Isolation and preliminary characterization of the transforming gene of a human neuroblastoma cell line

(DNA-mediated gene transfer/human oncogene/tRNA suppressor)

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ABSTRACT DNA from the human neuroblastoma cell line SK-N-SH is capable of inducing foci of transformed NIH 3T3 cells after DNA-mediated gene transfer. Using genetic selection with the Escherichia coli sup F gene, we have isolated human sequences from mouse cells responsible for the oncogenic transformation. These sequences are present in all human DNAs surveyed and no gross rearrangements of these sequences are found in SK-N-SH cells. Although clearly distinct from two other human transforming genes present in bladder, lung, and colon carcinoma cell lines, all three transforming gene sequences may be related members of the ras gene family.

Several types of experiments have indicated the existence of oncogenes of cellular origin. One set, the oncogenes present in the rapidly transforming retroviruses (the v-onc genes), has been detected by their homology to cellular genes (1–6). These cellular genes (c-onc) presumably have normal physiological functions when residing unperturbed in their usual chromosomal setting. Another set of oncogenes, or cellular transforming genes, has been detected in the DNA of tumors and tumorigenic cell lines by their ability to induce foci of transformed cells after DNA-mediated gene transfer into NIH 3T3, a normal growth-controlled mouse fibroblast cell line (7–11). These transforming genes presumably arise from normal cellular genes by mutation (12–14). Several different human transforming genes have been reported and recently the transforming gene of a human bladder carcinoma cell line has been isolated by molecular cloning (12–14). We report here the isolation and preliminary characterization of another transforming gene, that present in the human neuroblastoma cell line SK-N-SH.

MATERIALS AND METHODS

Animal Cells. NIH 3T3 cells (15) were obtained from R. Weinberg and co-workers (7) in 1978. These cells have undergone several subclonings in our laboratory since that time to reduce spontaneous transformation. They are maintained at low cell densities in Dulbecco’s medium containing antibiotics (streptomycin at 50 μg/ml and penicillin at 50 units/ml) and 10% calf serum (Flow Laboratories). Human tumor cell lines used, which have been described (10), were: neuroblastomas SK-N-SH and IMR-32; lung carcinomas, SK-LU-1 and Calu-1; colon carcinoma, SK-B7-1; hepatoma, SK-HEP-1; and bladder carcinoma, T24. GM 2998 and XPVA-Het are low-passage human fibroblast lines and were provided by L. Grossman. PR371 is a human lung tumor that has been maintained in nude mice and was provided by J. Fogh. The various NIH 3T3 transformants are described below.

Preparation of DNA, Hybridization, and Restriction Endonuclease Digestion. High molecular weight DNA was prepared from animal cells as described (10). DNA was prepared from A phage Charon 4A and its derivatives by phage banding in CsCl followed by phenol extraction (16). Plasmid DNA was prepared from chloramphenicol-amplified cultures by detergent lysis (17). DNA restriction endonuclease fragments were purified by agarose gel electrophoresis and extracted from the agarose by the KI/glass powder method (18). Agarose gel electrophoresis and Southern blotting were carried out as described (19). 32P-Labeled probes were prepared by nick-translation (20) and blot hybridization was carried out at 71°C in 0.90 M NaCl/0.09 M Na citrate, pH 7.0. The final blot wash was 75 mM NaCl/7.5 mM Na citrate, pH 7.0 at 71°C. Restriction endonuclease digestions were in buffers recommended by vendors (New England Biolabs, Bethesda Research Laboratories) and restriction endonuclease mapping was done by analysis of double digestion products.

The bacterial plasmids used were blurs 5 and pK5. Blur 8 contains a 300-base-pair BamHI insert in pBR322 that is a member of the human Alu family repeat (21) and was provided by W. Jelink. pK5 is a pBR322 derivative containing the sup F gene. Its complete restriction map will be published in another paper. The sup F gene was originally provided by I. Kudo and U. RajBhandary.

Suppressor Rescue. DNA from NP-1, an NIH 3T3 transformant derived from SK-N-SH DNA (see Fig. 1), was partially digested with EcoRI (0.1 unit of enzyme per μg of DNA) at 37°C for 30 min; 150 μg of DNA was loaded onto 10–40% sucrose gradients in 100 mM NaCl/50 mM Tris-HCl/10 mM EDTA, pH 8.0, and centrifuged at 20,000 rpm in a SW27 rotor for 24 hr at 20°C. Gradients were fractionated, and the 12- to 30-kilobase-pair (kbp) fractions were pooled and ethanol precipitated.

DNA was dissolved, mixed (1:1, mol/mol) with a 1.5-kbp EcoRI-cleaved sup F-containing fragment from pK5 (see above), ligated at 100 μg/ml with T4 DNA ligase (Bethesda Research Laboratories) in the buffer recommended by vendor. Ligated DNA was mixed (1:3) with NIH 3T3 carrier DNA and used to transfect NIH 3T3 cells by a modification of the calcium phosphate method (22) as described (10). Transformed foci were picked after 3 wk and expanded for analysis. DNA was prepared from NIH 3T3 transformants containing sup F, partially digested with EcoRI, and size fractionated as described above. DNA in the 15- to 25-kbp range was mixed (1:1, mol/mol) with EcoRI “arms” of Charon 4A and ligated as described above. Ligated product was packaged into phage particles (23) and titered on the sup E sup F coli strain BNN45. To select sup F-containing phage derivatives, 106 packaged phage were plated onto a suppressor-free E. coli host strain, K5624. Phage yielding plaques on K5624 were picked and tested for the presence of the sup F gene.

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ence of suppressor by plating on a lac Z<sup>+</sup> strain, JG139, in the presence of the β-galactoside indicator dye X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) (24). Phage containing the sup F gave blue plaques and were picked and expanded for further analysis. A detailed description of these techniques will be given in another paper.

Recombinant DNA experiments were conducted under EK1/ P2 containment conditions as specified by the National Institutes of Health guidelines.

RESULTS

Molecular Cloning of Sequences with Transforming Activity from Transformed Cells. DNA from SK-N-SH, a cell line derived from a human neuroblastoma, can efficiently transform NIH 3T3 cells (10). DNA from the NIH 3T3 transformants can in turn transform fresh NIH 3T3 cells, due to the serial passage of a transforming principle by DNA-mediated gene transfer. To isolate this transforming principle, we began with DNA from a line of NIH 3T3 cells transformed with DNA from SK-N-SH, NP-1, that had a higher specific activity of transformation than DNA from SK-N-SH itself.

We used the same strategy for gene isolation that we had used in isolating the transforming gene of the human bladder carcinoma cell line T24 (12). This method (suppressor rescue), which has been described briefly (12) and will be described in greater detail in another paper, entails ligating restriction endonuclease-digested DNA from NP-1 to a restriction endonuclease fragment containing a bacterial tRNA amber suppressor gene (sup F). Because the transforming activity of NP-1 DNA was sensitive to cleavage with EcoRI, BamHI, and HindIII (10), we partially digested NP-1 DNA with EcoRI and then ligated DNA to a 1.5-kbp EcoRI fragment containing sup F. The ligated DNA was used to transform NIH 3T3 cells and DNA from the resulting transformants was used in a second round of gene transfer to yield cells containing only a single copy of sup F flanking the transforming gene. We used DNA from these transformants to construct EcoRI partial libraries in the bacteriophage λ vector Charon 4A, which contains two amber mutations. Only those phage that have undergone mutation at their amber loci or contain sup F can grow in a suppressor-free bacterial host. We selected in this way several phages that contained sup F and flanking DNA from the NIH 3T3 transformants. The lineage of four of these phages and the NIH 3T3 transformants used is shown in Fig. 1.

DNA was prepared from all four λ phages and analyzed by EcoRI digestion. In addition to the Charon 4A arms, all phage DNA had a 1.5-kbp EcoRI fragment containing sup F (see Fig. 2). Also, all four contained a common 7.0-kbp EcoRI fragment and two phage, λ NPS-1-1-1 and λ NPS-1-1-2, derived from NPS-1-1, contained a common 5.8-kbp EcoRI fragment. DNA from all four phages were assayed for transforming activity on NIH 3T3 cells. Only DNA from two phages, λ NPS-1-1-1 and λ NPS-1-1-2, were active. They had a specific activity of transformation (about 10<sup>5</sup> foci per μg of phage DNA) equal to that of DNA from phage containing the transforming gene of the bladder carcinoma cell line T24. These results suggested that we had obtained a biologically active clone of the transforming sequences of SK-N-SH.

Use of Transforming Sequences as Hybirdization Probes. Restriction endonuclease sites in λ NPS-1-1-1 DNA were mapped, and the presence and positions of human or mouse repeated sequences in this DNA were determined (Figs. 2 and 3). DNA digests were electrophoresed in agarose gels, blotted onto nitrocellulose filters, and hybridized with 32<sup>P</sup>-labeled nick-translated total human DNA or total mouse DNA. λ NPS-1-1-

![Fig. 1. Lineage of transformants and phage derivatives. DNA from the human cell line SK-N-SH was used to induce foci of transformed NIH 3T3 cells. Three foci were picked and expanded to yield the primary transformants, NP-1, NP-2, and NP-3. DNAs from each of these were then used to derive secondary transformants. In addition, DNA from NP-1 was used to isolate the transforming sequences. DNA from NP-1 was ligated to sup F DNA and the ligated DNA was used to induce transformed foci. DNA from one of these, NPS-1, was used to derive the additional transformed foci NPS-1-1 and NPS-1-2. From these, we derived a phage containing sup F. ](image)
the same DNAs. In this experiment, two EcoRI fragments appeared in all human DNAs: the 9.2-kbp fragment observed previously and a 7.0-kbp fragment. Two EcoRI fragments were also seen in most NIH 3T3 cells transformed with SK-N-SH DNA. The apparent exceptions, NP-1 and NP-1-2 (lanes C and E), contained two coincident EcoRI fragments that were separately hybridizable to fragments L and R (data not shown). Again, no strong hybridization was seen in either NIH 3T3 cells or NIH 3T3 cells transformed with other human transforming genes. Taking these two experiments together, the results imply that the transforming sequences of SK-N-SH are contained in EcoRI fragments of 9.2 and 7.0 kbp, that these transforming sequences are human in origin, and that the transforming gene of SK-N-SH is distinct from the transforming genes of T24 and SK-LU-1 (10–12).

We have inferred the restriction pattern of the chromosomal transforming gene of SK-N-SH (Fig. 3). We show that the gene coincides with the sequences of λ NPS-1-1-1 from the left-most EcoRI site to the left-most Bgl II site. We are confident of the left-most EcoRI site because the left-most EcoRI/Xba I fragment of λ NPS-1-1-1 hybridizes to the same EcoRI DNA segment as does fragment L (data not shown) and of the Bgl II site because Bgl II cleavage destroys the transforming activity of λ NPS-1-1-1 DNA (data not shown).

**Fig. 2.** Analysis of λ phage containing sup F. DNA (0.2–1.0 μg) from the four phage λ NPS-1-2-1 (lanes a), λ NPS-1-1-2 (lanes b), λ NPS-1-1-3 (lanes c), and λ NPS-1-1-1 (lanes d) was digested with EcoRI and electrophoresed in 1.0% agarose gels. Each phage is an independent isolate. (A) Ethidium bromide-stained gel. (B and C) DNA was denatured, transferred to nitrocellulose filters, and hybridized with total 32P-labeled nick-translated human DNA (B) or mouse DNA (C). Digested DNAs contain in common 19.9- and 11.0-kbp λ arms, a 1.5-kbp fragment containing sup F, and a 7.0-kbp fragment hybridizing to repetitive human DNA. The 1.5-kbp fragment is not readily visible in lanes b and d, which contain less DNA than lanes a and c. In addition, λ NPS-1-1-1 (lanes d) and λ NPS-1-1-2 (lanes b) contain a common 5.8-kbp fragment that hybridizes to repetitive human DNA. λ NPS-1-1-1 contains a 3.7-kbp fragment that hybridizes to repetitive mouse DNA. Arrowheads indicate the 19.9-, 11.0-, and 1.5-kbp fragments.

**Fig. 3.** Structure of the transforming sequences in SK-N-SH cells and λ NPS-1-1-1. The restriction endonuclease sites of phage λ NPS-1-1-1 insert were mapped. Regions containing human Alu sequences as determined by blotting with plasmid blur 8; L and R, fragments used in Southern hybridization experiments. The structure of the transforming sequences in SK-N-SH cells as inferred from the structure of λ NPS-1-1-1 and the hybridization experiments is also shown. RI, EcoRI; H, HindIII; Ba, BamHI; X, Xba I; K, Kpn I; Bg, Bgl II; S, Sma I.
mouse DNA. We tentatively conclude from this that the R fragment, which spans the Bgl II site disrupting transforming activity, contains sequences that are conserved in evolution and that a single mouse homolog of this human gene exists. In humans, as many as three additional homologous genes may exist.

Finally, we note that, in NIH 3T3 cells transformed with DNA from the human lung carcinoma cell line Calu-1, an additional weakly hybridizing EcoRI fragment, 3.6 kbp in size, is observed. Curiously, a comigrating species is not detected by hybridization in human DNA. We therefore examined two additional NIH 3T3 transformants for the presence of this band: NIH 3T3 cells transformed with DNA from SK-LU-1, another human lung carcinoma cell line, and NIH 3T3 cells transformed with DNA from SK-CO-1, a human colon carcinoma cell line. DNA from these transformants also contained a 3.6-kbp EcoRI fragment that hybridized to fragment R (data not shown). Previous work has shown that SK-LU-1, SK-CO-1, and Calu-1 contain a common transforming gene (10). We interpret this result to mean that the transforming gene common to these three human carcinoma cell lines is distantly related to the transforming gene of SK-N-SH. We attribute our ability to observe this fragment in transformants, but not in human DNA, to an amplification of the transforming sequences in NIH 3T3 cells that raises the hybridization signal above background levels. Although other interpretations are possible, other work justifies this conclusion (unpublished data).

**DISCUSSION**

Several human transforming genes have been identified by using DNA-mediated gene transfer and focus induction in NIH 3T3 cells as an assay. Transforming genes have been isolated by molecular cloning from T24 (12, 14), a human bladder carcinoma cell, and from EJ (13), another human bladder carcinoma cell. The two genes appear to be identical to each other and also to the transforming gene of yet a third human bladder carcinoma cell line, J82 (27). However, all three cell lines used in those studies were probably derived from a common cell line and represent only a single instance of activation of that gene (unpublished data). A second human transforming gene has been identified that is common to a colon carcinoma cell line and several other lung and colon carcinoma cell lines (10, 11). Several other
distinct human transforming genes are associated with a breast
carcinoma cell line, MCF-7 (28), a leukemic cell line, HL60
(11), and a neuroblastoma cell line, SK-N-SH (10).

In this paper, we have reported the isolation by molecular
cloning of the transforming gene from SK-N-SH. Biologically
active transforming sequences were obtained by genetic selec-
tion. Hybridization studies showed that these sequences were
found in each NIH 3T3 cell transformed by SK-N-SH DNA.
Two EcoRI fragments, of approximate sizes 9.2 and 7.0 kbp,
comprise the gene and are found in every human cell line we
have examined. Thus, like the transforming gene of T24, the
transforming gene of SK-N-SH is likely to have arisen without
gross sequence rearrangement from a normal cellular gene.

The SK-N-SH transforming sequences hybridize to other
human sequences. In particular, a 1.0-kbp HindIII fragment
(fragment R) hybridizes to EcoRI fragments in human DNA of
sizes 4.5, 10.5, and 15.0 kbp. Thus, as many as three other hu-
man genes may be closely related to the SK-N-SH gene. In
addition, the 1.0-kbp HindIII fragment hybridizes, although
less strongly, to the human sequences present in NIH 3T3 cells
transformed with the common lung/colon carcinoma trans-
forming gene. Thus, the transforming gene of SK-N-SH may belong
to a still larger gene family. Other work (unpublished) confirms
this conclusion and shows that this family contains at least three
distinct transforming genes: the transforming gene of SK-N-
SH, the transforming gene common to lung and colon carcin-
a cell lines, and the transforming gene present in the bladder
carcinoma cell line T24. Recent work also indicates that these
genes are related to Kirsten and Harvey ras genes (refs. 27 and
29; unpublished data), the oncogenes of Kirsten and Harvey
sarcoma viruses (30).

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