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Mechanism of Viral Carcinogenesis by DNA Mammalian Viruses, VII. Viral Genes Transcribed in Adenovirus Type 2 Infected and Transformed Cells*

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Abstract. DNA-RNA hybridization-competition experiments were used to compare the virus-specific RNA sequences synthesized during productive infection with human adenovirus type 2 with those synthesized in virus-free adenovirus type 2 transformed cells. The "early" virus-specific RNA present at six hours after infection, prior to the onset of viral DNA synthesis, represents 8–20 per cent (2 to 10 genes) of the viral genome. All viral RNA sequences synthesized early are also present "late," at 18 hours after infection. The base sequences transcribed in transformed cells are homologous to approximately 50 per cent of the sequences transcribed early after infection. Thus only 4 to 10 per cent of the viral genome, representing 1 to 5 viral genes, are transcribed in adenovirus type 2 transformed cells. The virus-specific RNA synthesized 18 hours after infection was not found in transformed cells, suggesting that either these late viral genes are not present or are not transcribed in adenovirus type 2 transformed cells.

Introduction. Cells transformed by ten members of human adenovirus (Ad) groups A, B, and C synthesize virus-specific RNA molecules with an average G + C content of 47 to 51 per cent.[1–5] Groups A and B adenovirus DNA's have G + C contents of 48 to 49 and 50 to 52 per cent, respectively, but group C DNA's have 56 to 59 per cent G + C. These results suggest that only a portion of the adenovirus genome with an average G + C content of 47 to 51 per cent is transcribed in adenovirus tumor and transformed cells. This paper reports the results of hybridization-competition experiments which determine the fraction of the viral genome transcribed in Ad 2 (group C) transformed cells and the relationship between the viral mRNA species synthesized in transformed cells and those synthesized "early" and "late" after productive infection.

Early Ad 2 mRNA (messenger ribonucleic acid), i.e., virus-specific RNA synthesized prior to the onset of viral DNA synthesis at seven hours after infection,[6–8] and late viral mRNA, i.e., virus-specific RNA synthesized subsequently, were characterized recently.[9, 10] All or nearly all, 80 to 100 per cent, of the viral genome is transcribed during Ad 2 infection.[10] All early mRNA sequences synthesized at 6 to 6.5 hours after infection are present late after infection (18 hr), but only a fraction of late mRNA sequences synthesized at 18 to 18.5 hours

after infection are present at the early times.[9] As described below, viral mRNA sequences transcribed in Ad 2 transformed cells represent one-half of the viral gene sequences transcribed early after infection; virus-specific RNA sequences transcribed late after infection were not detected in transformed cells.

Materials and Methods. Cell culture and virus infection: Cultures of Ad 2 transformed rat embryo cells[11] (8617) kindly provided by Dr. A. E. Freeman were grown in suspension cultures in Eagle's minimum essential medium[12] with 5% calf serum. Suspension cultures of KB cells at 2–3 × 10^5 cells/ml were infected with Ad 2 (strain 38-6) at an input multiplicity of 100 plaque-forming units/cell as described previously.[10] In this system, infectious virus is formed between 13 and 24 hr and viral DNA is synthesized between 7 and 20 hr after infection.[6–8]

Viral DNA: The growth and purification of Ad 2 and the isolation of viral DNA have been described.[13] Viral DNA was purified further by CsCl density gradient centrifugation.

RNA: [H^3]RNA was prepared from infected KB cells labeled with [H^3] uridine (1 μCi/ml, 20 Ci/mmole or from Ad 2 transformed cells labeled with [H^3]uridine (4 μCi/ml, 20 Ci/mmole) by the hot phenol-SDS (sodium dodecyl sulfate) method including a DNase treatment.[10]

DNA-RNA hybridization: DNA-RNA hybridizations were performed by the procedure of Gillespie and Spiegelman[14] as described previously.[5]

DNA-RNA hybridization inhibition and hybridization competition: Two sequential annealing reactions were performed. In step 1, viral DNA immobilized on membrane filters was annealed with increasing amounts of unlabeled RNA from Ad 2 infected cells or Ad 2 transformed cells in 1.0 ml of 2 × SSC (SSC = 0.15 *M* NaCl–0.015 *M* Na_3 citrate) — 0.1% SDS for 20 hr at 66°. In step 2 of the hybridization-inhibition reaction, the RNA solution was removed, the DNA filter was rinsed with 1 ml of 2 × SSC, and annealed further with saturating amounts of [H^3]RNA (late, early, or transformed) in 1 ml of 2 × SSC — 0.1% SDS for 20 hr at 66°. In the hybridization-competition reaction, saturating amounts of [H^3]RNA (0.4 ml) were added without removal of the unlabeled RNA and the incubation carried out for an additional 20 hr at 66°.

Results. Are the virus-specific RNA molecules synthesized in adenovirus transformed cells also present in cells productively infected by Ad 2? The relationship between Ad 2 early and early plus late virus-specific RNA and Ad 2 transformed cell RNA was analyzed by hybridization-inhibition experiments. As shown in Table 1, hybrid formation between labeled virus-specific RNA from Ad 2 transformed cells and Ad 2 DNA was reduced to the same extent, 20, 16, and 18 per cent of the control levels, by preannealing with 3.2 to 4.0 mg of unlabeled early, early plus late, and transformed cell RNA. These results indicate that the virus-specific base sequences synthesized in Ad 2 transformed cells are present both early and late after productive infection with Ad 2.

The fraction of the viral genome transcribed in Ad 2 transformed cells: Virus-specific RNA molecules present in KB cells 18 hours after infection are transcribed from 80 to 100 per cent of the viral genome and contain all base sequences synthesized early (6 to 6.5 hr) after infection.[9] Hybridization-competition experiments with RNA labeled with [H^3]uridine from 2 to 18 hours after infection (early plus late RNA, representing 80 to 100 per cent of the viral gene transcripts) were used to determine the fraction of the viral genome transcribed early after infection and the fraction of the viral genome transcribed in Ad 2 transformed cells.

(*a*) **Saturation of viral DNA with labeled RNA from Ad 2 infected cells:**

TABLE 1. *The presence of adenovirus type 2 transformed cell virus-specific RNA early and late after productive infection.*

1st hybridization reaction unlabeled RNA	2nd hybridization reaction [H³]RNA	Ad 2 DNA (μg/filter)	Bound (cpm)	Control (%)[a]
Ad 2 early RNA[b] 3200 μg	Ad 2 transformed cell RNA[c] 577,000 cpm/filter	0.5	74[d]	20
		None	28	
Ad 2 early plus late RNA[e] 2500 μg	Ad 2 transformed cell RNA[c] 577,000 cpm/filter	0.5	63[d]	16
		None	26	
Ad 2 transformed cell RNA[f] 4050 μg	Ad 2 transformed cell RNA[c] 577,000 cpm/filter	0.5	69[d]	18
		None	28	
KB cell RNA[g] 4050 μg	Ad 2 transformed cell RNA[c] 577,000 cpm/filter	0.5	235[d]	97
		None	11	
2 × SSC − 0.1% SDS (control)	Ad 2 transformed cell RNA[c] 577,000 cpm/filter	0.5	268[d]	100
		None	36	

[a] Background (cpm bound to an empty filter) subtracted. Counts bound to DNA filter not prehybridized with unlabeled RNA (control) normalized to 100%.
[b] RNA from Ad 2 infected KB cells, 6 hr after infection.
[c] RNA from Ad 2 transformed rat embryo cells labeled for 180 min with [H³]uridine (4 μCi/ml, 20 Ci/mmole).
[d] Average of duplicate hybridization reactions.
[e] RNA from Ad 2 infected KB cells, 18 hr after infection.
[f] RNA from Ad 2 transformed rat embryo cells.
[g] RNA from uninfected KB cells

Saturating amounts of both unlabeled and labeled RNA species were used so that results were interpretable in terms of base sequences transcribed. First the amounts of labeled early and early plus late RNA necessary to saturate viral DNA were determined (Fig. 1). Filters containing 0.05 μg of Ad 2 DNA were annealed with increasing amounts of [H³]RNA from infected KB cells labeled with [H³]uridine (1 μCi/ml) from 2 to 18 hours after infection (early plus late RNA) (Fig. 1*a*). Saturation was reached at an input of 2×10^6 cpm of [H³]RNA (18,700 cpm/μg). Saturation of 0.1 μg of Ad 2 DNA was reached with RNA from cells labeled from 2 to 6 hours after infection (early RNA) with an input of 2×10^6 cpm (14,100 cpm/μg) (Fig. 1*b*).

(*b*) **The fraction of the viral genome transcribed early after infection:** Hybridization-competition experiments were performed by annealing Ad 2 DNA (0.05 μg) first with increasing amounts of unlabeled RNA and then with RNA from KB cells labeled from 2 to 18 hours after infection. RNA from KB cells 18 hours after infection completely blocked the hybridization of labeled RNA with Ad 2 DNA as was expected (Fig. 2). Uninfected KB cell RNA did not inhibit significantly hybrid formation between labeled RNA and Ad 2 DNA (less than 5%). Early RNA competed with 10 to 20 per cent of the base sequences of 2 to 18 hours [H³]RNA. Transformed cell RNA competed with some of the base sequences of labeled virus-specific RNA (less than 10%); the exact amount is difficult to estimate in this experiment but can be more accurately estimated by competition with labeled early RNA (see below). Since 80 to 100 per cent of the viral genome is transcribed during the first 18 hours after infection,[10] we conclude that 8 to 20 per cent of the viral genome is transcribed early after infection.

(*c*) **The fraction of the viral genome transcribed in Ad 2 transformed cells:** The competition of transformed cell RNA for sites on Ad 2 DNA with early RNA labeled from 2 to 6 hours after infection was measured using saturating

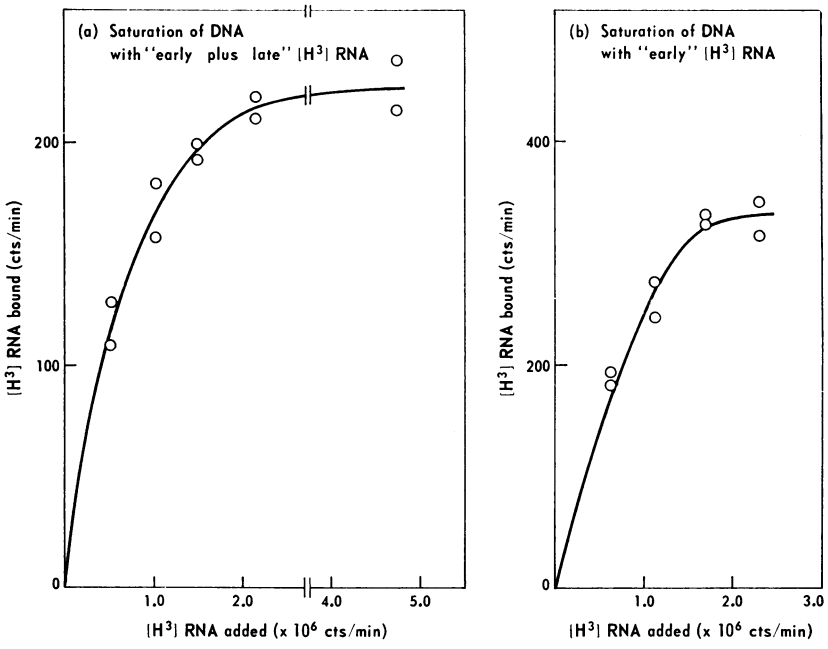

FIG. 1.—Saturation of DNA with early and early plus late [H³]RNA labeled from 2 hr after infection. (*a*) Filters containing 0.05 μg of Ad 2 DNA were annealed for 20 hr at 66° with increasing amounts of [H³]RNA from Ad 2 infected KB cells labeled with 1 μCi/ml of [H³]uridine from 2 to 18 hr after infection (early plus late RNA). Specific activity of [H³]RNA was 18,700 cpm/μg. (*b*) Filters containing 0.10 μg of Ad 2 DNA were annealed for 20 hr at 66° with increasing amounts of [H³]RNA from Ad 2 infected KB cells labeled with 1 μCi/ml of [H³]uridine from 2 to 6 hr after infection (early RNA). Specific activity of [H³]RNA was 14,100 cpm/μg.

amounts of both unlabeled and labeled RNA (Fig. 3). Unlabeled 18 hour RNA (early plus late) completely blocked hybrid formation between early [H³]RNA and viral DNA indicating that all early RNA sequences are present also late after infection. This agrees with previous observations in which RNA sequences synthesized 6 to 6.5 hours after infection were studied.[9]

The most significant finding is that transformed cell RNA competed with 50 per cent of early RNA sequences labeled with [H³]uridine from 2 to 6 hours after infection (Fig. 3). Similar results were obtained using RNA labeled with [P³²]orthophosphate. Since early RNA is transcribed from 8 to 20 per cent of the viral genome, as shown above, we estimate that virus-specific RNA in Ad 2 transformed cells is transcribed from 4 to 10 per cent of the viral genome.

Are the virus-specific RNA sequences transcribed in transformed cells also synthesized late after productive infection? Viral DNA was annealed first with unlabeled RNA from Ad 2 transformed cells or from KB cells 6 hours after infection and then with RNA from KB cells pulse labeled for 30 minutes at 18 hours after infection. The amount of labeled RNA needed to saturate 0.05 μg of viral DNA was 1.84×10^5 cpm of RNA (4200 cpm/μg) (Fig. 4). Unlabeled RNA from uninfected cells and from Ad 2 transformed cells did not com-

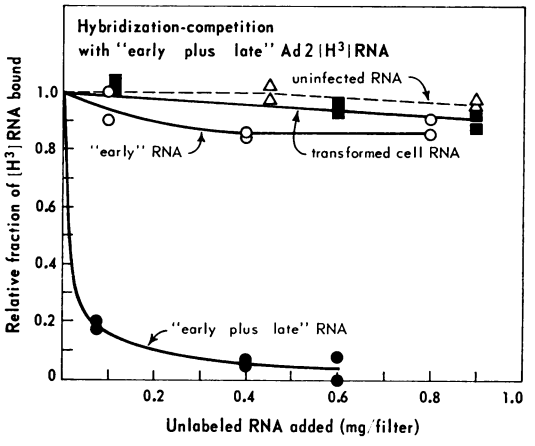

FIG. 2.—Hybridization competition between labeled Ad 2 early plus late virus-specific RNA and unlabeled Ad 2 early virus-specific RNA or Ad 2 transformed virus-specific RNA. In step 1, membrane filters containing 0.05 μg of Ad 2 DNA were incubated with different amounts of unlabeled RNA for 20 hr at 66°. In step 2, the DNA filters were incubated further for 20 hr at 66° with 3.24×10^6 cpm (140 μg)/filter of [H^3]RNA from Ad 2 infected cells labeled with 1 μc/ml of [H^3]-uridine (20 Ci/mmole) for 2 to 18 hr after infection. The filters were washed, treated with RNase, washed, and counted. Counts bound to DNA filters incubated with 2 × SSC–0.1% SDS instead of unlabeled RNA in step 1 were normalized to 100%.

pete significantly with pulse labeled late RNA (Fig. 5). Thus the virus-specific RNA sequences synthesized late after productive infection are not detected in Ad 2 transformed cells. In agreement with previous findings,[9] early RNA competed with 10 to 20 per cent of the base sequences of pulse labeled late RNA indicating that some early genes are transcribed both before and after the onset of viral DNA synthesis.

Discussion. Studies reported here are based on viral DNA-RNA hybridization-competition measurements using saturating amounts of both unlabeled and labeled RNA. In this manner the fraction of the base sequences held in common is measured. This method assumes that each gene product, i.e., the virus-specific RNA molecule, has the same specific activity. When virus-specific RNA is labeled for a long time, 4 to 16 hours as reported here, these conditions are probably met.

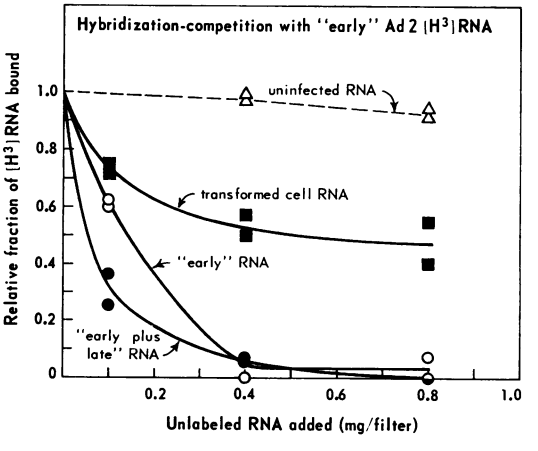

FIG. 3.—Hybridization-competition between labeled Ad 2 early virus-specific RNA and unlabeled Ad 2 transformed virus-specific RNA or unlabeled Ad 2 early plus late virus-specific RNA. Filters containing 0.1 μg of Ad 2 DNA were incubated with different amounts of unlabeled RNA from Ad 2 transformed rat embryo cells, Ad 2 infected KB cells, 6 hr (early) or 18 hr (early plus late), or uninfected KB cells 20 hr at 66°. The filters were incubated further for 20 hr at 66° with 3.09×10^6 cpm (219 μg)/filter of [H^3]RNA from Ad 2 infected cells labeled with 1 μCi/ml of [H^3]uridine (20 Ci/mmole) from 2 to 6 hr after infection and processed as described in the legend to Figure 2.

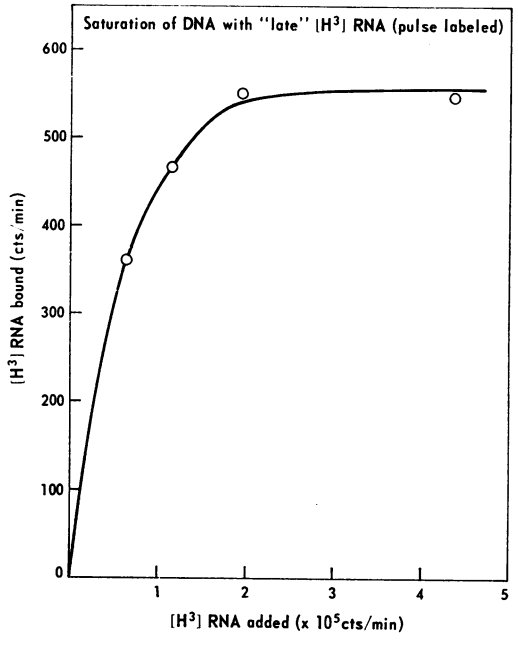

FIG. 4.—Saturation of DNA with pulse-labeled late [H³]RNA. Membrane filters containing 0.05 μg of Ad 2 DNA were incubated with increasing amounts of [H³]RNA from Ad 2 infected KB cells labeled with 1 μCi/ml of [H³]uridine for 30 min, from 18 to 18.5 hr after infection. After incubation for 20 hr at 66°, the filters were washed, treated with RNase, washed, and counted. The specific activity of [H³]RNA was 4200 cpm/μg.

The fractions of the viral genome transcribed during productive infection with Ad 2 and in Ad 2 transformed cells are diagrammed in Figure 6. During productive infection of KB cells with Ad 2, 80 to 100 per cent of the viral genome is transcribed.[10] DNA-RNA hybridization-competition show that: (1) 8 to 20 per cent of the viral genome is transcribed early after infection, the remainder

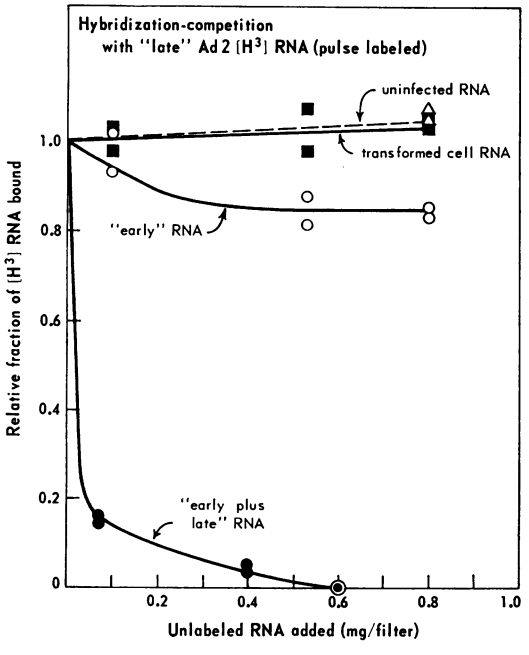

FIG. 5.—Hybridization-competition between pulse-labeled Ad 2 late virus-specific RNA and unlabeled Ad 2 early virus-specific RNA or unlabeled Ad 2 transformed virus-specific RNA. Membrane filters containing 0.05 μg of Ad 2 DNA were incubated for 20 hr at 66° with different amounts of unlabeled RNA from Ad 2 transformed rat embryo cells, Ad 2 infected KB cells, 6 hr (early) or 18 hr (early plus late), or uninfected KB cells. The filters were incubated further for 20 hr at 66° with 1.84×10^6 cpm (44 μg)/filter of [H³]RNA from Ad 2 infected cells labeled with 1 μCi/ml of [H³]uridine (20 Ci/mmole) from 18 to 18.5 hr after infection and processed as described in the legend to Figure 2.

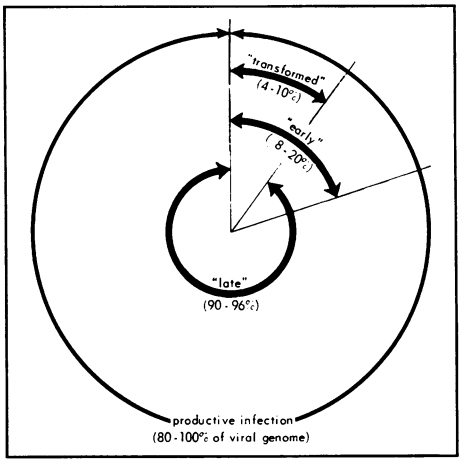

FIG. 6.—Diagram representing the fractions of the viral genome transcribed in Ad 2 infected cells and in Ad 2 transformed cells.

being transcribed late, and (2) 4 to 10 per cent of the viral genome is transcribed in Ad 2 transformed rat embryo cells.

The Ad 2 RNA base sequences synthesized in transformed cells are present both early and late after productive infection. They represent 50 per cent of the early viral gene transcripts. Virus-specific RNA transcribed relatively late after infection with Ad 2 (at 18 hours) were not detectably transcribed in Ad 2 transformed cells. Aloni *et al.*[15] and Oda and Dulbecco[16] have detected late virus-specific RNA in SV40 transformed cells. The physical state of the viral genome in adenovirus and SV40 transformed cells may be different since infectious virus can be rescued by fusion of permissive cells with SV40 transformed cells[17, 18] but not with adenovirus transformed cells.[19] It will be of interest to determine whether cells transformed by polyoma virus and group A and B adenoviruses transcribe late viral genes.

The Ad 2 genome is a duplex DNA molecule of molecular weight 23 million daltons,[20] which can code for 23 polypeptides of average molecular weight 50,000 or for 46 polypeptides of average molecular weight 25,000. Assuming that the viral genome consists of 23 to 46 genes, we estimate 18 to 46 genes are transcribed during productive infection (80–100% of the genome), 2 to 10 genes prior to DNA synthesis (early genes), and only 1 to 5 genes in Ad 2 transformed cells.

The small number of viral genes functioning in Ad 2 transformed cells specify several viral-coded proteins which probably function in maintaining the transformed cell phenotype. Ad 12 T^{21a} antigens isolated from Ad 12 transformed cells were resolved into four polypeptide components which react with antibody from tumor bearing hamsters.[21] However, the function of these virus-specific proteins is not known nor has it been rigorously established that they are viral coded.

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