Multiple Copies of Human Adenovirus 12 Genomes Are Integrated in Virus-Induced Hamster Tumors

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Tumors induced in hamsters by highly oncogenic human adenovirus 12 contain multiple copies of 90 to 100% of the viral genome in an integrated form.

DNA tumor virus-cell interactions have been extensively studied as a model for understanding virus replication, eukaryotic cell biology, and cell transformation (Cold Spring Harbor Symposium on Quantitative Biology 39, parts 1 and 2, 1974). The first human virus shown to possess oncogenic properties was adenovirus 12 (Ad12) (12, 15). The human adenoviruses comprise three oncogenic groups: highly oncogenic group A, which induces tumors in newborn hamsters with high frequency (Ad12, -18, and -31); weakly oncogenic group B, which induces tumors infrequently (Ad3, -7, -11, -14, -16, and -21); and group C (Ad1, -2, -5, and -6), which does not induce tumors but transforms cells *in vitro* (7). It is believed that the Ad transforming gene(s) functions to initiate and maintain the transformed phenotype. The transforming gene(s) of Ad2 and Ad5 is located at the left end (guanine plus cytosine rich) of the linear viral DNA molecule (4, 6, 14). Ad12-transforming genes are present in the *Eco*RI-C fragment (6; Graham and Mak, personal communication) representing the left 16% of the viral genome (C. Mulder, personal communication).

Studies on Ad-transformed cells have dealt predominantly with cell lines established by *in vitro* transformation and have shown that Ad2-, Ad5 (4, 14)-, and Ad7 (3)-transformed cells contain portions of but never the entire viral genome. Hamster cells transformed by highly oncogenic Ad12 (group A), in contrast, contain multiple copies of all or nearly all of the viral genome in an integrated state (11). Unlike group B and group C adenoviruses (7), Ad12 readily produces tumors in newborn hamsters and thus provides the unique opportunity to compare *in vitro* cell transformation with tumor induction. In this report, we demonstrate that tumors induced in newborn hamsters by Ad12 contain multiple copies of 90 to 100% of the viral genome in an integrated state. This integration pattern is similar to that found with Ad12 *in vitro* transformed hamster cells

(11) and supports the belief that *in vitro* transformed cell systems are realistic models for analyzing viral tumorigenesis. The integration of all or nearly all of the viral genome may possibly explain the high oncogenicity of Ad12, since only a fraction of the viral genome (generally less than 50%) is present in cells transformed by weakly and nononcogenic human adenoviruses.

We have analyzed Ad12-induced hamster tumors to determine (i) the fraction of the viral genome present, (ii) the number of copies of viral DNA, and (iii) whether viral DNA is integrated into host DNA. Tumors (generously provided by William T. Lane) were produced by inoculation of Ad12 (strain Huie) into newborn NIH hamsters. In our initial experiments, DNA (6 mg/ml) from two primary tumors were hybridized with *in vitro* labeled Ad12 [³²P]DNA (2×10^8 to 4×10^8 cpm/ μ g), as described in the legend to Fig. 1. After 24 h of annealing, 93 and 94% of viral DNA hybridized with tumor DNA

FIG. 1. Saturation-hybridization of *in vitro* labeled Ad12 [³²P]DNA with hamster tumor DNA. Ad12 DNA was isolated (8, 9) and labeled with ³²P-deoxyribonucleoside triphosphates to specific activities of 2×10^8 to 4×10^8 cpm/ μ g by the nick translation reaction of *Escherichia coli* DNA polymerase I (13) essentially as described by Paul Berg and col-

(not shown). These data suggested that all or nearly all of the viral genome was present in each tumor, since reconstruction experiments with added unlabeled Ad12 DNA hybridized 90 to 98% (legend to Fig. 1).

Large quantities of tumors were produced by subcutaneous transplantation of two additional Ad12 primary tumors into NIH hamsters to obtain sufficient tumor DNA for multiple analyses. DNA isolated from one of these tumors was used for the experiments in Fig. 1 and 2.

leagues (personal communication), scaled down to a 4- μ l reaction mixture (11). Heat-denatured Ad12 [32 P]DNA (800 cpm) was annealed at 68°C with 6 or 9 mg of tumor DNA per ml in 5 μ l of 0.72 M NaCl, 10 mM PIPES (piperazine-N,N'-bis-(2-ethanesulfonic acid) (pH 6.7), 1 mM EDTA, and 0.05% sodium dodecyl sulfate in sealed siliconized capillary tube (Fisher Scientific Co., Pittsburgh, Pa.; catalog no. 2-668-40). At the indicated times, samples were removed, cooled at 4°C, and assayed for double-stranded DNA by hydroxyapatite chromatography (11). As controls, Ad12 [32 P]DNA was incubated with: (i) 6 mg of salmon sperm DNA per ml to determine self-annealing of labeled Ad12 DNA (generally less than 8% after 72 h), (ii) 10 μ g of unlabeled Ad12 DNA and 6 mg of salmon sperm DNA per ml to determine the maximum amount of labeled DNA that could hybridize under these conditions (85 to 97% actual values), (iii) the equivalent of one copy of unlabeled Ad12 DNA per diploid quantity of cell DNA and 6 mg of salmon sperm DNA per ml (86% double stranded after 72 h, normalized value), and (iv) eight copies of unlabeled Ad12 DNA per diploid quantity of cell DNA and 6 mg of salmon sperm DNA per ml (98% double stranded after 72 h, normalized value). The hybridization values were normalized to a 0 to 100% scale based upon the self-annealing of the probe and the maximum amount of the probe that was driven into duplex DNA. All reactions were performed in duplicate and the average of the duplicates are plotted. Tumor DNA was prepared from minced tumors suspended in 0.01 M Tris-hydrochloride (pH 8.1), 0.01 M EDTA, 0.15 M NaCl, and 1% sodium dodecyl sulfate. Pronase (Calbiochem, Los Angeles, Calif.; self-digested at 37°C for 2 h) was added to 500 μ g/ml, and the suspension was incubated at 37°C overnight and then extracted three times with phenol-chloroform-isoamyl alcohol (2:1:0.04). DNA was precipitated with 2.5 volumes of 95% ethanol and 0.1 M potassium acetate for 2 h at -20°C. The precipitate was collected by centrifugation and redissolved in 0.01 M Tris-hydrochloride (pH 8.0). The DNA preparation was sonically treated using a Branson Sonifier (model W350) calibrated to give 400 nucleotide-length fragments. Any contaminating RNA was hydrolyzed by incubation with 0.3 M NaOH at 50°C for 30 min. DNA fragments were purified by exclusion chromatography on a Sephadex G-50 column (2.5 by 55 cm). The excluded volume was collected, and the DNA was precipitated with alcohol and dissolved in an appropriate buffer.

The second transplanted tumor was used in the network analyses of Fig. 3 and 4.

Tumor DNA (6 and 9 mg/ml) saturated nearly 100% of Ad12 [32 P]DNA (4×10^{-7} to 8×10^{-7} mg/ml) after 24 to 70 h of annealing (Fig. 1). Thus, we conclude that all or nearly all of the viral genome is present in Ad12 transplanted tumor cells.

The number of copies of viral DNA in tumors was estimated from the acceleration of the reannealing rate of labeled Ad12 DNA in the presence of tumor DNA (5). DNA sequences reassociate at a rate proportional to their initial concentration (2, 17). The plot of (concentration of duplex DNA)/(concentration of single-stranded DNA) versus time is linear (Fig. 2); this result is consistent with the presence of the entire viral genome (3). Based upon (i) reconstruction experiments with 2.5 and 12.5 copies of added Ad12 DNA and (ii) the accelerated reannealing rate in the presence of tumor DNA, we estimate that six copies are present per diploid quantity of Ad12 hamster tumor DNA.

The presence of most of the viral genome could reflect the synthesis of infectious virus by a small number of cells, although this seems unlikely since hamster cells are nonpermissive for Ad12 replication (7). To exclude this possibility, Z. Veres-Molnar in our laboratory analyzed portions of each tumor for infectious virus by a sensitive plaque titration (10). No infectious virus was found under conditions that would detect one virus particle per 10^5 to 10^6 cells. Since our hybridization measurements estimate six genome equivalents per cell, these results exclude infectious virus as the source of viral DNA sequences in tumor DNA.

To determine whether Ad12 DNA is covalently integrated into the cell genome, we analyzed tumor cell DNA "networks." DNA networks were prepared by the rapid reassociation of interspersed reiterated DNA sequences in high-molecular-weight cellular DNA (1) by the procedure originally used to detect integrated provirus in Rous sarcoma virus-transformed cells (16). We have shown that networks isolated by brief centrifugation are minimally contaminated with free adenovirus DNA (11). Network DNA (6 mg/ml) prepared from an Ad12 tumor (different from those used in Fig. 1 and 2) was annealed with *in vitro* labeled Ad12 [32 P]DNA. Hybridization of over 90% of the viral DNA occurred, reaching a plateau in 24 h (Fig. 3). Network DNA from the same tumor (Fig. 3), analyzed by reassociation kinetics, contained three copies of integrated viral DNA per diploid quantity of cell DNA (Fig. 4). As

FIG. 2. Reassociation of Ad12 [^{32}P]DNA with hamster tumor DNA. *In vivo* labeled Ad12 [^{32}P]DNA was prepared by infecting 40 ml of KB cells in Eagle minimal essential medium without serum (3×10^6 cells/ml) with 3 PFU of purified virus per cell. After 1 h at 37°C, the cells were diluted to 400 ml with Eagle minimal essential medium plus 5% horse serum and incubated at 37°C. At 8.5 h after infection, the cells were washed with phosphate-free Eagle minimal essential medium, buffered with 1 mM N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid (HEPES) buffer (pH 7.2) containing 2% dialyzed calf serum and resuspended in 400 ml of the same medium. At 10 h after infection, the cells were centrifuged and resuspended in phosphate-free medium containing 200 mCi of $^{32}\text{PO}_4$. At 30 to 35 h after infection, cells were harvested, the virus was purified, and viral DNA was isolated. [^{32}P]DNA preparations with specific activities of 10^7 to 2×10^7 cpm/ μg were sonically treated to 400 nucleotide fragments, and 400 to 1,000 cpm of DNA was denatured and reannealed under the hybridization conditions described in the legend to Fig. 1 in the presence of 5 mg of (i) salmon sperm DNA, (ii) tumor DNA, (iii) salmon sperm DNA plus 0.345 ng (2.5 copies) of unlabeled Ad12 DNA, or (iv) salmon sperm DNA plus 1.7 ng (12.5 copies) of unlabeled Ad12 DNA per ml. At the indicated times the reaction tubes were cooled to 4°C and analyzed by hydroxyapatite chromatography as in Fig. 1. The maximum percentage of labeled DNA that could form a duplex (generally 90 to 98%) was determined in a control reaction containing unlabeled Ad12 DNA annealed to C_0t values of over 300 times the expected $C_0t_{1/2}$ for Ad12 DNA (5×10^{-3} mol $\times$ s/liter). All reactions were performed in duplicate, and the data were corrected using a computer program (M. R. G., unpublished) for: (i) scintillation counter background; (ii) systematic hydroxyapatite errors, i.e., about 1% retention and 1% crossover; (iii) zero time duplex DNA (less than 5%); and (iv) lack of complete hybridization (90 to 98% duplex formation was maximal in reconstruction experiments using Ad12 DNA). Concentration of duplex DNA, C_{ds} ; concentration of single-stranded DNA, C_{ss} .

a control, the network supernatant, which contained about 20% of the DNA preparation, was shown to contain about two genome equivalents per diploid quantity of cell DNA (not shown), eliminating the possibility that viral DNA in the network pellet resulted from non-specific trapping of 10 to 15% of putative unintegrated sequences (11, 16) in the cellular DNA preparations. These results are also incompatible with the possibility that the three Ad12 genomes exist as a concatamer of molecular weight approximately 75×10^6 sedimenting with the network DNA pellet; DNA molecules of 100×10^6 daltons have been shown to distribute 20% in the network pellet and 80% in the network supernatant (11).

We found three and six copies of the viral genome in two different Ad12 tumors (Fig. 2 and 4). Identical numbers of viral genomes would not be expected to be present in individual tumors. Since the reassociation kinetics for both tumor DNA preparations were second order, different regions of the viral genome may be present in equal frequency. To establish this point decisively would require reassociation measurements with Ad12 DNA restriction frag-

FIG. 3. Saturation-hybridization of *in vitro* labeled Ad12 [^{32}P]DNA with hamster tumor network DNA. The procedure is as described in the legend to Fig. 1, except that tumor network DNA was used instead of unfractionated tumor DNA. Network DNA was prepared by a slight modification of the procedure reported by Varmus *et al.* (16). Cellular DNA was prepared as described in Fig. 1, with precautions to avoid shear degradation and without alkali treatment or sonication. DNA was dialyzed against $0.1 \times \text{SSC}$ (0.15 M NaCl plus 0.015 M sodium citrate) adjusted to 4 optical density units at 260 nm per ml, and denatured at 100°C for 5 min. Immediately after denaturation, the solution was adjusted to 0.6 M NaCl and annealed at 68°C. After 1 h, the solution was cooled in an ice-water bath and centrifuged in a Spinco Ti 50.2 rotor at 35,000 rpm for 15 min at 4°C. The supernatant was decanted, and the clear DNA pellet (network) was resuspended in $0.1 \times \text{SSC}$. From 80 to 95% of the DNA in the preparation was recovered in the network pellet. The DNA was then treated with alkali, sonically treated, and passed through a Sephadex G-50 column prior to hybridization analysis (see Fig. 1).

FIG. 4. Reassociation of Ad12 [^{32}P]DNA with hamster tumor network DNA. The procedure described in the legend to Fig. 2 was used, except that tumor network DNA was analyzed instead of unfractionated tumor DNA.

ments, as reported with in vitro transformed hamster embryo cells (11).

These results demonstrate that multiple copies of most, and possibly all, of the viral genome are integrated into the cellular genome of Ad12 primary tumors and tumor transplants. To our knowledge, this is the first analysis and demonstration of integrated viral DNA sequences in animal tumors produced by virus infection. Our results are consistent with a model of tumorigenesis that confers a growth advantage to cells containing multiple copies of all or most of the viral genome. Transformation by Ad12 (group A) may differ fundamentally from transformation by group B and C adenoviruses, which generally integrate a much smaller fraction of the viral genome into host cell DNA (3, 4). The similar pattern of integrated viral DNA sequences in Ad12-transformed cells (11) and in Ad12-induced tumors supports the belief that in vitro viral-transformed cells provide meaningful systems for studying viral oncogenesis. This is particularly significant since it is difficult to evaluate which properties of morphologically transformed cells define malignancy. The cor-

respondence between a specific and characteristic parameter of viral transformation (e.g., viral DNA integration pattern) in in vitro and in vivo transformed cell systems reinforces the notion that the molecular events established with cells transformed by viruses in vitro are relevant to viral-induced neoplasia in animals.

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