Selection for Precise Chromosomal Targeting of a Dominant Marker by Homologous Recombination

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The antibiotic resistance gene neomycin phosphotransferase (*neo*) has been precisely targeted to a chromosomal region close to the cystic fibrosis (CF) locus on chromosome 7. The chromosomal target was the expressed SV40 array integrated at chromosome 7, band q31–q35 in a human-mouse hybrid cell line that contains chromosome 7 as the only human component. Stringent selection for *neo* expression by homologous recombination (3 of 11 correctly targeted) was achieved by fusing the SV40 large T antigen gene, in frame, to *neo* in a promoterless construct, such that G418 resistance depended on endogenous promoter function and read-through transcription. Chromosome-mediated gene transfer (CMGT) with G418 selection was then used to generate mouse hybrids that carried the targeted locus intact, but retained only a fragment of human chromosome 7. This gene targeting strategy will access new regions of the human (or other mammalian) genome, create precise mutations efficiently by gene disruption, and potentially restore normal gene function by mutation correction.

THE DEFECTIVE GENE PRODUCT IS only known for a minority of developmental defects and inherited diseases in mouse and human. However, recent advances in physical and recombinational mapping procedures have allowed an increasing number of important phenotypic traits to be mapped to particular chromosome regions. A variety of strategies have been proposed and applied to the "reverse genetic" process of moving from linked markers to disease genes (*1*). Chromosome-mediated gene transfer (CMGT) can be used to isolate subchromosomal fragments of human DNA in rodent cells (or vice versa) that span the region of interest and thus provide an enriched source of molecular clones for the region (*2–4*). The success of this approach depends on the chromosome donor cell line carrying a dominant selectable marker that maps close to the locus of interest. Despite the recent development of novel selection strategies (*3–5*), exploitable markers are still limited. It has been shown that specific endogenous loci in mammalian somatic and embryonal stem cells can be modified by introducing exogenous DNA via homologous recombination (*6–8*). However, this process is very inefficient (*6, 8*). Consequently, unless integration at the target locus itself confers a selectable phenotype, screening for the rare homologous recombinant becomes a limiting factor. In particular, a substantial increase in the efficiency of precise targeting is necessary to exploit this attractive approach for gene replacement therapy.

We therefore devised a strategy, based on endogenous promoter control and production of read-through transcripts, as a stringent selection for precise chromosomal targeting. We chose the SV40 gene array in the human-mouse hybrid cell line Cl21 as a target locus to test the strategy for the following reasons: (i) the complete sequence of SV40 is available, which facilitated vector construction; (ii) our strategy relies on a functional promoter at the target site and SV40 large T antigen is constitutively expressed in Cl21; (iii) each of the one to four copies of human chromosome 7 that are retained as the sole human component in Cl21 carries an identical and stable array of SV40 integrated at a single chromosomal site (*9*), increasing the potential target size and facilitating the identification of putative homologous integrants; and (iv) the site of SV40 integration lies between chromosome 7 (q31–q35) (*10*), close to the cystic fibrosis (CF) locus at chromosome 7 (q22–q31) (*11*).

Vector design and construction (*12*) involved the in-frame fusion of a promoterless *neo* 3′ to a 2.7-kbp portion of SV40 large T antigen (pTAGNEO), such that only integration events close to a functional promoter and maintaining an open reading frame into *neo* could generate G418-resistant colonies. Homologous recombination can occur by a simple insertion event with no loss of genomic DNA, or by a replacement event where the genomic DNA lying between the two sites of recombination is lost (*8*). pTAGNEO is similar in general design to the insertional vector type as described by Thomas and Capecchi (*8*) in which the ends of the linearized vector lie adjacent to one another on the target sequence and recombination, via the double-stranded break, results in the vector being inserted into the endogenous gene. In the alternative replacement vector design, linearized vector is colinear with the endogenous DNA. Because our target is made up of a head-to-tail linear array of SV40 genomes, our "insertional" vector has the potential for legitimate se-

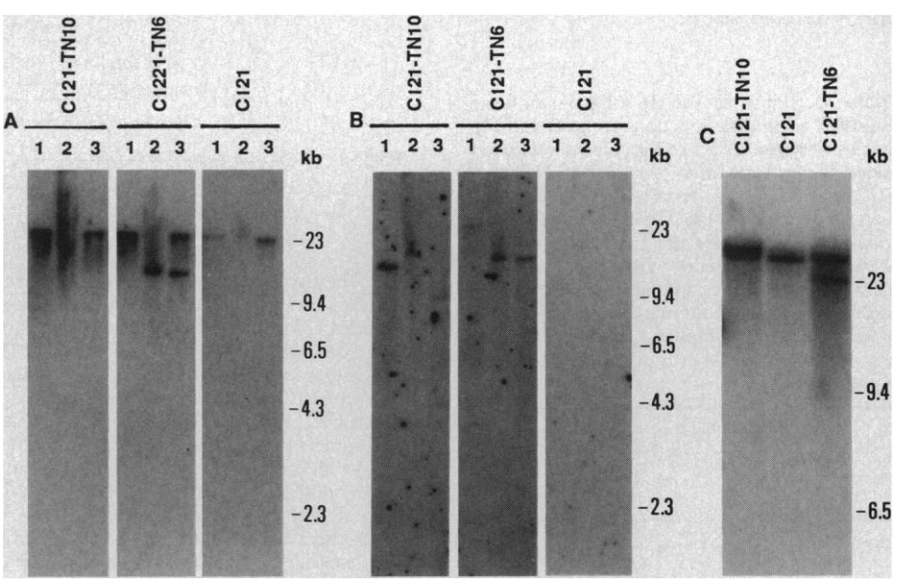

Fig. 1. Screening Cl21 G418-resistant clones for correct targeting of pTAGNEO by restriction mapping. (**A**) DNAs (10 μg) from Cl21 and G418-resistant clones generated by pTAGNEO transfection were digested with restriction endonuclease and loaded onto an agarose gel as indicated. DNAs were separated by electrophoresis, transferred to Hybond N (Amersham), and hybridized to a ^{32}P-labeled probe A (*15*). (**B**) The blot shown in (A) was washed and reprobed with ^{32}P-labeled *neo* probe. (**C**) DNA from two G418-resistant colonies and Cl21 were digested with Bgl II and separated by electrophoresis on a 0.5% agarose gel. Gels were blotted and probed as for (A). Lane 1, Bgl II; lane 2, Sma I; and lane 3, Bgl II and Sma I.

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quence replacement.

To confirm that pTAGNEO could produce functional neomycin phosphotransferase, a vector modified only by the addition of SV40 early promoter and enhancer sequences (designated p(pro+)TAGNEO (*12*)) was electroporated as circular plasmid DNA into mouse Cl27 cells (containing target SV40 DNA) (Table 1). G418-resistant colonies arose at a frequency at least equal to that obtained with pSV2neo (*13*). The G418-resistant mouse Cl27 cells were also positive for SV40 T antigen [as detected by monoclonal antibody (*14*)], and DNA probes specific to both SV40 and *neo* hybridized to the same sized RNA transcript (*14*). Digestion with Bst XI linearizes the vector within the T antigen moiety, dissociating the promoter elements from *neo* and reducing substantially the frequency of G418-resistant clones (Table 1). Substituting p(pro$^+$)TAGNEO with pTAGNEO further reduces the number of G418-resistant clones, consistent with the imposition of a strong selection for integrants into com-

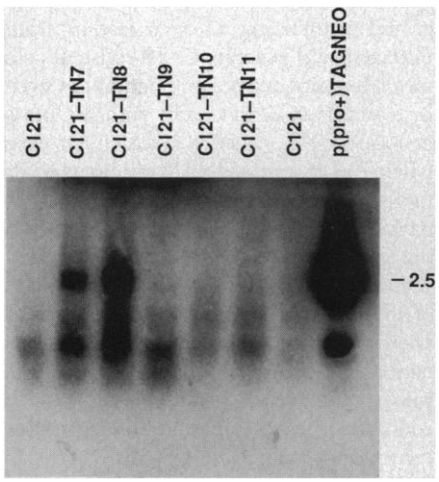

Fig. 2. Screening Cl21 G418-resistant clones for correct targeting of pTAGNEO by PCR. PCR reactions were carried out (*16*) and 1/10 of each sample was subjected to electrophoresis. The DNA was then transferred onto Hybond N and hybridized to oligonucleotide 116 (*16*). The marker size is in kilobases.

Table 1. Ability of the T antigen–*neo* fusion transcript to produce functional enzyme and confer G418 resistance to sensitive cells was determined by electroporating p(pro$^+$)TAGNEO into Cl27 or Cl21 cells as described (*20*). Linearizing the construct as the Bst XI site in the T antigen coding region reduces the frequency of G418-resistant colonies obtained from both cell types. pTAGNEO generates significantly fewer G418-resistant colonies (about one-third as many) than p(pro$^+$)TAGNEO, implying that the promoterless construct will enrich for integration into expressed sequences.

Plasmid	Cell line	Number of G418-resistant colonies per 10^6 cells
Intact p(pro$^+$)TAGNEO	Cl27	5×10^2
	Cl21	3.6×10^3
Linear p(pro$^+$)TAGNEO	Cl27	19
		74
	Cl21	600
		600
Linear pTAGNEO	Cl27	0
		1
	Cl21	18
		6

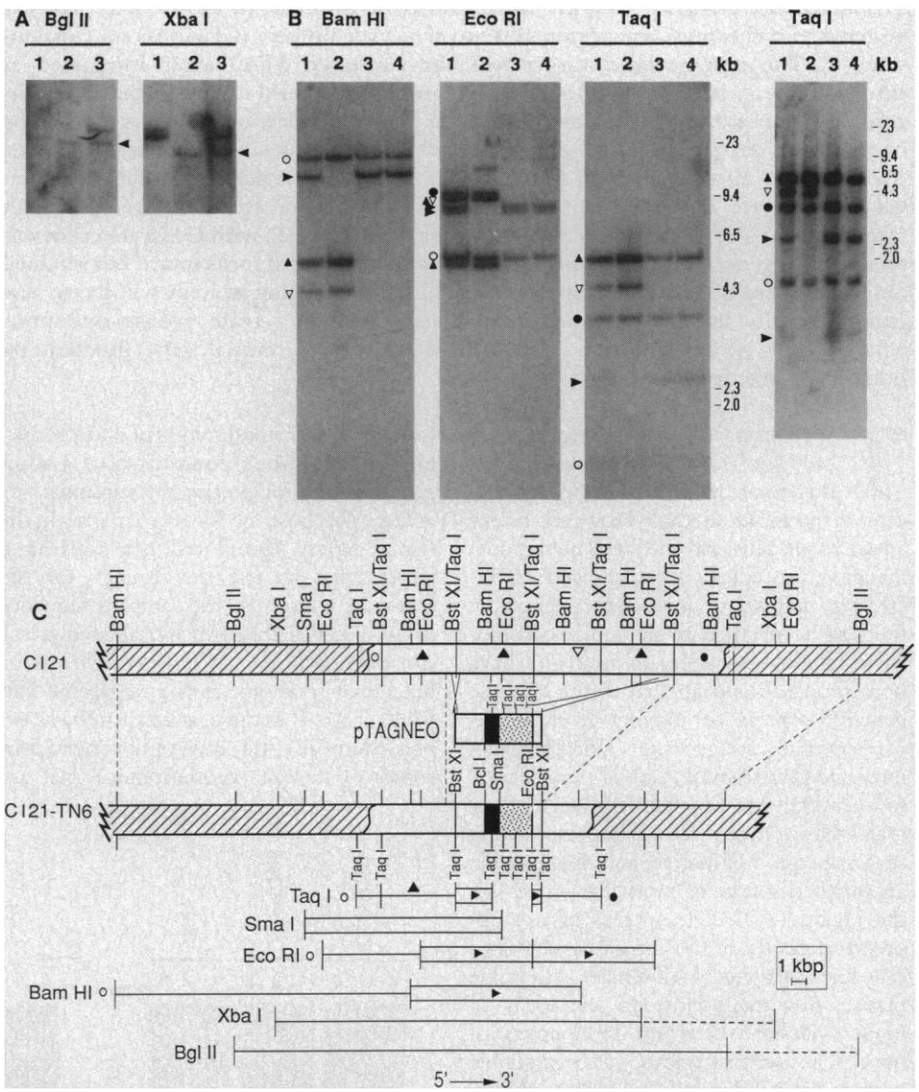

Fig. 3. The hybridization pattern of SV40 to the TN6-CMGT transfectants. (**A**) DNAs from Cl21, Cl21-TN6, and one of the CMGT-derived transfectants TN6-CMGT1 were digested with restriction endonuclease, loaded onto a 0.5% agarose gel, and subjected to DNA blot analysis as indicated. Blots were probed with ^{32}P-labeled intact SV40. The novel Bgl II and Xba I gel fragments caused by pTAGNEO integration are arrowed. These fragments were transferred intact to TN6-CMGT1. The Bgl II fragment in Cl21 is reduced in size because of a gel artifact that is not evident in Fig. 1C. Lane 1, Cl21; lane 2, TN6-CMGT1; lane 3, Cl21-TN6. (**B**) DNAs from both CMGT transfectants, the chromosome donor and homologous recombinant Cl21-TN6, together with the progenitor Cl21 were digested with various restriction enzymes and analyzed as in (A). Gel fragments representing unit length SV40 are indicated with a closed triangle and deleted SV40 with an open triangle. The gel fragments contiguous with human DNA are indicated with an open circle for the 5′ side of the array and a closed circle for the 3′ side of the array. Novel fragments generated by pTAGNEO integration are arrowed. The orientation of the array is designated by direction of SV40 T antigen transcription. The same symbols are used to identify corresponding fragments in (C). The Taq I gel on the far right reveals the 400-bp novel fragment not evident on the full-length gel. Bands in the Eco RI digests that are not annotated are caused by partial digestion. Lane 1, Cl21-TN6; lane 2, Cl21; lane 3, TN6-CMGT-1; and lane 4, TN6-CMGT-2. (**C**) A restriction map of Cl21 (indicating the DNA fragments generated by Taq I digestion) and Cl21-TN6 (indicating the DNA fragments generated by digestion with the enzymes in Figs. 1 and 3). DNA from human chromosome 7, hatched; SV40 blank; *neo*, solid; and pBluescribe, stippled. SV40 hybridizing restriction fragments that are preserved (solid line), truncated (dotted line), or novel (double solid line), in Cl21-TN6 and in TN6-CMGT1 and CMGT-2, compared with Cl21.

patible expressed sequences.

Of the 24 Cl21 G418-resistant colonies arising from electroporation with pTAGNEO, six were grown up and analyzed by restriction mapping to determine whether a correctly targeted event had occurred. There are no restriction sites for Bgl II, Sma I, or Xba I in SV40. The SV40 array in Cl21 flanked by genomic DNA sites for Bgl II (which generates an ~40-kbp fragment); Sma I (which generates a >50-kbp fragment); and Xba I (which generates an ~34-kbp fragment). Homologous recombination will introduce the Sma I site from *neo* into this array. By whatever mechanism (insertional or "replacement"), digestion with both Bgl II and Sma I will generate new restriction fragments of predictable size (Fig. 1A).

In Cl21-TN6 (one of the six colonies analyzed in detail), probe A (*15*) (an SV40 fragment not present in pTAGNEO) hybridizes to an additional Bgl II fragment and a novel 12-kbp Sma I fragment. When digested with both Bgl II and Sma I together, the 12-kbp Sma I fragment is still the only visible band not present in Cl21. This novel Sma I fragment hybridizes to a probe specific for *neo* (Fig. 1B), but not to one for plasmid DNA. Cl21-TN10 and the other four G418-resistant clones all have a single vector-specific Bgl II band, indicating single sites of integration in these clones but with unresolvable Sma I fragments, indicating nonhomologous integration. A low percentage gel with Bgl II–digested Cl21-TN6 DNA hybridized with probe A (*15*) is shown in Fig. 1C, where the size reduction of the new Bgl II fragment can be seen more clearly. The fact that this fragment has reduced in size by ~9 kbp is compatible only with homologous recombination by the replacement mechanism.

Five independent G418-resistant colonies were isolated from a further experiment carried out under identical conditions. These colonies were analyzed for homologous recombination by the polymerase chain reaction (PCR) (*16*). Two amplifying oligonucleotides, separated by 2.5 kbp on p(pro$^+$)TAGNEO, were prepared (*16*): one complementary to the coding strand of *neo*; and the other to the opposite strand of the SV40 promoter, and not contained in pTAGNEO. Thus only those G418-resistant clones targeted with pTAGNEO and generated by homologous recombination will amplify a 2.5-kbp fragment that can hybridize to an internal oligonucleotide probe (*16*). By this criteria, two further transfectants (TN7 and TN8) were identified as homologous recombinants (Fig. 2), giving a total of 3 of the 11 G418-resistant clones analyzed.

Analysis of Cl21-TN6 is complicated by the presence of additional nontargeted copies of chromosome 7. We therefore transferred chromosomes from Cl21-TN6 to mouse Cl27 cells under selection for G418 resistance (*2–5, 17*). This enabled us to (i) confirm and extend the restriction map of the homologous recombination event, and (ii) test the CMGT strategy for specific fragmentation and isolation of human chromosome 7 DNA. Two Cl21-TN6–derived CMGT cell lines (TN6-CMGT1 and TN6-CMGT2) retained a subset of the Cl21-TN6 SV40 hybridizing fragments that is specific and complete for the targeted chromosome. The large, unique Bgl II and Xba I fragments described above were transferred intact to TN6-CMGT1 and TN6-CMGT2 (Fig. 3A). Digestion with enzymes that cut within SV40 mapped the pTAGNEO recombination event precisely. In Cl21, the SV40 array consists of three complete SV40 genomes, a deleted SV40 genome (which spans the Eco RI but not the Bam HI restriction site), and regions of SV40 that run into flanking DNA (*9*). Digestion of Cl21 with Eco RI produces fragments of unit length SV40 (5.2 kbp), unit length plus deleted SV40 (9.4 kbp), and two flanking fragments of ~5.5 and 9.6 kbp (Fig. 3B). Digestion with Bam HI produces a unit length SV40, a deleted SV40 (fragment of ~4.2 kbp), and a 5′ flanking band (of ~19 kbp). The 3′ flanking fragment is not resolvable on DNA blot analysis. Analysis of Eco RI– and Bam HI–digested TN6-CMGT1 and TN6-CMGT2 showed that the targeted chromosome retains the 5′ flanking sites, but that the intact and deleted SV40 genomes are lost. A single new band was observed in the Bam HI digest containing the exogenous DNA (there is no Bam HI site in the targeting vector) and the SV40 DNA lying between the retained Bam HI sites. Two new bands were observed in the Eco RI digests. Only the larger of these new Eco RI fragments hybridized with probe A. The 5′ flanking Eco RI fragment was retained and one new fragment lies between the retained SV40 site and the Eco RI site in the vector. The second novel Eco RI fragment is from the vector site to the 3′ flanking human DNA. Taq I digests reveal that both 3′ and 5′ flanking fragments are present and, more importantly, unit length SV40 is also present. This places the recombination event precisely within the head-to-tail array of SV40 on the human chromosome 7 of Cl21. The novel 2.4-kbp fragment observed in the Taq I digests (Fig. 3B) lies between the site in the vector-derived SV40 and a site in *neo*, 430-bp downstream from Bcl I. The other SV40-containing Taq I fragment, derived from the plasmid to endogenous SV40, is only 400 bp in size (evident in Fig. 3B). A graphic interpretation of the results is shown in Fig. 3C.

One of the CMGT transfectant lines probed with pBK1.8, a cloned member of the human L1 class of dispersed repeated sequences (*3, 18*) is shown in Fig. 4. The hybridization to Cl21 and Cl21-TN6 represents the L1 profile of intact chromosome 7. TN6-CMGT1 shows a reduced complexity of hybridization. This confirms the site of pTAGNEO integration in human chromosome 7 and that only a small fragment was cotransferred by G418 selection. We estimate that TN6-CMGT1 retains 2 to 3 Mbp of human chromosome 7. A preliminary screen for cotransfer of CF-linked single copy genes (pJ311, *met* TCR-β, and P-glycoprotein) in TN6-CMGT1 was negative. Further CMGT hybrids, more appropriate for approaching CF and other loci on the long arm of chromosome 7, can be isolated. This strategy should complement the *met*-selected CMGT approach of Scambler *et al.* (*4*).

We conclude that the double requirement for endogenous promoter control and read-

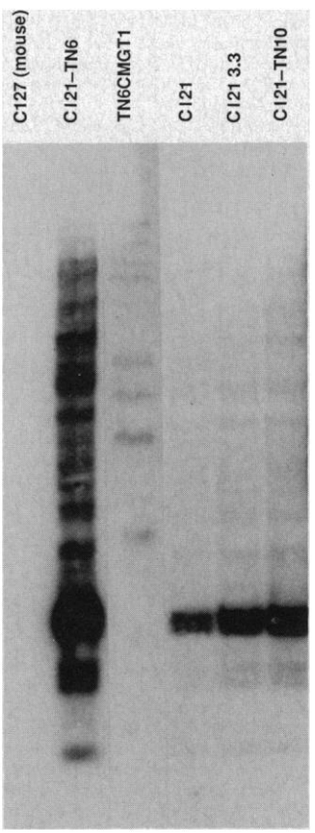

Fig. 4. L1 fingerprint analysis of CMGT transfectants from Cl21-TN6. DNA from relevant cell lines were digested with Bgl II, separated by electrophoresis, subjected to DNA blot analysis with a ^{32}P-labeled pBK1.8 L1 repeat (*18*) as a probe. Cl21 3.3 is a subclone of Cl21 and Cl21-TN10 is a pTAGNEO Cl21 random integrant.

through transcripts applies a stringent selection for integration by homologous recombination. Our strategy can now be tested for isolating other chromosomal regions of interest by targeting the *neo* selectable marker to any closely linked, expressed gene for which sequence information is available. We might expect to see significant variation between different target sequences in the ability to promote homologous recombination. In this regard, the high efficiency of targeting with pTAGNEO may reflect in part the possible recombinogenic function of SV40 T antigen. However, as a large number of other SV40-transformed human and human-mouse hybrid cells are already available, we can use pTAGNEO immediately to access many new regions of DNA. If comparable frequencies of precise homologous integration can be achieved at other loci, then our approach should provide an efficient strategy by which specific gene mutations can be created or repaired.

Note added in proof. Two recent papers describe strategies in which enhancer- or promoter-deficient genes were used to select for homologous recombination events (*19*).

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12. Vectors were constructed in Bluescribe (Vector Cloning Systems) by standard cloning methods. The bacterial *neo* was cloned initially into M13mp8 on a Bcl I–Hind III fragment. To remove a stop codon 5′ to the *neo* methionine, 0.2 pmol of single-stranded DNA was heated for 10 min to 70°C in buffer (50 m*M* NaCl, 10 m*M* $MgCl_2$, and 10 m*M* tris-HCl, *p*H 8.0) containing 200 pmol of antisense oligonucleotide (5′-ACGATCCTCCTCCTGTCTC) containing the required mutation. *Escherichia coli* JM 101 were then transformed with the precipitated DNA, and colonies were screened with the oligonucleotide to identify clones containing the mutation. Positive clones were transferred to a strain of *E. coli* deficient in adenine methylase, and the Bcl I–Hind III *neo* was isolated and spliced onto the 3-kbp Kpn I–Bcl I fragment of SV40 T antigen to create p(pro$^+$)TAGNEO. The deletion of the Avr II–Kpn I DNA fragment that contains the SV40 early promoter, origin of replication, and 72-bp enhancer boxes 5′ to T antigen results in the construction of pTAGNEO. Both vectors contain a unique BstXI site that lies 428 bp downstream from the Avr II site in the SV40 T antigen sequence, and a unique Sma I site in the *neo* gene ~1 kbp 3′ to the Bcl I site. DNA sequencing confirms the SV40 T antigen splice region to be as shown below. This construct results in the deletion of the last 27 amino acids of the SV40 antigen, which now terminates with valine (V) and histidine (H). The methionine (M) indicated is the start of the normal *neo* open reading frame. The asterisked base was altered by the site-directed mutagenesis (described above) so that there would not be a termination codon when translated in-frame from the T antigen sequence. The *neo* gene contains the HSV-tk poly (A) addition signal.

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        ↓ Bcl I
GTT CAT GAT CAA GAG ACA GGA ĠGA GGA TCG TTT CGC ATG ATT
V   H                                       M   I
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15. Probe A is a subclone of SV40 DNA not contained in pTAGNEO. The 1.4-kbp fragment lies between the Kpn I site (position 294) and the Eco RI site (position 1782) numbering clockwise from the Bgl I site in the direction of late genes to early genes [W. Fiers *et al.*, *Nature* **273**, 113 (1978)].
16. The amplifying oligonucleotides were specific for the *neo* mutation (*12*) and the SV40 promoter region (5′-CGCCTCGGCCTCTGAGCTA). Primers for PCR were synthesized on an Applied Biosystems DNA Synthesizer. DNA was prepared from 10^6 cells and PCR was carried out on an Intelligent Heating Block (Hybaid Ltd.) with *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus), buffer, and oligonucleotide concentrations according to the manufacturer's instructions and the method of R. K. Saiki *et al.* [*Science* **230**, 1350 (1985)]. The DNA was denatured at 95°C for 7 min, enzyme added, and then 30 cycles of 45 s at 92°C, 1 min at 55°C, and 5 min at 72°C were carried out. Samples were then run on a 1% agarose gel and blotted onto Hybond N (Amersham). Gels were probed at 58°C with oligonucleotide 116, specific for T antigen (5′-ATCCGAGAAGCCTCCAAAG), to distinguish specific from spurious bands.
17. CMGT was performed exactly as described by J. E. N. Morten *et al.* [*Anticancer Res.* **7**, 573 (1987)].
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20. Cl21 and Cl27 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum, under 10% CO_2. Cells to be electroporated were harvested with trypsin-versene when rapidly growing, washed in DMEM, counted, and resuspended in phosphate-buffered saline at a concentration of 2×10^6 cells per milliliter. Just before electroporation, the recombination vector was added to a concentration of 20 μg/ml and 0.5 ml of cells, and these were exposed to a single 3000 V/cm pulse, 4-μF capacitance direct discharge through 22-ohm resistance in series (at room temperature on a home-made apparatus), and then allowed to recover on ice for 10 min. The current supplied was 8.8 A and the current density 0.44 A/mm^2. The survival rate was ~40 to 60% for both cell lines. G418 selection was applied after 24 hours at 200 μg/ml, and after 5 days the concentration was increased to 400 μg/ml. Colonies arising were subcloned into G418 at 800 μg/ml. G418-resistant cell lines were propagated and analyzed as described in the text.

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Phylogenetic Stains: Ribosomal RNA–Based Probes for the Identification of Single Cells

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Rapid phylogenetic identification of single microbial cells was achieved with a new staining method. Formaldehyde-fixed, intact cells were hybridized with fluorescently labeled oligodeoxynucleotides complementary to 16*S* ribosomal RNA (rRNA) and viewed by fluorescence microscopy. Because of the abundance of rRNA in cells, the binding of the fluorescent probes to individual cells is readily visualized. Phylogenetic identification is achieved by the use of oligonucleotides (length 17 to 34 nucleotides) that are complementary to phylogenetic group-specific 16*S* rRNA sequences. Appropriate probes can be composed of oligonucleotide sequences that distinguish between the primary kingdoms (eukaryotes, eubacteria, archaebacteria) and between closely related organisms. The simultaneous use of multiple probes, labeled with different fluorescent dyes, allows the identification of different cell types in the same microscopic field. Quantitative microfluorimetry shows that the amount of an rRNA-specific probe that binds to *Escherichia coli* varies with the ribosome content and therefore reflects growth rate.

CLASSICAL CHEMICAL STAINS FOR VISUALIZING microorganisms seldom provide much information beyond the morphology of the organisms detected. Reagents such as fluorescent antibodies can be used for the identification of particular organisms, but their high specificity limits their utility to well-studied organisms. We have developed a staining method that provides phylogenetic information on single microbial cells and requires no previous knowledge of the organisms detected. The approach is based on oligodeoxynucleotide hybridization probes complementary to ribosomal RNA (rRNA) sequences that are diagnostic for selected phylogenetic groups. When these probes are labeled with fluorescent dyes, they can be used for the detection and phylogenetic characterization of orga-

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