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Human *trk* Oncogenes Activated by Point Mutation, In-Frame Deletion, and Duplication of the Tyrosine Kinase Domain

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Malignant activation of the human *trk* proto-oncogene, a member of the tyrosine protein kinase receptor family, has been implicated in the development of certain human cancers, including colon and thyroid papillary carcinomas. *trk* oncogenes have also been identified in cultured cells transfected with various DNAs. In this study, we report the characterization of three in vitro-generated *trk* oncogenes, *trk*2, *trk*4, and *trk*5 (R. Oskam, F. Coulier, M. Ernst, D. Martin-Zanca, and M. Barbacid, Proc. Natl. Acad. Sci. USA 85:2964–2968, 1988), in an effort to understand the spectrum of mutational events that can activate the human *trk* gene. Nucleotide sequence analysis of cDNA clones of *trk*2 and *trk*4 revealed that these oncogenes were generated by a head-to-tail arrangement of two *trk* tyrosine protein kinase domains connected by a purine-rich region. These oncogenes code for cytoplasmic molecules of 67,000 ($p67^{trk2}$) and 69,000 ($p69^{trk4}$) daltons. In contrast, the product of the *trk*5 oncogene, $gp95^{trk5}$, is a cell surface glycoprotein of 95,000 daltons. This oncogene was generated by a 153-base-pair in-frame deletion within sequences coding for the extracellular domain of the *trk* receptor. This activating deletion encompasses a triplet coding for one of the nine cysteine residues that the *trk* receptor shares with the product of the highly related *trk*B tyrosine protein kinase gene. Introduction of a single point mutation (TGT→AGT) in this codon resulted in a novel *trk* oncogene whose product, $gp140^{S345}$, differs from the nontransforming *trk* proto-oncogene receptor in a single amino acid residue, Ser-345 instead of Cys-345. These results illustrate that multiple molecular mechanisms, including point mutation, internal deletion, and kinase domain duplication, can result in the malignant activation of the human *trk* proto-oncogene.

Tyrosine protein kinases are emerging as key regulatory elements in signal transduction (17, 21). Unfortunately, their pivotal position within the cellular machinery may have a negative side effect. Mutations within many tyrosine protein kinase loci lead to malignant transformation, presumably as a result of imbalances within those signal transduction processes that they are meant to control (17, 21). Oncogenic tyrosine protein kinases transduced by retroviruses exhibit multiple and diverse mutations, a reflection of the strong selection process that occurs during viral replication (19, 20). In contrast, oncogenic tyrosine protein kinases of nonviral tumors, including those of humans, exhibit single mutations, perhaps a consequence of the limited number of mutagenic events allowed within the cellular genome. For instance, *neu* oncogenes activated in carcinogen-induced tumors exhibit a single point mutation within their transmembrane coding domain (3, 18). Similarly, the c-*abl* oncogenes of human leukemias owe their transforming properties to reproducible rearrangements with specific sequences within the *bcr* locus (14).

Malignant activation of *trk* oncogenes in human tumors results from genetic rearrangements in which its catalytic tyrosine kinase domain is fused to sequences derived from various unrelated loci (4, 10, 13). These rearrangements lead to the generation of chimeric molecules and allow the ectopic expression of the *trk* kinase in different tissues, depending upon the nature of the locus involved in this mutational event. To date, *trk* oncogenes have been detected in a tumor of the ascending colon and in a significant fraction of thyroid papillary carcinomas (4, 10). In addition, *trk* oncogenes have been generated during the course of gene transfer (8, 13). Transfection of NIH 3T3 cells with DNA isolated from a mammary carcinoma cell line resulted in the fusion of the *trk* kinase to sequences derived from the L7a ribosomal protein (8, 22).

We have previously reported the frequent generation of *trk* oncogenes as a result of transfection of NIH 3T3 cells with nontransforming *trk* proto-oncogene cDNA sequences (13). Some of these *trk* oncogenes code for cytoplasmic kinases reminiscent of the products of *trk* oncogenes identified in human tumors. Other oncogenes code for cell membrane glycoproteins. These results indicate that the *trk* locus can participate in the generation of distinct classes of transforming proteins (13). We undertook the present studies to analyze the molecular structure of these *trk* oncogenes and their respective gene products in an effort to understand the mechanisms underlying the oncogenic activation of the human *trk* proto-oncogene.

MATERIALS AND METHODS

Isolation of cDNA clones. Total cellular RNA was prepared by the guanidinium isothiocyanate-cesium chloride method, and the poly(A)-containing fraction was isolated by affinity chromatography on oligo(dT)-cellulose columns (9). cDNA was synthesized by oligo(dT) priming on poly(A)-containing RNAs (cDNA Synthesis System; Amersham International) isolated from third cycle NIH 3T3 transformants derived from the *trk*2 (E29-913 cells) and *trk*4 (E18-93 cells) oncogenes. cDNA libraries were prepared in lambda ZAP vectors (Stratagene Cloning Systems), and 10^6 bacteriophages were plated on a lawn of *Escherichia coli* BB4 cells. Phages were absorbed onto nitrocellulose filters and lysed, and their

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DNAs were hybridized under stringent conditions (65 h at 42°C in 5× SSC [1× SSC is 0.1 M NaCl, 0.015 M sodium citrate], 50% formamide, 1× Denhardt solution) with a nick-translated 1.2-kilobase-pair (kbp) *BalI-EcoRI* DNA fragment of the *trk* oncogene that encompasses its entire tyrosine protein kinase catalytic domain (10). Positive lambda clones were plaque purified as described previously (9) and subsequently converted to plasmid clones by the automatic excision process (Stratagene Cloning System).

Construction of pRK25. Poly(A)-containing RNA was isolated from E67-52 cells, a second cycle NIH 3T3 transformant derived from the *trk5* oncogene by the guanidinium isothiocyanate-$CsCl_2$ procedure (9) followed by oligo(dT) enrichment. A 1-μg portion of this poly(A)-containing RNA was converted into double-stranded cDNA by the Gubler and Hoffman (6) procedure by using a reagent kit (Invitrogen). One-tenth of this cDNA (5 μl) was amplified by the polymerase chain reaction (PCR) technique (16). The 5′ primer (no. 1709), 5′-GGCTGGATCCTCACAGAGCTGGA-3′, overlaps a *Bam*HI cleavage site (underlined) located at positions 764 to 769 in the *trk* cDNA (11). The 3′ primer (no. 1712), 5′-TCGGGTCCATGGGATCGGAGG-3′, overlaps an *Nco*I cleavage site (underlined) at positions 1875 to 1880. A control PCR amplification was set up by using pDM38 DNA, a plasmid containing our longest *trk* proto-oncogene cDNA clone (2,673 base pairs [bp]) as a template (11). Thirty cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and polymerization at 72°C for 3 min. The polymerization time was extended by 5 s after each cycle. A 10-μl portion of the total reaction (200 μl) was tested by agarose gel electrophoresis. The remainder was subjected to proteinase K digestion, organic extractions, and ethanol precipitation. DNA was resuspended in water and digested with *Bam*HI and *Nco*I. After phenol extraction, the restricted DNA was resuspended in water and subcloned into a pDM38 vector prepared by partial *Bam*HI and total *Nco*I digestion. The resulting plasmid, pRK25, was verified by restriction endonuclease digestions and nucleotide sequencing (both strands) of the insert.

Construction of pRK26. A single point mutation (T→A) was introduced in nucleotide 1117 of the *trk* proto-oncogene cDNA clone by PCR-aided mutagenesis. This change converts a TGT sequence coding for a cysteine residue (Cys-345) into a serine-coding triplet, AGT. Two segments of the *trk* proto-oncogene cDNA clone present in pDM38 were amplified by PCR. Primers no. 1709 (see above) and no. 2039 (5′-GAGGCGCAGACTCCCGTGCCGCAC-3′) amplified a 342-bp fragment. Primers no. 1712 (see above) and no. 2040 (5′-GTGCGGCACGGGAGTCTGCGCCTC-3′) amplified a 760-bp segment. Primers no. 2039 and 2040 (nucleotides 1105 to 1128) overlap an *Hin*fI cleavage site (underlined) created by designing an A/T mispriming over the first base of the wild-type TGT codon. This created *Hin*fI site is unique in the amplified 1.1-kbp DNA segment located between the *Bam*HI and *Nco*I cleavage sites. Amplified DNAs were processed as described above, separately subjected to *Hin*fI digestion, and ligated to each other by T4 DNA ligase (Bethesda Research Laboratories, Inc.). The ligated products were digested with *Nco*I and *Bam*HI and electrophoresed in a 1.0% agarose gel. A 1.1-kbp DNA fragment was purified by electroelution and subcloned into pDM38 previously digested with *Bam*HI (partial) and *Nco*I. The resulting plasmid, pRK26, was verified by *Hin*fI digestion and nucleotide sequence analysis.

Cell labeling and immunoprecipitation. Subconfluent cultures (10-cm dishes) were preincubated for 30 min and labeled with [^{35}S]methionine (50 μCi/ml, 1,200 Ci/mmol; ICN Radiochemicals) for 3 h in methionine-free Dulbecco modified Eagle medium containing 10% dialyzed calf serum. Cells were washed with phosphate-buffered saline, lysed in radioimmunoprecipitation buffer, and immunoprecipitated with polyclonal rabbit antibodies raised against either a bacterially made $p70^{trk}$ protein (12) or a synthetic peptide corresponding to the 14 carboxy-terminal residues of the *trk* proto-oncogene product (11). The resulting immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia, Inc.) and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8% polyacrylamide slab gels.

Protein kinase assays. Subconfluent cultures (10-cm dishes) were washed twice with phosphate-buffered saline and lysed in radioimmunoprecipitation buffer containing 100 μM sodium vanadate, 5 mM phenylmethylsulfonyl fluoride, and 0.2 U of aprotinin per ml. Clarified lysates were incubated with the polyclonal antisera indicated above. The resulting immunocomplexes were precipitated with protein A-Sepharose beads and resuspended in 50 μl of 50 mM HEPES (*N*-2-hydroethylpiperazine-*N*′-2-ethanesulfonic acid)-HCl buffer containing 20 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM ATP, and 200 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) per ml. After incubating for 10 min at 30°C, the immunoprecipitates were washed with radioimmunoprecipitation buffer and the ^{32}P-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis on 8% slab gels.

RESULTS

Generation of novel *trk* oncogenes during gene transfer. Transfection of NIH 3T3 cells with nontransforming plasmids carrying either the tyrosine protein kinase domain or the entire *trk* proto-oncogene coding sequences results in the frequent generation of *trk* oncogenes (13). As summarized in Table 1, we have characterized the products of 42 independently generated *trk* oncogenes by using two antisera raised against bacterially synthesized $p70^{trk}$ (12) and against a synthetic peptide corresponding to the 14 carboxy-terminal residues of the normal *trk* protein (11). Twelve of these in vitro-generated oncogenes have been described in a previous study (13). Each of the *trk* oncogenes derived from plasmids containing the catalytic domain of the *trk* kinase (pDM17 and pDM22) codes for cytoplasmic proteins of sizes ranging between 60,000 and 79,000 daltons (Table 1). In contrast, most of the oncogenes generated during transfection of cDNA sequences encoding the entire *trk* proto-oncogene product (pDM38) code for glycoproteins of sizes ranging between 83,000 to 178,000 daltons (Table 1). The peptidic backbones of these glycoproteins were found to be in the range of 69,000 to 100,000 daltons. Since the molecular mass of the nonglycosylated form of the *trk* proto-oncogene product is 80,000 daltons (11), it is likely that loss of *trk* sequences as well as gain of additional genetic information may have played a role in the generation of these pDM38-derived *trk* oncogenes.

Molecular cloning of *trk2* and *trk4* oncogenes. In order to characterize these in vitro-generated *trk* oncogenes, we isolated cDNA clones from two representative pDM22-derived oncogenes, *trk2* and *trk4*. cDNA libraries were prepared in λ ZAP vectors by reverse transcription of poly(A)-containing RNAs isolated from third cycle NIH 3T3 transformants derived from the *trk2* and *trk4* oncogenes. Third cycle transformants were used to eliminate the presence of additional *trk*-related transcripts unrelated to the

TABLE 1. Generation of novel *trk* oncogenes during gene transfer

Donor DNA[a]	*trk* oncogenes	Frequency (oncogenes per 10^4 transfected cells)	*trk* oncogene products (size in daltons)
pDM22 + carrier DNA	17 oncogenes (*trk*1, *trk*2, *trk*3, *trk*4, *trk*7, *trk*8, *trk*10, *trk*11, *trk*12, *trk*13, *trk*17, *trk*18, *trk*19, *trk*20, *trk*21, *trk*22, *trk*23)	3.7	60,000–79,000
pDM22	11 oncogenes (*trk*24, *trk*25, *trk*26, *trk*27, *trk*28, *trk*29, *trk*30, *trk*31, *trk*32, *trk*33, *trk*34)	3.7	63,000–79,000
pDM17 + carrier DNA	3 oncogenes (*trk*39, *trk*40, *trk*41)	1.1	70,000–130,000
pDM17	1 oncogene (*trk*42)	0.6	70,000
pDM38 + carrier DNA	7 oncogenes (*trk*5, *trk*6, *trk*9, *trk*35, *trk*36, *trk*37, *trk*38)	1.9	62,000–178,000[b]
pDM38	2 oncogenes (*trk*14, *trk*15)	0.7	89,000–175,000[b]

[a] pDM22 directs the synthesis of a nontransforming 36,000-dalton polypeptide which corresponds to the kinase catalytic domain of the human *trk* oncogene (10, 12), under the control of a murine sarcoma virus long terminal repeat. pDM17 is identical to pDM22, except it does not contain any promotor sequences. pDM38 codes for the entire human *trk* proto-oncogene product, under the control of a murine sarcoma virus long terminal repeat (11, 13).

[b] The products of these oncogenes are glycoproteins, except for $p62^{trk6}$ (13).

transformation process. About 10^6 recombinant phages from each cDNA library were screened with a *trk* tyrosine kinase-specific probe and were plaque purified, and those exhibiting the longest cDNA insert were converted into plasmid clones as described in Materials and Methods and subsequently subcloned in the mammalian expression vector pMEX (11).

Two expression plasmids—pFC38, which carries a 3.0-kbp cDNA insert of the *trk*2 oncogene, and pFC36, which contains a 2.9-kbp cDNA insert of the *trk*4 oncogene—were next assayed in gene transfer assays to determine whether they possessed transforming activity. Both plasmids efficiently induced morphologic transformation of NIH 3T3 cells with a specific activity (>2×10^5 focus-forming units per μg) comparable to that of pDM16, an expression vector carrying a cDNA clone of the original *trk* oncogene isolated from a human colon carcinoma (10). Representative foci of transformed cells were isolated, subcloned in agar, and submitted to immunoprecipitation analysis by using a polyclonal rabbit antiserum raised against a peptide corresponding to the carboxy terminus of the *trk* proto-oncogene product (11). As shown in Fig. 1, NIH 3T3 cells transformed by pFC38 (*trk*2 oncogene) expressed a *trk*-related protein of 67,000 daltons, indistinguishable from $p67^{trk2}$, the product of the *trk*2 oncogene present in the NIH 3T3 transformant used to prepare the *trk*2 cDNA library (13). Similarly, NIH 3T3 cells transformed by the expression vector pFC36 carrying the *trk*4 oncogene cDNA clone expressed a *trk*-related protein of 69,000 daltons, indistinguishable in size from $p69^{trk4}$, the product of the *trk*4 oncogene (Fig. 1) (13). These results demonstrate that the cDNA inserts present in pFC38 and pFC36 expression vectors represent biologically active clones of the in vitro-generated *trk*2 and *trk*4 oncogenes.

Structural analysis of cDNA clones of *trk*2 and *trk*4 oncogenes. We next submitted these *trk*2 and *trk*4 oncogene cDNA clones to structural analysis. Both clones exhibited almost identical restriction endonuclease maps (Fig. 2). Many of the mapped restriction sites were repeated in both halves of these cDNA clones, suggesting a tandem arrangement. Partial nucleotide sequence analysis confirmed this structure. As shown in Fig. 2, the *trk*2 oncogene consists of a head-to-tail tandem of the *trk* tyrosine kinase catalytic domain present in pDM22, the expression plasmid that originated the *trk*2 and *trk*4 oncogenes (13). Translation of the *trk*2 oncogene product, $p67^{trk2}$, is likely to initiate at the same methionine used to express the pDM22 product, $p36^{trk}$. This methionine is followed by four amino acid residues (Ala-Gly-Ile-Ser) derived from a linker used to generate pDM22 and by a 321-amino-acid-long kinase catalytic region derived from the *trk* proto-oncogene (residues 458 to 778) (13). The *trk* proto-oncogene sequences end at position 779, just 12 residues from its carboxy terminus (Fig. 2). These 5′ *trk* sequences are followed by a purine-rich 87-bp-long DNA segment derived from the 5′ noncoding region of pDM22. These sequences have their origin in the 5′ noncoding domain of the tropomyosin gene involved in the generation of the original human *trk* oncogene isolate (11). Since this G+A-rich region lacks in-frame terminators, it serves to

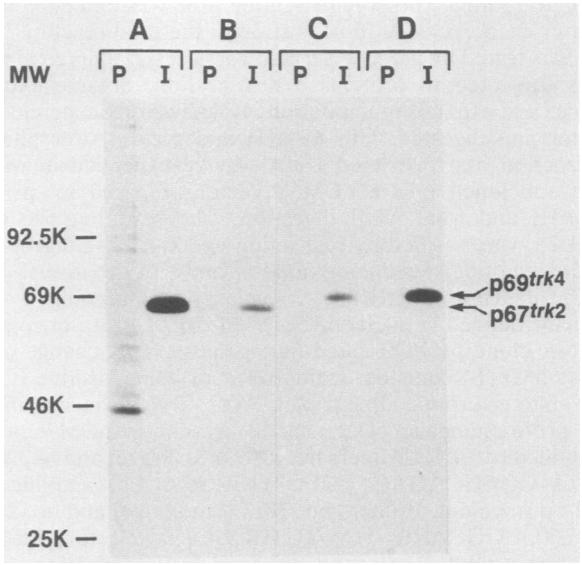

FIG. 1. Immunoprecipitation analysis of the *trk*2 and *trk*4 oncogene products. [^{35}S]methionine-labeled cell extracts of NIH 3T3 cells transformed by the *trk*2 oncogene (E29-913 cells) (A); pFC38 DNA, a plasmid carrying a cDNA clone of the *trk*2 oncogene (E98-1711 cells) (B); the *trk*4 oncogene (E18-93 cells) (C); and pFC36 DNA, a plasmid carrying a cDNA clone of the *trk*4 oncogene, (E98-1111 cells) (D) were incubated with either preimmune rabbit serum (P) or a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the 14 carboxy-terminal amino acid residues of the human *trk* proto-oncogene product (11) (I). The resulting immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Gels were exposed to Kodak XAR film for 24 h at −70°C with the help of an intensifier screen. The migration of the products of the *trk*2 oncogene ($p67^{trk2}$) and the *trk*4 oncogene ($p69^{trk4}$) is indicated by arrows. Coelectrophoresed molecular size markers included phosphorylase B (92,500 daltons [Da]), albumin (69,000 Da), ovalbumin (46,000 Da), and α-chymotrypsinogen (25,000 Da).

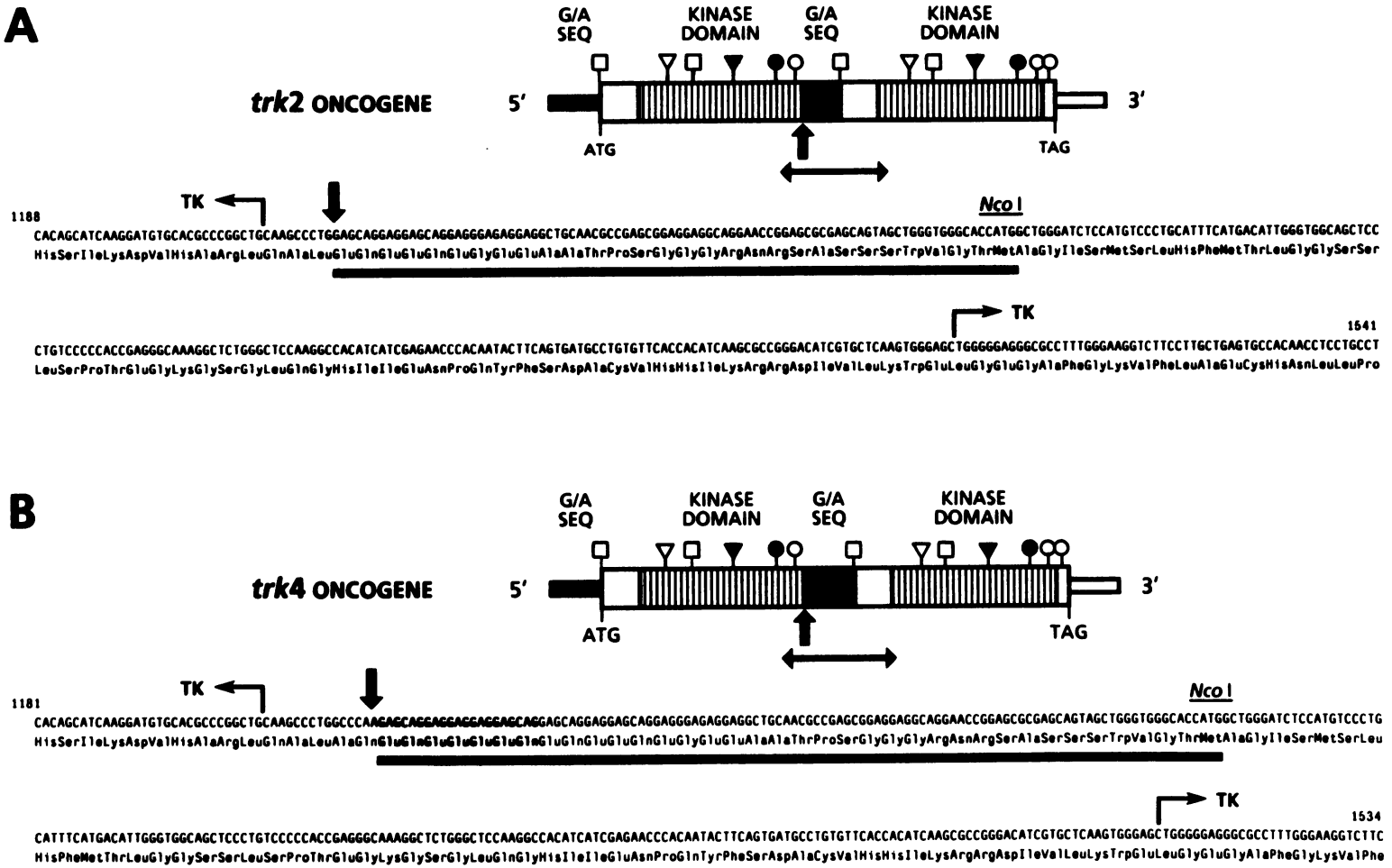

FIG. 2. Schematic representation of cDNA clones of the *trk*2 and *trk*4 oncogenes. The thick bar, flanked by the initiator (ATG) and terminator (TAG) codons, represents coding sequences. The shaded boxes represent G+A-rich sequences derived from the 5′ untranslated sequences of the tropomyosin gene involved in the generation of the human *trk* oncogene isolated for a colon carcinoma (10). The open boxes correspond to sequences derived from the cytoplasmic region of the *trk* proto-oncogene other than those encompassing the tyrosine protein kinase domain. The hatched boxes correspond to sequences derived from the tyrosine protein kinase domain. The horizontal arrows indicate the region of the *trk*2 (nucleotides 1188 to 1541) and *trk*4 (nucleotides 1181 to 1534) oncogenes whose nucleotide and deduced amino acid sequences are depicted below. The 3′ end of the first kinase domain and the 5′ end of the second kinase region is also indicated by arrows. The vertical arrows indicate the break point between the two subsets of pDM22-derived *trk* sequences (13). The G+A-rich sequences that separate the two *trk* domains are underlined by a shaded bar. Restriction endonuclease cleavage sites include *Nco*I (open squares), *Pst*I (open inverted triangle), *Bal*I (solid inverted triangle), *Kpn*I (closed circle), and *Acc*I (open circles).

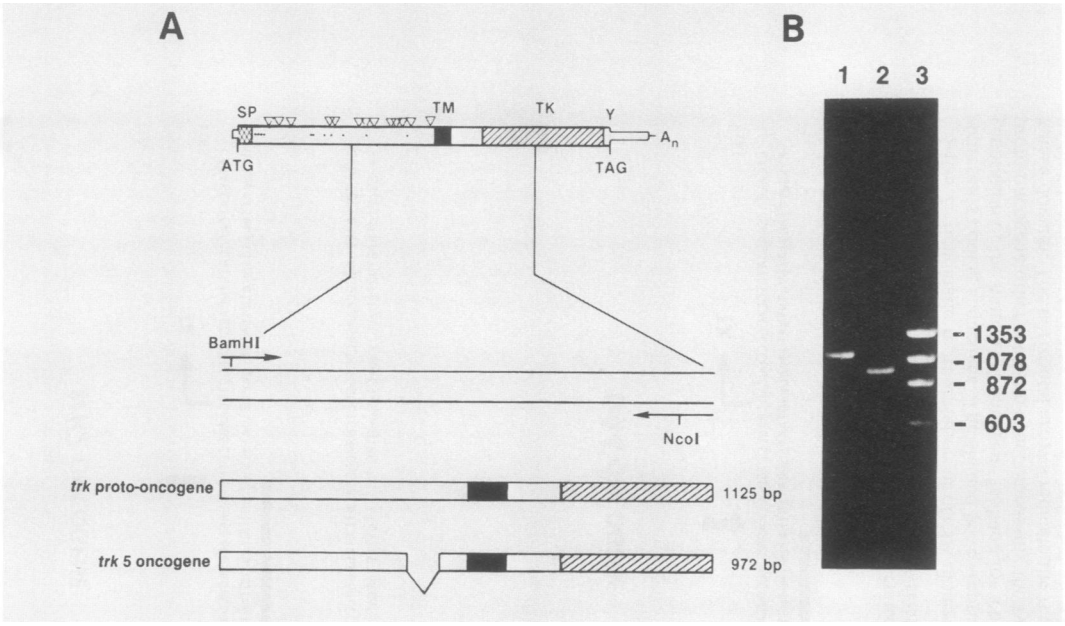

FIG. 3. (A) Schematic representation of the strategy used to generate a cDNA clone of the *trk5* oncogene. Poly(A)-containing RNA isolated from a second cycle NIH 3T3 transformant (E67-52 cells) was reverse transcribed. Sequences contained between a *Bam*HI cleavage site present in the extracellular domain and an *Nco*I cleavage site located in the kinase region were amplified by PCR as described in Materials and Methods. The thick bar represents *trk* proto-oncogene coding sequences, including the signal peptide (crosshatched bar), transmembrane domain (solid bar), and kinase domain (hatched bar). The cysteine residues (small dots) and potential sites for N-glycosylation (inverted triangles) are also indicated. The amplified mutant 972-bp *Bam*HI-*Nco*I fragment was used to replace the wild-type 1,125-bp *Bam*HI-*Nco*I DNA fragment present in the *trk* proto-oncogene cDNA clone, as described in Materials and Methods. (B) Agarose gel electrophoresis analysis of *Bam*HI-*Nco*I DNA fragments amplified from the *trk* proto-oncogene cDNA clone present in the expression vector pDM38 (lane 1) and DNA complimentary to *trk5* oncogene transcripts present in E67-52 cells (lane 2). Lane 3 contains *Hae*III-cleaved ΦX174 DNA used as molecular size markers.

connect the 5′ and 3′ kinase domains of the *trk2* oncogene (Fig. 2). The carboxy-terminal catalytic domain, unlike the one located at the amino terminus, contains an intact carboxy terminus, as deduced from direct nucleotide sequencing and by the ability of the antiserum elicited against the *trk* carboxy-terminal peptide to immunoprecipitate $p67^{trk2}$ (Fig. 1).

The structure of the *trk4* oncogene was found to be very similar to that of *trk2*. The upstream tyrosine kinase catalytic domain was terminated just five nucleotides 3′ from the breakpoint in the *trk2* oncogene (Fig. 2). In addition, the *trk4* oncogene product contains a slightly longer stretch of the G+A-rich, tropomyosin-derived sequences (22 nucleotides longer than the corresponding segment in the *trk2* oncogene). The remaining 3′ sequences in *trk4* are the same as in *trk2*, including a complete tyrosine kinase catalytic domain and an intact carboxy-terminal tail (residues 458 to 790 of the *trk* proto-oncogene) (11). The additional nine amino acid residues present in $p69^{trk4}$ (two residues derived from *trk* sequences and seven encoded for by the additional purine-rich connecting sequences) are likely to account for its slightly larger molecular weight (Fig. 1).

Molecular characterization of the *trk5* oncogene. Transfection of NIH 3T3 cells with an expression plasmid (pDM38) containing a cDNA clone of the *trk* proto-oncogene also results in the frequent generation of transforming genes (Table 1). Unlike the pDM22 DNA-derived oncogenes, those generated during transfection of pDM38 DNA code for glycoproteins associated with cellular membranes. In this study, we have characterized one of these oncogenes, *trk5*. The *trk5* oncogene codes for a 120,000-dalton cell surface glycoprotein slightly smaller than the mature product of the *trk* proto-oncogene, $gp140^{trk}$ (13). These results suggest that a small deletion may account for the malignant activation of the *trk5* oncogene.

To test this hypothesis, we isolated poly(A)-containing RNA from E67-52 cells, a second cycle NIH 3T3 transformant containing the *trk5* oncogene. This RNA was submitted to S1 nuclease analysis by using probes derived from the transmembrane and extracellular domain of the *trk* proto-oncogene. This strategy was based on the assumption that deletions within the tyrosine kinase catalytic domain would likely result in inactive nontransforming molecules. Poly(A)-containing RNA isolated from E67-52 cells fully protected an end-labeled antisense probe derived from the 5′ end of the extracellular domain of the *trk* proto-oncogene (nucleotides 1 to 757). In contrast, two similar probes encompassing the 3′ half of the extracellular domain and the transmembrane region (nucleotides 771 to 1420 and nucleotides 957 to 1420) were cleaved by the S1 enzyme, yielding identical fragments of 220 bp (data not shown). These results mapped the putative deletion in the *trk5* oncogene within these extracellular domain sequences somewhere between nucleotide 757 (3′ end of the fully protected probe) and nucleotide 1200 of the *trk* proto-oncogene cDNA clone.

On the basis of this information, we utilized a PCR-aided cloning strategy to isolate a partial cDNA clone of the *trk5* oncogene to precisely define its structural alteration(s). For this purpose, we synthesized a 23-mer 5′ sense amplimer corresponding to nucleotides 760 to 782 of the *trk* proto-oncogene which encompassed a *Bam*HI cleavage site and a 21-mer 3′ antisense amplimer complementary to nucleotides 1866 to 1886 of the *trk* proto-oncogene which included an *Nco*I cleavage site. As shown in Fig. 3, these primers

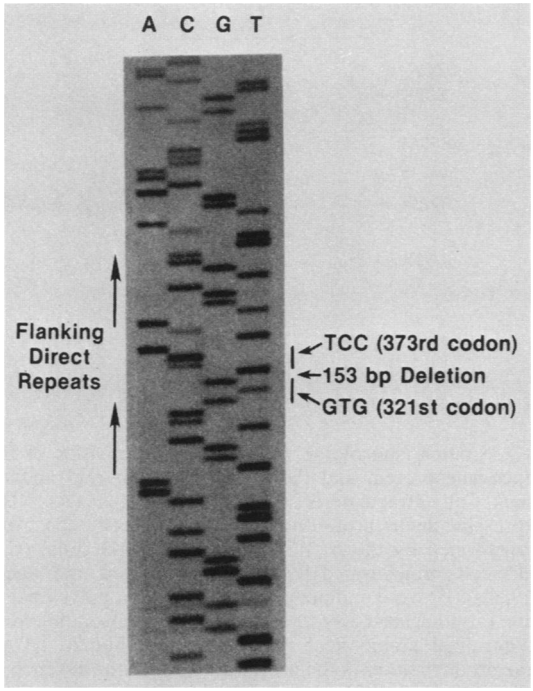

FIG. 4. Nucleotide sequence analysis of the region surrounding the 153-bp deletion in the *trk5* oncogene reveals flanking direct repeats 5'ATGGCTCC3' and 5'ATGGCTGCC3' (vertical arrows). Codon numbers correspond to those of the *trk* proto-oncogene. The position of the endpoints of the 153-bp in-frame deletion in the *trk5* oncogene is indicated by a horizontal arrow.

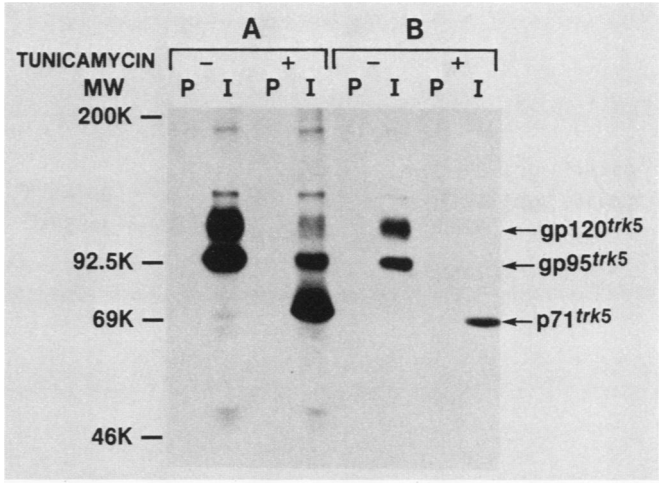

FIG. 5. Immunoprecipitation analysis of the *trk5* oncogene products. [^{35}S]methionine-labeled cell extracts of NIH 3T3 cells transformed by the *trk5* oncogene (E67-52 cells) (A) and pRK25 DNA, a plasmid carrying a *trk* proto-oncogene cDNA clone containing the 153-bp deletion present in the *trk5* oncogene, (B38-91 cells) (B) were incubated with either preimmune rabbit serum (P) or a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the 14 carboxy-terminal amino acid residues of the human *trk* proto-oncogene product (11) (I). The resulting immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Parallel cultures were metabolically labeled either in the absence (−) or in the presence (+) of 10 μg of tunicamycin per ml. Gels were exposed to Kodak XAR film for 24 h at −70°C with the help of an intensifier screen. The migration of the unglycosylated (p71^{trk5}), partially glycosylated (gp95^{trk5}), and mature (gp120^{trk5}) forms of the *trk5* oncogene product is indicated by arrows. Coelectrophoresed molecular size markers included myosin (200,000 Da), phosphorylase B (92,500 Da), albumin (69,000 Da), and ovalbumin (46,000 Da).

amplified the expected 1.1-kbp DNA fragment from cDNA prepared from NIH 3T3 cells overexpressing the *trk* proto-oncogene (E25-427 cells). Instead, the same set of primers amplified a 970-bp DNA fragment from the corresponding *trk5*-transformed E67-52 cells (Fig. 3). Nucleotide sequence analysis of this amplified DNA fragment revealed that the *trk5* oncogene contains a 153-bp-long in-frame deletion corresponding to nucleotides 1048 to 1200 of the *trk* proto-oncogene. Interestingly, these sequences are flanked by an 8-bp direct repeat, ATGGCT(G)CC, that may have facilitated their deletion during the course of gene transfer (Fig. 4). No other differences with the previously published sequence of the *trk* proto-oncogene were found in this amplified DNA fragment. These results predict that the *trk5* oncogene will code for a glycoprotein identical in sequence to that encoded by the *trk* proto-oncogene, except for the deletion of residues 322 to 372 (11).

To demonstrate that this 51-amino-acid deletion was directly responsible for the transforming properties of the *trk5* oncogene, we replaced the wild-type 1.1-kbp *Bam*HI-*Nco*I segment of the *trk* proto-oncogene cDNA clone pDM38 by the amplified 970-bp *Bam*HI-*Nco*I DNA fragment. The resulting plasmid, pRK25, was capable of transforming NIH 3T3 cells with an efficiency of at least 10^5 focus-forming units per μg of DNA, a transforming activity comparable to that of the original human *trk* oncogene (10). NIH 3T3 cells transformed by pRK25 DNA were isolated, subcloned in agar, and submitted to immunoprecipitation analysis by using the rabbit antiserum elicited against the *trk* carboxy-terminal sequences. As shown in Fig. 5, pRK25-derived transformants expressed two glycoproteins of 120,000 and 95,000 daltons, indistinguishable in size from those expressed in *trk5*-transformed E28-381 cells. More importantly, immunoprecipitation of tunicamycin-treated cells revealed that the polypeptidic backbones of the products of the recombinant pRK25 DNA and the *trk5* oncogene also had identical electrophoretical mobilities (Fig. 5). These results demonstrate that pRK25 codes for a representative cDNA clone of the in vitro-generated *trk5* oncogene.

A single amino acid substitution can generate a *trk* oncogene. The 51-amino-acid-long deletion responsible for the malignant activation of the *trk5* oncogene encompasses a domain highly conserved between the two members of the *trk* subfamily of cell surface receptors, *trk* and *trk*B (7, 11). This suggests that this region may play an important role in determining the structure of these receptors. One of the conserved residues is Cys-345, one of nine cysteines shared by the extracellular domains of the *trk* and *trk*B gene products and the only cysteine residue present in this deleted domain (7). Since cysteine residues play an important role in determining the secondary and tertiary structure of growth factor receptors, we decided to investigate whether mutation of this particular residue might result in the malignant activation of the *trk* proto-oncogene.

For this purpose, we replaced the TGT triplet coding for Cys-345 (nucleotides 1117 to 1119) by the serine-coding AGT sequence. To engineer this mutation, we amplified two DNA fragments from the *trk* proto-oncogene cDNA clone. One of these fragments extended from the *Bam*HI cleavage site described above (nucleotides 764 to 769) to sequences overlapping the TGT triplet. The second DNA fragment extended from sequences overlapping this TGT codon to the

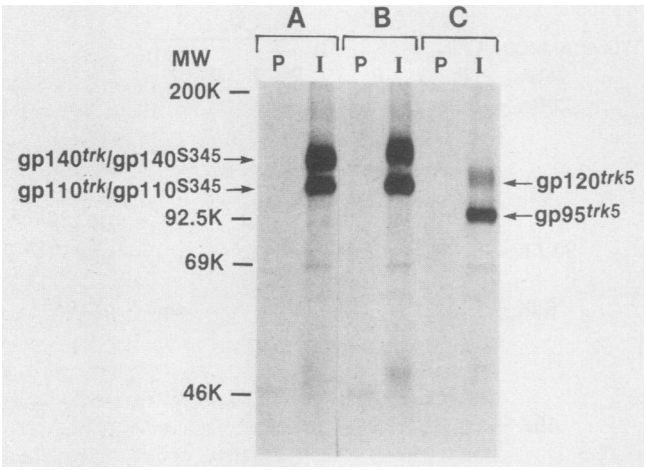

FIG. 6. Immunoprecipitation analysis of the product of the trk^{S345} oncogene. [^{35}S]methionine-labeled cell extracts of NIH 3T3 cells expressing the *trk* proto-oncogene (E25-427 cells) (A); NIH 3T3 cells transformed by pRK26 DNA, a plasmid carrying the trk^{S345} oncogene, (B38-42 cells) (B); and NIH 3T3 cells transformed by the reconstructed *trk5* oncogene (B38-91 cells) (C) were incubated with either preimmune rabbit serum (P) or a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the 14 carboxy-terminal amino acid residues of the human *trk* proto-oncogene product (11) (I). The resulting immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Gels were exposed to Kodak XAR film for 24 h at −70°C with an intensifier screen. The migration of the products of the *trk* proto-oncogene ($gp140^{trk}$ and $gp110^{trk}$), the trk^{S345} oncogene ($gp140^{S345}$ and $gp110^{S345}$), and the *trk5* oncogene ($gp120^{trk5}$ and $gp95^{trk5}$) is indicated by arrows. Coelectrophoresed molecular size markers were those described in the legend to Fig. 5.

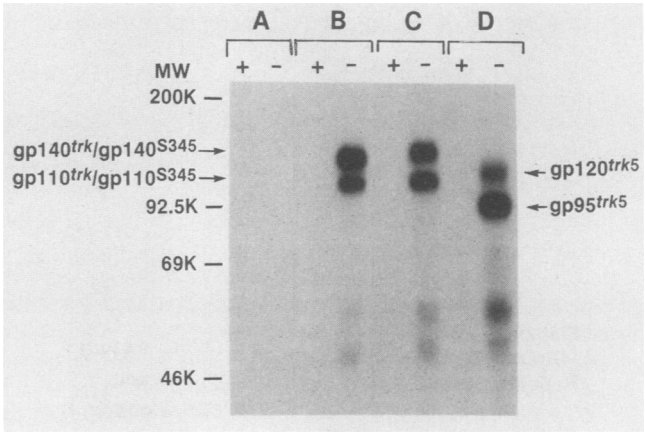

FIG. 7. Comparison of the protein kinase activities of the *trk* proto-oncogene protein and the products of the *trk5* and trk^{S345} oncogenes. Cell extracts derived from NIH 3T3 cells (A), NIH 3T3 cells expressing the *trk* proto-oncogene (E25-427 cells) (B), NIH 3T3 cells transformed by the trk^{S345} oncogene (B38-42 cells) (C), and NIH 3T3 cells transformed by the reconstructed *trk5* oncogene (B38-91 cells) (D) were immunoprecipitated with a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the 14 carboxy-terminal amino acid residues of the human *trk* proto-oncogene product either in the presence (+) or in the absence (−) of 10 μg of competing peptide and analyzed for protein kinase activity as described in Materials and Methods. ^{32}P-labeled samples were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were exposed to Kodak XAR film for 12 h at −70°C with an intensifier screen. The migration of the products of the *trk* proto-oncogene ($gp140^{trk}$ and $gp110^{trk}$), the trk^{S345} oncogene ($gp140^{S345}$ and $gp110^{S345}$), and the *trk5* oncogene ($gp120^{trk5}$ and $gp95^{trk5}$) is indicated by arrows. Coelectrophoresed molecular size markers were those described in the legend to Fig. 5.

*Nco*I cleavage site used to generate the *trk5* oncogene (nucleotides 1875 to 1880). The primers utilized in these amplifications carried a T→A mismatch at position 1117 of the *trk* proto-oncogene cDNA clone that resulted in the creation of a unique *Hin*fI cleavage site in the amplified DNA fragments (see Materials and Methods). Ligation of these amplified DNAs after digestion with *Hin*fI generated a 1.1-kbp *Bam*HI-*Nco*I DNA fragment identical to that present in the *trk* proto-oncogene, except that it contained a serine-coding AGT sequence instead of the wild-type TGT codon.

Replacement of the wild-type 1.1-kbp *Bam*HI-*Nco*I DNA fragment of pDM38 by this PCR-amplified fragment yielded pRK26, a plasmid capable of directing the synthesis of a *trk* protein carrying a single amino acid substitution (Cys→Ser) in residue 345. Transfection of NIH 3T3 cells with pRK26 DNA resulted in their malignant transformation with an efficiency of 10^2 to 10^3 focus-forming units per μg of DNA. This transforming activity is 100- to 1,000-fold lower than that of the *trk5* oncogene. Whether mutations other than Ser-345 may confer higher levels of transformation to this oncogene remains to be tested. Representative foci of transformed NIH 3T3 cells were isolated, cloned in semisolid agar, and submitted to immunoprecipitation analysis. As shown in Fig. 6, the products of pRK26 were indistinguishable from those encoded by its nontransforming allele, the *trk* proto-oncogene. These results indicate that replacement of a single amino acid residue is sufficient to confer transforming activity to the *trk* proto-oncogene and suggest that the conserved Cys-345 residue may play an important role in defining the appropriate tertiary structure of the normal *trk* cell surface receptor.

Finally, we examined the effect of the activating Cys-345→Ser-345 mutation on the tyrosine protein kinase activity of the *trk* proto-oncogene product. Cell lysates from normal NIH 3T3 cells expressing the *trk* proto-oncogene (E25-427 cells), NIH 3T3 cells transformed by the reconstructed *trk5* oncogene (B38-91 cells), and NIH 3T3 cells transformed by pRK26 DNA (B38-42 cells) were immunoprecipitated with a rabbit antiserum raised against a peptide corresponding to the carboxy-terminal domain of the *trk* proto-oncogene, in either the presence or absence of competing peptide. The resulting immunoprecipitates were then incubated with [γ-^{32}P]ATP in the presence of divalent cations. As shown in Fig. 7, the product of the *trk* oncogene activated by the Cys-345→Ser-345 miscoding mutation exhibits a kinase activity comparable to that of the product of the *trk5* oncogene $gp120/gp95^{trk5}$ and the *trk* proto-oncogene $gp140/gp110^{trk}$. Similar findings were obtained with a polyclonal antibody raised against the bacterially expressed *trk* oncogene product $p70^{trk}$ (12) (data not shown). These results indicate that the product of the in vitro-engineered trk^{S345} oncogene retains kinase activity.

DISCUSSION

The *trk* locus codes for a receptorlike molecule whose transcripts appear to be exclusively localized in certain ganglia of the peripheral nervous system (9a). These observations suggest that this proto-oncogene may have a highly specialized role in the nervous system. Yet, the *trk* proto-oncogene can undergo genetic rearrangements that activate it as a transforming oncogene in at least two types of human

malignancies, colon carcinoma and papillary thyroid cancer (4, 10). All human *trk* oncogenes characterized so far result from the fusion of the kinase domain to sequences derived from unrelated loci. Two of the genes known to participate in the generation of human *trk* oncogenes have been identified as those coding for a nonmuscle tropomyosin and the ribosomal protein L7a (10, 22). The latter corresponds to a *trk* oncogene activated during transfection of human breast carcinoma DNA (8). The contribution of these unrelated genes to the malignant activation of the *trk* kinase is likely to involve not only those coding sequences present in the chimeric oncogene product but also their regulatory elements. This property will allow the ectopic expression of the *trk* tyrosine kinase outside its restricted physiological environment, a property required for the involvement of *trk* oncogenes in tumors of epithelial origin (4, 10).

The mechanism by which foreign coding sequences contribute to the activation of the *trk* kinase is not well understood (5). Preliminary analysis of the tropomyosin and L7a ribosomal protein-derived domains present in two human *trk* oncogenes did not reveal any obvious common structural features. In both cases however, the tropomyosin and L7a sequences replaced the extracellular domain of the *trk* receptor (10, 22). This results in chimeric molecules that cannot interact with cell membranes. However, cytoplasmic localization is not sufficient to confer neoplastic properties to *trk* oncogenes, since cytoplasmic *trk* chimeras in which tropomyosin sequences were replaced by those of actin or globin did not exhibit transforming activity (5).

The human *trk* proto-oncogene frequently becomes activated as a transforming gene. Transfection of NIH 3T3 cells with either the *trk* catalytic domain (with or without promoter sequences) or the entire *trk* proto-oncogene results in the generation of *trk* oncogenes in 1 out of 3,000 to 1 out of 10,000 transfected cells. Molecular characterization of some of these in vitro-generated transforming genes has revealed additional mechanisms by which the *trk* proto-oncogene can acquire transforming properties. Partial nucleotide sequence analysis of cDNA clones derived from two of these oncogenes, *trk*2 and *trk*4, revealed that association of the *trk* kinase with heterologous sequences is not essential to become oncogenic. Both *trk*2 and *trk*4 code for proteins that consist of a head-to-tail tandem arrangement of two tyrosine kinase catalytic domains. We have previously shown that expression of a single *trk* catalytic domain possessing a structure identical to the carboxy-terminal half of the products of the *trk*2 and *trk*4 oncogenes results in an enzymatically active but nontransforming protein (13). Therefore, it is likely that the amino-terminal half of the $p67^{trk2}$ and $p69^{trk4}$ oncoproteins can activate the carboxy-terminal kinase by the same mechanism with which tropomyosin and L7a sequences activate their respective *trk* oncogenes.

One such mechanism may involve *trans*-phosphorylation of their kinase domains. In the case of the colon carcinoma *trk* oncogene, the tropomyosin sequences may induce dimerization of its gene product (1), leading to the formation of homodimers in a fashion reminiscent of the oligomerization of growth factor receptors induced by their cognate ligands (17). In the case of the *trk*2 and *trk*4 oncogene products, the proposed *trans*-phosphorylation may simply occur intramolecularly. In the case of the *trk* oncogene activated by the L7a ribosomal protein, the amino acid sequence is not sufficiently informative to predict the formation of homodimers. The validity of such a model must await direct experimental analysis.

Molecular characterization of oncogenes generated by transfection of the entire *trk* cDNA clone indicated that the *trk* proto-oncogene can also become activated without losing its basic receptor structure (11). The nucleotide sequence of a partial cDNA clone of the *trk*5 oncogene revealed a 153-bp deletion in the region coding for the extracellular domain. The missing sequences are likely to have been looped out during transfection, a mutation that may have been facilitated by the presence of an 8-nucleotide-long direct repeat flanking the deleted sequences. The 51 amino acid residues coded for by these deleted sequences encompass a stretch of 26 amino acids, of which 20 are also present in the corresponding region of *trk*B, a highly related neurogenic receptor (7). This 51-amino-acid-long domain also contains 3 of the 13 putative N-glycosylation sites (two are conserved in the *trk*B product) and 1 of the 9 cysteines shared between the *trk* and *trk*B receptors (7, 11). Thus, this deleted region is likely to play an important role in regulating the *trk* receptor. Whether deletion of other sequences within the extracellular domain of the *trk* proto-oncogene product also results in its malignant activation awaits the molecular characterization of other pDM38-derived *trk* oncogenes.

The conserved nature of cysteine residues present in the ligand-binding domain of tyrosine protein kinase receptors has underscored their important role in maintaining the overall structure of these receptors (17, 21). Therefore, it was not a complete surprise that substitution of serine for Cys-345, the conserved cysteine residue deleted in the *trk*5 oncogene, resulted in its malignant activation. Activation of tyrosine protein kinase receptors by miscoding mutations has been previously documented for the *neu* (2,3) and CSF-1 receptors (15). In the case of the *neu* gene product, only certain substitutions within its transmembrane domain appear to have neoplastic consequences (3). In the case of the CSF-1 receptor, the activating mutation present in its transforming allele v-*fms* has been mapped in the extracellular domain but involved a leucine residue (15). Whereas the transmembrane mutation renders the *neu* gene a fully transforming oncogene, the extracellular mutations present in v-*fms* and engineered in the *trk* cDNA clone confer only moderate transforming activities. Nevertheless, these results demonstrate that subtle changes in the extracellular domain of the *trk* receptor can have profound consequences in its ability to induce malignant transformation.

In summary, our studies indicate that the human *trk* locus can become an oncogene by a variety of mutations. Formation of chimeric molecules expressed under the control of heterologous promoters appears to be the most favored mechanism encountered in human tumors (4, 10). These observations may reflect the selective advantage of such rearrangements, considering the otherwise limited range of expression of the endogenous *trk* promoter (9a). However, the high transforming activity of the *trk*5 oncogene illustrates that small mutations within this locus can also lead to its malignant activation. Whether such putative mutations are involved in the development of neural tumors or in some other type of neurological abnormalities remains to be determined. Finally, the recent results of Bongarzone et al. describing the presence of *trk* oncogenes in a significant fraction of papillary thyroid carcinomas (4) demonstrate that malignant activation of *trk* sequences in human cancer is not limited to sporadic cases (10). Considering the multiple mechanisms by which this tyrosine protein kinase proto-oncogene can acquire neoplastic properties, its contribution to human neoplasia may be wider than previously suspected.

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