Proc. Natl. Acad. Sci. USA
Vol. 73, No. 12, pp. 4657–4661, December 1976
Medical Sciences

Do highly oncogenic group A human adenoviruses cause human cancer? Analysis of human tumors for adenovirus 12 transforming DNA sequences

(DNA·DNA hybridization/transforming genes/human tumor DNA)

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Communicated by Robert M. Chanock, September 20, 1976

ABSTRACT. **Adenovirus 12 (Ad12) (Huie) (highly oncogenic group A) readily induces tumors in newborn rodents. Since Ad12 is isolated from human fecal samples, we investigated whether it plays a role in the etiology of human gastrointestinal cancer. If Ad12 is a causal agent of human cancer, then human tumors should contain Ad12 transforming genes, as indicated by studies of cells transformed *in vitro* and *in vivo* by oncogenic viruses. Ad12 DNA and the Ad12 transforming restriction fragment (*Eco*RI-C fragment, left 16% of the viral genome) were labeled *in vitro* to 10^7 to 4×10^8 cpm/μg by the nick translation reaction of DNA polymerase of *Escherichia coli*. The fidelity and sensitivity of these probes were established by (*i*) analysis of DNA from Ad12-transformed cells and from hamsters with tumors induced by Ad12, (*ii*) reconstruction experiments with added Ad12 DNA and *Eco*RI restriction fragments, and (*iii*) comparison of annealing characteristics with Ad12 probes labeled *in vivo*. With Ad12 [^{3}H]DNA as probe, no viral DNA sequences were detected in 18 normal gastrointestinal tissues and 34 gastrointestinal tumors, including cancers of the colon, rectum, small intestine, and stomach, under conditions that would detect 0.1 copy of the Ad12 genome per tumor cell. Similar analyses of Ad12-transformed hamster cells and Ad12 primary hamster tumors indicated 6–18 copies per cell of over 90% of the viral genome. With the Ad12 *Eco*RI-C transforming fragment as probe, no hybridization was detected with 32 human gastrointestinal tumors and five normal tissues; this result excludes 1–2% of the Ad12 genome per tumor cell. Our data are strong evidence that Ad12 is not a major cause of human gastrointestinal cancer. The Ad12 transforming *Eco*RI-C fragment hybridized (50–68% efficiency) with other Ad12 isolates and with Ad18 and 31 (members of oncogenic group A), but not at all with 28 other human Ad serotypes (manuscript in preparation). Thus other group A members *probably* are also not involved in human gastrointestinal cancer. No viral DNA sequences were detected in 12 normal lungs and 22 lung tumors, suggesting that respiratory cancer does not involve an Ad12 etiology.**

Human adenoviruses (Ad) commonly cause respiratory and other infections in children and adults, frequently become latent in lymphoid tissues, and persist for long periods often without recurrent illness. Thirteen of the 31 human Ad have been classified into three oncogenic groups: highly oncogenic group A (Ad12, 18, and 31), which rapidly induces tumors in most inoculated newborn hamsters; weakly oncogenic group B (Ad3, 7, 11, 14, 16, and 21), which induces tumors (all but Ad11) in a small fraction of animals; and nononcogenic group C (Ad1, 2, 5, and 6), which does not induce tumors in newborn rodents but transforms cells in culture (reviewed in ref. 1). The tumorigenicity of Ad12, 18, 31, 3, and 7 was initially established with crude virus preparations (2–5). Green (6) confirmed this, using highly purified preparations of several strains of these serotypes, and established that Ad14, 16, and 21 were tumorigenic. Cells transformed by members of the three oncogenic human Ad groups contain viral DNA sequences (7–11) in an integrated form (12) and synthesize viral mRNA (7, 13, 14).

Most humans are infected early in life by several of the common human Ad. Since Ad have oncogenic properties, it is possible that they are involved in human cancer. Although there is no direct evidence that cancer occurs as a result of virus infection, or as an infectious disease or in epidemics, a viral etiology of cancer is conceivable. For example, a rare abortive Ad infection early in life could result in the integration of Ad transforming gene(s) into a cell chromosome. However, these might remain dormant, and not be expressed for some reason possibly associated with the hormonal balance or the immune system of the individual. At later stages of life, when these systems undergo modifications associated with aging or perhaps bombardment by environmental carcinogens, the Ad transforming gene(s) could be induced, transforming proteins expressed, and cancer could develop. If such an Ad12 etiology of cancer exists, then tumors should contain Ad12 DNA representing transforming and probably other genes. This assumption is based upon current concepts in tumor virology, which hold that all viral transformed cells retain viral transforming genes and express these as mRNA and protein, and are based upon the following observations. (*i*) All Ad-transforming cells examined retain viral DNA and mRNA; in cases where transforming DNA sequences have been identified, these are present in transformed cells and expressed as mRNA. With Ad12 (Huie), all or almost all of the viral genome is covalently integrated in hamster cells transformed *in vitro* by Ad12 (12) and hamster tumors induced by Ad12 (Green *et al.*, in preparation), and both *in vitro* and *in vivo* Ad12-transformed cells express viral mRNA. (*ii*) Transformation-defective, temperature-sensitive mutants of RNA tumor viruses, simian virus 40, and polyoma virus transform cells at permissive temperatures (but not restrictive temperatures), and these cells lose their transformed phenotype when shifted to restrictive temperatures. This indicates that the constant functioning of a viral-coded protein (transforming protein) is necessary to maintain cell transformation (Ad mutants of this type have not yet been described).

To test an Ad12 etiology of human cancer we have developed highly sensitive Ad12 DNA probes to attempt to detect by molecular hybridization Ad12 DNA sequences in human tumors. Ad DNA and DNA restriction endonuclease fragments containing transforming gene(s) have been labeled by the nick translation reaction of *Escherichia coli* DNA polymerase I using ^{3}H- or ^{32}P-labeled deoxyribonucleoside triphosphates; viral DNAs of specific activity 1×10^7 to 4×10^8 cpm/μg are routinely prepared. These probes can detect one copy of 5–7% of the viral genome per diploid quantity of tumor cell DNA with labeled whole viral DNA molecules as probe, or one copy of

Abbreviations: Ad, adenovirus(es); nick, single-strand break; C_0t, concentration of DNA × time in mol-sec/liter; GI, gastrointestinal.

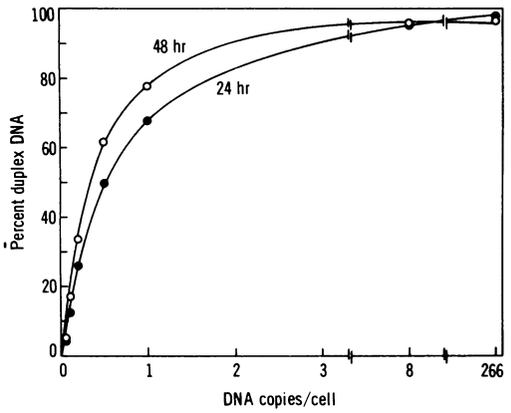

FIG. 1. Reassociation of Ad12 [^{3}H]DNA, labeled *in vitro*, with 0.05–266 copies of Ad12 DNA per diploid cell. Ad12 [^{3}H]DNA (1.0×10^7 cpm/μg; 500 cpm/50 μl) was annealed with varying amounts of unlabeled Ad12 DNA in the presence of 6 mg/ml of calf thymus DNA. Ad12 [^{3}H]DNA was incubated with 6 mg/ml of calf thymus DNA to determine self-annealing of the probe. At 24 and 48 hr, samples were removed and assayed for duplex DNA by batchwise hydroxylapatite chromatography. The data were corrected for self-annealing of the probe (6.6% at 24 and 11.3% at 48 hr) and normalized to the maximum percentage of labeled DNA able to form duplex (96%). The number of equivalent viral DNA copies per diploid cell is based on a cell DNA concentration of 6 mg/ml and viral and diploid mammalian cell DNA molecular weights of 2.2×10^7 and 3.9×10^{12}, respectively.

1–2% of the genome with restriction fragments containing Ad transformation genes as probe. In this communication we investigate whether highly oncogenic group A human Ad12 is associated with human cancer. Since Ad12 is isolated from human fecal samples, we have concentrated our initial efforts on human gastrointestinal (GI) tumors. We have also analyzed lung tumors, because of the association of Ad with respiratory infections. In addition to evaluating the role of human Ad in cancer, these studies should assist the development of the methodology and concepts to evaluate the possible etiological role of other oncogenic DNA viruses in human cancer.

MATERIALS AND METHODS

Cells and Virus. Ad12 (strain Huie, plaque 9), free of Ad associated virus, was grown on KB cells and purified as described (12, 15–17). Viral DNA was isolated and purified by the method of Green and Piña (15, 16). HE C19 cells were derived from HE cells (hamster embryo cells transformed by Ad12 strain Huie) (13) by two sequential clonings in soft agar. Cells were grown and DNA was purified as reported (12). Endonuclease *Eco*RI restriction fragments were prepared as described (12).

Preparation of Labeled Viral DNA and DNA Restriction Fragments. Ad12 DNA and Ad12 *Eco*RI-C fragment were labeled with [^{32}P]dNTPs to specific activities of 2 to 4×10^8 cpm/μg and with [^{3}H]dTTP to 1 to 1.5×10^7 cpm/μg by the nick translation reaction of *E. coli* DNA polymerase I (18), essentially as described by Rigby *et al.* (19), scaled down to 4 μl of reaction mixture (J. K. Mackey, K. H. Brackmann, M. R. Green, and M. Green, manuscript in preparation). *E. coli* DNA polymerase I binds at single-stranded breaks ("nicks") in the DNA molecule, and in the presence of four dNTPs extends the 3′-hydroxyl primer terminus with simultaneous hydrolysis of preexisting strands by the 5′ to 3′ exonuclease activity of the polymerase. The net result is the removal of a portion of the DNA strand at the site of the nick and concurrent resynthesis of removed segments. To introduce nicks, Ad12 DNA was treated at room temperature with a concentration of pancreatic DNase I to produce 1 nick per 400 nucleotides. Nicked DNA was then incubated in a reaction mixture consisting of *E. coli* DNA polymerase I [purified as described by Jovin *et al.* (20)], $MgCl_2$, and four dNTPs. The reaction was monitored by removing 0.1-μl aliquots and measuring trichloroacetic acid-precipitable radioactivity, and stopped when 20–30% resynthesis of DNA had occurred (3–8 hr). Labeled DNA was purified on Sephadex G-50; its size was determined by centrifugation in alkaline sucrose gradients. α-^{32}P-Labeled dATP and dCTP were synthesized by the anhydrous condensation of high specific activity $^{32}PO_4$ with the deoxyribonucleoside (21), followed by phosphorylation of the labeled mononucleotide with a mixture of nucleotide kinases prepared from *E. coli* (ref. 22; Mackey *et al.*, in preparation).

Isolation of Human and Hamster Tissue DNA. Tissue was homogenized and treated with Pronase and sodium dodecyl sulfate. Material was extracted with chloroform-phenol, and nucleic acid was precipitated with ethanol. After sonication, RNA was hydrolyzed with alkali and the DNA was purified on Sephadex G-50 (M. R. Green, J. K. Mackey, and M. Green, in preparation).

Hybridization Conditions. Hybridizations were performed in 0.72 M NaCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 6.7, 1 mM EDTA and 0.05% sodium dodecyl sulfate. Viral probe concentrations were 0.1–1 ng/ml, and cell and tissue DNA concentrations were 6 mg/ml unless otherwise specified. Reaction mixtures (10–100 μl) were sealed in siliconized capillaries and incubated at 68°. The fraction of duplex DNA was determined by batch elution from hydroxylapatite (12).

RESULTS

Fidelity, Sensitivity, and Specificity of Ad12 DNA Probe Labeled *In Vitro*. In our standard assay of tumor DNA for Ad12 information, the concentrations of tumor DNA and radioactive DNA probe were chosen to give a ratio of 50 to 100 cell genome equivalents per viral DNA molecule. We used 6 mg/ml of tumor DNA and hybridization times to yield an equivalent C_0t of 2 to 5×10^4 (C_0t is the concentration of DNA $\times$ time in mol-sec/liter); the labeled probe would hybridize if one copy of viral DNA was present in every tenth tumor cell.

The efficacy and sensitivity of the probe and hybridization procedure were tested by three different reconstruction experiments: Ad12 [^{3}H]DNA was annealed to (*i*) different amounts of unlabeled Ad12 DNA, (*ii*) DNA from Ad12-transformed hamster cells (HE C19) that contain 8 to 10 integrated copies of 90–100% of the viral genome (12), and (*iii*) DNA from virus-free primary hamster tumors induced by Ad12 and containing 6 to 18 copies of 90–100% of the viral genome in an integrated state (Green *et al.*, in preparation). Fig. 1 illustrates a reconstruction experiment in which 0.05 to 266 copies of Ad12 DNA [22×10^6 daltons (23)] was added to 6 mg/ml of calf thymus DNA and the mixture annealed with Ad12 [^{3}H]DNA probe [copy number was calculated assuming a human diploid cell genome of 3.9×10^{12} daltons (24)]. After 24 and 48 hr, 0.1 copy of Ad12 DNA hybridized 12% and 18%, respectively, and 0.2 copy hybridized 25% and 35%, respectively. Thus, 0.1 copy per cell is readily detected in 24 hr.

The effect of cell DNA concentration (30–6000 μg/ml) on hybridization of the Ad12 DNA probe is shown in Fig. 2 (Ad12-transformed cell DNA) and Fig. 3 (Ad12 hamster tumor DNA). Viral DNA hybridized 20–30% with 60 μg/ml of cell DNA after 48 hr. We used 6 mg/ml of human tumor DNA for the analyses

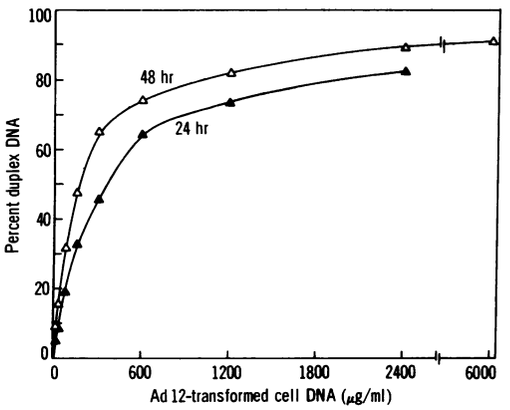

FIG. 2. Hybridization of Ad12 [^{3}H]DNA, labeled *in vitro*, with varying concentrations of Ad12-transformed cell (HE C19) DNA. Ad12 [^{3}H]DNA (1.0×10^7 cpm/μg; 500 cpm/50 μl) was annealed with 30–6000 μg/ml of HE C19 cell DNA. Calf thymus DNA was added as required to maintain a cell DNA concentration of 6 mg/ml. Aliquots were removed at 24 and 48 hr and assayed for duplex DNA. Data were normalized as in Fig. 1.

described below, a concentration 100 times that necessary to detect viral DNA in Ad12-transformed and tumor cells.

Fig. 4 shows the time course of Ad12 [^{3}H]DNA hybridization with (*i*) 2.4 mg/ml of Ad12-transformed cell DNA, (*ii*) 2.4 mg/ml of Ad12 tumor DNA, and (*iii*) one copy of Ad12 DNA per diploid quantity of human cell DNA (based on 6 mg/ml of DNA). After 3 hr, one copy of Ad12 DNA hybridized to 15% of the probe, tumor cell DNA to 25%, and transformed cell DNA to 35%. Routinely 48 to 72-hr hybridizations were used to analyze human tumors. Calculations based on C_0t ½ of the number of copies of the Ad12 genome present in HE C19 and the Ad12-induced hamster tumor showed eight and five copies, respectively, in agreement with previous results (ref. 12; Green *et al.*, in preparation).

Analyses of Human Tumors for Ad12 Specific DNA Sequences. Analyses of 18 normal GI tissues and 34 GI tumors (five stomach, seven rectum, five small intestine, and 17 colon) detected no viral DNA sequences with Ad12 [^{3}H]DNA as probe (Fig. 5). Hybridizations were within ±2–3 SD (standard deviation) of the background values to calf thymus and *E. coli* DNA. Reconstruction experiments showed that 0.2 copy per cell (one copy of the entire genome per five tumor cells) gave 54% hybridization and one copy gave 77%. Simultaneous analyses of Ad12-transformed hamster cells gave 85–95% hybridization (Fig. 5). Thus, neither normal GI tissue nor GI tumors contained detectable (less than 0.1 copy per cell) Ad12 DNA.

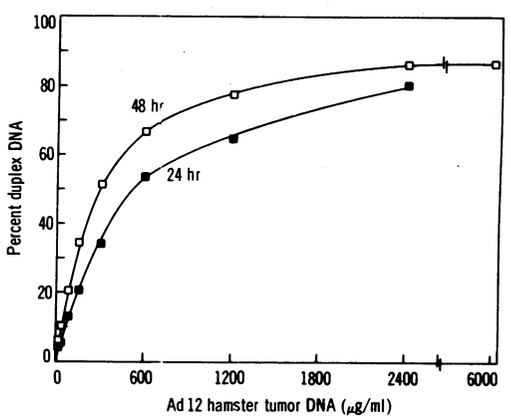

FIG. 3. Hybridization of Ad12 [^{3}H]DNA, labeled *in vitro*, with varying concentrations of Ad12-induced primary hamster tumor DNA. The procedure was the same as in the legend of Fig. 2.

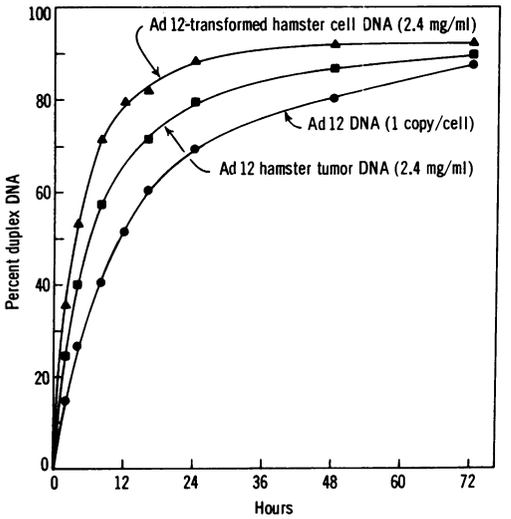

FIG. 4. Time course of hybridization of Ad12 [^{3}H]DNA, labeled *in vitro*, with unlabeled Ad12 DNA and Ad12-transformed cell and tumor DNAs. Ad12 [^{3}H]DNA (1.0×10^7 cpm/μg; 500 cpm/50 μl) was annealed with (*i*) 1 equivalent of Ad12 DNA per diploid cell plus 6 mg/ml of calf thymus DNA, (*ii*) 2.4 mg/ml of HE C19 cell DNA plus 3.6 mg/ml of calf thymus DNA, and (*iii*) 2.4 mg/ml of Ad12-induced hamster tumor DNA plus 3.6 mg/ml of calf thymus DNA. Controls were identical to those in Fig. 1. Aliquots were removed at indicated times and assayed for duplex DNA. Data were normalized as in Fig. 1.

We have also analyzed DNA from 22 lung tumors, 12 normal lungs, four Hodgkin's tumors, four cancers of the kidney, three breast, two brain, three lymphomas, and two sarcomas using

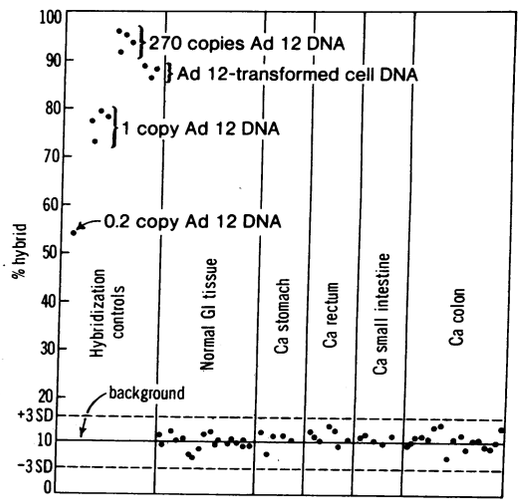

FIG. 5. Molecular hybridization analyses of human GI tumor (Ca, cancer) and normal tissue DNA for Ad12 DNA sequences. Ad12 [^{3}H]DNA, labeled *in vitro* (0.9 to 1.4×10^7 cpm/μg; 500 cpm/50 μl), was annealed with 6 mg/ml of human DNA for 48 hr, and samples were assayed for duplex DNA. As controls, Ad12 [^{3}H]DNA was annealed with (*i*) 6 mg/ml of calf thymus DNA, (*ii*) 0.2 equivalent per cell of unlabeled Ad12 DNA plus 6 mg/ml of calf thymus DNA, (*iii*) 1 equivalent per cell of unlabeled Ad12 DNA plus 6 mg/ml of calf thymus DNA, (*iv*) 270 equivalents per cell of Ad12 DNA plus 6 mg/ml of calf thymus DNA, and (*v*) 6 mg/ml of DNA from HE C19 transformed cell line. The background value (10% hybridization) was an average of the values obtained for calf thymus, *E. coli*, and KB cell DNAs. The standard deviation (SD) was determined for DNAs from 17 samples of normal GI tissues. Data plotted are actual values. Any values less than 3 SD above background are considered negative.

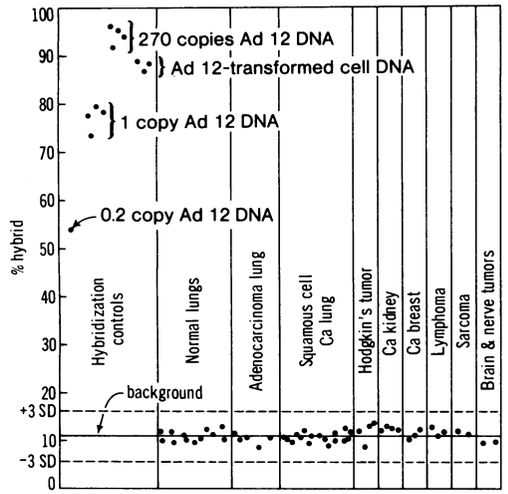

FIG. 6. Hybridization analyses of human lung and several other tumors and normal host DNAs for Ad12 DNA sequences. The procedure is as described in the legend to Fig. 5. The background value was 11%, and the SD was determined for DNAs from 12 samples of normal lung tissue.

labeled Ad12 DNA as probe. No tumor samples gave significant hybridization (Fig. 6). Reconstruction experiments showed that 0.2 copy per cell of Ad12 DNA yielded 55% and one copy 75–80% hybridization. The data in Fig. 6 with lung tumors (too few tumors in the other tumor categories were analyzed) probably exclude the presence of Ad12 DNA.

The studies with the entire Ad12 genome indicate the absence of Ad12 genetic information from human GI normal and tumor tissue. Most convincing were analyses using as probe ^{32}P-labeled Ad12 *Eco*RI-C fragment (left 16% of the viral genome), which has been reported (ref. 25; F. L. Graham and S. Mak, personal communication) to transform cells by transfection. *Eco*RI cleaves Ad12 DNA into six unique fragments, with the left to right sequence of C-D-B-E-F-A on the conventional map (ref. 26; Delius and Mulder, personal communication). Ad12 *Eco*RI-C fragment was prepared by digestion of Ad12 DNA with *Eco*RI and separation of fragments by electrophoresis on 1.4% agarose gels (12). The fragment was labeled to 1×10^8 cpm/μg by nick translation using [α-^{32}P] dCTP. Table 1 presents analyses of DNAs from 32 GI tumors and 5 normal GI tissues for *Eco*RI-C sequences; none was detected, i.e., each hybridization value was within 3 SD of background. The sensitivity is sufficient to exclude 1–2% of the Ad12 genome per cell. Thus, our data provide strong evidence that Ad12 is not a major cause of GI cancers, and confirm the analysis of 33 GI tumors with whole Ad12 DNA as probe (Fig. 5).

Table 1. Analysis of human GI tract tumors for DNA sequences specific to the Ad12 *Eco*RI-C transforming fragment

Tissue	No. tested	% Hybridization* (mean ± SD)
Normal colon	5	0.6 ± 1.5
Ca colon	18	0.5 ± 0.9
Ca stomach	5	1.7 ± 1.7
Ca rectum	5	−0.2 ± 2.1
Ca small intestine	4	1.0 ± 1.3

* [^{32}P]DNA of Ad12 *Eco*RI-C fragment that had been labeled *in vitro* (500 cpm/10-μl aliquot, 1×10^8 cpm/μg) was annealed in the presence of 6 mg/ml of human GI DNA in duplicate under standard conditions to an equivalent C_0t of 15,000–25,000. Combined results of two experiments are shown. Background due to self-annealing of the probe (8–11%) was subtracted and resulting values were normalized to the maximum percentage of DNA that formed duplex with Ad12 (Huie) DNA (90–95%). One copy per cell of added Ad12 DNA hybridized 45–68%; HE C19 cell DNA (6 mg/ml) hybridized 86–96%. Ca, cancer.

DISCUSSION

We have examined the possible involvement of human Ad12 (Huie) (highly oncogenic group A) in human cancer by using highly sensitive Ad12 DNA probes to analyze human tumor DNA for Ad12 genetic information. In analyses of 34 GI tumors, 22 lung tumors, and a number of other tumors, we were unable to detect Ad12 genetic information, suggesting that Ad12 (Huie) is not involved in human GI or lung cancer. However, to completely exclude Ad12 as a causal agent of these cancers it is necessary to know (*i*) the number of malignant cells per tumor, (*ii*) the fraction of the Ad12 genome represented by transforming gene(s), and (*iii*) the number of copies of transforming gene(s) per tumor cell that could be detected by our probes. Usually 50% or more of the cells in our tumors were malignant, as estimated by pathological examination. The Ad12 genome fraction encoding transforming genes is not known, but with Ad2 (genome of 23×10^6 daltons), the transforming gene(s) is at most 6.5%. Our probes representing the entire genome would detect one copy of 7% of the Ad12 genome per tumor cell, whereas the *Eco*RI-C fragment probes would detect one copy of 1.5% of the Ad12 genome per cell. Since all Ad12 hamster tumors and transformed cells contain multiple copies per cell of greater than 90% of the genome, and all Ad2-transformed cells examined contain multiple copies of 14% of the genome (in many cell lines more than 14%), our results are very strong evidence against an Ad12 (Huie) etiology of human GI and lung cancer. It is conceivable that Ad12 transforming gene(s) could represent as little as 1% of the genome, since we have identified an Ad2-specific, 15,000-dalton candidate transforming protein (27). If tumors contain 50% tumor cells and these have one copy of 1% of the genome, we would not have detected Ad12 sequences even with our *Eco*RI-C probe. Therefore, our data do not formally exclude Ad12 as an etiological agent of cancer. However, because of the integration pattern of Ad12 DNA in hamster cells it seems unlikely that human cancer cells would integrate only one copy of 1% of the genome.

The transforming Ad12 (Huie) *Eco*RI-C fragment is 50–68% homologous to other Ad12 isolates and to Ad18 and 31, other members of group A Ad (manuscript in preparation). We have observed similar homologies using the entire Ad12 (Huie) genome (28). Therefore, it is quite likely that our results rule out all members of group A Ad as causal agents of GI and lung cancer. It is possible that the portion of Ad12 (Huie) *Eco*RI-C fragment that is *not* homologous to the other group A Ad represents the Ad12 (Huie) transforming gene(s); if so, then our current data do not pertain to other group A Ad and human cancer. Finally, Ad12 *Eco*RI-C fragment has no homology to group B or C Ad, or to any of the unclassified Ad (manuscript in preparation), and therefore we emphasize that our tumor analyses presented here are not germane to a group B or C Ad etiology of GI or lung cancer.

Several years ago we analyzed human tumors for Ad genetic information by hybridization-competition, and concluded that human tumors contained less than 1000 molecules per cell of Ad mRNA (6). We have now shown that human GI tumors contain less than one copy of 1.5% of the genome per cell. It is

clear that the methodology we have used can test whether other DNA tumor viruses are involved in human cancer. In the case of papovaviruses, which have smaller genomes (3×10^6 daltons), the sensitivity of our probes would be adequate for an unequivocal answer (29). In the case of the larger herpesviruses with genomes of 10^8 daltons in which transforming gene(s) could represent 0.2–1.0% of the genome, it will be necessary to use selected probes containing mainly herpesvirus transforming gene(s) to rule out herpesviruses as causes of human cancer.

Note Added in Proof: Recently we have analyzed DNAs or RNAs from 25 additional GI tumors, 57 lung tumors, and 14 tumors of the breast, ovary, lymphomas, and Hodgkins tissues using ^{32}P-labeled Ad12 transforming fragment as probe and found they contain less than one copy of 1% of the Ad12 genome.

We thank Dr. W. S. M. Wold and M. R. Green for critical discussion and reading of the manuscript, Dr. H. Thornton for cell culture assistance, and Constance Self for technical assistance. We are grateful to the following individuals for supplying human tumors: Drs. J. Gruber and I. Sekely (Office of Program Resources and Logistics, NCI), Dr. E. Harrison (deceased) (Mayo Clinic), Dr. M. Gardner (University of Southern California), and Dr. H. Pinkerton (St. Louis University). This work was supported by Contract NOI CP 43359 within the Virus Cancer Program of the National Cancer Institute and Contract 223-75-1022 from the Bureau of Biologics, Food and Drug Administration. M.G. is the recipient of a Research Career Award from the National Institutes of Health (5K6-AI-4739).

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