells possess a low C and high A content compared to mammalian ribosomal and transfer RNA molecules.[8]

DNA-DNA homology between group A, B, and C viral DNA's: DNA-DNA homology measurements were used to estimate the genetic relatedness among group C adenoviruses and between group C and group A and B adenoviruses. As shown in Table 8, group C members are closely related since [^{3}H]Ad 2 DNA hybridized with Ad 1, 5, and 6 DNA's 83 to 93 per cent as efficiently as it did with Ad 2 DNA. Ad 12 [^{3}H]DNA (group A) and Ad 7 [^{3}H]DNA (group B) hybridized with Ad 1, 5, and 6 DNA only 20 to 26 per cent as efficiently as with homologous DNA (Table 8). Thus, most viral DNA nucleotide sequences of group C adenoviruses differ from those of group A and B adenoviruses.

TABLE 6. *Hybridization of purified Ad 6 specific RNA with different DNA's.*

RNA	DNA from*	Oncogenicity	Bound† (cpm)	Bound† (%)	Relatedness‡ (%)
Purified Ad 6 specific [^{3}H]RNA§	Ad 1	Nononcogenic[7]	354	63	89
	Ad 2	"	366	65	92
	Ad 4	"	65	12	16
	Ad 5	"	398	71	99
	Ad 6	"	400	71	100
	Ad 7	Weakly oncogenic	36	<10	<10
	Ad 12	Highly oncogenic	18	<10	<10
	Ps. aeruginosa	—	2	<10	<10

* 3 μg/filter used throughout.
† Average of duplicate hybridization reactions corrected for nonspecific binding to DNA-free filter (1×10^{-2} of input counts).
‡ Binding to Ad 6 DNA normalized to 100%.
§ [^{3}H]RNA from Ad 6 transformed cells labeled for 180 min with [^{3}H]uridine (4 μc/ml, 20 c/mmole) purified by hybrid formation with Ad 6 DNA; input 560 cpm/filter.

TABLE 7. *Base composition of adenovirus specific RNA isolated from transformed cells.*

Virus-specific RNA* from	No. of determinations	Molar Percentage ± Standard Deviation				
		Cytosine	Adenine	Guanine	Uracil	G + C†
Ad 2 transformed cells	8	23.1 ± 0.4	24.4 ± 0.6	25.7 ± 0.3	26.9 ± 0.5	48.8 ± 0.4
Ad 5 transformed cells	5	22.2 ± 0.9	23.1 ± 0.5	28.6 ± 0.9	26.1 ± 0.5	50.8 ± 0.8
Ad 6 transformed cells	5	21.3 ± 0.3	22.9 ± 0.7	28.8 ± 0.2	27.0 ± 0.8	50.1 ± 0.7

* RNA isolated from transformed cells labeled for 180 min with [^{32}P]orthophosphate (50 μc/ml), purified by two cycles of hybrid formation and elution.
† Guanine + cytosine content.

TABLE 8. *DNA-DNA homology among C group adenoviruses.*

DNA on filter (3 μg)	[^{3}H]DNA in solution (cpm/ml)	Counts bound*	Homology (%)	DNA on filter (3 μg)	[^{3}H]DNA in solution (cpm/ml)	Counts bound*	Homology (%)	DNA on filter (3 μg)	[^{3}H]DNA in solution (cpm/ml)	Counts bound*	Homology (%)
Ad 2	Ad 2 (980)	764†	100‡	Ad 12	Ad 12 (590)	335§	100	Ad 7	Ad 7 (1790)	1534§	100
Ad 1	"	691	92	Ad 1	"	81	24	Ad 1	"	384	25
Ad 5	"	635	83	Ad 5	"	71	21	Ad 5	"	399	26
Ad 6	"	699	93	Ad 6	"	68	20	Ad 6	"	374	24

* Binding to empty filter subtracted (7–10 cpm).
† Average of triplicate filters.
‡ Homologous reaction normalized to 100%.
§ Average of duplicate filters.

Discussion.—A new class of adenovirus-specific RNA molecules was found in rat embryo cells transformed by group C Ad 2, 5, and 6. Group C adenovirus-specific RNA's hybridized equally well with Ad 1, 2, 5, and 6 DNA's but not significantly with group A and B DNA's. Thus, different viral coded RNA molecules are transcribed in cells transformed by group A, B, and C adenoviruses. However, the experimental data do not rule out a small proportion of common sequences (less than 10%).

Group C adenoviruses are closely related. Ad 1, 2, 5, and 6 share 83 to 93 per cent of their DNA nucleotide sequences. Group A (Ad 12, 18, and 31) are 80 to 85 per cent related[17] and group B (Ad 3, 7, 11, 14, 16, and 21) are 70 to 100 per cent related.[18] Group A, B, and C DNA's share only 10 to 26 per cent of their nucleotide sequences but the shared nucleotide sequences are not transcribed detectably in adenovirus-transformed cells. These results strongly suggest that the viral genes involved in transformation by group C adenovirus differ from those of group A and B adenoviruses.

Ad 2 specific RNA differs in base composition from Ad 5 and 6 specific RNA although Ad 2, 5, and 6 specific RNA hybridize with all group C DNA's with equal efficiency. These data may be explained by different proportions of individual virus-specific RNA molecules in different group C transformed cells or by the transcription of dissimilar viral genes in different group C transformed cells.

DNA regions with an average G + C content of 47 to 51 per cent are incorporated or transcribed preferentially in adenovirus-tumor and transformed cells. As described previously,[8] group A and B virus-specific RNA's contain 47 to 48 per cent G + C. The G + C content of virus-specific RNA from group C transformed cells is 49 to 51 per cent which is 7 to 9 per cent lower than that of the respective viral DNA's. These data suggest that only a portion of the viral genome is transcribed in group C transformed cells. Recent hybridization competition studies show that only 4 to 10 per cent of the viral genome is transcribed in Ad 2 transformed rat embryo cells.[19] Since the molecular weight of adenovirus duplex DNA is 23 million,[20] the portion of the viral genome transcribed in transformed cells represents less than 3 million daltons of adenovirus DNA. This amount of DNA could code for only six polypeptides of molecular weight 25,000.

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