

Transforming gene in human atherosclerotic plaque DNA

(monoclonal hypothesis/transfection/coronary artery/NIH 3T3/nude mouse)

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ABSTRACT The monoclonal hypothesis equates atherosclerotic plaques with benign smooth muscle cell tumors and proposes that plaques can arise via mutational or viral events. Here, we provide direct evidence that molecular events, heretofore associated only with tumor cells, are common to plaque cells as well. Three distinct groups of human coronary artery plaque (hCAP) DNA samples transfected into NIH 3T3 cells gave rise to transformed foci. DNA samples from a panel of normal noncancerous human tissues, including coronary artery, were negative in the assay. Southern-blotted focus DNA yielded positive signals when hybridized to the ³²P-labeled nick-translated repetitive human "Alu" DNA sequence. The DNA from cloned foci was used successfully in a second round of transfection. Focus DNA hybridized to nick-translated v-Ki-ras, v-Ha-ras, or N-ras probes failed to detect human fragments of these genes. Primary focus cells from each of five clones elicited tumors after injection into nude mice (6/42). Several distinct high molecular weight (>6.6 kilobases) bands were detected after BamHI-digested tumor DNA was hybridized to Alu. Preliminary characterization of these hCAP DNA-associated tumors indicates that they are similar to the fibrosarcomas that arise after injection of ras-transformed cells into nude mice. We propose that transforming genes in plaque cells behave in a manner analogous to the way in which oncogenes behave in cancer cells.

Three distinct sequences of cellular events have been associated with atherosclerotic plaque development: cellular proliferation, thrombosis, and lipid accumulation. Of these, proliferation of smooth muscle cells in response to an as-yet-unknown stimulus is thought to be an early event that is indispensable for plaque development. The monoclonal hypothesis was proposed by Benditt and Benditt to explain this proliferation of smooth muscle cells. They provided evidence that human atherosclerotic plaques are monoclonal in origin (1). It was inferred that plaques are benign smooth-muscle-cell tumors of the artery wall. A key element of the monoclonal hypothesis is that plaques can arise via mutational or viral events. Experimental studies with cockerels subsequently provided indirect support for this hypothesis. Weekly injections of the carcinogens dimethylbenz[a]anthracene or benzo[a]pyrene elicited the appearance of large, focal, fibromuscular plaques in the abdominal aorta (2, 3). Both dimethylbenz[a]anthracene and benzo[a]pyrene have been used extensively to initiate carcinogenesis in experimental animals (4, 5). The enzymes that metabolize these carcinogens are present in the artery wall (6, 7).

In a separate series of studies, 15-week-old cockerels, injected at 4 days of age with the oncogenic Marek disease virus, displayed focal, microscopic plaques in the thoracic aorta that were not present in controls (8, 9). Additionally, dimethylbenz[a]anthracene and methoxamine administered to cockerels in an initiation-promotion protocol elicited the

appearance (via scanning EM) of small focal mounds of cells in the thoracic aortas, which were absent from aortas of controls (10). While these results suggest that gross phenomenological similarities may exist during the development of tumors and plaques, they do not provide direct evidence for mechanistic similarities between the origins of plaques and tumors. We reasoned that it should be possible to test the monoclonal hypothesis directly and determine whether there are molecular events, heretofore associated only with tumor cells, that are common to plaque cells as well.

During the past few years, an *in vitro* DNA-mediated gene transfer assay (11) has been used to demonstrate the activation of oncogenes in the genome of a variety of tumors and transformed cells (12-17). The assay relies on the incorporation and expression of dominant, transforming DNA sequences by NIH 3T3 fibroblasts, a cell line that has proved to be susceptible to transformation by genes of the *ras* complementation group. The most direct concomitant *in vivo* method for assessing oncogene activation is to inject the putatively transformed cells into suitable hosts, usually athymic nude mice, and to determine whether tumors subsequently arise (18).

We now demonstrate that human atherosclerotic plaque DNA is capable of completing the transformation of NIH 3T3 cells via DNA transfection. The DNAs from transfectants contain human DNA sequences. After injection into nude mice, the transformed cells elicit the appearance of tumors that contain human sequences. The similarities in molecular alterations between human plaque cells and tumor cells lead us to suggest that somatic-cell gene alterations play an essential role in atherosclerotic plaque development.

MATERIALS AND METHODS

Tissue Samples. The human DNA samples tested in the NIH 3T3 cell transfection assay were obtained from human coronary artery plaques (hCAP), coronary artery, liver, spleen, lung, kidney, and trachea. DNAs from untreated NIH 3T3 cells and from T24 bladder carcinoma cells were used as negative and positive controls, respectively.

The hCAP samples were obtained by one of us (B.M.) during the course of endarterectomies carried out at the Cardiovascular Surgery Unit at Mt. Sinai Hospital, NY. A small piece of grossly normal coronary artery was obtained from one patient as well. The samples of nonvascular tissue were obtained at autopsy from a 20-year-old Black-Hispanic male who had no history of disease. Three groups of hCAP DNA were tested. In group A, DNA was obtained from pooled portions of plaques from six different patients. In

Abbreviations: hCAP, human coronary artery plaque; kb, kilobase(s).

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group B, plaques from three patients were pooled for DNA extraction. Group C was a single plaque that was sufficiently large to provide adequate DNA for the transfection assay. Patient anonymity was maintained throughout the study. Upon surgical removal, all tissue samples were frozen in liquid N₂ and stored at -85°C.

DNA Transfection. All tissues were pulverized in liquid N₂. DNA extraction was carried out with standard (phenol/chloroform and isoamyl alcohol) procedures (19). Ethanol-precipitated DNA was resuspended in Tris/EDTA buffer, pH 8.0. Cell monolayers were trypsinized and pelleted into sterile tubes before DNA extraction was carried out.

The standard calcium phosphate transfection protocol of Wigler *et al.* (11) was followed. For each sample, 40 µg of DNA was transfected into each of three 25-cm² flasks containing $\approx 1 \times 10^6$ NIH 3T3 cells grown in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum (KC Biological, Lenexa, KS). Forty-eight hours after transfection, cells were trypsinized and replated at a 1:3 ratio in randomly coded flasks to allow for double-blind scoring by 2 or 3 individuals. Plates were scored for foci after 24 days. Foci were picked with cloning cylinders or stained with Camco Quick Stain II (American Scientific Products, McGaw Park, IL). Those foci whose cells maintained the morphological alterations characteristic of the focus after subculturing were grown in mass culture. The DNAs from these transfectant cell lines were used in a second round of transfection.

Southern Blot Hybridization. The DNA samples were digested with either *EcoRI*, *BamHI*, or *HindIII* according to manufacturers' (New England Biolabs; International Biotechnologies, New Haven, CT) instructions.

Digested DNA was subjected to electrophoresis overnight in 0.7% or 0.8% agarose gels (Bio-Rad) at 20 V and 15 mA per gel and were Southern-blotted (20) to nitrocellulose (Schleicher & Schuell).

³²P-labeled probes (*v-Ha-ras*, *v-Ki-ras*, *N-ras*, and *Alu*) were purchased from ONCOR (Gaithersburg, MD). Hybridization to *Alu* was also accomplished by use of the BLUR8 probe (a gift of Warren Jelinek), which was nick-translated (21) with ³²P[CTP] (New England Nuclear). Filters were hybridized to labeled probes at 65°C for 18–48 hr and washed to a final stringency of 0.5× NaCl/Cit (1× NaCl/Cit = 150 mM NaCl/15 mM sodium citrate) with 0.1% NaDodSO₄ for the oncogene probes and 0.1× NaCl/Cit with 0.1% NaDodSO₄ for the *Alu* probes. The hybridization mixture to which the labeled probes were added consisted of 6× NaCl/Cit containing 2× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin), 10 mM NaH₂PO₄, 10% dextran sulfate, and 0.067 mg of salmon sperm DNA per ml. In some cases, hybridization was at 45°C in 45% formamide for 16 hr. Filters were air-dried and exposed, with Cronex intensifying screens (DuPont) to x-ray film (Kodak X-Omat) for 7 days (oncogenes) or 1–2 days (*Alu*) at -70°C.

Nude Mouse Assay. Transformant cells (5×10^6) were injected subcutaneously into the scapular area of nude (*nu*⁺/*nu*⁺) male mice (Charles River Breeding Laboratories) 52–56 days old. Animals were checked for the presence of visible tumors daily for the first 2 weeks and three times each week thereafter. Tumors arising in the experimental groups were measured weekly with calipers. When tumor-bearing animals were sacrificed, samples of all tumors were taken for histological observation, DNA isolation, and growth in cell culture. Samples of selected tumors were also removed and processed for ultrastructural characterization (results to be presented elsewhere).

RESULTS

DNA-Mediated Gene Transfer. Foci arose in cells transfected with each of the hCAP DNA samples (Table 1). The transfection efficiency (no. of foci per µg of DNA) for hCAP DNAs ranged from 0.016 to 0.060 (mean = 0.036); for NIH 3T3 cell DNA, the value was 0.004, and for T24 DNA, 0.100. The transfection efficiencies for DNAs from human coronary artery, liver, spleen, lung, kidney, and trachea were ≤ 0.008 each.

Foci arising as a result of transfection of NIH 3T3 cells with hCAP DNA were generally similar morphologically to foci arising from transfection by T24 cell DNA (Fig. 1). hCAP foci were compact, round or oval, and highly refractile. The hCAP focus cells were smaller and rounder than the surrounding untransformed 3T3 cells. These results show that hCAP DNA contains transforming sequences.

In some cases, when focus cells were trypsinized and replated, the dispersed cells from a single focus gave rise to additional foci. Each of these subclones was picked and grown in batch culture. Nine hCAP foci arising from transfection with group A DNA gave rise to 18 cell lines. The DNAs isolated from 10 of these cell lines were used in a second round of transfection. Nine of these 10 DNA samples gave rise to multiple foci. The efficiency of secondary transfection ranged from 0.008 to 0.075 foci per µg of DNA (mean = 0.038). The morphology of foci and the appearance of cells within the foci were similar for primary and secondary transfectants. These results show that the transforming elements in hCAP DNA can be transmitted serially.

Characterization of DNAs from hCAP Foci. After overnight digestion with restriction enzymes, DNAs from the first round of transfection were electrophoresed through 0.7% agarose, Southern-blotted onto nitrocellulose, and then hybridized to a ³²P-labeled probe of *Alu*, the human repetitive sequence (22). Each of the plaque-derived focus DNAs (Fig. 2 *Left*, lanes 2–6) displayed a strong hybridization signal with the *Alu* probe, compared with DNA from NIH 3T3 cells (lane 1). Thus, the focus DNAs contain human sequences.

Table 1. Transfection efficiency of hCAP DNAs

Transformant	Ratio foci/ total plates	Foci per µg of DNA	Nude mouse tumors
Primary			
NIH 3T3	1/18	0.004	0/30
T24	12/9	0.100	5/5
hCAP A	16/36	0.033	6/42
hCAP B	7/9	0.060	
hCAP C	2/9	0.016	
Coronary artery	1/9	0.008	
Liver	1/9	0.008	
Spleen	0/9	<0.008	
Kidney	0/9	<0.008	
Lung	0/9	<0.008	
Trachea	0/9	<0.008	
Secondary			
hCAP A1	8/9	0.067	
hCAP A2	2/9	0.017	
hCAP A3	2/9	0.017	
hCAP A4	9/9	0.075	
hCAP A5	3/9	0.025	
hCAP A6	1/9	0.008	
hCAP A7	4/9	0.033	
hCAP A8	4/9	0.033	
hCAP A9	8/9	0.067	
hCAP A10	5/9	0.042	

hCAP A1–A10 are cell lines that arose from group A hCAP primary foci.

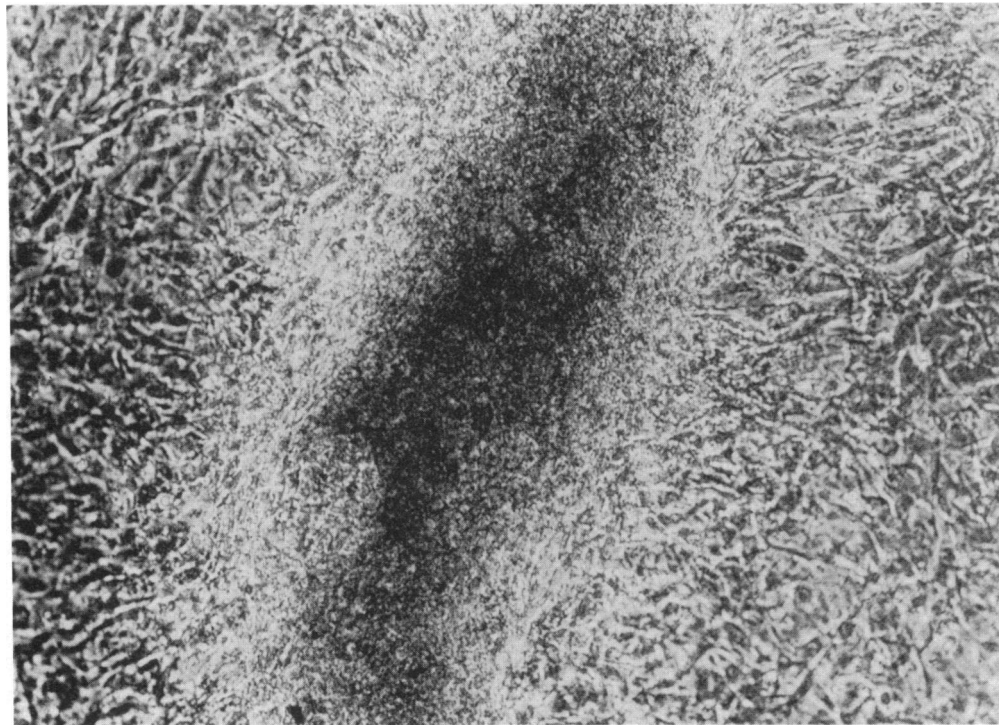


FIG. 1. Morphology of a focus arising from transfection of NIH 3T3 cells by hCAP DNA. ($\times 30$.)

In the great majority of cases in which transfection of DNA into NIH 3T3 cells has yielded positive results, the effective gene sequences have been members of the *ras* complementation group. Therefore, we tested DNAs from both primary and secondary transformants vs. *v-Ki-ras*, *N-ras*, and *v-Ha-ras* probes. None of the experimental samples displayed hybridization patterns with *v-Ha-ras* (Fig. 2 Right), *N-ras*, or *v-Ki-ras* (data not presented) that were different from that of the negative control, NIH 3T3 cell DNA. These results show that the transforming nature of hCAP DNA cannot be attributed to the presence of activated human *c-Ha-ras*, *N-ras*, or *c-Ki-ras* oncogenes (called *HRAS*, *NRAS*, and *KRAS* in human gene nomenclature).

Tumorigenicity. The nine primary transformant lines that

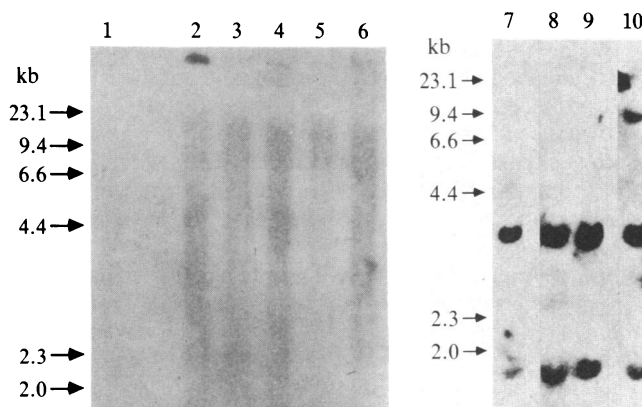


FIG. 2. Southern analysis of hCAP NIH 3T3 transfectants. DNA was isolated from individual foci, digested with *Bam*HI, Southern blotted, and hybridized to a 32 P-labeled nick-translated *Alu* probe (Left) or *v-Ha-ras* probe (Right). Ten micrograms of DNA was loaded on each lane. Lanes: 1 and 7, NIH 3T3 cell DNA; 2-6, 8, and 9, DNA from primary hCAP foci; 10, T24-derived primary focus DNA. The two lower molecular weight bands in lanes 7-10 represent endogenous mouse *c-Ha-ras*. The high molecular weight band in lane 10 represents human *c-Ha-ras* that is present in T24 transformed NIH 3T3 cells.

yielded multiple foci in secondary transfections were tested for their ability to elicit tumor formation after injection into 8-week-old male *nu⁺/nu⁺* mice. Initially there were five mice in each experimental group. Five of the injected cell lines were tumorigenic. In one group, two of the injected mice developed large (>15-mm diameter at sacrifice) encapsulated tumors under the dorsal surface. One mouse in each of the other four groups developed a similar tumor. In the first case, the tumors appeared at 7 and 9 weeks after injection, and in the other cases, at 10, 13, 14, and 16 weeks. They were small (2- to 3-mm diameter) for 17-21 days and then grew rapidly.

In contrast, tumors arose in 5 of 5 mice injected with cells transformed by T24 DNA within 17 days after injections. The first of these tumors arose 6 days after injection. No tumors arose in the negative control groups by 16 weeks after injection. Tumor-bearing mice in all groups were sacrificed when tumors exceeded 15 mm in diameter.

The hCAP-associated tumors (Fig. 3 Upper) were generally similar to the fibrosarcomas (Fig. 3 Lower) that arise in nude mice after injection of *ras*-transformed cells. Via light microscopy, the only major differences were that cells in hCAP-associated tumors were smaller and more densely packed than cells in *ras*-associated tumors. A detailed histological and ultrastructural examination of the hCAP-associated tumors will be presented elsewhere.

hCAP-derived tumor DNAs were extracted, digested with *Bam*HI, Southern-blotted, and hybridized to the human repetitive probe *Alu*. Several distinct high molecular weight bands were visible (Fig. 4). These results demonstrate that hCAP DNA is tumorigenic in the same test system and under the same general conditions used to test for the tumorigenicity of putative oncogenes.

DISCUSSION

The results described provide direct evidence for similarities on the molecular level in the development of plaques and tumors. We have presented six discrete findings: (i) hCAP DNA contains sequences capable of completing transformation of NIH 3T3 cells; (ii) this transforming capability can be

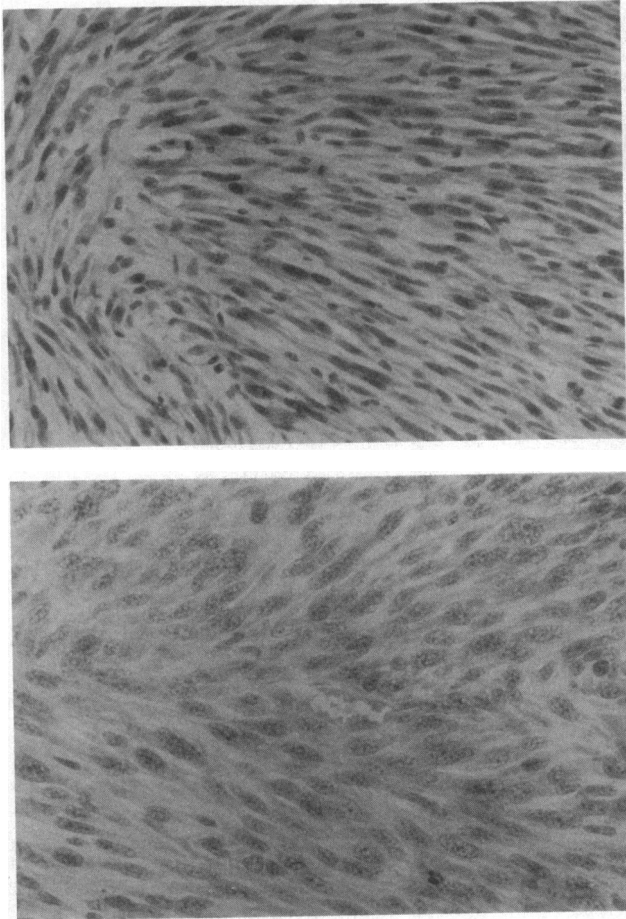


FIG. 3. Histological comparison of tumors. Tumors were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. ($\times 100$.) (Upper) Tumor taken from a nu^+/nu^+ mouse injected with hCAP DNA-transformed cells. (Lower) Tumor taken from a nu^+/nu^+ mouse injected with T24 DNA-transformed cells.

transmitted serially; (iii) the DNA of the transformed cells hybridizes with a human repetitive probe; (iv) the hCAP-transforming gene does not hybridize to *v-Ki-ras*, *N-ras*, or *v-Ha-ras* probes; (v) cells transformed by hCAP DNA give rise to tumors after injection into nude mice; and (vi) DNA from these nude mice tumors hybridizes to the human repetitive probe.

The transfection assay we used has three major limitations: (i) it selects only dominant genes; (ii) most of the genes identified with this assay to date have been members of the *ras* complementation group; and (iii) in the case of human samples, only $\approx 20\%$ of all tumors of a type that would be expected to be positive in this assay (e.g., bladder carcinomas) actually give rise to foci (23). Whether the latter

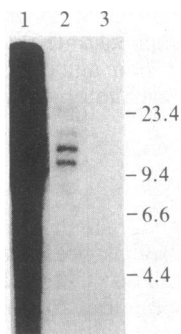


FIG. 4. Southern analysis of tumors arising in nu^+/nu^+ mice per after inoculation of 5×10^6 hCAP-transformed NIH 3T3 cells per mouse. Methods are described in Fig. 2. Hybridization was to the ^{32}P -labeled nick-translated BLUR8 probe. Lanes: 1, T-24 genomic DNA; 2, DNA from a tumor that arose after injection of hCAP DNA-transformed NIH 3T3 cells; 3, DNA from untransformed NIH 3T3 cells. Ten micrograms of DNA was loaded on each lane.

observation is due to tumor heterogeneity, variations in tumor etiology, or other factors is not known. However, in animal models with tumors of known etiology, the percentage of tumor DNAs that give rise to foci approaches 100% (16, 17). In addition, there is growing evidence that non-*ras*-genes can be readily discerned by using this assay. Included among these non-*ras*-genes are: *neu*, an oncogene derived from neuroblastomas (24); *met*, derived from chemically transformed human cells (25); and an as-yet-unidentified oncogene isolated from methyl methanesulfonate-induced rat nasal carcinomas (26). Thus, despite its limitations, the transfection assay has been useful in helping to identify alterations in specific DNA sequences that are associated with tumor development and/or cellular transformation. Therefore, we selected this assay as an initial screen for the presence of transforming sequences in human plaque DNA.

In all cases, the transfection assay yielded foci that could be picked and grown in culture. Transfection efficiencies were intermediate (0.008–0.075 foci per μg of DNA) between those for positive (0.100 focus per μg of DNA) and negative (0.004 focus per μg of DNA) controls. The efficiencies of primary and secondary transfections were very similar. This is consistent with results from transfection studies in which genes other than those of the *ras* family were involved (26). The implication is that in contrast to the case with *ras* genes, the hCAP transforming gene is not amplified in the primary transfectants.

It is not possible to determine whether the transforming DNA in the pooled samples arose from only one or from more than one of the donor DNAs. If only one donor DNA in each pooled sample had transforming activity then, at the very least, 30% of all samples (1 of 6, 1 of 3, and 1 of 1) had transforming activity. This is slightly higher than the $\approx 20\%$ value generally ascribed to human DNA in the transfection assay. If more than one of the DNAs in the pooled samples had transforming ability, the percentage of transforming DNAs would obviously increase. Thus, the transfection efficiency of at least 30% for hCAP DNA is consistent with transfection efficiencies for DNAs from human tumors of unknown etiology. In contrast, human DNAs from six grossly normal tissues, including coronary artery, were negative in the transfection assay.

All foci that were picked were ≥ 1 mm in diameter, round-to-oval, and densely packed. Focus morphology and the appearance of cells within a focus were similar to foci arising from T24 DNA-transfected NIH 3T3 cells.

We next demonstrated that the transformed NIH 3T3 cell foci contained human DNA. Hybridization of the ^{32}P -labeled *Alu* probe with five of the DNAs derived from primary foci is evident in Fig. 2 Left. These *Alu*-positive signals varied both in intensity and location from one digested DNA sample to another. However, they were present in the DNAs of all primary and secondary cell lines that we have analyzed. These *Alu*-positive signals were not present in DNA from untransformed NIH 3T3 cells. Distinct high molecular weight *Alu*-positive signals were detected in the DNAs from two of the tumors that arose after injection of hCAP-associated focus cells into nude mice (see the discussion of Fig. 4 below). Therefore, we conclude that the transforming sequences in our studies are of human origin.

Since the preponderance of reported successful transfections has been with members of the *ras* gene family, we tested three of the most often studied members of this family, *v-Ha-ras*, *N-ras*, and *v-Ki-ras*, for their ability to hybridize to hCAP DNA. In all three cases, the bands that appeared in the lanes with focus DNA comigrated with bands characteristic of *c-Ha-ras*, *N-ras*, and *c-Ki-ras* in the mouse. Thus, activation of human *c-Ha-ras*, *N-ras*, or *c-Ki-ras* cannot explain the positive transfection results that we have obtained.

The most unequivocal test of transformation is to determine whether cells that are morphologically altered and apparently transformed *in vitro* are tumorigenic when injected *in vivo*. Our results demonstrate that this is the case with cells morphologically transformed by hCAP DNA. Three aspects of hCAP-associated tumor development were notable. The time to first appearance of tumors (7–16 weeks) was considerably longer than for many activated oncogene systems. Moreover, after appearing, the tumors grew more slowly, and tumor incidence was low (6 of 42). Brief tumor latent periods and short development times are characteristic of many oncogenes that are positive in the transfection assay. However, in a case involving rat nasal tumors induced by inhalation of the direct-acting carcinogen methyl methanesulfonate, there was a broad time continuum for tumor development after injection of transfected cells into nude mice (26). Some tumors did not appear until 16 weeks after injection, while others appeared within 2 weeks. In this case, *ras* genes were not involved.

At present, it is not clear why only a relatively small number of tumors arose in our studies. In most systems studied to date, cells or cell lines that yield positive results in the NIH 3T3 cell transfection assay also give rise to tumors at a relatively high incidence [e.g., human and mouse bladder carcinomas (23) and rat neuroblastoma (24)]. Our results show that tumors arose from 5 of 9 injected cell lines, no two of which were derived from the same focus, and in 6 of 42 animals. This ratio is much lower than that for the positive control T24 DNA-transfected cells (5/5) but is significantly higher ($\chi^2 = 4.67$, $P < 0.05$) than that for the negative controls (0 of 30).

Of further interest is the hybridization pattern of hCAP-associated nude mouse tumor DNA with the human repetitive sequence probe *Alu*. As shown in Fig. 4, several distinct high molecular weight (>6.6 kb) *Alu*-positive bands are present in the tumor DNA (Fig. 4, lane 2). No *Alu*-positive signal is present in the DNA from untransformed mouse cells (Fig. 4, lane 3). Development of nude mouse tumors after injection of primary transfectant cells is analogous to a secondary round of transfection. In both cases, there is a further purification of the original transforming donor sequences. The probability that the human DNA in the tumors or secondary transformants is not associated with a functional transforming gene is extremely low (24, 27). Thus, the detection of specific *Alu*-positive restriction fragments in the genome of a nude mouse tumor provides very strong evidence that human coronary artery plaque DNA contains transforming gene(s).

Although the results clearly demonstrate that human plaque DNA has transforming ability, the temporal expression of this activity *in vivo* is not known. The plaques were taken from adult patients in late stages of vascular disease. Thus, we cannot determine from these samples whether the manifestation of transformation is a relatively late event in plaque development or an early but stable event. Other studies suggest strongly that oncogene activation/expression is an important early event in transformation and tumorigenesis (28, 29).

The data presented here identify specific molecular events that may underlie the proliferation of smooth muscle cells that is a hallmark of atherosclerotic plaque development. In addition, these results demonstrate that plaque cells exhibit molecular alterations that until now have been implicated only in cancer-cell transformation and tumorigenesis. In cancer cells the active entities are oncogenes. By analogy, we propose that one or more as yet unidentified transforming genes plays a similar role in plaque cells. Our results provide direct experimental support for the monoclonal hypothesis.

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