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Transforming Genes in Human Tumors

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DNAs isolated from a variety of human tumor cell lines as well as from naturally occurring human carcinomas and sarcomas were shown to induce morphologic transformation upon transfection into NIH/3T3 cells. All tested transformants contained human DNA sequences, some of which specifically cosegregated with the malignant phenotype in additional cycles of transfection. Southern blot analysis of second cycle transformants derived from T24 human bladder carcinoma cells showed the presence of a single 15 kbp EcoRI fragment of human DNA. These sequences were molecularly cloned utilizing λ Charon 9A as the cloning vector. The resulting recombinant DNA molecule, designated λ T24-15A, was shown to contain an internal 6.6 kbp Bam HI fragment of human DNA that transformed NIH/3T3 fibroblasts with a specific activity of 5×10^4 focus forming units per picomol. These results indicate that we have moleculary cloned an oncogene present in T24 bladder carcinoma cells. Comparison of molecular clones containing the T24 oncogene and its normal homologue did not reveal biochemical differences that helped to explain the malignant properties of this oncogene. Finally, we report preliminary results indicating that the T24 bladder carcimoma oncogene is highly related to the transforming gene of BALB-MSV, an acute transforming retrovirus.

Key words: NIH/3T3 cells, carcinoma, sarcoma, T24 bladder carcinoma cells, transfection

Selectable phenotypes encoded by single copy dominant genes can be transmitted to appropriate recipient cells by transfection with genomic DNA [1,2]. This approach has recently been utilized to investigate whether human tumor cells contain dominant transforming genes. DNAs isolated from a variety of human tumor cell lines as well as from human tumor specimens have been tested for their ability to transform morphologically normal NIH/3T3 mouse cells in culture [3–9]. Human tumor DNAs representing most types of human cancers have been able to confer neoplastic properties to transfected NIH/3T3 cells. DNA sequences responsible for the malignant transformation of the recipient mouse cells have been biochemically identified by utilizing probes specific for a family of highly repetitive human sequences [3,6,7,9]. These findings have made it possible to attempt the isolation of these human transforming genes by utilizing classical recombinant DNA techniques.

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Recently, a gene capable of transforming NIH/3T3 cells in culture has been molecularly cloned from two human bladder carcinoma cell lines [9–11]. Preliminary characterization of this human oncogene has revealed that it is small in size and structurally similar to homologous sequences present in normal human DNA. Moreover, this bladder carcinoma oncogene has been found to be highly related to the transforming genes of two acute transforming retroviruses, the BALB and Harvey strains of murine sarcoma viruses [12a,b,c]. The present manuscript summarizes our current knowledge regarding the genetic nature of transforming genes present in a variety of human tumors and describes a detailed characterization of the T24 bladder carcinoma oncogene.

MATERIALS AND METHODS

Cells

Human cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. NIH/3T3 mouse fibroblasts [12], derived from a single clone (A6109) selected for its flat morphology, were maintained in the same medium except that it was supplemented with 5% calf serum. Human tumor tissues were stored at −70°C.

Isolation of High Molecular Weight DNA

Cells were disrupted in a 10 mM Tris-HCl buffer containing 150 mM NaCl, 2 mM EDTA, pH 7.8, and 0.5% SDS, and incubated overnight at 37°C in the presence of 100 μg/ml of pronase. Partially deproteinized nucleic acids were extracted with 1 volume of phenol, 1 volume of phenol:chloroform:isoamylalcohol (25:24:1) and 1 volume of chloroform:isoamylalcohol (24:1), and precipitated at −20°C with 2.5 volumes of ethanol. In some cases, DNA was further purified by consecutive digestions (37°C for 30 min) with 10 μg/ml of RNase A and 100 μg/ml of proteinase K. To isolate high molecular weight DNA from human tumor tissues, they were minced into small pieces after removal of necrotic material, and treated with a phosphate buffered saline solution containing 0.1% (w/v) of trypsin for 1 hr. Cells were disaggregated with the help of a Dounce homogenizer and processed as indicated above. In all cases, DNA was stored at 4°C in sterile water containing 0.1 mM EDTA.

Transfection Assays

Transfection assays were carried out by the calcium phosphate precipitation technique of Graham and van der Eb [13] as modified by Wigler et al [14]. Transfecting DNAs, either 30 μg of high molecular weight cellular DNA or the required amount of cloned DNA mixed with 20 μg of NIH/3T3 carrier DNA, contained in 1 ml of 0.5 M $CaCl_2$ was mixed with an equal volume of 250 mM Hepes (pH 7.1)/1.5 mM sodium phosphate. After allowing formation of the calcium-phosphate precipitate, this DNA solution was added to a 10-cm Petri dish in which 2×10^5 clonal NIH/3T3 mouse fibroblasts had been seeded the day before. Following a 22-hr incubation, the DNA was removed and 10 ml of fresh Dulbecco modified Eagle medium supplemented with 5% calf serum were added. Foci were scored after 14 days of incubation.

Analysis of Clonal Transformed Cells

Twenty micrograms of high molecular weight DNA were digested with appropriate restriction endonucleases and applied to horizontal 0.6% (w/v) agarose gels. Samples were electrophoresed at 30 V for 20 hr, blotted to nitrocellulose sheets, and hybridized for 48 hr to 2×7^7 cpm of the corresponding nick-translated [^{32}P]-labeled DNA as described by Southern [15].

Molecular Cloning

EcoRI fragments containing human DNA sequences were partially purified by preparative sucrose gradient centrifugation. They were mixed with EcoRI cleaved λ Charon 9A DNA at 1:1 molar ratio in the presence of 0.04 units of T24 ligase per microgram of DNA and packaged in vitro into phage particles as described [16]. Plaques containing human DNA were identified by the method of Benton and Davis [17] using ^{32}P-labeled human DNA as a probe. The normal human sequence homologous to the T24 oncogene were isolated from a λ Charon 4A Hae III/Alu I library of human fetal liver DNA [18].

RESULTS

The development of DNA-mediated gene transfer technology has made it possible to transmit selectable phenotypes encoded by dominant genetic elements, even should they be present as single copy genes within the cellular genome [1,2]. This approach has been recently utilized to study the induction of morphologic transformation by genomic DNAs containing retroviral *onc* genes [19,20]. Transforming efficiencies of around 0.5 foci per microgram of donor cellular DNA can be routinely obtained when morphologically normal NIH/3T3 mouse fibroblasts are utilized as recipient cells. Thus, we have utilized this experimental approach to examine whether human tumor cells contain dominant transforming genes. DNA was isolated from a variety of solid human tumors as well as from established human tumor cell lines. They included carcinomas of the bladder, breast, colon, gall bladder, kidney, lung, ovary, pancreas, and vulva, as well as various sarcomas such as fibrosarcomas, liposarcomas, osteosarcomas, and rhabdomyosarcomas. Other types of tumors including glioblastomas, melanomas, and teratocarcinomas were also utilized as sources of DNA. As summarized in Table I, only 15% of these DNAs, independently of whether they were derived from solid tumors or from established tumor cell lines, were able to transform NIH/3T3 cells in transfection assays. It should be noted that in most cases human tumor DNAs transfected NIH/3T3 cells with efficiencies close to the detection limits of our assays. Thus, it is possible that certain human tumor DNAs that scored as negative in the experiments summarized in Table I may also contain dominant transforming genetic elements. Whether this is the case or not, our results indicate that samples capable of transforming NIH/3T3 cells were found in half of the tumor types investigated.

The tumorigenicity of NIH/3T3 transformants induced by human tumor DNAs was examined by determining their ability to grow in semisolid agar and to induce tumors in athymic as well as immunocompetent mice. Representative transformants derived from each of the human tumor DNAs listed in Table I formed large colonies in semisolid agar with efficiencies ranging from 25% to 90% of the plated cells.

TABLE I. Human Tumor DNAs Tested for Transformation of NIH/3T3 Mouse Cells

Donor DNA		Transformation of NIH/3T3 cells by human tumor DNA (No. tested/No. positive)	
Tumor type	Tissue	Cell lines	Solid tumors
Carcinoma	bladder	6/2	2/0
	breast	3/0	2/0
	colon	1/1	2/2
	gall bladder	1/1	—
	kidney	—	3/0
	lung	4/1	3/1
	ovary	1/0	4/0
	pancreas	—	2/1
	vulva	1/0	—
Sarcoma	fibrosarcoma	3/1	4/0
	liposarcoma	1/0	—
	osteosarcoma	5/0	2/0
	rhabdomyosarcoma	4/0	2/1
Glioblastoma		1/0	—
Melanoma		3/0	—
Teratocarcinoma		2/0	—

Moreover, when 10^6 cells of each of these transformants were injected into either athymic and/or immunocompetent NIH Swiss mice, solid tumors developed in 3 weeks. These results illustrate the tumorigenic properties of NIH/3T3 transformants induced by human tumor DNA.

We next investigated whether the acquisition of the transformed phenotype by NIH/3T3 was mediated by transfection of human DNA sequences. Representative NIH/3T3 transformants derived from each of the human tumor DNAs were characterized by Southern blot analysis utilizing a probe specific for a family of human repetitive sequences (designated as *Alu* sequences, see ref. 21). All transformants tested exhibited multiple human DNA sequences, suggesting that transformation of these NIH/3T3 cells was a direct consequence of the acquisition of oncogenic sequences present in human tumor DNAs [9]. Definition of human DNA fragments encompassing these transforming genes required elimination of the large number of nonspecifically cotransfected human DNA sequences by additional cycles of transfection [22]. Second and/or third cycle transformants derived from each human tumor DNA were routinely submitted to Southern blot analysis after digestion with appropriate restriction enzymes. Representative results obtained with transfectants derived from T24 bladder carcinoma [23] and HT-1080 fibrosarcoma [24] human tumor cell lines, as well as those derived from a naturally occurring carcinoma of the colon are shown in Figure 1. In each case, only a discrete number of human DNA fragments were found to cosegregate with the malignant phenotype suggesting that these sequences contain the transforming genes (Fig. 1). Moreover, the different pattern of human DNA fragments observed in transformants derived from each tumor DNA supports the concept that their oncogenes have an independent genetic origin.

Identification of human DNA sequences that cosegregated with the malignant

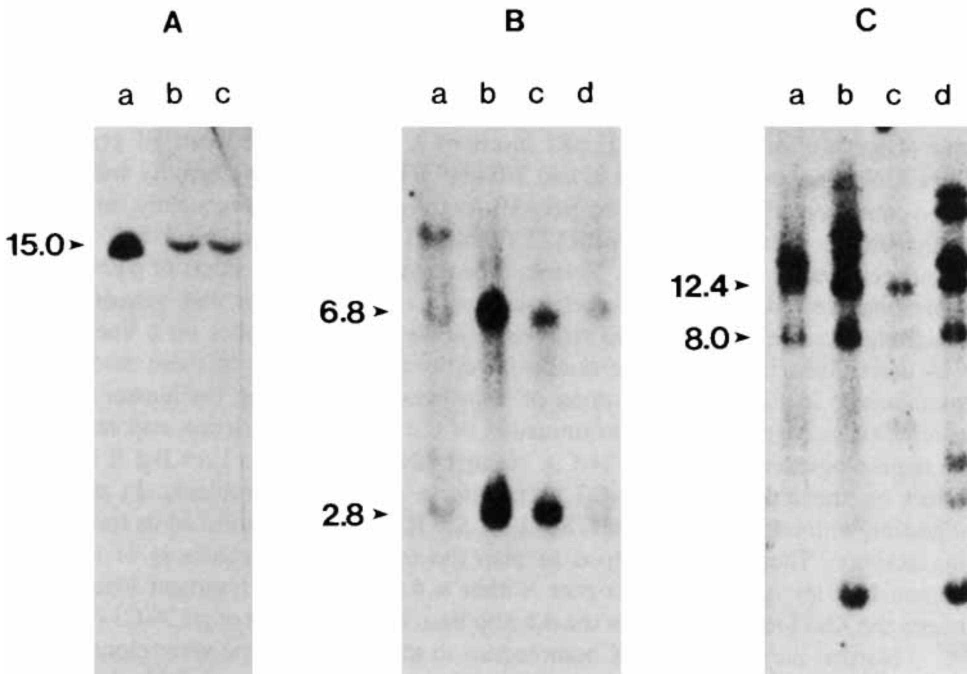

Fig. 1. Human DNA sequences in second cycle transformants derived from (A) T24 bladder carcinoma cells, (B) HT-1080 fibrosarcoma cells, and (C) a naturally occurring colon carcinoma (#1665). Twenty micrograms of DNA isolated from representative second cycle transfectants were digested with EcoRI and submitted to Southern blot analysis for the presence of human *Alu* sequences, as described in Materials and Methods. Human DNA fragments common to transformants derived from each human tumor DNA (labeled in kilobase pairs) are indicated by arrows.

phenotype made it possible to attempt their isolation by molecular cloning techniques. However, in order to clone human oncogenes in their biologically active form it is necessary to investigate the effect of restriction enzymes on their transforming activity. DNAs isolated from transformants derived from HT-1080 fibrosarcoma cells and from carcinomas of the colon, lung, and pancreas, lost their transforming activity when they were digested with several restriction enzymes including EcoRI, Hind III, Bam HI, and Sac I. In contrast, the transforming activity of DNAs isolated from T24-derived transformants was only abolished by incubation with Sac I, but not after cleavage with EcoRI, Hind III, or Bam HI. Moreover, digestion of these T24-derived DNAs with either EcoRI or Hind III did not separate the human marker sequences from the DNA fragment containing the T24 oncogene. These findings established that EcoRI or Hind III could be utilized to clone the transforming gene present in T24 bladder carcinoma cells molecularly.

DNA isolated from 44-91 cells, a representative T24-derived second cycle transformant, was digested with the enzyme EcoRI and partially purified by sucrose gradient centrifugation. Fractions containing human sequences were ligated to EcoRI-cleaved DNA purified from the Charon 9A strain of λ phage. Of 5×10^5 plaques screened for the presence of human repetitive sequences, one plaque scored as positive. This phage, designated λ T24-15A, was plaque-purified and shown to

contain a 15 kbp EcoRI insert of human DNA. As little as 1 ng of recombinant λ T24-15A DNA was sufficient to induce morphologic transformation in NIH/3T3 recipient cells (Table II). These results demonstrate that we have cloned a biologically active human oncogene.

Digestion of the 15 kbp EcoRI insert of λ T24-15A with Bam HI generated three DNA fragments of 6.8, 6.6, and 2.0 kbp. The transforming activity was found to be contained within the 6.6 kbp Bam HI fragment that was subsequently subcloned at the unique Bam HI site of pBR322 (Table II). A physical map of these human sequences was next established. Restriction enzyme cleavage products of pT24-C3, a representative plasmid, were electrophoresed on agarose gels and visualized by ethidium bromide staining. The relative location of cleavage sites on a linear map was determined by appropriate double digestions. The results of these studies are summarized in Figure 2. Definition of sequences encompassing the human bladder carcinoma oncogene required examination of the effect of restriction endonucleases on the biological activity of pT24-C3. Neither Xho I, Sph I, Cla I, or Bgl II had any effect on the ability of pT24-C3 to transform NIH/3T3 fibroblasts. In contrast, digestion with either Kpn I, Xba I, Sac I, or Sac II completely abolished its transforming activity. These results helped to map the transforming sequences of the T24 human bladder carcinoma oncogene within a 4.6 kbp DNA fragment located between the Xho I and Sph I site of the 6.6 kbp Bam HI DNA insert of pT24-C3 (Fig. 2).

Normal human sequences homologous to the T24 oncogene were cloned in an effort to understand the molecular basis for the malignant properties of this transforming gene. We isolated a recombinant λ Charon 4A phage possessing a 17 kbp EcoRI human DNA insert from a library of human fetal liver DNA. Sequences related to the T24 oncogene were located within an internal 6.4 kbp Bam HI fragment that was subsequently subcloned at the unique Bam HI site of pBR322. Neither the recombinant phage, designated λ bc-N, nor a representative plasmid designated pbc-N1, possessed transforming activity when tested in transfection assays (Table II). Comparison of the restriction endonuclease maps of pT24-C3 and pbc-N1 DNA did not reveal any significant biochemical differences that helped to explain their different oncogenic properties (Fig. 3). Thus, our results support the concept that very minor

TABLE 2. Biologic Activity of Recombinant DNA Molecules Containing the T24 Bladder Carcinoma Oncogene and Its Normal Homologue

Donor DNA	Nanograms added	No. of foci/plate
λ T24-15A	100	32
	10	4
	1	0.5
pT24-C3	100	>50
	10	26
	1	3
6.6 kbp Bam HI insert of pT24-C3	10	>50
	1	14
λ bc-N	1000	0
pbc-N1	1000	0
6.4 kbp Bam HI insert of pbc-N1	500	0

biochemical alterations must be responsible for the acquisition of malignant properties by the T24 bladder carcinoma oncogene.

We considered it of interest to determine whether the T24 oncogene was related to oncogenes present in tumors other than bladder carcinomas. DNAs from representative second cycle NIH/3T3 transformants derived from carcinomas of the colon, gall bladder, lung, and pancreas, as well as from a fibrosarcoma and a rhabdomyosarcoma, were submitted to Southern blot analysis for the presence of sequences related to pT24-C3. As shown in Figure 4, none of the transformants tested exhibited a

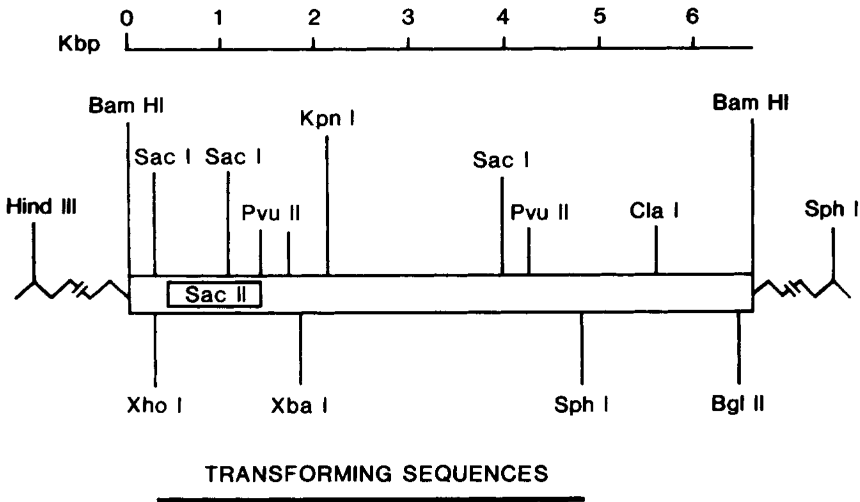

Fig. 2. Restriction enzyme analysis of the 6.6 kbp Bam HI human DNA insert of pT24-C3. Sequences encompassing the T24 bladder carcinoma oncogene are indicated by the solid bar. The location of the six Sac II cleavage sites is indicated by the open box located between the Xho I and Xba I cleavage sites. Wavy lines represent pBR322 sequences.

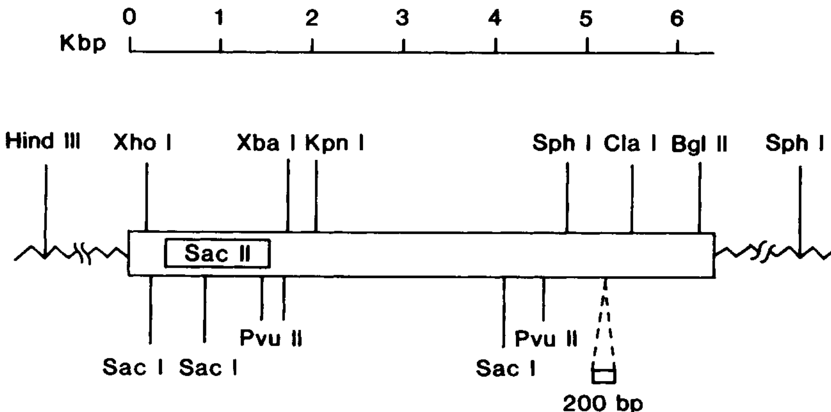

Fig. 3. Restriction enzyme analysis of the 6.4 kbp Bam HI human DNA insert of pbc-N1, a plasmid that contains the normal human homologue of the T24 oncogene.

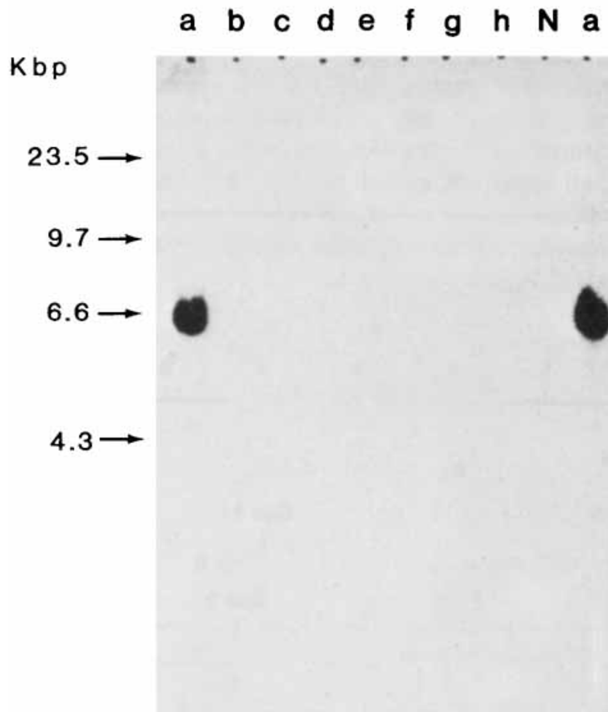

Fig. 4. Relationship between the T24 bladder carcinoma oncogene and transforming genes present in other human tumor DNAs. Twenty micrograms of DNA isolated from either control NIH/3T3 cells (lane N) or from second cycle transfectants derived from human (a) T24 bladder carcinoma, (b) HT-1080 fibrosarcoma, (c) A2182 lung carcinoma, and (d) A1604 gall bladder carcinoma cell lines as well as from naturally occurring human tumors including, (e) colon carcinoma (#1665), (f) colon carcinoma (#2033), (g) pancreatic carcinoma (#1189), and (h) rhabdomyosarcoma (#1085), were digested with Bam HI and submitted to Southern blot analysis for the presence of sequences related to the T24 oncogene as described in Materials and Methods. Coelectrophoresed DNA fragments of Hind III-digested λ c1857 DNA served as size standards (labeled in kilobase pairs).

significant degree of homology with the transforming sequences of the T24 bladder carcinoma oncogene. These results add further support to the concept that different types of human tumors have different transforming genes.

Another family of genes possessing oncogenic properties are those present in acute transforming retroviruses [25]. These retroviral *onc* genes arose by recombination of replication competent type-C viruses with cellular genetic information. *Onc* gene sequences have been highly conserved during evolution and, as a consequence, they are normal constituents of the genomes of all vertebrate species, including humans [25]. Thus, we examined whether the T24 oncogene may be related to retroviral *onc* sequences. Recombinant plasmids containing *onc*-specific sequences of several retroviruses including Rous sarcoma virus [26], Abelson murine leukemia virus [27], the BALB [28], and Moloney [29] strains of murine sarcoma virus (MSV), the Snyder–Theilen strain of feline sarcoma virus [30], and simian sarcoma virus [31] were submitted to Southern blot analysis to determine whether they contained sequences related to the T24 oncogene. As shown in Figure 5, the 6.6 kbp Bam HI

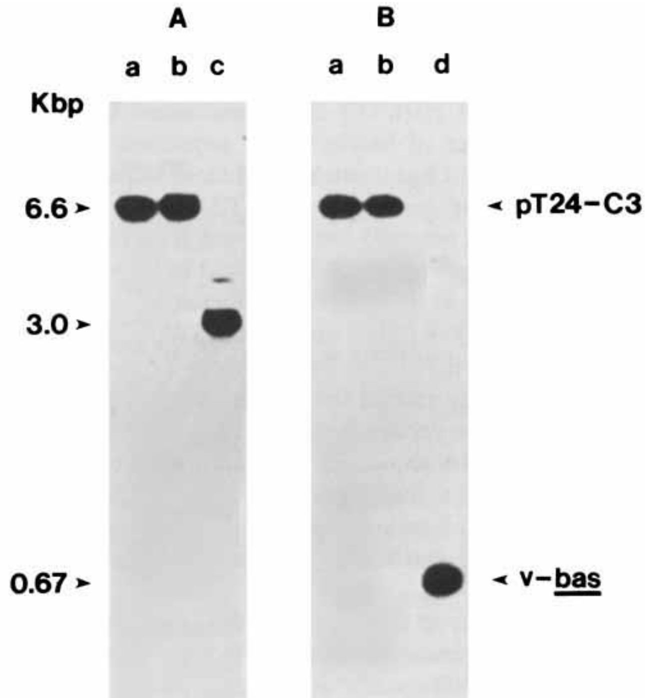

Fig. 5. Relationship between the T24 bladder carcinoma oncogene and v-*bas*, the transforming gene of BALB-MSV. Molecularly cloned DNAs including (a) 100 ng of the 6.6 kbp Bam HI insert of λ T24-15A, (b) 100 ng of the 6.6 kbp Bam HI insert of pT24-C3, (c) 200 ng of the 6.6 kbp Bam HI insert of pT24-C3 digested with Sac I, and (d) 100 ng of 0.67 kbp Hind III to Bam HI fragment of v-*bas* were submitted to Southern blot analysis for the presence of sequences related to (A) v-*bas* and (B) pT24-C3 DNAs, as described in Materials and Methods. Migration of the 6.6 kbp Bam HI and 3.0 kbp Sac I fragments of pT24-C3 and of the 0.67 kbp Hind III to Bam HI fragment of v-*bas* is indicated by arrows.

human DNA insert of pT24-C3 readily hybridized with a 675 bp of DNA fragment containing v-*bas*, the *onc* gene of the BALB-MSV. These v-*bas* related sequences were mapped within the internal 3.0 Sac I fragment, a region of the T24 oncogene required for malignant transformation. No such relationship was observed between the T24 oncogene and any of the other retroviral *onc* genes utilized (data not shown). These results established that the T24 human bladder carcinoma oncogene is highly related to the transforming gene of BALB-MSV, an acute transforming retrovirus.

DISCUSSION

The development of DNA-mediated gene transfer techniques has made it possible to identify transforming genes in a variety of human neoplasias [3–9]. These results have suggested that human cancers may be the result of the activation of dominant genetic elements. However, only a small percentage of tumors tested were shown to possess transmissible oncogenes [3,4,7,9]. Whether this is a consequence of the limited sensitivity of present day transfection assays or is an inherent property of human tumors remains to be determined. In either case, DNA isolated from at

least one sample from representatives of the major forms of human cancers has been shown to induce morphologic transformation upon transfection into NIH/3T3 cells [3–9].

Southern blot analysis of NIH/3T3 cells transformed by human tumor DNA revealed that a limited number of human DNA sequences cosegregated with the transformed phenotype [6,7,9]. Characterization of these sequences has allowed us to determine that the transforming genes present in T24 bladder carcinoma cells, HT-1080 fibrosarcoma cells, and a naturally occurring colon carcinoma have an independent genetic origin. Similar results have been reported by Murray et al [6] when they studied the oncogenes present in three different human tumor cell lines established from a bladder carcinoma (EJ), a colon carcinoma (SW 480), and a promyelocytic leukemia (HL-60). However, it appears that different types of human tumors may also possess the same or highly related oncogenes. Perucho et al [7] have reported that transformants derived from certain lung and colon carcinoma cell lines exhibited the same pattern of human DNA sequences. Similarly, transforming genes present in cell lines established from carcinomas of the lung and gall bladder, as well as in two solid tumors diagnosed as a pancreatic carcinoma and a rhabdomyosarcoma, are also related [32]. Thus it is possible that human tumors may possess a limited number of different transforming genes.

Among human oncogenes so far identified, those present in bladder carcinoma cell lines exhibited the least genetic complexity [6,7,9]. This has greatly facilitated their molecular cloning in a biologically active form [9,11]. In the present studies, the cloned T24 bladder carcinoma oncogene exhibited a specific transforming activity of more than 10^4 ffu/pmol when assayed on NIH/3T3 cells. Sequences required for the establishment and maintenance of the transformed phenotype were located within a human DNA fragment not larger than 4.6 kbp. The molecular basis for the transforming activity of the T24 bladder carcinoma oncogene remains unknown. Comparative restriction enzyme analysis of this oncogene with a molecular clone of its normal allele isolated from a library of human fetal liver DNA failed to reveal any detectable differences. These results strongly imply that the acquisition of transforming activity by the T24 oncogene must be the result of subtle genetic alterations.

Comparison of the T24 bladder carcinoma oncogene with other known transforming genes, such as those detected in other types of human tumors, and the cell-derived *onc* genes present in acute transforming retroviruses indicated that the T24 oncogene was highly related to v-*bas*, the *onc* gene of BALB-MSV [28]. In collaborative studies with S. Tronick and S.A. Aaronson we have demonstrated that the T24 oncogene is an activated form of c-*bas*, the normal human homologue of the transforming genes of BALB and Harvey strains of MSV [12a,b,c]. Moreover, we have determined that the transcriptional and translational products of the T24 oncogene are highly related to those of BALB- and Harvey-MSV. These findings should make it possible to apply the large body of knowledge concerning these retroviruses to determine the mechanism by which the T24 bladder carcinoma oncogene induces malignant transformation.

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