

# Isolation of a Transforming Sequence from a Human Bladder Carcinoma Cell Line

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## Summary

**We have isolated the component of human bladder carcinoma cell DNA that is able to transform mouse fibroblasts. The oncogenic sequence was isolated initially from a lambda phage genomic library made from DNA of a transfected mouse cell carrying the human oncogene. A subcloned insert of 6.6 kb that carried transforming activity was amplified in the plasmid vector pBR322. The subcloned oncogene has been used as a sequence probe in Southern blot analyses. The oncogene appears to derive from sequences present in normal cellular DNA. Structural analysis has failed so far to reveal differences between the oncogene and its normal cellular homolog. The oncogene is unrelated to transforming sequences detected in a variety of other types of human tumor cell lines derived from colonic and lung carcinoma and from neuroblastoma. In contrast, the EJ bladder oncogene appears closely related to one that is active in the human T24 bladder carcinoma cell line. The oncogene appears to have undergone little, if any, amplification in several bladder carcinoma cell lines.**

## Introduction

The role of DNA in oncogenesis has been indicated by many studies that have demonstrated that carcinogens are generally DNA-damaging agents (McCann et al., 1975; Bouck and diMayorca, 1976; Bridges, 1976; Barrett et al., 1978). More recent studies have been designed to detect DNA sequences in transformed cells whose alteration is critical for creation of the tumor phenotype. These experiments have shown that in many cases, the phenotype of transformation can be passed from cell to cell by transfer of naked tumor-cell DNA (Shih et al., 1979, 1981; Cooper et al., 1980; Krontiris and Cooper, 1981; Perucho et al., 1981). Since normal cellular DNAs, studied in parallel, seemed to lack such competence, it was concluded that the actively oncogenic sequences of tumor cell DNA arose during the process of carcinogenesis, and that these oncogenic sequences are responsible for encoding part of the phenotype of transformation in the cells from which these DNAs were isolated.

These studies utilized DNAs from a variety of cell lines that had either been transformed by chemical agents or derived by in vitro passage of explants from

spontaneously occurring tumors. The tumor types included fibrosarcomas, neuroblastomas and carcinomas from organs including the gut, urinary bladder, lung and breast (Shih et al., 1979, 1981; Cooper et al., 1980; Krontiris and Cooper, 1981; Lane et al., 1981; Murray et al., 1981). With the exception of certain mouse mammary carcinomas, no oncogenic viruses were known to be involved in the induction of the parental tumors.

Our experiments have focused on oncogenic sequences found to be present in the DNA of the EJ human bladder carcinoma cell line. Application of DNA of this cell line induces transformation of mouse fibroblasts (Krontiris and Cooper, 1981; Shih et al., 1981). Progress in understanding the nature of the EJ transforming sequences depended on their isolation by molecular cloning. We report the isolation of a molecular clone of actively oncogenic sequences from the DNA of this cell line. Use of this clone in sequence hybridization has provided insights into the origin of this sequence and its function in oncogenesis.

## Results

### Preliminary Characterization of the Bladder Transforming Sequences

Initial experiments were designed to ascertain whether the transforming trait induced by the EJ bladder carcinoma DNA was encoded by sequences carried within a single, contiguous segment of DNA. To this end, we measured the yield of foci as a function of the amount of EJ tumor cell DNA used in a transfection assay. In this and subsequent experiments, DNA was applied to monolayer cultures of NIH/3T3 mouse fibroblasts, and any resulting foci were scored 14-21 days later. As seen in Table 1, the dose response of foci was roughly linear, which strongly suggested that the foci were not being induced by two or more unlinked, cooperating DNA sequences whose concomitant transfer was required for transformation.

Having concluded that the transformation trait was likely encoded by a single genetic segment, we attempted to define the limits of a DNA fragment that would encompass the transforming sequences fully. Various transforming DNAs were exposed to one or another restriction endonuclease prior to transfection, and were tested to see whether the biological activity of the DNA survived enzyme treatment. Survival of biological activity would indicate that the functionally essential sequences were carried entirely within one of the many DNA fragments generated after enzyme treatment.

After screening with a series of restriction enzymes, we found that Kpn I and Pvu II inactivated the transforming potency of DNAs prepared from EJ tumor cells. Eco RI was found to spare the biological activity. Identical results were obtained when cultures were grown up from transfected foci and their DNAs were

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Table 1. Dependence of Focus-Inducing Activity on DNA Concentration

Dose of Transforming DNA ( $\mu$ g)	Dose of Added Carrier DNA ( $\mu$ g)	No. of Foci Induced by	
		EJ DNA <sup>a</sup>	EJ-6-2 DNA <sup>b</sup>
5	70	2	6
10	65	3	13
19	56	4	25
38	38	10	60
75		20	73

Seventy-five micrograms of DNA was applied to  $1.5 \times 10^6$  NIH/3T3 cells in each case. The transfection protocol is described in the Experimental Procedures.

<sup>a</sup> EJ DNA was prepared directly from a culture of the tumor cell line.

<sup>b</sup> EJ-6-2 DNA was prepared from a culture grown up from a secondary focus.

tested by enzyme treatment prior to a second round of transfection (data not shown). These data allowed us to conclude that the transforming sequences were contained entirely within one Eco RI-generated fragment of EJ bladder carcinoma DNA.

#### Detection and Identification of the Transforming DNA Segment by Sequence Hybridization

As shown in previous reports, human DNA segments can be detected by sequence hybridization after they have been introduced into a rodent cell (Gusella et al., 1980; Shih et al., 1981). This detection is made possible by the fact that the human genome contains 300,000 or more copies of a sequence that is interspersed widely throughout cellular DNA (Schmid and Deininger, 1975; Rubin et al., 1980; Rinehart et al., 1981). Consequently, virtually every human gene is closely linked to one or more copies of this sequence (Tashima et al., 1981). These human sequence blocks, sometimes termed Alu sequences, have diverged from their rodent homologs sufficiently to allow their specific detection in a Southern blot.

In previous work, we used a molecular clone of a human Alu sequence as probe (Jelinek et al., 1980) in Southern blots to detect the presence in mouse cells of introduced human oncogenes derived from bladder and colonic carcinoma and from promyelocytic leukemia (Murray et al., 1981). Each of these genes, when resolved from the mouse sequence background, was seen to be affiliated with its own characteristic array of human Alu blocks. In the present work, we use as probe that fraction of human DNA that self-anneals at a rate of  $Cot = 1$  or less. This probe was used with the hope of detecting repeated human sequences besides those represented by the cloned Alu fragment. For the purposes of the present experiments, these two types of probe appear to function equivalently.

The  $Cot = 1$  sequence probe was used initially to analyze DNAs present in secondary foci induced by two serial passages of the EJ transforming sequence.

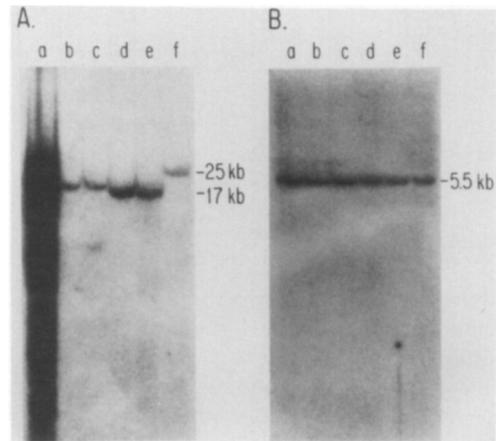


Figure 1. Detection of Human-Alu-Containing DNA Fragments in Mouse Transfectants

(A) Detection of Eco RI-generated segments in a series of independently derived secondary foci. Primary and secondary foci were induced by serial passage of the EJ transforming sequence through NIH/3T3 cells. Cultures were grown out from selected foci, and 11  $\mu$ g of DNA of each culture was digested by endonuclease Eco RI, resolved by agarose gel electrophoresis and transferred to a nitrocellulose filter. A human repetitive-sequence DNA ( $Cot = 1$ ) was used as probe in sequence hybridization. (Lane a) DNA of primary focus EJ-3. (Lanes b-f) DNAs of five different, independently induced secondary foci, termed EJ-3-1, EJ-3-2, EJ-6-1, EJ-6-2 and EJ-6-3. (B) Detection of human repetitive sequences in tertiary foci derived by transfection of Eco RI-cleaved, secondary focus DNA. DNA of secondary focus EJ-6-2 was treated with endonuclease Eco RI prior to a round of transfection used to induce a series of tertiary foci. These tertiary foci were picked and grown into mass cultures. The DNAs of these cultures were cleaved by endonuclease Pvu II prior to Southern blot analysis as above. (Lane a) DNA of secondary focus EJ-6-2. (Lanes b-f) DNAs of tertiary foci, termed EJ-6-2-(R)5, EJ-6-2-(R)4, EJ-6-2-(R)3, EJ-6-2-(R)2 and EJ-6-2-(R)1.

Two cycles of transfection through mouse cells ensured that virtually the only human sequences present in a secondary transformant were those that were closely linked to the gene encoding the scored transforming trait. Figure 1A, lane a, shows that a primary transfectant, which picked up the EJ transforming sequence after transfection of EJ tumor cell DNA, concomitantly acquired a large number of other human DNA sequences. But serial passage of the transforming sequence in a second round of transfection led to secondary foci, whose DNAs carried only a single large Eco RI fragment that is reactive with the human-specific probe (Figure 1A, lanes b-f; Murray et al., 1981).

This large Eco RI fragment, of about 25 kb, represents a characteristic signature of the presence of the introduced bladder carcinoma gene. All cells that acquired the gene carried this fragment or one of very similar size (Figure 1A; Murray et al., 1981). This cosegregation of the large Eco RI DNA fragment and the transforming sequence indicated that the two elements were closely linked, but did not prove that the functional sequence was contained within the Eco RI

fragment. An alternative theory would place the transforming sequence on an adjacent DNA fragment that lacks Alu sequences and that therefore escapes detection by blot hybridization.

We wished to determine whether the transforming sequence was carried within the large Alu-containing Eco RI segment. To do this, we treated the DNA of secondary transfectant EJ-6-2 (Figure 1A, lane e) with Eco RI prior to a third round of transfection. As indicated above, this enzyme does not inactivate the biological activity of the DNA. We asked if this enzyme were able to break the linkage between the transforming and the Alu sequences. DNAs of resulting tertiary foci were analyzed for the presence of Alu sequences. In this particular experiment, the enzyme Pvu II was used to cleave DNAs prior to Southern analysis because of the exonucleolytic destruction of many Eco RI sites that followed the previous Eco RI treatment and transfection. Figure 1B, lanes b-f, indicate that all such tertiary foci still contained Alu sequences. Since the linkage between the oncogene and these Alu sequences could not be broken by Eco RI, we concluded that these Alu blocks and the transforming sequences resided within the same Eco RI fragment. This fragment was, by necessity, the single large, Alu-positive fragment seen in Figure 1A, lanes b-f.

#### Molecular Cloning of the Transforming Segment

Our strategy for molecular cloning of the large Eco RI fragment and associated oncogene began with insertion of Eco RI fragments of a secondary transfectant into lambda phage vectors. We planned to screen for those components of the resulting library that exhibited sequences reactive with the Cot = 1 probe. Unfortunately, the Eco RI fragment containing the oncogene was seen to have a size (about 25 kb; Figure 1A, lanes b, c and f) that exceeded the carrying capacity of available lambda phage vectors. However, in occasional secondary transfectants we observed smaller versions of this large Alu-containing Eco RI fragment (Figure 1A, lanes d and e). These smaller fragments appeared to have resulted from mechanical breakage of the DNAs prior to transfection. As a consequence, truncated DNA fragments carrying the transforming and Alu sequences appeared to have become fused after transfection with new arrays of Eco RI sites present in cotransfected DNA (Perucho et al., 1980).

An example of this was DNA of the secondary focus line EJ-6-2, which was found to carry an Alu-positive Eco RI segment of only 15-20 kb (Figure 1, lane e), a size that is within the length limits of inserts allowed by the lambda phage vector Charon 4A. This DNA fragment was the only human segment that we could detect in this cell line. Moreover, the DNA of this cell line was shown to be potent upon transfection, yielding as much as 1 focus per 2 micrograms of transfected DNA. We prepared DNA of this cell line, cleaved

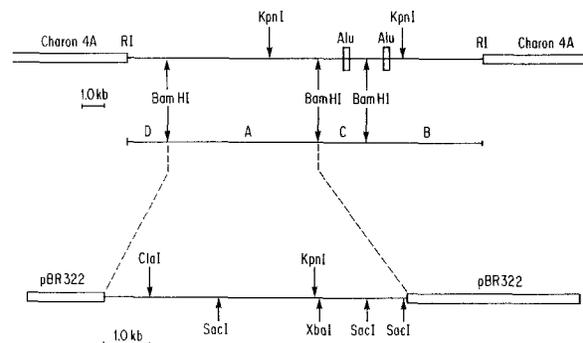


Figure 2. Endonuclease Cleavage Map of Transforming DNA Segment Isolated from EJ Human Bladder Carcinoma DNA

(Top) A map of the 15.5 kb insert present in the Charon 4A vector. A, B, C and D: the four Bam HI fragments of the insert, subcloned into pBR322. (Bottom) A 6.6 kb biologically active subclone (fragment A) carried by a pBR322 plasmid vector. The positions of the two Alu blocks were not precisely mapped within the Bam HI-Bam HI and Bam HI-Kpn I fragments in which they are found. The 6.6 kb insert is not cleaved by Hind III or Sal I endonucleases.

the DNA with Eco RI and enriched this DNA fragment by preparative sucrose gradient centrifugation. Fractions that gave strong signals in Southern blots with the Cot = 1 probe were pooled together (data not shown). This enriched-fragment mixture was linked to the genomic ends of the Charon 4A bacteriophage vector and packaged in vitro (Blattner et al., 1977). The resulting chimeric phage stock contained an estimated  $5 \times 10^5$  plaque-forming units. These were all plated, and the resulting plaques were screened for Alu-containing bacteriophage by the procedure of Benton and Davis (1977). Two phage stocks were isolated that retained reactivity with the human probe through three cycles of plaquing. These two phage stocks had identical inserted DNAs, and only one will be described further here. Figure 2 illustrates the cleavage site map of the inserted DNA, along with the location of the human repetitive-sequence blocks.

DNA of this phage stock was able to induce foci at a frequency of approximately  $10^4$  foci per microgram of transfected DNA. DNAs of several of these foci were analyzed. These DNAs carried copies of the transfected chimeric phage DNA, thus ensuring that the observed foci did not arise from spontaneous transformation of cells in the transfected monolayer (Figure 3, lanes b-d). Moreover, these acquired cloned DNAs carried arrays of restriction sites indistinguishable from those seen in the homologous DNA fragment present in EJ-6-2, the cell line from which the genomic library was constructed (Figure 3, lane a). This confirmed the fidelity of the molecular cloning process.

#### Subcloning of the Inserted Sequences of the Bacteriophage

The 16 kb insert present within the bacteriophage was suspected to carry far more sequences than those



Figure 3. Comparison of the Donor DNA Fragment Carrying the Transforming Sequence with Homologous Fragments Carried by Cells Transformed by Cloned DNA

Alu-containing, Eco RI-cleaved DNA fragments were detected as described in Figure 1. (Lane a) DNA of secondary transfectant EJ-6-2; (lanes b-d) DNAs from three primary foci transformed by cloned chimeric phage DNA; (lane e) DNA of untransfected NIH/3T3 cells.

necessary to induce transformation. We attempted subcloning of this segment to localize the essential transforming region and to discard sequences that were irrelevant to further analysis of the gene. Among the several restriction enzymes previously used to treat uncloned cellular DNAs, Bam HI was seen to spare the transforming activity. We found as well that Bam HI did not reduce the biological activity of the cloned phage DNA (data not shown). This indicated the presence of the functionally essential region of the gene entirely within one of the four Bam HI fragments of the insert (Figure 2).

These four fragments were subcloned by excision from the bacteriophage with Bam HI and ligation to Bam HI-treated pBR322 plasmid DNA. Since the transforming activity was inactivated by Kpn I endonuclease, we suspected that either Bam HI fragments A or B carried the transforming sequence (Figure 2); furthermore, the gene could occasionally become unlinked from Alu sequences by mechanical shearing (data not shown), which suggested that it was located on the more distantly linked Bam HI fragment A. These predictions were vindicated by transfection assay of plasmids carrying different Bam HI fragments. Only the chimeric plasmid DNA of 6.6 kb Bam HI fragment was found to have biological activity, which was measured at approximately  $10^3$  focus-inducing units per microgram in one transfection and  $10^4$  focus-inducing units per microgram in a second experiment. The chimeric plasmid DNA was of great utility, since it

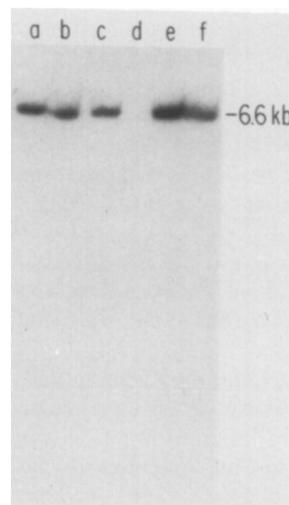


Figure 4. Detection of Oncogenic DNAs in a Series of Independent Primary and Secondary Foci Transfected with EJ DNA

DNAs were prepared from foci, cleaved with endonuclease Bam HI and analyzed by the Southern procedure with the plasmid-borne 6.6 kb subclone as probe. DNAs analyzed were from the parental EJ bladder carcinoma line (lane a); secondary focus EJ-1-1, a derivative of primary focus EJ-1 (lane b); secondary focus EJ-3-1, a derivative of primary focus EJ-3 (lane c); NIH/3T3 cells (lane d); secondary focus EJ-6-2, a derivative of primary focus EJ-6 (lane e); and primary focus EJ-7 (lane f).

carried no repeated sequences and could be used as a specific probe for the transforming gene.

#### Presence of the Active Bladder Oncogene in EJ DNA before Transfection

We wished to rule out the possibility that the cloned sequence represents an oncogene that was inadvertently activated either during the derivation of the EJ-6-2 transfectant or during the subsequent cloning (Cooper et al., 1980). To do this, we analyzed the DNAs of a series of independently induced transfectants for the presence of this gene. As seen in Figure 4, lanes a, b, c, e and f, these DNAs all carried copies of the acquired EJ bladder carcinoma oncogene. In contrast, strongly reactive sequences were absent in NIH/3T3 cells prior to transfection (Figure 4, lane d). Since these transfectants derived originally from four independent primary transfection events, we conclude that the active oncogene existed in the EJ bladder carcinoma DNA prior to the experimental manipulations that led to the derivation of these transfected cell lines.

#### Use of Cloned Sequence to Examine Normal Human DNA

The plasmid subclone was used to examine normal human DNA for the presence of homologous sequences. As seen in Figure 5A, lane a, normal human diploid fibroblast DNA contains a DNA fragment that is strongly reactive with the bladder carcinoma onco-

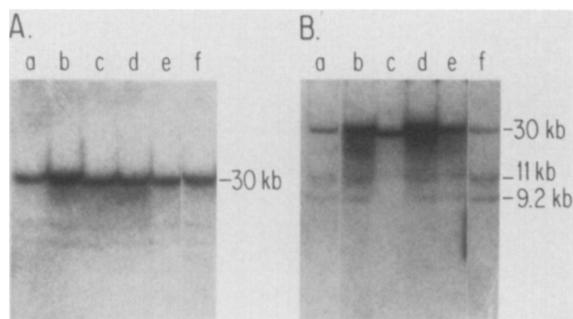


Figure 5. Use of the 6.6 kb Oncogene Subclone as Probe to Compare Normal and Tumor DNAs

DNAs were cleaved with endonuclease Eco RI prior to Southern analysis, with the 6.6 kb plasmid-amplified subclone as probe. Hybridization was performed at normal stringency conditions in the presence of 50% formamide (A), or at lowered stringency (45% formamide; B). (A) DNAs analyzed were from cultures of FS4 normal human diploid fibroblasts (lane a); EJ human bladder carcinoma (lanes b and c); T24 human bladder carcinoma (lane d); HT1376 human bladder carcinoma (lane e); and Lx1 human small-cell lung carcinoma. (B) DNAs analyzed were from cultures of FS4 normal human diploid fibroblasts (lane a); EJ human bladder carcinoma (lane b); EJ-(RI, religated)<sub>2</sub> primary focus prepared by Eco RI cleavage of EJ tumor cell DNA followed by religation before transfection (lane c); T24 human bladder carcinoma cell line (lane d); J82 human bladder carcinoma cell line (lane e); and HT1376 human bladder carcinoma cell line (lane f).

gene probe. This indicates that the active oncogene could derive from a very closely related sequence that is present in the normal human genome. The result argues against the possibility that the oncogene is of exogenous origin, such as a sequence introduced into the cell by viral infection. This conclusion is reinforced by further structural analysis described below.

This survey of the normal human genome revealed that the crossreacting Eco RI fragment was of a size that was similar to that which had been acquired from the tumor cell by transfection (Figure 5B, lane c). Moreover, a similarly sized, large Eco RI fragment was detected in a variety of human tumor DNAs, including the EJ, T24, J82 and HT1376 bladder carcinoma cell lines (Figure 5B, lanes b, d, e and f).

Of additional interest is the presence of homologous fragments of 9.2 kb and 11 kb that are present in normal human DNA (Figure 5B, lanes a, b, d-f). These presumably represent related sequences present in the human genome. These related sequences are not detected after more stringent hybridization and washing conditions, and are therefore not visualized in subsequent figures. As expected, these two other fragments are not present in the DNA of a transfection-derived focus (Figure 5B, lane c).

We have undertaken extensive comparisons between normal and EJ tumor DNAs using the cloned oncogene sequence as probe. These surveys have so far revealed no difference between the following restriction sites that surround or are embedded within the gene: Eco RI, Bam HI, Hind III, Bgl II, Sac I, Kpn

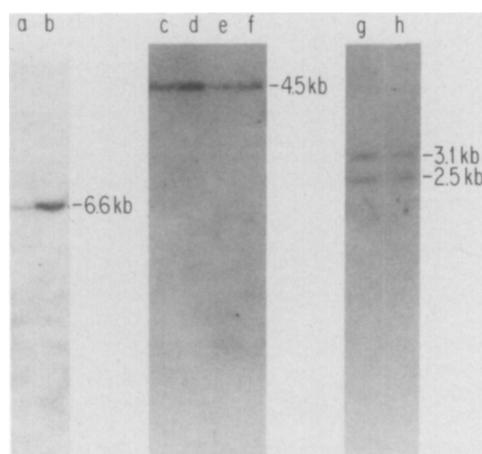


Figure 6. Comparison of EJ Bladder Carcinoma and FS4 Normal Human Diploid Fibroblast DNA with the 6.6 kb Oncogene Subclone as Probe

DNAs of EJ and FS4 origin were treated with the indicated endonucleases prior to Southern analysis with the plasmid-borne 6.6 kb subclone as probe. (Lanes a and b) Bam HI-digested FS4 and EJ DNAs; (lanes c and d) Xba I-Bam HI-double digested FS4 and EJ DNAs; (lanes e and f) Kpn I-Bam HI-double digested FS4 and EJ DNAs; (lanes g and h) Sac I-Bam HI-double digested FS4 and EJ DNAs. The 1.0 kb Sac I fragment is too faint to be seen here.

I, Xba I and Pvu II. Data from some of these comparative mappings are presented in Figure 6. Although attempts to detect differences are continuing, we may already conclude that the differences between the normal sequence and its transforming counterpart are subtle and elude detection by these rough structural analyses.

#### Possible Amplification of the Oncogenic Sequences in Tumor Cells

Some of the Southern blot studies suggested a possible increase in copy number of the oncogenic sequences in certain tumor cell DNAs. The increased intensity of bands detected in these analyses could have been due to a variety of extraneous factors that surround manipulations used in the Southern procedure. To control for these factors, we constructed an internal control by measuring the sequences reactive with a human insulin probe in these various DNA samples. The Eco RI fragment carrying the insulin sequences has a size (14 kb; Bell et al., 1980) that causes it to migrate closely with the oncogene-containing restriction fragment.

Probes reactive with the oncogene and insulin sequences were mixed and incubated with a filter carrying DNAs from normal diploid fibroblasts, from foreskin epidermal cells and from bladder carcinoma cells of EJ, T24 and HT1376 origin (Figure 7, lanes a-h). Although the intensities of the observed bands varied between lanes, it was clear that within each lane the intensities of the two bands were comparable. Stated differently, the ratio of oncogene to insulin sequence

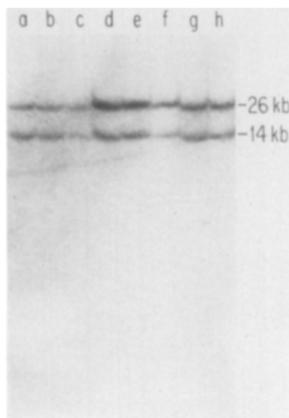


Figure 7. Analysis of Possible Amplification of Oncogene Sequences in a Series of Normal and Bladder Carcinoma DNAs

DNAs from indicated cultures were treated with endonuclease *Eco* RI before Southern analysis, with a mixture of a cloned human insulin gene and the 6.6 kb oncogene subclone as probe. (Lanes a and b) Two separate preparations of FS4 human diploid fibroblast DNA; (lane c) early-passage human foreskin epidermal cells; (lanes d, e and f) three different preparations of EJ human bladder carcinoma DNA; (lane g) T24 human bladder carcinoma DNA; (lane h) HT1376 human bladder carcinoma DNA.

did not vary significantly from one DNA to another. A similar result was obtained when other endonucleases were used to cleave DNA of the EJ bladder carcinoma cell line and of FS4 normal diploid fibroblasts (data not shown). We conclude that the maintenance of oncogenic phenotype in the EJ bladder carcinoma cell line does not depend on significant amplification of the oncogenic sequences studied here.

#### Relation of the EJ Transforming Gene to Other Tumor Transforming Genes

In previous experiments, we had found that the array of *Alu* sequences surrounding the bladder carcinoma gene was different from arrays surrounding a colonic adenocarcinoma transforming sequence and a promyelocytic leukemia sequence (Murray et al., 1981). On the basis of these data, we concluded that the three transforming sequences were likely to be structurally distinct.

A more direct assay of sequence relatedness is now made possible by use of the cloned segment. The segment can be used as sequence probe to analyze the DNAs of transfected mouse cells that have acquired various tumor transforming genes. In this manner, one can ascertain whether these transfectants have acquired DNA fragments reactive with the bladder carcinoma sequence probe following the transfections that led to their transformation.

As seen in Figure 8A, lane d, the cloned probe detected a newly acquired 6.6 kb segment in cells transfected with EJ bladder carcinoma DNA. This positive control demonstrated the reactivity of the cloned probe with the transfected gene from which it was derived. In contrast, the probe was not reactive

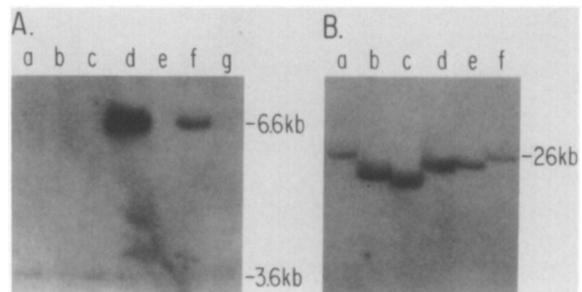


Figure 8. Relationship of the EJ Bladder Oncogene to Other Tumor Transforming Genes

Primary and secondary foci were induced by transfection of DNAs from a variety of human tumor cell lines. Cultures were expanded from these foci, and their DNAs were analyzed by the Southern procedure with the 6.6 kb oncogene subclone as probe.

(A) DNAs were treated with endonuclease *Bam* HI prior to Southern analysis. Sequence hybridization was performed at low stringency (35% formamide). DNAs analyzed were prepared from SW-2-2, a secondary transfectant induced by DNA of SW480, a human colonic adenocarcinoma cell line (lane a); Lx1-2, a primary transfectant induced by DNA of Lx1, a human small-cell lung carcinoma cell line (lane b); A549-1, a primary transfectant induced by DNA of A549 human lung adenocarcinoma cell line (lane c); EJ-6-2, a secondary transfectant induced by DNA of EJ human bladder carcinoma cell line (lane d); SH-1-1, a secondary transfectant induced by DNA of SK-N-SH, a human neuroblastoma cell line (lane e); T24-8-5, a secondary transfectant induced by DNA of T24 human bladder carcinoma cell line (lane f); and HL60-1-6, a secondary transfectant induced by DNA of HL60 human promyelocytic leukemia cell line (lane g).

(B) DNAs were treated with endonuclease *Eco* RI before Southern analysis. Sequence hybridization was performed at high stringency conditions (50% formamide). DNAs were from independent primary and secondary foci induced by passage of the transforming sequence of the T24 human bladder carcinoma cell line. These foci were termed T24-8 (lane a), T24-7 (lane b), T24-6 (lane c), T24-5 (lane d), T24-8-2 (lane e) and T24-8-5 (lane f).

with DNA of cells that had acquired oncogenic sequences from the following human tumor cell lines: colonic adenocarcinoma, small-cell lung carcinoma, lung adenocarcinoma, neuroblastoma and promyelocytic leukemia (Figure 8A, lanes a, b, c, e and g), even at low stringency conditions.

One exception to this group of negative correlations was the detection of a novel DNA fragment in mouse cells transfected with the T24 human bladder carcinoma sequences (Figure 8A, lane f). We and others have found DNA of this cell line to be active in focus induction (Perucho et al., 1981; C. Shih and R. Weinberg, unpublished results). The present results (Figure 8A, lanes d and f) indicate that the oncogenic sequence of this tumor cell line is either structurally related or extremely closely linked to the oncogene of the EJ bladder carcinoma cell line.

Further analysis of the DNAs of primary and secondary transfectants induced by passage of the T24 bladder carcinoma sequence (Figure 8B, lanes a-f) indicated that they all carried a DNA fragment reactive with the EJ bladder carcinoma probe, and that the sizes of these fragments were reminiscent of those seen in EJ-derived transfectants (Figure 1A, lanes b-

f). In both cases, a single high molecular weight Eco RI segment was seen, a pattern that is distinct from that seen with a variety of other types of human oncogenes (Murray et al., 1981; Perucho et al., 1981). Since the two oncogenic sequences appeared to be resistant or sensitive to inactivation by the same restriction enzymes (Perucho et al., 1981; C. Shih and R. Weinberg, unpublished results), we would suggest that the two oncogenes derive from activation of the same normal cellular sequence. This result bears on evidence gathered previously (Lane et al., 1981; Shilo and Weinberg, 1981; Padhy et al., 1982; C. Shih and R. Weinberg, unpublished results), we would suggest that the two oncogenes derive from activation of the same normal cellular sequence. This result bears on

## Discussion

We have provided direct proof that the transforming sequences of a human bladder carcinoma cell line are contained in a discrete, contiguous segment of DNA. This was suggested only indirectly by previous experiments (Shih et al., 1979, 1981; Cooper et al., 1980; Krontiris and Cooper, 1981; Shilo and Weinberg, 1981). This result excludes the possibility that a group of unlinked genes scattered about the tumor cell genome are required to encode the phenotype that we detect by transfection. Moreover, the behavior of this sequence allows us to refer to it tentatively as a gene that can eventually be defined further by genetic criteria.

The transforming gene contains sequences that are present as well in normal cellular DNA. This finding provides strong support for a somatic-mutation theory of cancer, following which preexisting cellular genes are said to be oncogenically activated as a consequence of insult by carcinogen (Bauer, 1928; Burdette, 1955; Burnet, 1978). Conversely, it becomes less likely that this gene has arisen from foreign genetic sequences introduced into the cell, as might be expected after transformation by a tumorigenic virus.

As previously reported, EJ tumor cell DNA yields foci upon transfection, while normal human DNA does not (Krontiris and Cooper, 1981; Shih et al., 1981). We have isolated the component of the tumor cell DNA that is responsible for this focus-inducing activity, and we would anticipate that structural differences will be found that distinguish the competent sequence from its allelic counterpart in normal cellular DNA. Such structural differences have not yet been found, but this initial comparison has relied only on endonuclease cleavage site mapping. This technique would not detect many minor structural alterations of DNA. We will pursue detection of two types of structural differences: those deriving from nucleotide sequence alteration, and those deriving from DNA modification by methylases.

Several conclusions have emerged from use of the

cloned oncogenic DNA as probe in the Southern blot analysis. The first is that the bladder carcinoma probe does not react with the DNAs of NIH/3T3 cells that have acquired, via transfection, a variety of other types of tumor transforming genes, including those from human colonic carcinoma, human neuroblastoma, human promyelocytic leukemia, human lung adenocarcinoma and human small-cell lung carcinoma. This shows directly the distinct structure of this EJ gene, a conclusion that had been made earlier on the basis of less direct structural analysis (Murray et al., 1981).

Second, it appears that DNA of the isolated EJ bladder carcinoma gene crossreacts with human DNA present in mouse cells that have taken up the T24 human bladder carcinoma gene. It is suggested that these two genes are structurally related. A less likely, although logical, alternative is that the two oncogenes are unrelated but lie adjacently on the genome, such that the T24 transfectant has adventitiously acquired a silent EJ homolog along with a selected, actively transforming, adjacent sequence. Resolution of this issue will be forthcoming, since the T24 oncogene has recently been isolated (M. Goldfarb, K. Shimizu, M. Perucho and M. Wigler, personal communication).

Third, the oncogenic sequences do not appear to be significantly amplified in several bladder carcinoma cell lines. This depends on the use of a cloned insulin probe whose genomic homolog serves as a standard for the calibration of the amounts of oncogene-containing fragment. The amplification enjoyed by the oncogene in these tumor cells would appear to be minimal, certainly less than threefold. It is thus likely that the carcinogenesis that led to the EJ bladder tumor did not depend on the large-scale amplification of the oncogene and surrounding sequences.

Many of the present conclusions are encumbered by an important reservation: that the gene described here has been isolated from a tumor cell line and not directly from a tumor biopsy. It remains possible that this gene became activated not during the initial tumorigenesis that led to the EJ tumor *in vivo*, but rather during the subsequent *in vitro* adaptation of the EJ cell line. The oncogene clone may be used to isolate homologous sequences directly from the DNAs of tumor biopsies to rule out this possibility. Such experiments would be able to demonstrate the presence of active homologous oncogenes in the bladder carcinoma cells, and would support the role of these genes as important agents in the induction of the malignancy phenotype *in vivo*.

## Experimental Procedures

### Tumor Cell Lines

The EJ human bladder carcinoma cell line (Marshall et al., 1977) was a gift from I. Summerhayes and L. B. Chen. The T24 human bladder carcinoma cell line (Marshall et al., 1977), the human neuroblastoma cell line SK-N-SH and the SW-480 human colonic carcinoma cell line

(Leibovitz et al., 1976) were gifts of J. Fogh. The human lung carcinoma cell line A549 was obtained from G. Todaro. R. C. Gallo provided the human promyelocytic leukemia cell line HL60 (Collins et al., 1977). The HT1376 human bladder carcinoma cell line was obtained from D. Senger. The Mason Research Institute provided the human small-cell lung carcinoma cell line Lx1.

#### Preparation of Genomic DNA

Confluent cultures of cells were washed twice with phosphate buffered saline. Lysis buffer (0.5% SDS, 0.1 M NaCl, 40 mM Tris-Cl, 20 mM EDTA [pH 7.0]) containing 0.2 mg/ml proteinase K (Boehringer Mannheim) was then applied directly onto cells. Cells were lysed, and the viscous lysate was incubated at 37°C with gentle shaking for at least 2 hr. This solution was extracted twice with an equal volume of redistilled phenol, followed by two extractions with chloroform and isoamyl alcohol (24:1). The DNA solution was then adjusted to a final concentration of 0.2 M NaCl before ethanol precipitation. Clumps of DNA precipitates were then removed with a Pasteur pipette and transferred to 10 mM Tris-Cl, 1 mM EDTA (pH 8.0).

#### DNA Transfection Assays

DNA transfection of NIH/3T3 cells was carried out by the calcium phosphate precipitation technique of Graham and van der Eb (1973), as modified by Andersson et al. (1979). High molecular weight DNAs were sheared once through a 20 gauge needle. Aliquots (75 µg) of sheared DNA were ethanol-precipitated and resuspended in 2.5 ml of transfection buffer (0.7 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 21 mM HEPES, 0.145 M NaCl [pH 7.0]). One hundred twenty-five microliters of 2.5 M CaCl<sub>2</sub> was added and mixed immediately by vortexing. As soon as a bluish fine precipitate was apparent, 1.25 ml of the solution was applied onto 7 × 10<sup>5</sup> NIH/3T3 cells in the presence of 10 ml Dulbecco's modified Eagle's medium containing 10% calf serum. Better transfection efficiencies were obtained if recipient NIH/3T3 cells were plated 12–20 hr before use.

We removed the calcium phosphate-DNA coprecipitate from the transfected culture dish by changing the medium 4 hr after initial application. Subsequent splitting of NIH/3T3 cells into subcultures was found not to be necessary. Medium was changed twice each week. Fourteen to twenty-one days later, morphologically transformed foci were scored as described by Shih et al. (1979). A twofold fluctuation of transfection efficiency was routinely observed. When cloned DNA was to be transfected, 50–100 ng of the DNA was mixed with 75 µg carrier DNA (usually of NIH/3T3 or FS4 human diploid fibroblast origin) before ethanol precipitation.

#### Preparation of Highly Repetitive Human Sequence Probes

Total cellular DNA was prepared from a human bladder carcinoma cell line, A1663. The DNA concentration was adjusted to 125 µg/ml in 0.01 M PIPES buffer (pH 6.8) and sonicated to ~400 bp fragments, which were denatured by boiling at 100°C for 10 min. The NaCl concentration was adjusted to 0.3 M, and reannealing was performed at 68°C for 40 min. The solution was quenched on ice, and an equal volume of 2× S1 buffer (0.50 M NaCl, 0.06 M sodium acetate, 2 mM ZnSO<sub>4</sub>, 10% glycerol [pH 4.5]) was added, followed by addition of 20 µl S1 enzyme (Boehringer Mannheim; 10<sup>5</sup> U/µl). This solution was incubated at 37°C for 1 hr. A 1 M sodium phosphate buffer (equimolar mixture of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>) was added to a final concentration of 0.12 M sodium phosphate, and the solution was passed through a hydroxyapatite column at 60°C. Double-stranded DNA was then eluted with a 0.5 M sodium phosphate buffer at 60°C. Salts were removed by extensive dialysis against 10 mM Tris-Cl, 1 mM EDTA (pH 8.0). This eluted DNA was termed Cot = 1 DNA.

#### Isolation of 16 kb Eco RI Fragment

EJ-6-2 Eco RI-cleaved DNA (280 µg) was extracted with phenol and chloroform, and then precipitated with ethanol. The total DNA pellet was then resuspended in 3 ml of 10% (w/v) sucrose, 20 mM Tris-HCl (pH 8.0), 10 mM EDTA and 1 M NaCl. An aliquot (70 µg) was then applied to a 15%–40% linear sucrose gradient (1 M NaCl, 20 mM Tris-Cl [pH 8.0], 10 mM EDTA) in a Beckman SW27 centrifuge tube. The gradient was centrifuged at 23,000 rpm for 30 hr at 20°C.

Fractions of 1.5 ml were collected and resolved by electrophoresis through a 1% agarose gel. DNA fragments were then transferred to a nitrocellulose filter via the Southern blot procedure (Southern, 1975). Cot = 1 DNA was used as a hybridization probe. Fractions containing the strongest signal were pooled, concentrated by ethanol precipitation and resuspended in 10 mM Tris-Cl, 1 mM EDTA (pH 7.0).

#### Creation and Screening of Bacteriophage Library

The isolated 16 kb Eco RI fragment was ligated to genomic arms of the Charon 4A vector and packaged *in vitro* according to procedures of Blattner et al. (1977). An estimated 5 × 10<sup>5</sup> plaque-forming units was plated and screened with the Cot = 1 probe for Alu-containing phage according to the procedure of Benton and Davis (1977).

#### Large-Scale Preparation of Phage DNA

A fraction (0.3 ml) of an overnight culture of *Escherichia coli* strain LE392 was mixed well with 0.3 ml adsorption solution (10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>). An aliquot (50–100 µl) of phage stock was then added and incubated for 15 min at 37°C. This phage adsorption mixture was inoculated into 1 liter of NZY medium, and allowed to grow overnight at 37°C. The phage pellet was collected by spinning at 9000 rpm at 4°C for 20 min, and then resuspended in 15 ml lambda buffer (10 mM Tris, 5 mM MgCl<sub>2</sub>, 100 mM NaCl [pH 7.5]). Two sequential cycles of CsCl step gradients (3 M–5 M) were used to purify phage particles, and the resulting bands were then dialyzed against lambda buffer at 4°C overnight. Dialyzed phage preparations were then phenol- and chloroform-extracted and precipitated by ethanol, and the pellet was redissolved in TE buffer for storage.

#### Subcloning into Plasmid pBR322

Bam HI-digested phage clone DNA was incubated with a 2 M excess of calf intestinal phosphatase-treated, Bam HI-cleaved pBR322 in the presence of T4 DNA ligase (New England BioLabs). This DNA was used to transform *Escherichia coli* strain C600 according to the procedure of Cohen et al. (1972). Recombinant clones were selected for ampicillin resistance (50 µg/ml tetracycline). Colonies responding appropriately to drugs were screened with both Cot = 1 and phage lambda DNA probes according to the protocol of Grunstein and Hogness (1975). Subclones of different sizes were picked. Bam HI-cleaved chimeric plasmid DNAs were analyzed on 1% agarose gels, and the identity of each subclone was determined by the size of the inserted sequences.

#### Preparation of Plasmid Subclone DNA

Large-scale preparation of plasmid DNA was performed as follows. Cells were allowed to grow overnight in 1 liter LB medium in the presence of ampicillin (50 µg/ml), and sedimented at 7000 rpm for 10 min at 0°–4°C. Cell pellets were resuspended in 12 ml of 25% sucrose, 50 mM Tris-Cl (pH 8.0) and 0.15 M NaCl. One milliliter of lysozyme (100 mg/ml) was added, and the mixture was incubated for 5 min on ice. One milliliter of 0.5 M EDTA (pH 8.0) was added, and the incubation was continued on ice for 5 min. Fifteen milliliters of a solution containing 1% Triton X-100, 50 mM Tris-Cl (pH 8.0) and 62.5 mM EDTA was added. The solution was mixed vigorously, and incubation was continued for another 15 min on ice. The viscous preparation was centrifuged for 30 min at 23,000 rpm at 0°C. The supernatant containing the plasmid DNA was then purified by banding in cesium chloride-ethidium bromide equilibrium centrifugation according to the method of Timmis et al. (1978). Ethidium bromide was extracted with pure isopropanol five times, and plasmid DNA was dialyzed overnight against 10 mM Tris-Cl (pH 8.0), 1 mM EDTA.

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