discarded. The samples were stored from 1 month to 8 years at −70°C prior to analysis; samples from schizophrenic and normal subjects were randomly distributed over this period whereas samples from most of the neurological patients were 6 to 8 years old. The research was done under a protocol approved by the Institutional Review Board of St. Elizabeths Hospital.
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Transformation Induced by Abelson Murine Leukemia Virus Involves Production of a Polypeptide Growth Factor

Abstract. *Rat embryo fibroblasts transformed by Abelson murine leukemia virus (MuLV) produce and release a transforming growth factor (TGF). Production of this factor is correlated with a tyrosine-specific protein kinase that is functionally active and is associated with the major Abelson MuLV gene product, P120. Transformation-defective mutants of Abelson MuLV do not transform cells, do not have their virus coded transforming gene product phosphorylated in tyrosine, and do not induce TGF production. Abelson MuLV–induced TGF morphologically transforms cells in culture, competes with ^{125}I-labeled epidermal growth factor (EGF) for binding to cell receptors, and induces phosphorylation of tyrosine acceptor sites in the 160,000-dalton EGF membrane receptor. After purification to homogeneity, Abelson virus–induced TGF migrates as a single polypeptide with an apparent size of 7400 daltons as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.*

Abelson murine leukemia virus (MuLV) is a prototype replication-defective mammalian transforming virus initially generated as a result of genetic recombination between the Moloney strain of MuLV and transformation-specific sequences of mouse cell origin (*1–4*). In the presence of an appropriate type C helper virus, Abelson MuLV transforms embryo fibroblasts in cell culture (*5*) and induces a rapid B cell lymphoid leukemia in vivo (*6*, *7*). A characteristic property of Abelson MuLV transformed embryo fibroblasts is a marked reduction in available binding sites for epidermal growth factor (EGF) (*8*). The major Abelson MuLV translational product has been identified as a 120,000-dalton polyprotein with Moloney MuLV amino terminal structural components (p15 and p12) and an acquired sequence encoded nonstructural component (*2–4*) with tyrosine-specific protein kinase activity (*9*, *10*). The involvement of this viral gene product and its associated enzymatic activity in transformation has been established through the analysis of Abelson MuLV transformation-defective (td) mutants (*11*, *12*).

In an effort to determine the significance of the reduced EGF binding observed in response to Abelson MuLV transformation (*8*), culture fluids from Abelson MuLV nonproductively transformed rat embryo fibroblasts were harvested, concentrated, extracted with acid ethanol, and subjected to analysis of molecular size by gel filtration chromatography (Fig. 1). Individual column fractions were assayed for competition with ^{125}I-labeled EGF for binding A431 cell membrane receptors and for transformation of normal rat kidney (NRK) cells as measured by ability to form progressively growing colonies in soft agar. As shown in Fig. 1, two peaks of EGF competing activity were identified; these eluted with apparent molecular sizes of approximately 10,000 daltons (fractions 75 to 85) and 20,000 daltons (fractions 45 to 55), respectively. Activities in both peaks morphologically transform cells in monolayer culture and support anchorage-independent growth of NRK cells in soft agar (Table 1). In contrast, EGF did not promote soft agar growth in this assay. Thus the activities produced and released into the culture fluids by Abelson MuLV transformed rat cells demonstrate many of the characteristics described for growth factors produced by Moloney murine sarcoma virus (MSV) transformed mouse fibroblasts (*13*, *14*), and certain human tumor cells (*15*).

Activities of Abelson MuLV transforming growth factor (TGF) are distinguished from EGF on the basis of several immunological and biochemical criteria. For instance, neither molecular size form of Abelson MuLV TGF exhibited detectable reactivity in a competition radioimmunoassay for mouse EGF (Table 1). Abelson MuLV TGF is further distinguished from EGF by its differential solvent elution profile in high-performance liquid chromatography (HPLC). Whereas mouse EGF elutes at 28.7 percent acetonitrile in 0.05 percent trifluoroacetic acid from a μBondapak C_{18} column, the 10,000-dalton Abelson MuLV TGF elutes at 19.5 percent acetonitrile concentration (Table 1); in this respect this latter factor closely resembles Moloney MSV transformed mouse cell and human tumor cell derived TGF's. After purification by HPLC of the smaller TGF of the Abelson MuLV transformed cells, a single polypeptide was obtained with an apparent molecular size of 7400 daltons, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). In contrast, when analyzed in parallel, purified EGF migrated at a distinctly lower apparent molecular size (6000 daltons).

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The availability of well-characterized td mutants of Abelson MuLV encoding a P120 polyprotein lacking detectable protein kinase activity (*8*, *11*) permitted us to examine whether TGF expression in Abelson MuLV transformed cells is associated with this viral encoded enzymatic activity. Culture fluids derived from rat embryo fibroblasts infected with an Abelson MuLV td mutant were purified and subjected to molecular size analysis. Neither soft agar colony stimulating activity nor EGF competing activity could be detected in any of the column fractions, including those corresponding to wild-type (wt) Abelson MuLV fractions containing high-titered TGF activity (Table 1). Thus a functionally active Abelson MuLV gene product is associated with the release of TGF into the culture fluids of Abelson MuLV transformed cells.

Transformation of mouse, rat, and mink cells by wt but not td variants of Abelson MuLV results in an eight- to tenfold overall elevation of cellular phosphotyrosine levels (*8*, *11*). Treatment of A431 human carcinoma cells with partially purified Abelson cell TGF resulted in an increase of cellular levels of phosphotyrosine levels of similar magnitude (Table 1). By SDS-PAGE analysis, the major cellular protein phosphorylated in response to Abelson cell TGF was identified as the 160,000-dalton EGF receptor (Fig. 3). Treatment of A431 cells with EGF (*16*, *17*) or TGF of either Moloney MSV transformed mouse cell (*17*), or human tumor cell (*17*) origin, also stimulates phosphorylation of the EGF receptor in tyrosine. By tryptic peptide analysis, phosphorylation of P160 in response to Abelson MuLV–induced TGF involves one major and several minor tyrosine acceptor sites (data not shown). This pattern is indistinguishable from that described for the EGF receptor phosphorylated in response to either EGF or human tumor cell TGF (*17*).

In view of the number of viral encoded transforming proteins with demonstrated tyrosine-specific protein kinase activity (*18*, *19*), the role of tyrosine phosphorylation in malignancy is of considerable interest. This is particularly true because of the established cellular origin of transforming sequences represented within the genomic RNA's of many of these viruses.

Our findings establish that a TGF of low molecular size is involved in trans-

Table 1. Properties of transforming growth factors isolated from nonproductively transformed rat cells.

Growth factor	Inhibition of EGF binding (percent)*	Growth in soft agar (Number of colonies per eight fields)†	Competition in EGF RIA (percent)‡	HPLC (percent of acetonitrile)§	A431 phosphotyrosine (percent)‖
Abelson MuLV					
wt					
10,000	47.5	466	< 1	19.5	2.0
20,000	34.0	250	< 1		2.0
td					
10,000	0	0	< 1		< 0.2
20,000	0	0	< 1		< 0.2
EGF					
6000 (per 2.5 ng)	50.0	0	70	28.7	1.9

*Values represent duplicate determinations of the amount of EGF competing activity (*13*) in 15 percent of each fraction (Fig. 1). Peak fractions corresponding to the 10,000- and 20,000-dalton Abelson cell TGF activities contained 20.6 and 10.7 ng of EGF equivalents, respectively, of EGF competing activity where one EGF equivalent is defined as the amount of protein that inhibits the binding of ^{125}I-labeled EGF to its receptor by 50 percent. †The soft agar colony assay was as described (*13*, *14*) with normal rat kidney cells (clone 49F) as indicators of progressive colony growth. The values represent the number of soft agar colonies per eight fields formed in response to concentrations of Abelson cell TGF's equivalent to the amount used to determine the percent EGF competition (column 1). ‡Both the 10,000- and 20,000-dalton Abelson cell TGF activities were tested in a competitive radioimmune assay (RIA) for ^{125}I-labeled EGF with rabbit antiserum to mouse submaxillary EGF. §Growth factors were analyzed by reverse phase HPLC on μBondapak C_{18} (*20*). Results are expressed as the percent of acetonitrile needed for elution of peak reactivity as measured by EGF competition. ‖Cells (2×10^6) were incubated overnight in 2 ml of medium containing [^{32}P]orthophosphate (1.0 mCi/ml) and growth factors as in column 1; they were extracted with phenol, hydrolyzed, and subjected to phosphoamino acid analysis (*11*). The results are expressed as [^{32}P]phosphotyrosine as a percentage of total ^{32}P-labeled cellular phosphoamino acids.

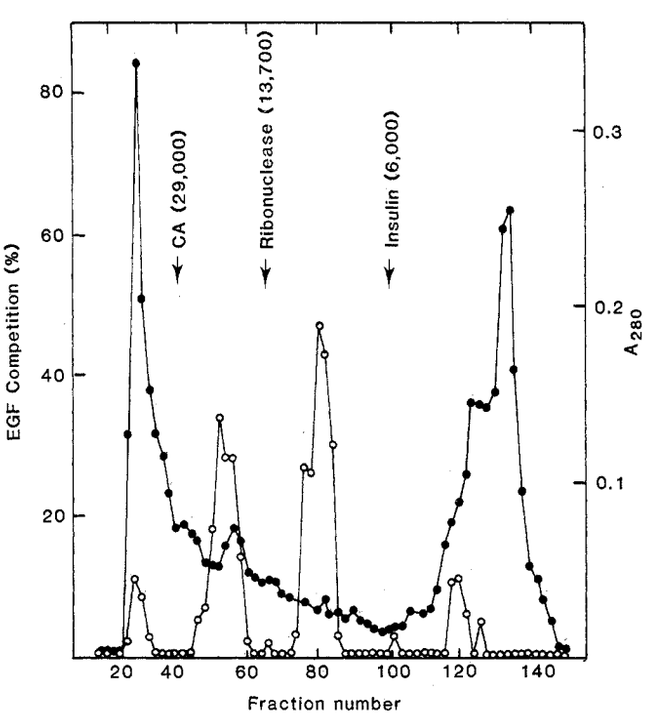

Fig. 1. Nonproductively transformed Abelson MuLV Fisher rat embryo cell monolayers were washed twice with serum-free medium; culture fluids were collected at three sequential 12-hour intervals, clarified by low-speed centrifugation, concentrated in a hollow fiber concentrator DC2 (Amicon, Scientific Systems Division), dialyzed against 1 percent acetic acid, and lyophilized. The lyophilized material from 6.0 liters of culture fluid was extracted according to a modification of the acid ethanol procedure (*24*, *25*) and applied to a Bio-Gel P100 (2.5 by 100 cm) column in 1*M* acetic acid. Individual fractions (3.5 ml) were collected and portions were tested for competition with ^{125}I-labeled EGF for binding to membrane receptors of A431 cells (*13*, *14*).

formation by Abelson MuLV. Production of TGF is correlated with a functionally active protein kinase activity associated with the Abelson MuLV–encoded transforming protein, P120. Whether TGF released by transformed cells is directly involved in the transformation process or is a consequence of the expression of the transformed phenotype is not resolved by the above experiments.

The low molecular weight growth factor released by Abelson MuLV transformed cells closely resembles the "sarcoma growth factor" identified (*14*) in culture fluids of MSV transformed cells. Growth factors associated with transformation by each of these viruses morphologically transform cells in vitro (*13*), compete with EGF for binding to homologous cell surface receptors (*14*), and induce tyrosine phosphorylation of EGF membrane receptors (*17*). TGF's produced by Abelson MuLV transformed cells further resemble the Moloney MSV associated growth factors in that under nondenaturating conditions they are observed as two distinct molecular species—one at approximately 10,000 and a second at 20,000 daltons. Whether these represent distinct factors or, as would seem more probable, multiple forms of the single 7400-dalton polypeptide purified from the lower molecular weight peak is not known. TGF's produced by Abelson MuLV and Moloney MSV transformed cells, respectively, resemble each other, although they can be distinguished from EGF by a number of biological and biochemical properties. The similarities in growth factors produced by cells transformed by retroviruses containing genetically distinct acquired cellular sequences favors the conclusion that TGF's represent cellular rather than viral gene products. The rat cell–derived TGF, when compared to TGF from human melanoma cells (*20*), shows identities in 17 of the first 19 positions in the amino acid sequence, while showing no obvious sequence homology with mouse or human EGF (*21*).

The mechanism by which cells convert to the transformed phenotype as a result of exogenous exposure to TGF's is still unclear. Since tyrosine phosphorylation of EGF receptors is induced by both EGF and TGF, this event, in itself, does not appear sufficient for transformation. Indeed, in certain situations EGF receptor phosphorylation can be distinguished from growth stimulation (*22*, *23*). Our findings indicate that TGF production may be a relatively common feature of retrovirus transformed cells, including those transformed by Abelson MuLV, and that such factors may be induced either directly or indirectly by phosphorylation of one or more cellular tyrosine acceptor sites. The recent demonstration of tyrosine phosphorylation of the EGF receptor by TGF's produced by human tumor cells (*17*) argues that identification of cellular pathways involved in the expression of the transformed phenotype will have significance for the understanding of cancer causation.

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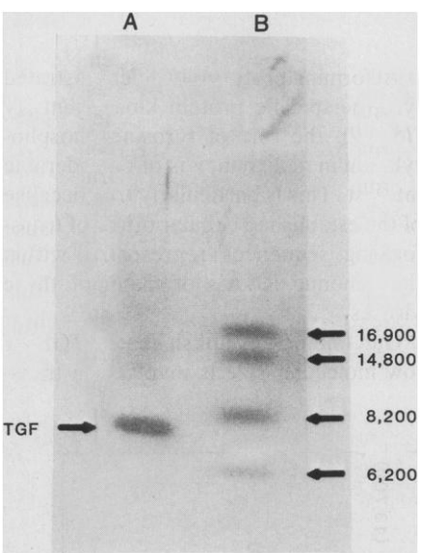

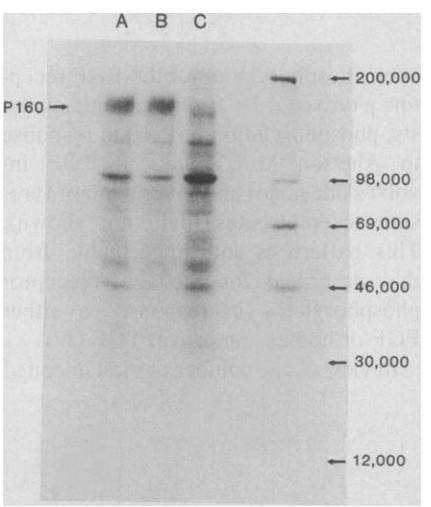

Fig. 2 (left). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of TGF produced by Abelson MuLV nonproductively transformed Fisher rat embryo cells. TGF was purified by reverse phase high-pressure liquid chromatography (*20*). The sample (100 ng) was dissolved in 10 μl of sample buffer containing 1 percent SDS and 2 percent 2-mercaptoethanol, incubated for 2 minutes at 100°C, and subjected to electrophoresis on an acrylamide (15 to 30 percent) gradient slab (*26*). After electrophoresis, gels were fixed in 50 percent methanol and 10 percent acetic acid for 2 hours and washed in 5 percent methanol. They were placed in 7 percent acetic acid overnight and then silver-stained (*27*). Molecular size standards include horse heart myoglobin (16,900 daltons) and three of its cyanogen bromide peptides (14,800, 8200, and 6200 daltons). Fig. 3 (right). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of substrates phosphorylated in A431 cell extracts in response to treatment with either mouse EGF or Abelson cell TGF. A431 human tumor cells were grown to confluency in Costar 24 well cluster plates. Culture fluids were aspirated, cells were rinsed with a buffer consisting of 10 m*M* sodium phosphate, *p*H 7.2, 200 m*M* NaCl, and 5 m*M* $MgCl_2$. The same buffer (0.05 ml) containing (A) 1.0 μg of EGF or (B) 200 ng of partially purified 10,000-dalton Abelson cell TGF (Fig. 1, fractions 75 to 85) or (C) 0.05 ml of buffer alone were added. The cells were incubated at 25°C for 1 minute and disrupted by treatment for 1 minute at 4°C in 0.05 ml of buffer consisting of 10 m*M* sodium phosphate, *p*H 7.2, 200 m*M* NaCl, 1 percent Triton X-100, 0.5 percent sodium deoxycholate, and 5 m*M* $MgCl_2$. [γ^{32}P]-ATP (50 μCi) was added and incubation was continued for 1.0 minute more. Reactions were terminated by addition of 0.05 ml of buffer consisting of 0.65*M* tris-HCl, *p*H 6.7, 1.0 percent SDS, 10 percent glycerol, 2.5 percent 2-mercaptoethanol, and 0.1 percent bromphenol blue, heated for 2 minutes at 90°C and analyzed by SDS-PAGE (*17*). Molecular size standards included ^{14}C-labeled myosin (200,000 daltons), phosphorylase (98,000 daltons), bovine serum albumin (69,000 daltons), ovalbumin (46,000 daltons), carbonic anhydrase (30,000 daltons), and cytochrome c (12,000 daltons).

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Nerve Fiber Growth on Defined Hydrogel Substrates

Abstract. *Cultured neurons become attached to hydrogel substrates prepared from 2-hydroxyethylmethacrylate but grow few nerve fibers unless fibronectin, collagen, or nerve growth factor is incorporated into the hydrogel. Antibodies to fibronectin inhibit nerve fiber growth on hydrogels containing fibronectin, which suggests that growing neurons interact directly with proteins trapped in the hydrogel. The adhesive requirements for attachment of neurons appear distinct and possibly less specific than those for fiber growth. Defined hydrogel substrates offer a controlled method for analyzing complex substrates that support nerve fiber growth and neuronal differentiation.*

The extent to which cultured neurons extend nerve fibers is known to depend on the culture substrate, but this dependence is poorly understood. Interactions of the cell surface with the substrate, especially at growth cones (that is, at the attached motile tips of growing nerve fibers), appear to determine the rate and direction of growth (*1*). Substrates that support fiber extension are thought to be suitably "adhesive" (*2*), but the factors responsible for this adhesiveness have not been defined in molecular terms. Attachment of neuronal somata to various substrates is commonly used as a measure of substrate adhesiveness (*3*), but it is not clear whether the molecular interactions involved in the attachment of somata are identical, or even related, to those that mediate nerve fiber extension.

The uncertainty results in part from difficulty in preparing defined substrates that support nerve fiber outgrowth. Dishes precoated with purified proteins and related macromolecules such as fibronectin, collagen, and polyamino acids support excellent nerve fiber outgrowth (*1, 2, 4*). However, analysis of the molecular interactions required for nerve fiber outgrowth is complicated by variability in the binding of macromolecules to the dishes (*5*) and in the subsequent dissociation of the macromolecules or their removal by cells (*6*), and by the difficulty that neurons from several sources extend nerve fibers when seeded on tissue culture dishes that have not been precoated. Moreover, the upper surfaces of nonneuronal cells (*7*) as well as materials deposited on the dish by such cells (*8*) have been shown to promote luxuriant fiber outgrowth. Thus, in primary neuronal cultures that are invariably contaminated by large numbers of nonneuronal cells (glia and fibroblasts), the substrate, whether it has been deliberately precoated or not, may be substantially modified by undefined products of nonneuronal cells.

We report here the results of an effort to elucidate the molecular properties of substrates necessary for nerve fiber outgrowth. Hydrogels prepared from 2-hydroxyethylmethacrylate (HEMA) permit neuron attachment, but support little nerve fiber growth. When the gels are modified by the inclusion of appropriate macromolecules within the gel matrix, neurons grow extensive nerve fibers. Small molecules such as mannose (molecular weight 180) can be easily incorporated into HEMA gels, whereas satisfactory, stable coating of culture dishes by incubation with solutions of small molecules is seldom feasible. As a result it is possible to study, in a controlled fashion,

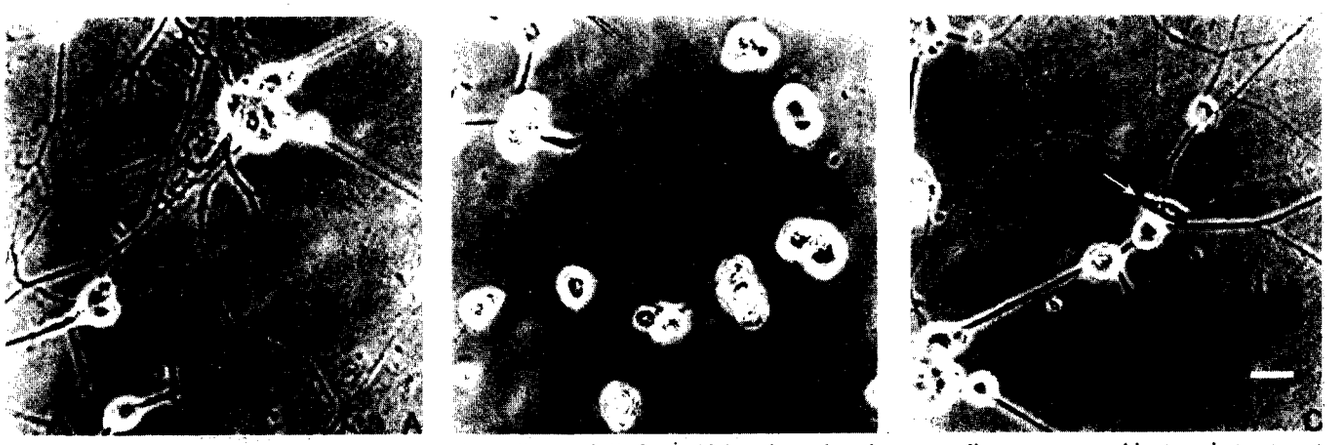

Fig. 1. Growth of neurons on HEMA gels containing collagen. Cells from chick embryo dorsal root ganglia were prepared by trypsin treatment and trituration of dorsal root ganglia from chick embryos (*14*). A suspension of cells in culture medium containing fibronectin-free horse serum was seeded onto circles of HEMA-collagen gels (1 mg/ml in tris-buffered saline, *p*H 7.4) or onto circles of HEMA with tris-buffered saline. There was no significant difference in the number of cells attached to the two types of gels. (A) On HEMA-collagen gels after 36 hours in culture 40 percent of the neurons had nerve fibers that averaged 208 μm in length per cell (N = 41). (B) After 36 hours 16 percent of the neurons on simple HEMA gels had grown nerve fibers that averaged 49 μm in length (N = 60). Total axon length per 100 neurons, determined by multiplying the fraction of growing neurons by the total nerve fiber length per cell, was 784 μm per 100 for neurons on simple HEMA gels and 8320 μm per 100 neurons on HEMA-collagen gels. After 36 hours the cells on simple HEMA gels were harvested from the gel by mild trypsin treatment (0.0125 percent trypsin in divalent cation-free Hanks balanced salt solution, *p*H 7.2, for 10 minutes at 23°C), then centrifuged and resuspended in culture medium (as above). These harvested cells were reseeded onto HEMA-collagen gels (1 mg/ml) and within 24 hours 30 percent of the neurons had extended fibers (C) that averaged 290 μm in length (N = 31; total axon length, 8600 μm per 100 neurons). A spread fibroblast (arrow) is visible in this photomicrograph.

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