Detection of Viral DNA Sequences in Adenovirus-Transformed Cells by In Situ Hybridization

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Received for publication 17 July 1973

Cytological preparations of cells transformed by members of three groups of human adenoviruses, adenovirus 12, 7, and 2, were annealed with radioactive complementary RNA (cRNA) (4×10^7 to 4.5×10^7 dpm/ μ g) prepared by copying viral DNA with the *Escherichia coli* DNA-directed RNA polymerase. These in situ hybridizations detected adenovirus-specific DNA sequences in interphase nuclei when transformed cells were annealed with homologous viral cRNA, but not with heterologous viral cRNA. The highest autoradiographic grain counts were found over adenovirus 7-transformed cell nuclei, next over adenovirus 12-, and the lowest over adenovirus 2-transformed cell nuclei. This is the same order as found by reassociation kinetic measurements (K. Fujinaga and M. Green, unpublished data).

Cells transformed by human adenoviruses transcribe virus-specific RNA sequences from viral genes that are most likely integrated into cellular DNA (8). Molecular hybridization and reassociation kinetic studies have detected from 1 to 100 copies of some viral DNA sequences in cells transformed by human adenoviruses (6, 8, 15, and K. Fujinaga and M. Green, unpublished data). A way to localize and quantitate relative amounts of adenovirus DNA sequences within the cell is provided by in situ hybridization (4). We report here studies in which highly radioactive complementary RNA (cRNA) was transcribed from viral DNA with the *Escherichia coli* RNA polymerase (7) and used as a probe to locate DNA of adenovirus 12, 7, and 2, members of human adenovirus groups A, B, and C, respectively, by annealing with cytological preparations of adenovirus-transformed cells. The results show that adenovirus gene sequences are present in interphase nuclei of cells transformed by three different human adenoviruses.

MATERIALS AND METHODS

Cell lines. Clonally derived cell lines of adenovirus 2-transformed rat (8617, clone 9), adenovirus 7-transformed hamster (5728, clone 15), adenovirus 12-transformed hamster (HE, clone 9), normal rat (F1853), and normal hamster (NIL-2E) were grown in monolayer in Eagle minimal essential medium containing

10% fetal calf serum. Cytological preparations were prepared from cells harvested by trypsinization and were fixed in 3:1 alcohol-acetic acid (4).

Viral DNA. Plaque-purified stocks of adenovirus 2 (strain 38-2, plaque 4), adenovirus 7 (Grider, plaque 6), and adenovirus 12 (Huie, plaque 9), free of adenovirus-associated viruses, were grown in KB cells (9). Viral DNA was purified as described (9).

Preparation of ³H-cRNA. Adenovirus 2, 7, and 12 DNA were transcribed in vitro (7) by the *E. coli* RNA polymerase purified through the glycerol gradient step (3). A ratio of enzyme protein to DNA of 1:7 was used in a reaction mixture containing 100 mM Tris-hydrochloride (pH 8.1), 200 mM KCl, 10 mM MgCl₂, 4 mM β -mercaptoethanol, 1.6 mM each of ATP, GTP, and CTP, and 40 μ Ci of ³H-UTP per ml (22 to 24 Ci/mmol). Incubation was performed for 120 min at 37 C. The RNA product was purified (7) and dissolved in 0.1 $\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate). The calculated specific activity of viral cRNA was 4×10^7 to 4.5×10^7 dpm/ μ g, assuming an equimolar content of the four ribonucleotides.

Analysis of ³H-cRNA by polyacrylamide gel electrophoresis. The size of in vitro-synthesized cRNA was analyzed by electrophoresis on 3.2% polyacrylamide gels (8 by 0.6 cm) for 3 h at room temperature with a constant current of 5 mA/gel (1). The gels were sliced into 2-mm segments, placed in scintillation vials, and solubilized overnight with 300 μ liters of NH₄OH at room temperature. Ten milliliters of scintillation fluid (1 g of 2,5-bis[2-(5-tert-butylbenzoxazolyl)]thiophene, 20 g of naphthalene, 100 ml of ethylene glycol monomethyl ether, 150 ml of toluene) was added to each vial, and the samples were counted in a liquid scintillation spectrometer.

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Preparation of cellular RNA for competition studies. Adenovirus 2-, 7-, and 12-transformed cells were lysed with 1% Na dodecyl SO_4 in 10 to 15 volumes of 0.05 M Na acetate buffer (pH 5.1), digested with Pronase (100 $\mu\text{g}/\text{ml}$) for 2 h at 37 C, and extracted twice with buffer-saturated phenol at 60 C for 3 min. The pooled aqueous phases were precipitated with two volumes of ethanol, suspended in 0.05 M Tris-hydrochloride (pH 7.5) and 10 mM MgCl_2 , and digested for 40 min at 37 C with DNase (50 $\mu\text{g}/\text{ml}$, Worthington Biochemical Co., RNase free). After extraction with chloroform-isoamyl alcohol (24:1) and precipitation with alcohol, the RNA preparation was dissolved in $0.1 \times \text{SSC}$ and dialyzed against $0.1 \times \text{SSC}$. RNA concentration was estimated by orcinol analysis (5). Possible contamination with DNA and protein was monitored by diphenylamine and Lowry protein analysis, respectively (5).

In situ hybridization. The procedure for in situ hybridization was basically that described by Gall and Pardue (4). Cytological preparations of cells on slides were denatured with 0.07 N NaOH for 2 min at room temperature, washed sequentially in 70 and 95% alcohol, and dried in air. From 100 to 150 μl of ^3H -cRNA in $2 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl}$ -0.015 M $\text{Na}_2\text{citrate}$) was placed on each slide, and annealing was performed at 66 C for 16 to 18 h. In some experiments annealing was performed in 50% formamide in $2.5 \times \text{SSC}$ for 24 h at 45 C. After hybridization, slides were treated with RNase A (20 $\mu\text{g}/\text{ml}$, Sigma Chemical Co.) and RNase T_1 (40 U/ml, Sigma Chemical Co.) in $2 \times \text{SSC}$ for 1 h at 37 C. Preparations were then dehydrated with alcohol as described above and used directly for autoradiography. Representative slides from each experiment were treated with 0.2 mg of DNase per ml (Worthington Biochemical Co., RNase free) in 10 mM Tris-hydrochloride (pH 7.3) and 1 mM MgCl_2 for 2 h at 37 C, as controls for the specificity of hybridization. Slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) and stored in 4 C for 16 to 24 weeks. Slides were developed in Kodak D 19 for 4 min and stained for 30 min with Giemsa.

RESULTS

Properties of adenovirus cRNA. ^3H -labeled cRNA preparations transcribed by the *E. coli* RNA polymerase with purified adenovirus 2, 7, and 12 DNA molecules as templates were characterized with regard to sequence specificity and size of RNA molecules. Preparations of cRNA were annealed with 3 ng of adenovirus DNA as described (7). The extent of hybridization and specificity for homologous adenovirus DNA was found as in the range previously reported (7).

Polyacrylamide gel electrophoresis was performed with adenovirus 2, 7, and 12 cRNA (Fig. 1). Markers consisted of ^{14}C -labeled 18 and 4S RNA isolated from the cytoplasm of KB cells. Most of the viral RNA migrated with *S* values greater than 4S, indicating the absence of

FIG. 1. Polyacrylamide gel electrophoresis of the ^3H -cRNA's synthesized *in vitro* by the *E. coli* RNA polymerase. Electrophoresis was for 3 h at 5 mA per gel tube (8 by 0.6 cm). The positions of 18S and 4S RNA were determined by parallel electrophoresis of cytoplasmic ^{14}C -RNA from KB cells.

appreciable degradation. Adenovirus 2 cRNA displayed four peaks of radioactivity with calculated *S* values of approximately 8, 12, 13, and 16. Adenovirus 7 and 12 cRNA contained only two major peaks with *S* values close to 9 and 12.

In situ hybridization of adenovirus 2-, 7-, and 12-transformed cells with viral cRNA. Cytological preparations of adenovirus 12-transformed hamster cells (HE, clone 9), adenovirus 7-transformed hamster cells (5728, clone 15), and adenovirus 2-transformed rat cells (8617, clone 9), members of group A, B, and C, respectively, were annealed with their homologous viral cRNA. Normal rat (F1853) and hamster cells (NIL-2E) were used as controls. Silver grains were detected over the interphase nuclei of transformed cells (Fig. 2, 3, 4) as well as over a few chromosomes. Background grains were determined over equal areas containing no cellular structures for each set of control and transformed cells. The number of grains per nucleus was then computed from 150 nuclei for each cell line and 150 corresponding blank areas.

Although the absolute grain density per nucleus is low, the differences between control and transformed cells are unequivocal (Table 1). Comparisons among mean grain counts were performed for each group of means after arrangement in sequential order. The pooled standard error was calculated for each set of means and the differences were tested at $P = 0.05$ (11). The adenovirus 2 results (Table 1, 2) show low background levels, that emphasized the significance of transformed cell grain counts. In the case of adenovirus 12, the differences between the four sets of means (Table 2) are significant for each pair, although the average grain count for the control cell line and its background were relatively high; correcting the

FIG. 2. Nuclei of adenovirus type 2-transformed rat cells (a) and normal rat cells (b) after annealing with adenovirus 2 ^3H -cRNA (specific activity 4×10^7 to 4.5×10^7 dpm/ μg) synthesized *in vitro* by the *E. coli* RNA polymerase. The slides were incubated for 16 to 18 h at 66 C. After hybridization the preparations were processed as described in Materials and Methods. Exposure time, 16 weeks. $\times 2,080$.

control cell for its own background grain value yields a figure of only 0.55 grains. With adenovirus 7-transformed cells the situation is similar, but the grain density was sufficiently high so that control grain counts are immaterial.

Specificity of *in situ* hybridization. The specificity for the *in situ* RNA-DNA hybridization with adenovirus ^3H -cRNA was confirmed by three procedures: (i) slides pretreated with DNase in each set of hybridizations showed no grains over cytological structures (data not

shown); (ii) hybridization with heterologous adenovirus ^3H -cRNA gave no significant grains over background values (e.g., adenovirus 12-transformed cells annealed with adenovirus 7 ^3H -cRNA in Table 1); (iii) homologous cellular RNA purified from adenovirus 2-, 7-, and 12-transformed cell lines effectively competed with adenovirus ^3H -cRNA for binding sites (Table 1).

DISCUSSION

The localization of specific DNA sequences within the nucleus and on the chromosomes of transformed cells is of particular interest in clarifying important aspects of virus-cell interaction. Few studies have been made on the detection of virus-specific DNA sequences in the genome of mammalian cells by *in situ* hybridization (A. R. Dunn, P. H. Gallimore, K. W. Jones, and J. K. McDougall, *in press*; references 12, 13). McDougall et al. (12) de-

FIG. 3. Nuclei of adenovirus type 7-transformed hamster cells (a, b) and normal hamster cells (c) after *in situ* hybridization with adenovirus 7 ^3H -cRNA. Conditions are the same as described in Fig. 2. $\times 2,080$.

FIG. 4. Nuclei of adenovirus type 12-transformed hamster cells (a, b, c) and normal hamster cells (d) after *in situ* hybridization with adenovirus 12 ^3H -cRNA. Conditions are the same as described in Fig. 2. $\times 2,080$.

tected adenovirus 12 DNA sequences in productively infected human cells, which contain a very large number of viral gene copies, and in adenovirus 2- and 12-transformed cells. From the mean autoradiographic grain count, they estimated roughly 50 to 150 viral genomes per cell, assuming that cRNA represents 20% of the viral genome, with 10% efficiency for *in situ* hybridization (10) and 10% for autoradiographic detection (14).

Our results with clonal lines of different adenovirus 2-, 7-, and 12-transformed cells give somewhat different estimates. Correcting for background grains over transformed cell preparations and over control cells, we obtained 0.85 grains per nucleus for adenovirus 2-, 3.31 grains for adenovirus 7-, and 1.72 grains for adenovirus 12-transformed cells. From the calculated specific activity for cRNA of 4×10^7 to 4.5×10^7 dpm/ μg , and an assumed efficiency of 10% for *in situ* hybridization (10) and 10% for autoradio-

graphic detection (14), and assuming that 20% of the viral genome is represented in the radioactive ^3H -cRNA probe (7), an exposure time of 16 weeks should give approximately 0.3 grains per viral DNA. According to this value, the estimated genome equivalent per cell would be 2.7, 10.7, and 5.5, respectively, for adenovirus 2-, 7-, and 12-transformed cells. Of course, these values could represent under- or overestimates if the assumed efficiencies or the amount of viral genome transcribed to cRNA were different or viral DNA sequences were integrated in tandem. Moreover, it might be premature to compare copies per cell estimated by cytological hybridization with that obtained by molecular hybridization (2). But it is interesting to note that an independent estimate of the number of viral gene equivalents by reassociation kinetics gave 1 to 5 for adeno 2, 20 to 25 for adeno 12, and 25 to 30 for adeno 7 (K. Fujinaga and M. Green, unpublished data; reference 6), values suggesting that the above assumptions are not too far off.

Further analysis of our data will provide evidence concerning localization of grains on

TABLE 1. Mean grain count per nuclei in control and adenovirus type 2-, 7-, and 12-transformed cells

Conditions	Grain density per nucleus (mean $\pm$ SEM) ^a
Adenovirus 2-transformed rat cells	1.71 $\pm$ 0.106
Background over equal areas	0.73 $\pm$ 0.065
Control rat cells (F-1853)	0.93 $\pm$ 0.082
Background	0.80 $\pm$ 0.072
Competing unlabeled homologous RNA ^b (100 $\mu\text{g}/2 \times 10^5$ counts/min)	0.02
Adenovirus 7 hamster tumor cells	6.40 $\pm$ 0.217
Background	1.85 $\pm$ 0.109
Control hamster cells (NIL-2E)	2.73 $\pm$ 0.120
Background	1.49 $\pm$ 0.107
Competing unlabeled homologous RNA ^b (100 $\mu\text{g}/4 \times 10^4$ counts/min)	0
Adenovirus 12-transformed hamster cells	3.70 $\pm$ 0.163
Background	1.43 $\pm$ 0.100
Control hamster cells (NIL-2E)	2.83 $\pm$ 0.137
Background	2.28 $\pm$ 0.140
Competing unlabeled homologous RNA ^b (100 $\mu\text{g}/8.5 \times 10^4$ counts/min)	0.02
Adenovirus 12-transformed hamster cells + adenovirus 7 ^3H -cRNA	0

^a Standard error of the mean. One hundred fifty nuclei were counted.

^b Background subtracted.

TABLE 2. Comparisons among mean grain counts

Determinations	BGtr	BGc	C ^a	TR ^a
Adenovirus 2				
BGc	0.85 ^b		2.42 ^b	11.84 ^c
BGtr			1.57 ^b	10.99 ^c
C				9.42 ^c
Adenovirus 7				
BGc	2.47 ^b		8.52 ^c	33.72 ^c
BGtr			6.05 ^c	31.25 ^c
C				25.20 ^c
Adenovirus 12				
BGtr		6.20 ^c	10.22 ^c	16.57 ^c
BGc			4.02 ^c	10.37 ^c
C				6.35 ^c

^a The studentized range $Q = \bar{x}_{\max} - \bar{x}_{\min} / s_{\bar{x}}$ (11). The quantity $(\bar{x}_{\max} - \bar{x}_{\min})$ represents the difference of any pair of means arranged in descending order of magnitude. The pooled standard error of each group of means ($s_{\bar{x}}$) is calculated. This standard error is based on $4(150-1) = 596$ degrees of freedom. Abbreviations: BGc, background in control cell line; BGtr, background in transformed cells; C, control cells; TR, transformed cells.

^b Significant.

^c Tested and found to be nonsignificant at $P = 0.05$.

chromosomes within chromosomes. Thus far, our observations indicate that grains are associated with chromosomes and that only a few chromosomes are labeled (M. C. Loni and M. Green, unpublished data). Whether label is associated with specific chromosomes or specific sites on chromosomes is being determined.

ACKNOWLEDGMENTS

We thank David Schlessinger for critical reviewing of the manuscript.

The investigation was supported by Public Health Service grant AI-01725 from the National Institute of Allergy and Infectious Diseases and by the Virus Cancer Program, contract no. PH43-67-692 from the National Cancer Institute.

M.C.L. was supported by funds provided by the University of Louvain, Belgium.

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