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Oncogenes in human tumor cell lines: Molecular cloning of a transforming gene from human bladder carcinoma cells

(transfection assays/*Alu* repetitive sequences)

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ABSTRACT **The presence of dominant transforming genes in human tumor cell lines has been investigated. High molecular weight DNAs isolated from cell lines established from carcinomas and sarcomas of various organs as well as from a glioblastoma and two melanomas were utilized to transfect NIH/3T3 mouse fibroblasts. The DNAs of T24 and A2182, two cell lines derived from a bladder and a lung carcinoma, respectively, and of HT-1080, a cell line established from a fibrosarcoma, were able to transform recipient NIH/3T3 cells. First-cycle transformants exhibited anchorage-independent growth and were tumorigenic in athymic and immunocompetent mice. Moreover, they contained human DNA sequences and were able to transmit their malignant phenotype in additional cycles of transfection. Southern blot analysis of T24-derived transformants showed that a single fragment of human DNA specifically cosegregated with the malignant phenotype, suggesting that it contained the T24 oncogene. Therefore, these human sequences were molecularly cloned with λ Charon 9A as the cloning vector. The resulting recombinant DNA molecule, designated λT24-15A, was shown to contain a 15-kilobase-pair *Eco*RI insert of human cellular DNA. λT24-15A DNA (either intact or *Eco*RI digested) transformed NIH/3T3 fibroblasts with a specific activity of 20,000 focus-forming units per pmol of cloned DNA. Our results indicate that we have molecularly cloned a biologically active oncogene present in T24 human bladder carcinoma cells.**

The origin of most human cancers remains unknown. A plausible theory predicts the existence of dominant genetic elements whose expression would be responsible for the onset of the neoplastic properties of tumor cells. The development of gene transfer and genetic engineering technologies has permitted the designing of experimental protocols to test such hypothesis (for reviews, see refs. 1 and 2). Transmission of the malignant phenotype from tumor to normal cells via transfection with discrete fragments of DNA provides direct evidence of the presence of dominant oncogenes in human tumor cells. Molecular cloning of these genes can then readily be achieved, provided that they are biochemically identified. Finally, characterization of these *in vitro* selected oncogenes will establish their role in the onset of human neoplasia.

Recently, several laboratories have been able to transform normal mouse cells with DNA isolated from various human tumor cell lines (3–7). In this paper, we describe three additional human tumor cells whose malignant phenotype can be transmitted to normal cells via transfection of their respective high molecular weight DNA: T24, a cell line derived from a carcinoma of the urinary bladder (8); A2182, a cell line established from a lung carcinoma (S. A. Aaronson, personal communication); and HT-1080, a fibrosarcoma cell line (9). In addition, we report here the isolation by molecular cloning techniques of the transforming gene of T24 human bladder carcinoma cells.

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MATERIALS AND METHODS

Cells. Human cell lines used in the present study are listed, along with their respective sources, in Table 1. They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The NIH/3T3 fibroblasts (18) utilized in transfection assays (see below) were derived from a single clone (A6109) selected on the basis of its particularly flat morphology as well as by its low incidence of spontaneous overgrowth upon prolonged periods of incubation. Approximately 10^8 NIH/3T3 A6109 clonal cells were frozen in groups of 2×10^6 cells and periodically thawed so that, when used in transfection experiments, they had not been in culture for longer than 2 weeks.

Transfection Assays. We utilized the basic protocol of Wigler *et al.* (19) with minor modifications (20). Approximately 30 μg of high molecular weight DNA in 1 ml of 0.5 M $CaCl_2$ was slowly added to 1 ml of 250 mM Hepes, pH 7.1/1.5 mM sodium phosphate while a gentle stream of nitrogen was bubbled through. After formation of the calcium phosphate precipitate, this DNA solution was added to a 10-cm Petri dish in which 1.5×10^5 NIH/3T3 cells had been seeded the day before. After a 22-hr incubation, the DNA was removed and 10 ml fresh Dulbecco's modified Eagle's medium containing 5% calf serum was added. Foci were counted 2 weeks later in an attempt to avoid scoring secondary foci that formed as a consequence of cell spread. Transformed cells were collected by the cloning cylinder procedure and transferred to soft agar (0.3%) plates, and individual colonies were selected for further characterization.

Analysis of Clonal Transformed Cells. DNA isolated from representative clones was subjected to Southern blot analysis (21) for detection of human marker sequences. Twenty micrograms of each DNA was digested with appropriate restriction endonucleases according to the conditions suggested by the manufacturer. Digested DNA was applied to 0.6% horizontal agarose gels, electrophoresed at 30 V for 20 hr, blotted to nitrocellulose sheets, and hybridized for 48 hr to 2×10^7 cpm of the corresponding nick-translated ^{32}P-labeled DNA.

Molecular Cloning. The Charon 9A strain of λ phage was propagated in *Escherichia coli* K-12 DP50 sup F (22). DNA was purified from CsCl-banded phage as described (23). *Eco*RI fragments containing human DNA sequences were partially purified by preparative sucrose gradient centrifugation. *Eco*RI-cleaved vector and cellular DNA were mixed at 1:1 molar ratio in the presence of 0.04 unit of T4 ligase per microgram of DNA and packaged *in vitro* into phage particles as described (24). Plaques containing human DNA were identified by the method

Table 1. Transforming activity of DNA isolated from human cell lines

Human donor DNA	Cell line: Name	Cell line: Source*	Foci and recipient cultures, no./no.
Carcinomas:			
Bladder	5637	G.C.	0/12
	J82	C.O.	0/ 8
	RT4	Ref. 10	0/12
	T24	Ref. 8	156/24
Breast	BT-20	Ref. 11	0/ 8
	MCF-7	Ref. 12	0/ 8
Lung	A549	Ref. 13	0/ 8
	A2182	S.A.	11/ 8
Ovary	A2780	S.A.	0/ 8
Vulva	A431	Ref. 13	0/24
Sarcomas:			
Fibrosarcoma	HT-1080	Ref. 9	6/16
	A1383	S.A.	0/16
Liposarcoma	SW872	A.L.	0/ 8
Osteosarcoma	SK-OS-10	Ref. 14	0/ 8
	TE-85	Ref. 15	0/16
Rhabdomyosarcoma	A204	Ref. 13	0/12
	A673	Ref. 13	0/ 8
Miscellaneous tumors:			
Glioblastoma	A172	Ref. 13	0/12
Melanoma	A101D	L.H.	0/ 8
	A1306	S.A.	0/ 8
Normal embryonic fibroblasts	M413	Ref. 16	0/24
Retrovirus-transformed cells	K-HOS	Ref. 17	212/24

NIH/3T3 cells (1.5×10^5 cells per 10-cm Petri dish) were transfected with ≈30 μg of high molecular weight human DNA. Cells were maintained in culture with twice weekly changes of Dulbecco's modified Eagle's medium supplemented with 5% calf serum. Foci of transformed cells were scored after 14 days of cultivation. Usually, four Petri dishes of NIH/3T3 cells were transfected per experiment.

* Originators of unpublished cell lines: G.C., G. Cannon; C.O., C. O'Toole; S.A., S. A. Aaronson; A.L., A. Leibovitz; L.H., L. E. Hooser.

of Benton and Davis (25) using ^{32}P-labeled human DNA as a probe. All work involving recombinant phage was performed in a P2 containment facility in accordance with National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Transformation of NIH/3T3 Cells by DNA from Human Tumor Cell Lines. High molecular weight DNA (>50 kilobase pairs) was isolated from various human tumor cell lines including those established from carcinomas and sarcomas of various organs, as well as from a glioblastoma and two melanomas (Table 1). These DNAs were tested for their ability to induce morphologic transformation upon transfection of NIH/3T3 mouse cells. The efficiency of our transfection assays was monitored by using a DNA extracted from K-HOS, a human cell line transformed by the Kirsten strain of murine sarcoma virus (17). K-HOS DNA induced ≈0.3 focus per μg of donor DNA, an efficiency comparable to that described in other biological systems in which the transfecting DNAs contained a single copy of a dominant selectable gene per cellular genome (1, 2). All tested K-HOS DNA-derived transformants demonstrated a rescuable sarcoma virus when superinfected with the Moloney strain of murine leukemia virus (data not shown).

Among the donor human tumor DNAs listed in Table 1, only those isolated from T24, A2182, and HT-1080 cells were able to alter the morphology of recipient NIH/3T3 cells. T24 cells were derived by Bubenik *et al.* (8) from a bladder carcinoma in an 82-year-old woman with long-standing urinary bladder papillomatosis that was treated by electrocoagulation. A2182 is a cell line established by S. A. Aaronson (unpublished data) from a lung carcinoma in a 48-year-old man. HT-1080 is a cell line established in 1974 by Rasheed and coworkers (9) from a fibrosarcoma in a 35-year-old man who died without receiving any chemotherapy or radiotherapy treatment. T24 DNA exhibited the highest transforming activity, with 0.2 focus per μg of donor DNA, an efficiency similar to that of K-HOS DNA. In contrast, HT-1080 DNA was found to be only 5% as efficient (0.01 focus per μg of DNA) in transforming NIH/3T3 fibroblasts. A2182 DNA had an intermediate transforming efficiency (0.05 focus per μg of DNA) causing the appearance of an average of 2 foci per transfected plate of NIH/3T3 cells.

The morphologic appearances of NIH/3T3 cells transformed by each of these human tumor DNAs were similar in several parameters. They were highly refractile, grew densely, and overlaid one another in a disoriented manner. These foci were clearly distinguishable from the morphologic variants of NIH/3T3 cells that overcame the characteristic contact-inhibition of these cells upon prolonged incubation.

Tumorigenic Properties of NIH/3T3 Cells Transformed by Human Tumor Cellular DNAs. The malignant properties of these NIH/3T3 transformants were next investigated. As an *in vitro* assay, we determined their growth efficiency in semisolid media. *In vivo*, we studied their ability to produce tumors in both athymic and immunocompetent mice. Representative transformants derived from each positive human tumor cell line were found to form large colonies in semisolid agar (0.3%) with efficiencies ranging between 40% (HT-1080-derived transformants) and 95% (T24-derived transformants). Each of these primary transfectants was highly tumorigenic when injected (10^5 cells) into athymic and immunocompetent weanling NIH/Swiss mice. All inoculated animals developed solid tumors at the site of injection in less than 3 weeks. Finally, DNA from these representative transfectants readily transferred the malignant phenotype to NIH/3T3 cells in a second cycle of transfection. Their transforming activities ranged from 0.05 to 0.3 focus per μg of donor DNA, independently of the original efficiency detected in the first cycle of transfection utilizing human tumor DNA.

Human Marker Sequences in NIH/3T3 Transformants. In order to demonstrate that the serial transmission of the transformed phenotype from human tumor cell lines to normal mouse cells was mediated by a human oncogene, we investigated the presence of human DNA marker sequences in the genome of NIH/3T3 transformants. We utilized an approach developed by Shih *et al.* (3) in which a probe for a family of highly repeated human sequences [designated "*Alu* sequences" (26)] was used to identify human DNA on a murine genomic background. First-cycle transformants derived from T24, HT-1080, or A2182 human cells contained a large number of DNA fragments that hybridized to a probe representative for the human *Alu* repetitive sequences (Fig. 1). None of these fragments was detected in control NIH/3T3 DNA, indicating that they were derived from the transfecting human DNA.

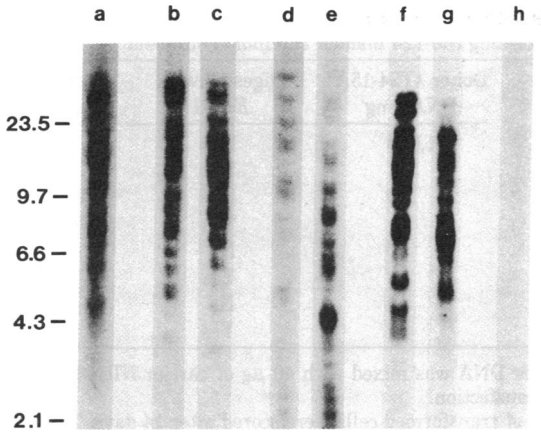

FIG. 1. Human DNA sequences in NIH/3T3 cells transformed by DNA isolated from human tumor cell lines. Lanes: a, K-HOS-derived first-cycle transformant; b and c, T24-derived first-cycle transformants; d and e, HT-1080-derived first-cycle transformants; f and g, A2182-derived first-cycle transformants; and h, normal NIH/3T3 A6109 clonal cells. Twenty micrograms of DNA was electrophoresed in a 0.6% horizontal agarose gel, blotted to a nitrocellulose sheet, and hybridized to 2×10^7 cpm of ^{32}P-labeled human DNA probe for 48 hr. Hybridized blots were exposed to Kodak XR-5 film at −70°C in the presence of intensifier screens for 2 days. Coelectrophoresed DNA fragments of *Hin*dIII-digested λ c1857 DNA served as size standards (labeled in kilobase pairs).

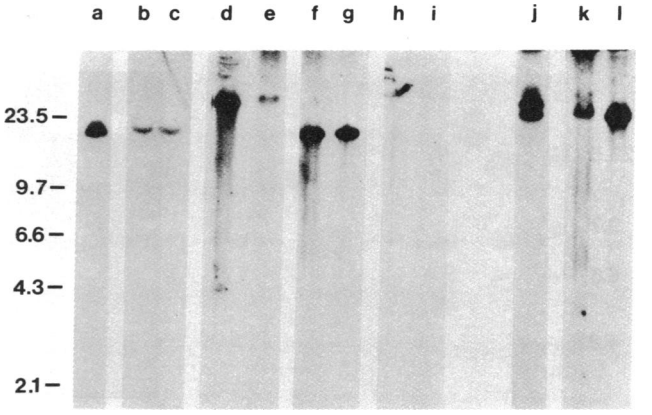

FIG. 2. Human DNA sequences in second- and third-cycle transformants derived from T24 bladder carcinoma cells. Lanes: a, second-cycle transformant 44-91; b–i, third-cycle transformants obtained by transfection with untreated 44-91 DNA (b, c), *Eco*RI-cleaved 44-91 DNA (d, e), *Hin*dIII-cleaved 44-91 DNA (f, g), or *Bam*HI-cleaved 44-91 DNA (h, i); j, second-cycle transformant 44-103; and k and l, third-cycle transformants obtained by transfection with untreated 44-103 DNA. Twenty micrograms of DNA was analyzed for the presence of human *Alu* sequences as described in the legend to Fig. 1. Coelectrophoresed DNA fragments of *Hin*dIII-digested λ c1857 DNA served as size standards (labeled in kilobase pairs).

A Single T24-Derived Human DNA Fragment Cosegregates with the Malignant Phenotype. The large fraction of donor DNA incorporated by transfection-competent NIH/3T3 cells (27) hampered the identification of those human DNA sequences harboring the transforming gene(s). However, cotransfected, nongenetically linked human sequences should be eliminated in subsequent cycles of transfection. Therefore, DNAs from representative transformants were used to transform NIH/3T3 cells in an effort to identify biochemically those sequences encompassing human transforming genes. Second-cycle transformants derived from T24 cells possessed one or two high molecular weight human DNA fragments as deduced from their ability to hybridize with a probe for human *Alu* marker sequences (Fig. 2). Only one of these DNA fragments was found to cosegregate with the transformed phenotype after an additional (third) cycle of transfection, indicating that the T24 transforming gene must be contained within these human sequences. When similar experiments were performed with transformants derived from A2182 and HT-1080 cells, several human DNA fragments were found to cosegregate with the transformed phenotype even after three cycles of transfection. These results suggest that the transforming genes of A2182 and HT-1080 human tumor cell lines must have a larger genetic complexity than that present in T24 bladder carcinoma cells.

Molecular Cloning of the T24 Oncogene. For molecular cloning of the T24 oncogene in a biologically active form, we selected a second-cycle transformant, designated 44-91, that contains a single human DNA fragment known to cosegregate with the transformed phenotype (see Fig. 2, lanes a–c). In addition, it was necessary to find a restriction endonuclease that would be compatible with available cloning vectors but would not inactivate the transforming activity of 44-91 DNA or physically separate the T24 oncogene from the human *Alu* marker sequences. Among those enzymes tested (Table 2), *Bam*HI, *Eco*RI, and *Hin*dIII did not affect the biologic activity of 44-91 DNA. Moreover, NIH/3T3 transformants obtained with either *Eco*RI- or *Hin*dIII-digested 44-91 DNA retained the human *Alu* sequences (Fig. 2, lanes d–g), making these two enzymes suitable for the molecular cloning of the T24 oncogene. The fact that transformants generated with *Bam*HI-cleaved DNA lacked such marker sequences suggested that they are not an integral part of the T24 transforming gene (Fig. 2, lanes h and i).

*Eco*RI-digested 44-91 was partially purified by sucrose gradient centrifugation in an effort to enrich for sequences of human origin. Fractions hybridizing to a human *Alu* probe were ligated to *Eco*RI-cleaved DNA purified from the Charon 9A strain of λ phage (22), packaged *in vitro* into phage particles, and plated onto *E. coli* K-12 LE392. Of the 5×10^5 plaques obtained, ≈50% were found to be recombinant phages as determined by hybridization with a ^{32}P-labeled NIH/3T3 DNA

Table 2. Effect of restriction endonuclease digestion on transforming activity of DNA isolated from human T24 cells or from 44-91, a T24-derived second-cycle transformant

Donor DNA	Digested with	Foci and recipient cultures, no./no.
T24	Control	77/8
	*Bam*HI	42/8
	*Eco*RI	51/8
	*Hin*dIII	40/8
	Kpn I	0/8
	Sst I	0/8
44-91	Control	32/4
	*Bam*HI	19/4
	*Eco*RI	17/4
	*Hin*dIII	13/4

High molecular weight DNA was incubated with the indicated restriction endonucleases according to the conditions recommended by the manufacturer. Digested DNA was extracted with 1 vol of phenol and then with 1 vol of chloroform:isoamyl alcohol, 24:1 (vol/vol). Deproteinized DNA was precipitated with ethanol, resuspended in distilled water, and used for transfecting 1.5×10^5 NIH/3T3 cells seeded the previous day in 10-cm Petri dishes (usually four plates per experiment and DNA). Foci were scored after 14 days of cultivation.

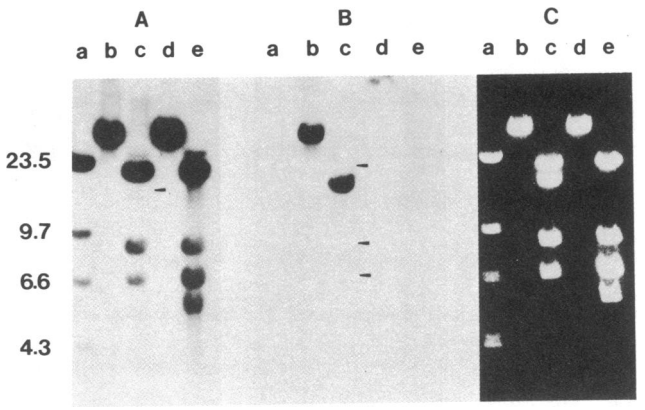

FIG. 3. Electrophoretic analysis of λT24-15A recombinant DNA. Lanes: a, *Hin*dIII-cleaved λ c1857 DNA (size standards; labeled in kilobase pairs); b, λT24-15A DNA; c, *Eco*RI-cleaved λT24-15A DNA; d, λ Charon 9A DNA; and e, *Eco*RI-cleaved λ Charon 9A DNA. One microgram of DNA was electrophoresed (20 hr; 30 V) in a 0.6% horizontal agarose gel, blotted to a nitrocellulose sheet, and hybridized for 4 hr to ^{32}P-labeled λ Charon 9A probe (*A*) or ^{32}P-labeled human DNA probe (*B*). (*C*) Ethidium bromide-stained gel photographed under UV light. Hybridized blots were exposed to Kodak XR-5 film at −70°C in the presence of intensifier screens for 3 hr.

probe. Of these, only one plaque hybridized to a ^{32}P-labeled human DNA probe. This phage was plaque-purified three times, grown to mass culture, and further characterized. This recombinant phage, designated λT24-15A, contained a 15-kilobase-pair *Eco*RI insert that replaced the 6.9- and 5.7-kilobase-pair *Eco*RI internal fragments of λ Charon 9A (Fig. 3). The human, rather than mouse, origin of this cloned 15-kilobase-pair *Eco*RI insert was demonstrated by the presence of human-specific *Alu* sequences (Fig. 3) and by its lack of hybridization to mouse cellular DNA (Fig. 4, lane c).

Biological Activity of Molecularly Cloned T24 Oncogene. To determine whether the cloned 15-kilobase-pair DNA fragment derived from T24 human tumor cells had transforming activity,

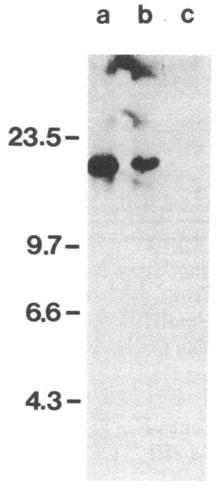

FIG. 4. Characterization of NIH/3T3 cells transformed by λT24-15A DNA. Lanes: a and b, representative NIH/3T3 transformants obtained by transfection with λT24-15A DNA; and c, control NIH/3T3 cells. Twenty micrograms of DNA was electrophoresed in a 0.6% horizontal agarose gel, blotted to a nitrocellulose sheet, and hybridized to a probe of ^{32}P-labeled 15-kilobase-pair *Eco*RI insert of λT24-15A DNA. Hybridized blots were exposed to Kodak XR-5 film at −70°C in the presence of intensifier screens for 2 days. Size standards were as in Fig. 1.

Table 3. Biologic activity of a recombinant DNA clone containing the T24 bladder carcinoma oncogene

Donor λT24-15A DNA,* ng	Digested with *Eco*RI	Foci,† no.
1,000	No	‡
	Yes	‡
100	No	32
	Yes	>50
10	No	4
	Yes	9
1	No	0.5
	Yes	1.5

* Donor DNA was mixed with 40 μg of carrier NIH/3T3 DNA prior to transfection.
† Foci of transformed cells were scored after 14 days. The number of foci scored represents the average from two Petri dishes.
‡ Too many to count.

we transfected NIH/3T3 cells with varying amounts of λT24-15A DNA. As little as 1 ng of this recombinant DNA molecule induced morphologic transformation of recipient cells (Table 3). Cleavage of λT24-15A DNA with *Eco*RI prior to transfection slightly enhanced its biological activity, yielding a transforming specific activity of ≈20,000 focus-forming units per pmol of cloned human insert. When representative NIH/3T3 transformants were picked and grown to mass culture, their DNA was found to contain those human sequences present in λT24-15A recombinant DNA (Fig. 4, lanes a and b). These results, taken together, demonstrate that we have cloned a DNA fragment that contains a biologically active human oncogene present in T24 bladder carcinoma cells.

DISCUSSION

The ability of human tumor cell lines to transmit their neoplastic properties to normal cells would support the concept that they contain dominant oncogenes. Previous studies have shown that inoculation into rats of cells established from various human tumors induced the formation of sarcomas of host rather than of human origin (28). Recently, DNAs isolated from cell lines derived from several types of human tumors have been found to transform mouse fibroblasts in *in vitro* transfection assays (3–7). In the present study we have shown that DNA isolated from three additional human cell lines derived from bladder and lung carcinomas and from a fibrosarcoma were able to transmit their malignant phenotype to NIH/3T3 cells. These findings not only provide evidence for the presence of transmissible oncogenes in human tumor cell lines but also indicate that such oncogenes are not restricted to cells derived from a particular type of neoplasia.

Emerging evidence indicates that oncogenes present in different human tumor cell lines have an independent genetic origin. For instance, second-cycle transformants derived from human cell lines established from various tumors including bladder and colon carcinomas, a neuroblastoma, and a myeloid leukemia exhibited different restriction enzyme patterns of human *Alu* marker sequences (6, 7). Similarly, we have found that restriction enzymes such as *Bam*HI, *Eco*RI, or *Hin*dIII which do not inactivate the T24 oncogene completely abolish transformation induced by HT-1080 DNA (unpublished data). Nevertheless, Perucho *et al.* (7) have reported that oncogenes present in human cell lines derived from a colon and two lung carcinomas may be identical or at least closely related. In similar studies, Lane *et al.* (5) observed that the transforming activities of DNAs isolated from human and mouse mammary tumor cell

lines were equally affected by digestion with several restriction endonucleases. Thus, it is possible that only a limited number of oncogenes are responsible for the appearance of at least certain types of naturally occurring tumors.

The ubiquitous presence of certain repetitive (*Alu*) sequences in mammalian genomes, along with their highly evolved nature (26), has made it possible to detect human DNA fragments biochemically in a murine genetic background (3, 6, 7). Cosegregation of any of these fragments with malignant transformation induced by human tumor DNAs served as a criterion for the identification of those sequences encompassing the corresponding human oncogenes. A combination of these experimental approaches has allowed us to clone molecularly the transforming gene of T24 human bladder carcinoma cells. In fact, it should be possible to apply a similar protocol to the isolation of any biologically detectable human oncogene with only the limitations imposed by current recombinant DNA technology (i.e., gene size, poisonous sequences, etc.). Characterization of the cloned T24 oncogene not only should provide definitive evidence regarding its genetic nature but, more importantly, it also should enable us to elucidate its involvement in bladder carcinomas as well as in other types of human malignancies.

Note Added in Proof. We have shown that the transforming sequences of the T24 bladder carcinoma oncogene are located within an internal 6.6-kilobase-pair *Bam*HI fragment of λT24-15A. Moreover, in collaborative studies with S. Tronick and S. A. Aaronson, we have found that this fragment contains sequences related to the *onc* gene of BALB murine sarcoma virus and closely resembles the normal human analogue of this retroviral *onc* gene.

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