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12. If channel insertion into the bilayer results from random all-or-none fusion of vesicles (packets of channels), then channel density and vesicle fusion rates can be obtained by counting the total number of Ca^{2+}-activated K^+ channels that incorporate into the bilayer [R. Ashley, *J. Membr. Biol.* **111**, 179 (1989)]. Our results indicate that on average a single vesicle contains a single Ca^{2+}-activated K^+ channel. To calculate the maximal number of protein kinase molecules in a vesicle, we assayed the PKC activity in our vesicle preparation. This kinase was chosen because it is the most abundant protein kinase in rat brain. The specific activity of PKC in our washed membrane vesicle preparation was less than 0.3 nmol of phosphate milligram protein^{-1} min^{-1}. Because the turnover number of this enzyme in rat brain is 180 to 500 s^{-1} [K. Huang *et al.*, *J. Biol. Chem.* **263**, 14839 (1988)], there are at most 0.2 to 1.0×10^9 molecules of PKC per microgram of vesicle protein. Because the total number of vesicles in 1 μg of protein is 10^9 to 10^{10} {assuming an average vesicle diameter of 100 nm and a protein to lipid ratio of 1:1 [V. P. Whittaker *et al.*, *Biochem. J.* **90**, 293 (1964)]}, the average number of protein kinase molecules per vesicle is 0.02 to 1.0. Thus, one Ca^{2+}-activated K^+ channel and at most one PKC molecule insert into the 100-μm-diameter bilayer with a single vesicle fusion. If the kinase and channel are inserted into the bilayer adjacent to each other and are free to diffuse randomly, then we can calculate the average distance (r) separating them after t (time) = 10^3 s [we always take at least a 20-min (10^3 s) control record before adding the ATP] by the equation, $r = 2(Dt)^{1/2}$. For proteins in lipid membranes the diffusion constant (D) is 3×10^{-9} cm^2 sec^{-1} [R. Cone, *Nature* **247**, 438 (1974); D. Tank *et al.*, *J. Cell Biol.* **92**, 207 (1982)]; r is approximately 20 μm. If we now put a constraint on the distance the kinase and channel can separate from one another by assuming that there is a reflecting boundary at $b = 2r$ (that is, every molecule that diffuses as far as $2r$, or 40 μm, diffuses back inward), assume a capture radius a of 1 nm of the channel for the kinase, and further assume that every hit between kinase and channel leads to a successful phosphorylation and consequent channel modulation, then we can solve the equation $\tau(r) = (b^2/2D)\ln(r/a)$ [H. Berg and E. Purcell, *Biophys. J.* **20**, 193 (1977)] for the average interaction time $\tau(r)$ to yield 8×10^4 s or approximately 1 day. As there is no such reflecting boundary in the bilayer, the interaction time is probably much longer. That is, it would take at least 1 day on average for an interaction between independently moving channel and kinase molecules to produce channel modulation. However, activation of the channel occurs routinely within 20 to 180 s after adding ATP. Only if kinase molecules and channels are distributed in different vesicle populations with different fusion rates or if the endogenous kinase is very much more abundant than PKC can our results be explained by random interactions between independent kinase and channel molecules in the bilayer.
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Selective Inhibition of Leukemia Cell Proliferation by BCR-ABL Antisense Oligodeoxynucleotides

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To determine the role of the BCR-ABL gene in the proliferation of blast cells of patients with chronic myelogenous leukemia, leukemia blast cells were exposed to synthetic 18-mer oligodeoxynucleotides complementary to two identified BCR-ABL junctions. Leukemia colony formation was suppressed, whereas granulocyte-macrophage colony formation from normal marrow progenitors was unaffected. When equal proportions of normal marrow progenitors and blast cells were mixed, exposed to the oligodeoxynucleotides, and assayed for residual colony formation, the majority of residual cells were normal. These findings demonstrate the requirement for a functional BCR-ABL gene in maintaining the leukemic phenotype and the feasibility of gene-targeted selective killing of neoplastic cells.

THE PHILADELPHIA CHROMOSOME (Ph[1]) translocation is the most common genetic abnormality in human leukemias (*1*). At the molecular level, the most notable feature is the translocation of the proto-oncogene ABL from chromosome 9 to the breakpoint cluster region (BCR) on chromosome 22, resulting in the formation of BCR-ABL hybrid genes (*2*). The ABL proto-oncogene normally encodes a protein with tyrosine kinase activity (*3*); this activity is augmented in cells carrying BCR-ABL hybrid genes (*4*). The BCR-ABL transcripts are found in the vast majority of chronic myelogenous leukemia (CML) patients and in Ph[1] acute lymphocytic leukemia patients (*5*).

CML invariably progresses from the chronic phase into the blast crisis. In chronic phase CML, the increase in mature and immature myeloid elements in bone marrow and peripheral blood is the most characteristic feature (*6*). Kinetic studies indicate that these abnormal cells do not proliferate or mature faster than their normal counterparts. Instead, the basic defect underlying the abnormal degree of granulopoiesis in CML appears to reside in the expansion of the myeloid progenitor cell pool in bone marrow and peripheral blood (*6*). Nevertheless, the generation of terminally differentiated cells indicates that the process of hematopoiesis retains some normal features. In contrast, during blast transformation, the leukemic cells exhibit a marked degree of differentiation arrest with a "blast" phenotype (7). The role of the BCR-ABL transcript in the pathogenesis of the abnormal hematopoiesis of CML has been investigated by introducing BCR-ABL constructs in mice and demonstrating the occurrence of a CML-like syndrome (*8*). To determine the functional relevance of the BCR-ABL protein for the proliferation of leukemia cells (CML-BC), we selectively inhibited BCR-ABL protein synthesis by an antisense strategy (*9*).

Clonogenic assays of leukemia cells freshly obtained from individuals with myeloid CML-BC often revealed the formation of numerous colonies. In most cases, a BCR-ABL transcript was amplified by the reverse transcriptase–polymerase chain reaction (RT-PCR) technique with a 5′ primer corresponding to 22 bases of BCR exon 2 and a 3′ primer complementary to 22 bases of ABL exon 2. Because blast colonies derived from cells of one patient were particularly numerous, they were pooled for RNA extraction; a region of 257 nucleotides corresponding to the BCR-ABL transcript was amplified by RT-PCR and cloned by blunt-end ligation into the Bluescript SK vector (Stratagene) that had been linearized by Sma I digestion. Sequence analysis of ten

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recombinant clones identified the same BCR-ABL junction (Fig. 1A). This junction corresponded precisely to the L-6–type breakpoint in which the BCR exon 2 is fused to ABL exon 2 (*10*). Four additional L-6–type breakpoints were identified by hybridization of RT-PCR–amplification products derived from CML-BC RNA with a synthetic oligomer specific for the L-6 BCR-ABL junction. Sequence analysis of the cloned breakpoint demonstrated that in each case the junction was identical to that shown in Fig. 1A. An 18-base synthetic oligomer complementary to the BCR-ABL junction (nine nucleotides corresponding to BCR and nine corresponding to ABL) was added to CML-BC cells, and the effects on clonogenic growth were evaluated 10 days later.

Untreated and CML-BC cells exposed to a mismatched BCR-ABL oligomer with four nucleotide substitutions formed numerous colonies of blast cells (Fig. 2, A and B), whereas a much smaller number of colonies, each of which had fewer cells, formed in the presence of BCR-ABL antisense oligodeoxynucleotides (Fig. 2C). Inhibition of formation of colonies derived from blast cells of these five patients with the L-6–type breakpoint ranged from 60 to 90% in repeated experiments. In contrast, the number of colonies formed from normal progenitors (MNCs) was essentially identical in the presence or absence of synthetic oligomers (*11*).

To identify CML-BC patients carrying the second most common breakpoint (in which BCR exon 3 is juxtaposed to ABL exon 2), we first used RT on total RNA derived from CML-BC cells with synthetic primers corresponding to BCR exon 3 and ABL exon 2 and then amplified by PCR. The amplification products were then hybridized with a synthetic oligonucleotide (18-mer) corresponding to the BCR exon 3–ABL exon 2 junction; five individuals with breakpoints within the b3a2 junction were identified and, after each breakpoint was cloned, sequence analysis of several recombinant clones demonstrated that the BCR exon 3–ABL exon 2 junction corresponded in each case to the K-28–type breakpoint (Fig. 1B). An 18-base synthetic oligomer complementary to this junction was added at 60 μg/ml (40 μg at 0 hours and 20 μg after 18 hours) to CML-BC cells, and colony formation on clonogenic assays was evaluated 10 days later.

Untreated and CML-BC cells exposed to a two-nucleotide mismatched BCR-ABL oligomer formed numerous colonies of blast cells (Fig. 3, A and B), whereas few colonies, each with a reduced number of cells formed with the authentic antisense oligomer (Fig. 3C). In marked contrast, clonogenic growth from normal hematopoietic progenitors was unaffected (*11*). In these five cases with the K-28–type breakpoint, the inhibition of colony formation with the authentic BCR-ABL antisense oligomer ranged from 60 to 80% in duplicate experiments; these CML-BC cells carrying the K-28–type breakpoint were also exposed to the antisense oligomer (5′-GAAGGGCTTCTTCCTTAT-3′) complementary to the L-6–type junction; no significant reduction of colony formation (2.5 to 5% inhibition relative to untreated cells) was observed,

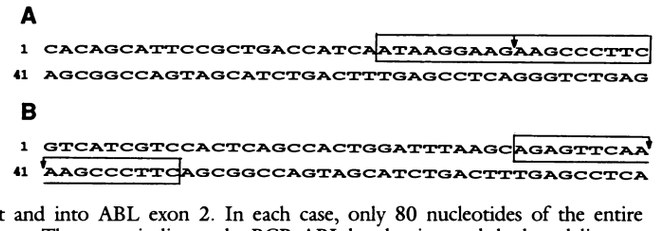

Fig. 1. Sequence of the BCR-ABL breakpoints in leukemic cells of CML-BC patients. Nucleotides are numbered in the 5′ to 3′ direction, beginning from the BCR exon 2 primer (**A**) or the BCR exon 3 primer (**B**) into the breakpoint and into ABL exon 2. In each case, only 80 nucleotides of the entire subcloned fragment are shown. The arrow indicates the BCR-ABL breakpoint, and the box delineates the 18 nucleotides corresponding to the BCR-ABL junction. (A) L-6–type breakpoint; (B) K-28–type breakpoint.

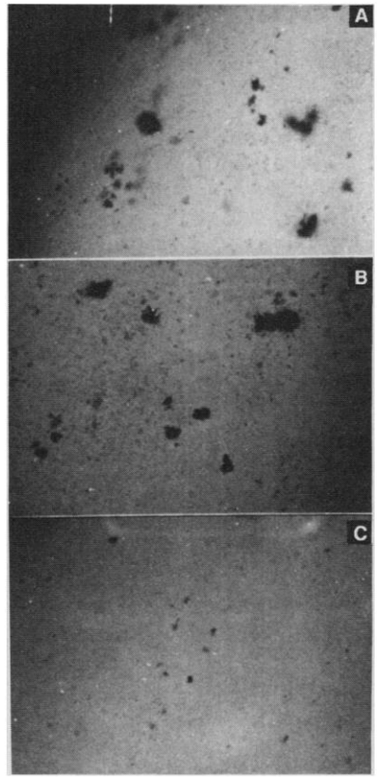

Fig. 2. Effect of BCR-ABL oligomers on leukemic colony formation from CML-BC cells with L-6–type breakpoints. Philadelphia chromosome–positive blast cells were isolated by Ficoll-Hypaque density-gradient sedimentation from patients in the blast phase of CML; blast cells (5×10^4) were placed in 0.4 ml of liquid suspension culture (Iscove's modified Dulbecco's modified medium with 2% human AB serum) in the presence of interleukin-3 (20 units/ml) and GM-CSF (5 ng/ml). Treated cultures received 40 μg/ml (1 μg/ml = 0.35 μM) of oligodeoxynucleotides at the start of the culture period and 20 μg/ml 18 hours later (control cultures were left untreated); 4 hours after the second addition of oligodeoxynucleotides to the cultures, cells were directly seeded into duplicate methylcellulose dishes containing interleukin-3 (20 units/ml) and GM-CSF (5 ng/ml). We placed cells into semisolid cultures and allowed them to grow for an additional 10 days. The plates were then scanned with an inverted microscope, and the total numbers of colonies and clusters were counted. Philadelphia chromosome blast cells were cultured in some cases in the absence of hematopoietic growth factors; under these conditions the colonies were fewer and smaller than in the presence of growth factors. (**A**) Untreated leukemia cells; (**B**) after treatment with a mismatched (four-nucleotide substitution) BCR-ABL antisense oligomer (5′-GAACGGCATCTACGTTAT-3′); and (**C**) after treatment with a BCR-ABL antisense oligomer complementary to the coding sequence of the identified BCR-ABL junction (5′-GAAGGGCTTCTTCCTTAT-3′).

Table 1. Effect of BCR-ABL oligomers on in vitro colony formation from partially purified marrow progenitors, CML-BC cells, and 1:1 mixtures of each. Values represent mean ± SD of duplicate control cultures (no oligodeoxynucleotide added) and duplicate experimental cultures from two separate experiments. Bone marrow mononuclear cells were isolated and cultured in the presence of interleukin-3 (20 units/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5 ng/ml) as described (*9, 13*); CML-BC cells were cultured under the same conditions. Exposure to oligomers are detailed in the text and legend to Fig. 4. CFU-L, colonies formed from CML-BC cells; CFU-GM + CFU-L, colonies formed from 1:1 mixtures of normal marrow progenitors and CML-BC cells. CFU-GM–derived colonies consisted of ≥50 cell aggregates, whereas CFU-GM–derived clusters were defined as aggregates of between 4 and 40 cells.

Oligodeoxynucleotide	Colonies or clusters		
	CFU-GM	CFU-L	CFU-GM + CFU-L
Control	263 ± 10	806 ± 70	978 ± 24
BCR-ABL antisense (four-nucleotide mismatches)	265 ± 6	786 ± 23	900 ± 46
BCR-ABL antisense	253 ± 35	180 ± 14	450 ± 42

further demonstrating that the effects were due to the targeting of a leukemia-specific sequence.

The inhibition of leukemia cell proliferation by synthetic oligomers against leukemia-specific genes could prove useful in a clinical context, as normal hematopoietic progenitor cells exposed to BCR-ABL antisense oligodeoxynucleotides should be unaffected and capable of generating mature descendants. To test this hypothesis, we mixed normal bone marrow progenitors and CML-BC (L-6–type breakpoint) cells at a 1:1 ratio, exposed them to the breakpoint junction–specific oligomer under standard conditions and plated them on methylcellulose to evaluate the resulting colonies. In addition, we exposed normal bone marrow progenitors and CML-BC cells separately to the breakpoint oligomer to reevaluate the effect on normal and leukemic cells. After 12 days in culture, it was apparent that the number of colonies arising from CML-BC cells exposed to the BCR-ABL antisense oligomer was much lower than the number arising in the presence of the mismatched BCR-ABL oligomer (Table 1). In contrast, the number of colonies formed from normal progenitors was essentially the same in the presence or absence of synthetic oligomers.

To determine whether residual colonies consisted of normal or leukemic cells, we used morphological and molecular criteria. Cells derived from residual colonies were isolated from methylcellulose plates and morphologically identified by Giemsa staining. Only blast cells were identified in colonies arising from CML-BC cells. In the 1:1 mix of normal MNCs and CML-BC cells exposed to a mismatched (four-nucleotide substitution) BCR-ABL antisense oligomer, the isolated cells were, as expected, heterogeneous and consisted of blast cells and myeloid elements in different stages of differentiation. In contrast, in the 1:1 mix of normal MNCs and CML-BC cells exposed to the authentic BCR-ABL antisense oligomer, the isolated cells consisted largely of differentiating elements, suggesting the persistence of normal progenitors and the selective depletion of leukemic blast cells (*11*).

To provide unambiguous evidence of the elimination of Philadelphia chromosome blast cells, we evaluated residual cells for the expression of BCR-ABL transcripts because the amounts of this transcript should reflect the number of surviving leukemia cells. Total RNA was isolated from a pool of colonies and the levels of BCR-ABL transcripts were evaluated by RT-PCR. No BCR-ABL transcript was detected in RNA isolated from colonies arising from mixed cell populations exposed to BCR-ABL antisense oligodeoxynucleotides; by contrast, the expression of β-microglobulin was clearly detectable in the antisense-treated colonies (Fig. 4), indicating the selective reduction of cells carrying the BCR-ABL translocation and the survival of a progeny arising from normal progenitors.

Our data establish, in the context of the natural disease, that a functional BCR-ABL gene is necessary to maintain the abnormal growth associated with the leukemic phenotype. The presence of residual leukemia colony formation after exposure to BCR-ABL antisense oligodeoxynucleotides (Figs. 2 and 3 and Table 1) might reflect a nonuniform uptake of oligodeoxynucleotides by CML-BC cells or the presence of additional genetic abnormalities in CML-BC cells that could replace the requirement for BCR-ABL expression for the maintenance of the leukemic phenotype during blast transformation. Table 1 shows that approximately 25% of CML-BC cells can survive the antisense treatment, but Fig. 4 indicates the complete elimination of BCR-ABL mRNA in cells surviving the antisense treatment. These results suggest that the antisense treatment can eliminate the BCR-ABL mRNA from all of the blast cells but might not affect the survival of

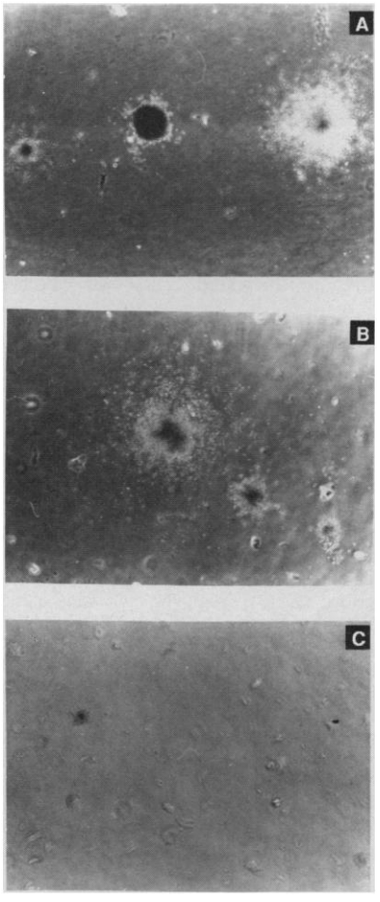

Fig. 3. Effect of BCR-ABL oligomers on leukemic colony formation from CML-BC cells with K-28–type breakpoints. Culture conditions and oligomer treatment were as described in Fig. 2. (**A**) Untreated leukemia cells; (**B**) after treatment with a mismatched (two-nucleotide substitution) BCR-ABL antisense oligomer (5′-GAAGTGCTGTTGAACTCT-3′); and (**C**) after treatment with a BCR-ABL antisense oligomer complementary to the coding sequence of the identified (K-28–type) BCR-ABL junction (5′-GAAGGGCTTTTGAACTCT-3′).

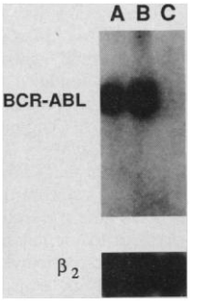

Fig. 4. Expression of BCR-ABL and β-2 microglobulin (β_2) mRNA in 1:1 mixtures of MNCs and CML-BC cells after exposure to BCR-ABL oligodeoxynucleotides. MNCs were obtained by aspiration from consenting volunteers and enriched for hematopoietic progenitors as reported (*9, 13*). In brief, marrow cells were subjected to Ficoll-Hypaque density gradient sedimentation and then depleted of adherent monocyte-macrophages and T lymphocytes by adherence to plastic petri dishes and by rosetting with neuraminidase-treated sheep red blood cells, respectively (*9, 13*). Ph^1 blast cells were isolated by Ficoll-Hypaque density gradient sedimentation from an individual with CML-BC. Morphological analysis revealed that >95% of the cells were blast. The residual nonblast cells had the morphological appearance of small lymphocytes. MNCs (25,000) and CML-BC cells (25,000) were incubated with a BCR-ABL antisense oligomer (40 μg/ml at 0 hours; 20 μg/ml 18 hours later), with a four-nucleotide mismatched BCR-ABL antisense oligomer (40 μg/ml at 0 hours; 20 μg/ml 18 hours later), or left untreated; 4 hours after the second addition of oligodeoxynucleotides to the cultures, we seeded cells into duplicate methylcellulose dishes containing interleukin (20 units/ml) and GM-CSF (5 ng/ml) and allowed them to grow for an additional 12 days. At the end of the incubation period, colonies were counted, and all colonies were removed from the methylcellulose plates for morphological examination and total RNA isolation. The BCR-ABL and β-2 microglobulin transcripts were reverse-transcribed and amplified in the presence of specific primers and Taq polymerase as described (*14*). The amplification products were separated on a 2% agarose gel and transferred to a nitrocellulose filter that was hybridized with a synthetic 40-base ABL fragment or a 40-base β-2 microglobulin fragment end-labeled with [γ-^{32}P]adenosine and triphosphate polynucleotide kinase. The ABL probe recognizes the amplified 257-bp sequence containing the BCR-ABL junction (Fig. 1A); the β-2 microglobulin probe recognizes the 195-bp sequence contained within the 5′ and 3′ β-2 microglobulin primers (*14, 15*). Lane A, untreated MNCs and CML-BC cells mixed 1:1; lane B, MNCs and CML-BC cells mixed 1:1, treated with a four-nucleotide mismatched BCR-ABL antisense oligodeoxynucleotide; and lane C, MNCs and CML-BC cells mixed 1:1, treated with a BCR-ABL antisense oligodeoxynucleotide.

those Ph[1] blast cells containing additional genetic alterations. The mechanism for the suppression of CML-BC colony formation by BCR-ABL antisense oligodeoxynucleotides is not yet known; inhibition of BCR-ABL protein synthesis could remove an essential component in the mitogenic pathway regulated by hematopoietic growth factors as reported (*12*). Less likely, down-regulation of BCR-ABL expression could determine aberrant differentiation of blast cells, resulting in a suppression of colony formation.

We have provided evidence that leukemia growth can be selectively inhibited by synthetic oligomers targeted against a tumor-specific gene involved in the maintenance of the leukemic phenotype. Synthetic oligodeoxynucleotides complementary to BCR-ABL hybrid genes can be synthesized on an individual basis once the specific BCR-ABL junction is identified; this can be done within a few days of diagnosis and offers the prospect, at least in vitro, of a gene-targeted anti-leukemic therapy.

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Differentiation of 3T3-L1 Fibroblasts to Adipocytes Induced by Transfection of *ras* Oncogenes

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Mammalian 3T3-L1 cells differentiate into adipocytes after continuous exposure to pharmacological doses of insulin or physiological doses of insulin-like growth factor I (IGF-1). Expression of transfected *ras* oncogenes led to differentiation of these cells into adipocytes in the absence of externally added insulin or IGF-I. Cells transfected with normal *ras* genes or the tyrosine kinase *trk* oncogene did not differentiate. Transfection with a dominant inhibitory *ras* mutant resulted in inhibition of differentiation. Exposure of untransfected 3T3-L1 cells to insulin stimulated formation of the active Ras·GTP complex. These observations indicate that Ras proteins participate in signal transduction pathways initiated by insulin and IGF-I in these cells.

THE EVOLUTIONARY CONSERVATION of the *ras* gene family suggests that its members have essential cellular functions (*1*). The mammalian Ras proteins are thought to be involved in signal transduction pathways of proliferation or differentiation. However, little is known about the components and mechanisms of the *ras* signaling pathway (*1*). Microinjection of transforming Ras proteins transiently transforms mammalian cells (*2*) and induces meiotic maturation in *Xenopus* oocytes (*3*). Microinjection into *Xenopus* oocytes of the neutralizing Ras antibody Y13-259 or cytosol-localized *ras* oncogene mutants (*4*) inhibits meiotic maturation induced by insulin or IGF-I but not by progesterone. These observations suggest a role of the Ras proteins in the insulin and IGF-I signaling pathways in these amphibian cells.

We examined the function of *ras* in signaling pathways of insulin and IGF in mammalian cells. Differentiation of the murine 3T3-L1 cell line (*5, 6*) into adipocytes shows an absolute requirement for insulin or IGF-I (*5–10*). Exponentially growing and preconfluent 3T3-L1 cells have the morphological and biochemical properties of fibroblasts; however, after reaching confluency, they can be converted to adipocytes (*7*) using protocols that involve prolonged, continuous exposure to pharmacological doses of insulin or physiological doses of IGF-I (*6,8, 9*). Insulin or IGF-I have been shown to be essential regulators of the differentiation process carried out in either serum-supplemented medium (*9*) or serum-free media (*10*). We introduced functionally active *ras* genes into 3T3-L1 cells by means of transfection in order to study their effect on insulin-dependent differentiation into adipocytes (*11*). To assess the specificity of these effects, other oncogenes were also transfected.

The plasmids analyzed in these transfection experiments (Table 1) included pMEXneo H-*ras*^{Gly12} and pMEXneo H-*ras*^{Lys12} (*12*) containing, respectively, the normal and transforming alleles of H-*ras* and pMEXneo H-*ras*^{Lys12,Ser186}, a cytosolic, nontransforming mutant that fails to localize to the plasma membrane because the Ser^{186} mutation precludes posttranslational farnesylation (*13*). Two vectors containing transforming variants of the tyrosine kinase oncogene *trk* were also used. These were pDM16, a pMEX-related plasmid containing the original *trk* oncogene activated through recombination with cellular tropomyosin sequences, and pDM78, a pMEXneo-derived construct containing *trk5*, a different transforming allele activated through a deletion in the extracellular domain of the *trk* proto-oncogene (*14*).

The efficiency of transfection in NIH 3T3 cells, as estimated by the number of neomycin-resistant (neo^r) colonies obtained after selection, ranged from 1 to 5%. As expected, pMEXneo H-*ras*^{Lys12} showed potent trans-

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