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Tumor Cell Growth Arrest Caused by Subchromosomal Transferable DNA Fragments from Chromosome 11

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A fundamental problem in the identification and isolation of tumor suppressor and other growth-inhibiting genes is the loss of power of genetic complementation at the subchromosomal level. A direct genetic strategy was developed to isolate subchromosomal transferable fragments (STFs) from any chromosome, each containing a selectable marker within the human DNA, that could be transferred to any mammalian cell. As a test of the method, several overlapping STFs from 11p15 were shown to cause in vitro growth arrest of rhabdomyosarcoma cells. This activity mapped between the β-globin and insulin genes.

The existence of tumor suppressor genes was first established by genetic complementation, which demonstrated that tumor cells fused to normal cells lose tumorigenicity (*1*). Studies have shown that suppression can also be detected by transfer of monochromosomes into tumor cells (*2*, *3*). However, direct expression cloning of suppressor genes in manageable vectors usually is not possible because growth suppression is normally selected against. Furthermore, although yeast artificial chromosomes (YACs) have been transferred to mammalian cells (*4*), success has been limited to small genes and specific cell types, and assaying for tumor suppression with the thousands of YACs needed for a whole chromosome is impractical. We therefore sought to develop a strategy for transferring subchromosomal fragments intermediate in size between YACs and chromosomes.

Our strategy involves three steps, outlined in Fig. 1: (i) transfection of a mammalian selectable marker gene into mouse cells containing a single independently selectable human chromosome; (ii) transfer of the chromosome by microcell fusion, followed by double selection for both the human chromosome and the marker gene; and (iii) isolation of individual marker-containing chromosomal subfragments by transfer of irradiated microcells from the pooled hybrid panel. Unlike conventional radiation hybrids (*5*), each resulting fragment can then be transferred independently to mammalian cells, owing to the presence of the selectable marker gene.

We isolated 150 separate hybrids of monochromosome 11 that were resistant to selection in hypoxanthine, aminopterin, and thymidine (HAT^R) plus G418 ($G418^R$). We transferred irradiated microcells from 90 of the hybrids, isolating 85 *neo*-containing subfragments of chromosome 11. Of these, 14 were positive by Southern (DNA) blotting for 11p15 sequences, representing 12 independent *neo*-integration sites (a result expected from random *neo* integration in the original transfection). Nine 11p15 subfragments were transferred from A9 cells to Chinese hamster ovary (CHO) cells by microcell fusion, and the pulsed-field gel electrophoresis (PFGE) pattern of donor cell DNA, digested with rare-cutting restriction enzymes, was compared to that of the recipient cells. Hybridization with a human repetitive sequence allowed visualization of the individual human PFGE fragments. The amount of human sequence (sum of the PFGE fragments) could be estimated in seven of nine cases with <10 megabase pairs (Mbp) and ranged from 3.5 to 9.5 Mbp (average, 6.8 Mbp). Eight of nine hybrids showed identical PFGE patterns in donor A9 and recipient CHO cells (Fig. 2), and one showed a different pattern of a single band, possibly resulting from methylation differences or rearrangement. Thus, 61 of 62 PFGE fragments within the chromosomal subfragments remained unchanged after transfer. In addition, pSV2neo always mapped to Alu-positive human PFGE fragments (Fig. 2). Thus, these chromosomal subfragments were intermediate in size between YACs and chromosomal bands, contained a selectable marker within the human DNA, and were stably transferable to mammalian cells. We therefore termed these chromosomal fragments "subchromosomal transferable fragments," or STFs, to distinguish them from conventional nontransferable radiation hybrid fragments.

We used rhabdomyosarcoma and 11p15 for experiments on tumor suppression for the following reasons: (i) 11p15 shows loss of allelic heterozygosity (LOH, implying the presence of a tumor suppressor gene) in many types of tumor, including rhabdomyosarcoma, Wilms tumor, and other embryonal tumors (6–9), as well as tumors of the bladder, lung, ovary, liver, and breast (*10*–

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15); (ii) 11p15 is a large band, and the LOH data as a whole do not provide precise sublocalization within the band and may represent multiple tumor suppressor genes (10–15) [similarly, experiments on chromosome deletion have localized a suppressor gene to the very large region p14-p15.5 (16)]; (iii) Wilms tumor also involves one or more 11p13 genes (17, 18); and (iv) rhabdomyosarcoma cells undergo in vitro growth arrest after introduction of chromosome 11 (19), unlike Wilms tumor (2), and thus the phenotype should be relatively easy to score.

To assay for growth suppression, we transferred ten 11p15 and two 11q STFs by microcell fusion into the rhabdomyocarcoma cell line RD. After an initial period of growth, RD cells that had received any of eight 11p15 STFs became flat, markedly enlarged, and elongated (Fig. 3A), in contrast to RD cells that had received the other four STFs (Fig. 3B). In all cases, morphologically altered RD cells stopped growing, usually within 2 to 4 weeks after transfer (five to seven population doublings), with a final colony size of $<10^2$ cells (Fig. 3C).

In each of six experiments with differing donor STFs and for which recipient DNA could be obtained, the pSV2neo gene was found by polymerase chain reaction (PCR) to be present in the growth-arrested colonies (data not shown). The pSV2neo was also present in growing colonies that had received nonsuppressing STFs, as shown by conventional Southern blotting. Thus, donor DNA was present in these cells. G418 toxicity due to impaired *neo* expression could not account for the growth arrest because withdrawal of G418 did not alter the growth-arrested phenotype. A specific mouse sequence could not have produced suppression because the mouse chromosome was presumably different for each suppressing human fragment. However, we confirmed this directly by karyotyping five RD-suppressing STFs with distinct *neo*-integration sites (19a).

Furthermore, we could demonstrate that specific human DNA sequences had been transferred to the tumor cells. Two growth-suppressing STFs contained the human β-globin (HBB) gene, which allowed us to exploit a DNA sequence polymorphism detected by single-stranded conformational polymorphism (SSCP)–PCR analysis (20) and distinguish between the donor and recipient DNA. In each case tested, the growth-arrested colonies contained the donor HBB gene (Fig. 4A). Similarly, growing RD cells that had received a nonsuppressing STF containing the H-*ras* gene retained the transferred gene, as shown by conventional Southern blotting, which detects a Taq I polymorphism in the H-*ras* gene (Fig. 4B). Thus, the colonies did not fail to proliferate simply as a result of loss of the selectable marker, and the introduced fragments were clearly present in the growth-arrested cells. The suppressor effect of the transferred STFs also appeared to be specific to the human DNA because STFs from 11q did not cause growth arrest and because a growth-suppressing STF from 11p15 did not suppress growth of a human cervical cancer cell line (3).

Many STFs were chimeras of smaller subfragments, each containing contiguous genetic markers, as is also seen with conventional nontransferable radiation hybrids. Our analysis revealed a statistically significant association of suppression with the presence of one or more of the contiguous markers D11S719, HBB, and TA9, the latter a clone isolated by inter-Alu PCR (21) from one of the STFs (Fig. 5A), although the remainder of 11p15.5 could not be excluded by statistical analysis. However, there was an inverse correlation between RD suppression and the presence of DNA from band 11p15.4 and band 11p13, the latter containing the WT1 gene (Fig. 5A), further implicating 11p15 rather than 11p13 in the suppression of RD cells.

Because of the apparent localization of a suppressor gene to a region near HBB, a long-range restriction map of this area was constructed. All but one of the growth-suppressing STFs contained DNA from the region previously identified as most likely by concordance analysis. Two nonchimeric

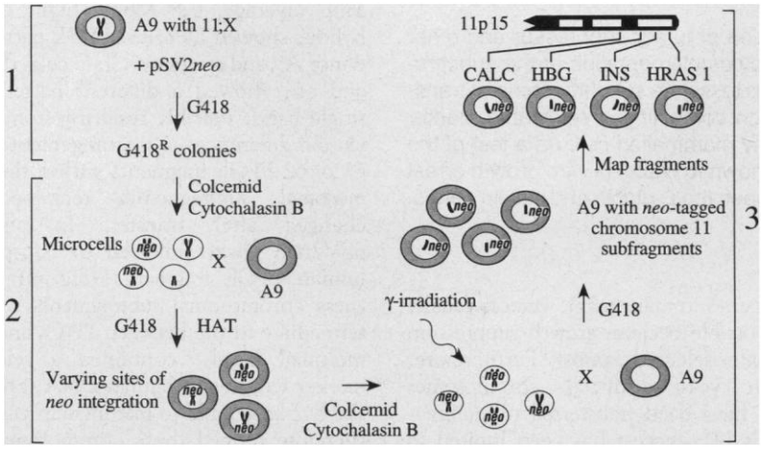

Fig. 1. Strategy for generating STFs. In step 1, a dominant mammalian selectable marker gene (pSV2neo) was transfected into an independently selectable human monochromosome hybrid on a mouse (A9) background [because chromosome 11 is one of the few nonselectable human chromosomes, we began with a t(11;X) chromosome containing the HRPT gene (18)]. After selection for G418, surviving colonies include those with *neo* integrated randomly throughout chromosome 11, as well as colonies with *neo* integrated onto one of the mouse chromosomes. In step 2, 18,000 G418-resistant colonies from step 1 were pooled, monochromosome transfer to A9 cells was done by microcell fusion (31), and colonies were double-selected in G418 plus HAT. Cells survived double selection if they received the human chromosome t(11;X) that contained a copy of *neo*. In step 3, 90 $G418^R$ HAT^R colonies were pooled, and microcells prepared from them were γ-irradiated with 10,000 rad and fused to A9 cells. Eighty-five $G418^R$ hybrids were then isolated and screened for chromosome 11 sequences by Southern blotting. In the experiments described here, the *neo*-containing 11p15 fragments were purified from other non–*neo*-tagged human DNA fragments by a subsequent transfer of microcells to A9 cells.

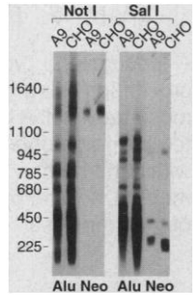

Fig. 2. Stable transfer of 11p15 STFs from mouse A9 to CHO cells, shown by hybridization of human Alu repeat and pSV2neo (32) probes to Not I and Sal I digests separated by PFGE. Cells (10^6 per block) were embedded in 1.25% low melting point agarose blocks containing 62.5 mM EDTA and treated with proteinase K (1 mg/ml), 1% N-lauroyl sarcosine, 0.5 M EDTA at 50°C, and stored in 0.5 M EDTA at 4°C until further use. Blocks were extensively dialyzed against reaction buffer and incubated with 22 U of Not I or 40 U of Sal I for 8 to 12 hours at 37°C. We separated DNA by field-inversion gel electrophoresis in 1% agarose using a DNAstar pulse electrophoresis programmer at 220 V for 36 hours at 4°C, with a 30- to 150-s ramp at a 3:1 ratio. Molecular size standards (shown on the left in kilobase pairs) were *Saccharomyces cerevisiae* AB1380 chromosomes in blocks. DNA was transferred to GeneScreen (NEN) filters under alkaline conditions (33). Radioactively labeled probes were prepared by the method of Feinberg and Vogelstein (34), and hybridization was prepared by the method of Church and Gilbert (33). The figure shows one of the largest STFs, containing 8.2 Mbp, with the greatest number of transferred PFGE fragments.

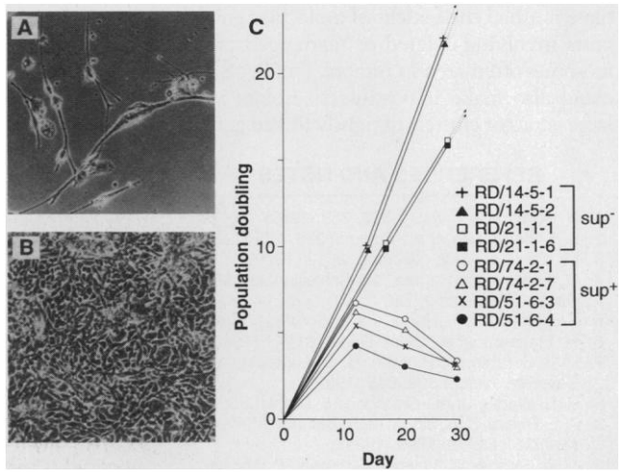

Fig. 3. In vitro growth arrest induced by specific STFs. Representative photomicrographs (magnification, ×100) are shown of RD cells that had received specific STFs. (**A**) Growth-arrested RD cells that had received a D11S719$^+$ HRAS$^-$ STF and (**B**) growing RD cells that had received a D11S719$^-$ HRAS$^+$ STF, at 2 weeks after transfer. (**C**) Growth rate of two colonies each of RD cells that had received either of two suppressing (sup$^+$) STFs (A9/74-2 and A9/51-6, both of which are D11S719$^+$ HRAS$^-$) or two nonsuppressing (sup$^-$) STFs (A9/21-1, which is D11S719$^-$ HRAS$^+$, and A9/14-5, which is D11S719$^-$ INS$^+$). We determined the number of population doublings by monitoring total cell number after transfer. Colony sizes of growth-arrested colonies was determined by observation. If the number of cells in a colony exceeded more than 200, total cell number of the colony was extrapolated from a portion of the colony. For growing colonies, cell number was determined by weekly passage of 10^5 cells per 10-cm^2 dish. The suppressing STFs cause growth arrest of RD cells within 4 weeks after transfer. All cells containing donor STFs as well as the recipient RD cells were tested negative for mycoplasma contamination.

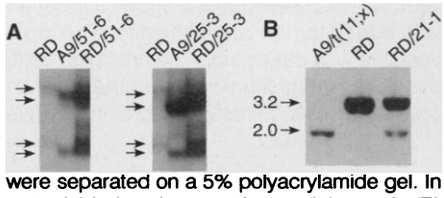

Fig. 4. Presence of STFs in recipient RD cells. (**A**) Detection by SSCP-PCR (*20*) of the donor human β-globin gene in pooled growth-arrested RD colonies (RD/51-6 and RD/25-3), which had received two different STFs (A9/51-6 and A9/25-3). The PCR primers were end-labeled 5′-ATGTTGATGCTGGATAGAGG-3′ and 5′-ATTCTCTTGAGACTACATTG-3′ (*20*). PCR products were separated on a 5% polyacrylamide gel. In the growth-arrested cells, both donor and recipient β-globin bands were detected (arrows). (**B**) Southern blot analysis of DNA from growing RD cells containing a nonsuppressing STF. DNA from the parent donor chromosome and a growing recipient colony (RD/21-1) that had received STF A9/21-1 was digested with Taq I, electrophoresed in 1% agarose, transferred to GeneScreen, and hybridized with an H-*ras* probe (*32*). The larger allele is significantly darker because the probe contains a variable number tandem repeat. Molecular size markers are indicated on the left (in kilobase pairs).

A

	11p13 CAT-WT1-S16-S441	11p15.4 PTH-CALC-S501-S431-S466-S776	11p15.5 TA9-HBB-S719	11p15.5 S12-Z104-S517-S648-S724	11p15.5 H19-IGF2-INS-HRAS
sup$^+$ colonies	0	18	39	30	24
total colonies	12	44	51	58	50
% sup$^+$	0	41	77	52	48
P	< 0.001 (sup$^-$)	< 0.001 (sup$^-$)	< 0.001 (sup$^+$)	NS	NS

B

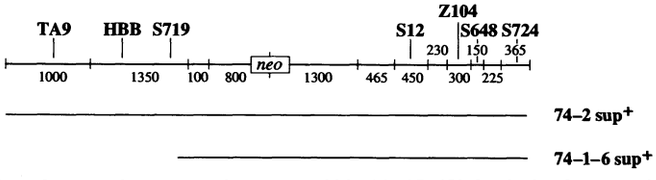

Fig. 5. Sublocalization of a growth-suppressing gene within 11p15. (**A**) Analysis of concordance of 11p markers with growth arrest. The total number of growing and nongrowing colonies after transfer of each STF was obtained from at least two separate experiments. Each STF was analyzed for the presence of a panel of 23 11p13 to 11p15 markers (*32*). The number of total and growth-suppressed colonies (sup$^+$) that had received each locus was determined. Statistical significance (*P*) of growth suppression (sup$^+$) or its absence (sup$^-$) was determined by chi-square analysis, which compared each region to the others taken as a group. NS, not significant. (**B**) Physical map of two nonchimeric STFs with a common *neo*-integration site. Agarose blocks (1.25%) containing STF donor cells were digested with Bss HII under complete (8 U, 16 hours at 50°C) or partial (0.32 U, from 5 min to 4 hours at 50°C) conditions and separated by clamped homogeneous electric fields (CHEF) with a Pulsaphor electrophoresis unit (Pharmacia) in 0.5× tris-borate EDTA at 14°C with constant voltage of 170 V for 25 hours. The stepped switching interval was 120 s for the initial 12.5 hours and 60 s for the next 12.5 hours. DNA was transferred to Hybond-N$^+$ (Amersham) and hybridized with D11S724, D11S648, D11S719, D11S12, Z104, HBB, TA9, and pSV2neo. All but four Alu-containing CHEF fragments of A9/74-2 were recognized by one of these probes, and the remaining four fragments were localized by ordered partial digests with the use of flanking markers. Numbers indicate sizes of restriction fragments (in kilobase pairs).

STFs further localized the suppressor gene between D11S719 and D11S724 (Fig. 5B). Thus, the gene appears to reside between, but is excluded from, two regions previously proposed to contain it, namely, the region near the calcitonin gene (*22*) and the insulin–insulin-like growth factor II (IGF2) cluster (*23, 24*). This same region also lies directly between two clusters of germline translocation breakpoints in patients with Beckwith-Wiedemann syndrome (BWS), which predisposes to rhabdomyosarcoma and other tumors (*25*), and in the region of uniparental disomy in BWS (*26*).

A question for further study is whether this gene can cause senescence of nonmalignant cells [although chromosome 11 is excluded from three of the four known senescence complementation groups (*27*)] or whether, like p53, it specifically inhibits the growth of tumor cells lacking a normal copy (*28*). Consistent with the latter idea is the finding that human mammary cells immortalized by SV40 DNA lose heterozygosity at 11p15 (*29*). Because breast cancers also commonly lose 11p15 (*11*), loss of an 11p15 gene may serve an immortalization function in breast cancer progression (*29*).

The ability to transfer STFs intermediate in size between YACs and whole chromosomes has several advantages over current technology. STFs shown to carry a functional gene could be used to generate a physical map as well as closely linked probes, as was done in the present study. The physical map agrees with a complete genomic physical map (*30*). STFs could also make it possible to dissociate complex phenotypes caused by a whole chromosome. For example, 11p15 may contain both tumor-inhibiting as well as tumor-promoting genes, such as IGF2. Because they are larger than YACs, STFs make it practical to perform complementation experiments where the precise localization is not known, a particular problem in the isolation of cellular senescence genes, which

have not had the benefit of molecular guideposts involving deleted or rearranged chromosomes often seen in tumors. Finally, STFs could also make it possible to isolate very large genes or clusters of tightly linked genes.

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Import of a Mitochondrial Presequence into Protein-Free Phospholipid Vesicles

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A synthetic mitochondrial presequence has been shown to translocate across pure phospholipid bilayers. The presequence was fluorescently labeled so that its association with membranes could be monitored spectroscopically. In the presence of large unilamellar vesicles, the presequence showed time- and potential-dependent protection from reaction with added trypsin and dithionite. The protection was rapidly reversed by treatment of the vesicles with detergent. If the vesicles contained trypsin, the added presequence became sensitive to digestion by the protease. The results show that a mitochondrial presequence can translocate across phospholipid bilayers that lack a hydrophilic translocation pore.

The translocation of proteins across biological membranes is a critical step in the biogenesis of organelles and in the secretion of proteins from prokaryotic and eukaryotic cells. It has been proposed that translocated proteins must pass through a hydrophilic environment in the interior of putative translocator proteins (*1*), but the precise mechanistic role of these proteins has not yet been demonstrated. Alternatively, it has been suggested that translocated proteins may be able to pass directly through the lipid bilayer without the aid of a translocation catalyst (*2*). Support for the role of lipids in protein translocation has come from studies with synthetic targeting sequences. Peptides corresponding to the signal sequences of secreted proteins and to the targeting sequences of mitochondrial and chloroplast proteins have been found to have substantial affinity for phospholipid monolayers and bilayers in model membranes (*3*, *4*). It could be argued that these models do not accurately reflect the behavior of transported proteins in biological systems. However, the recent demonstration that synthetic mitochondrial presequences are rapidly and efficiently imported into isolated mitochondria (*5*, *6*) in a process that involves an initial association of the presequence with the lipid bilayer (*6*) also suggests that affinity for the bilayer is required. At the least, these results suggest that the synthetic molecules can serve as useful probes of the normal protein translocation pathway. In the experiments described here, we have shown that a fluorescently labeled, synthetic mitochondrial presequence can be imported in a potential-dependent manner into pure phospholipid vesicles. The results suggest that mitochondrial presequences may have the inherent ability to pass through phospholipid bilayers and that this ability may be essential for the proper sorting of precursors destined for the mitochondria.

The synthetic peptide used in these studies corresponds to the amino-terminal 25 residues that form the presequence of the precursor of yeast cytochrome oxidase subunit IV (CoxIV). The peptide was labeled with *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ethylenediamine (IANBD amide) at its unique cysteine (residue 19) and purified by high-performance liquid chromatography (HPLC). Binding of the labeled presequence to phospholipid vesicles was rapid and resulted in a large enhancement of the fluorescence of the 7-nitro-2,1,3-benzoxadiazolyl (NBD) group. Measurement of this enhancement as a function of the concentration of the vesicles allowed the affinity of the presequence for the vesicles to be determined (Fig. 1).

Translocation of the presequence into the vesicles was assayed by three independent methods. In the first, suspensions of vesicles with bound presequences were treated with trypsin to digest all presequences remaining outside the vesicles. This treatment resulted in a rapid decrease in the fluorescence, because the fluorescent fragment generated by the proteolysis does

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