Molecular Cloning and Expression of a Human B-Cell Growth Factor Gene in *Escherichia coli*

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A human B-cell growth factor (BCGF) (12 kilodaltons) supports the clonal proliferation of B lymphocytes. A clone was isolated that contained the proper structural sequence to encode biologically active, 12-kilodalton BCGF in *Escherichia coli* and to hybridize to a specific messenger RNA, identified by in vitro translation in *Xenopus laevis* oocytes. A relatively hydrophobic region of 18 amino acids was found at the amino terminal of the 124–amino acid–long polypeptide. The carboxyl terminal is composed of at least 32 amino acids that are derived from nucleotide sequences bearing significant homology to the Alu repeat family.

AFTER INITIAL ACTIVATION MEDIATED by agents such as antigen, lectin, or antibodies to immunoglobulin, B cells become responsive to putative lineage-specific growth factors (*1*). One B-cell growth factor (BCGF), which represents the predominant molecular species released by normal lectin-activated T cells, has been defined by its comitogenic effects on activated B lymphocytes derived from either peripheral blood or tonsils (*2*). The mature secreted form of this BCGF is 12 kD and has a major isoelectric point of 6.3 to 6.6 (*3*). Multiple human BCGF species of higher molecular weight (60-kD BCGF) have also been detected in the supernatants of hybridomas (*4*), malignant T cells (*5*), and normal lectin-activated T cells costimulated with phorbol myristate acetate (PMA) (*1*). In addition, immortalized human B cells secrete a high molecular weight (60-kD) material with BCGF activity (*5*). These observations suggest that there are either multiple genes encoding BCGF-like activities [as has been seen in the mouse (*6*)], or that the mature secreted 12-kD BCGF is a by-product of higher molecular weight BCGF(s). Two murine BCGF genes have recently been identified by recombinant technology and the human homolog to one [B-cell stimulatory factor–1 (BSF–1)] has also been cloned (*7*). Here we report on the molecular cloning of a human BCGF variant related to the 12-kD secreted product.

Enrichment of BCGF-specific message was obtained by isolating polyadenylated [poly(A)$^+$] messenger RNA (mRNA) from [0.75% phytohemagglutinin (PHA)-activated, v/v, 18 hours] T cells (in the presence of 5% monocytes) by the guanidinium isothiocyanate method (*8*). The mRNA was further size-fractionated on a 1% low-melting agarose methyl mercury hydroxide gel. Fractionated mRNA samples were microinjected into *Xenopus laevis* oocytes, and secreted products were bioassayed for BCGF activity with a BCGF-responsive, B-cell line (BD 9) and for interleukin-2 (IL-2) activity with a long-term cultured IL-2–dependent T-cell population (*1, 9*). The B-cell targets utilized are refractory to interleukin-1 (IL-1), IL-2, and interferon γ. The bioassay utilizing these B cells is significantly more sensitive (>10:1) to limiting quantities of BCGF than the conventional assay involving peripheral blood B cells activated with antibodies to the Ig-μ chain (anti-μ). Identification of IL-2 mRNA served as an internal marker for the fidelity of in vitro translation. Fractions showing the highest BCGF activity (that is, those derived from 13*S* to 17*S* mRNA) were subsequently used for construction of the complementary DNA (cDNA) library. Partially enriched BCGF mRNA (2 μg) was used as a template to synthesize double-stranded DNA that could be inserted into a Pst I–digested and oligo-(dT)-tailed pUC9 vector (*10*) and expressed under the control of the promoter of the β-galactosidase gene. Initial screening revealed approximately 700 clones containing cDNA inserts of appropriate length.

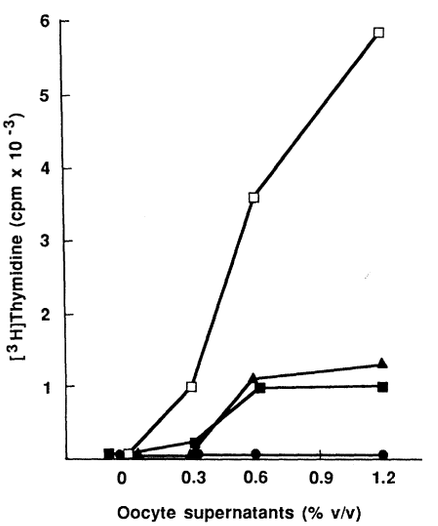

Fig. 1. Translation of hybrid-selected BCGF mRNA in *Xenopus laevis* oocytes. Plasmid DNAs (1 μg) were isolated and hybridized to poly(A)$^+$ RNA (400 μg) from activated T cells, as described, onto nitrocellulose filters. Bound mRNA was eluted by boiling for 1 minute, and it was microinjected in *Xenopus* oocytes. Secreted products were assayed on long-term factor-responsive human B cells. Results are represented in terms of [^{3}H]thymidine incorporation of 12 × 10^3 B cells. Symbols represent DNA from (■) pUC9; (□) pARJ43, (▲) pARJ55, and (●) pARJ36.

Complementary DNA–containing colonies were pooled into groups of 17 colonies and cell extracts were tested for BCGF activity. The BCGF activity of extracts was measured as the ability to stimulate [^{3}H]thymidine incorporation in B-cell lines (*1*). Only two pools consistently produced lymphokine activity when compared to negative control extracts from bacteria containing only the parental vector pUC9. Each pool contained only a single colony that exhibited BCGF activity. For example, treatment with extracts from colony 43 in pool 4 and from colony 45 in pool 11 resulted in an incorporation of 7608 ± 357 cpm and 2298 ±186 cpm, respectively, when used at 20% v/v. The [^{3}H]thymidine incorporation induced by negative control *Escherichia coli* extracts was 504 ± 79 cpm, whereas the positive control human BCGF standard (Cellular Products, Inc.) gave 16,420 ± 624 cpm. Complementary DNA inserts in the plasmids pARJ43 (pool 4) and pARJ45 (pool 11) were approximately 700 bp and 350 bp, respectively.

To further demonstrate that plasmid pARJ43 contained a BCGF-specific cDNA insert, we used the plasmid to select BCGF-specific mRNA (*11*) (Fig. 1). As a negative control, pUC9 and two other plasmids containing inserts were also assayed. The mRNA that hybridized to the plasmid DNAs was injected into *Xenopus* oocytes, and secreted products were assayed for BCGF activity. Only mRNA that hybridized to plasmid pARJ43 encoded BCGF bioactivity.

The nucleotide sequence of the cDNA insert in pARJ43 (Fig. 2) was determined by a combination of the M13 dideoxy and chemical degradation methods (*12*). This sequence predicts an mRNA size of at least 620 nucleotides. A single open reading frame of 372 nucleotides was found that encoded a polypeptide of 124 amino acids. The initiation *ATG* codon is located at position 67 of the nucleotide sequence and is associated with the true initiator codon–conserved sequences CCACAGGCATGG

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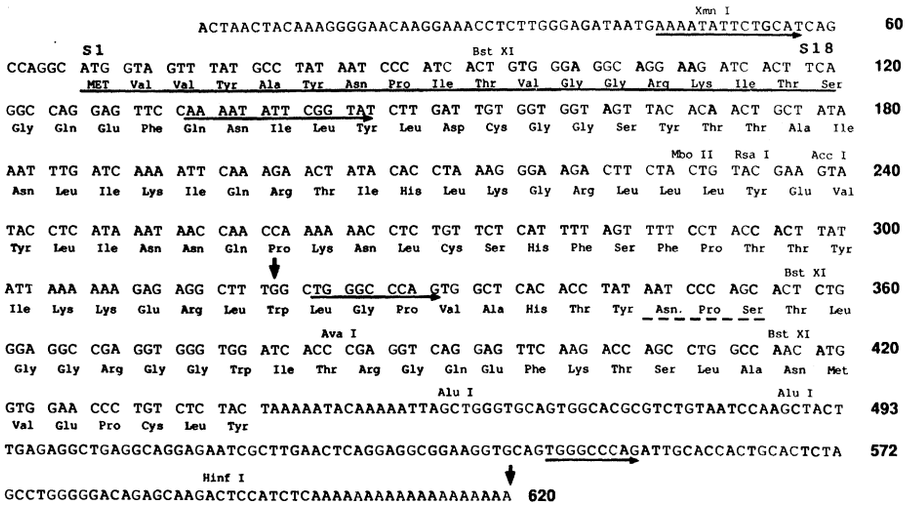

Fig. 2. Nucleotide sequence and predicted amino acid sequence of the human 12-kD BCGF cDNA insert. Position 1 of the nucleotide sequence is shown by the first nucleotide succeeding the oligo(dG) segment. The putative signal peptide (residue S1–S18) is underlined with a heavy line. A potential site for *N*-glycosylation is indicated by a dashed line. The horizontal arrows indicated direct repeats in the nucleotide sequence. The Alu-like segment in the BCGF cDNA is shown by vertical arrowheads.

(*13*). The termination codon *TAA* is located at position 439 of the nucleotide sequence.

Primer extension analysis was subsequently performed as described (*14*). In a series of experiments a 240-bp primer Pst I–Acc I fragment containing the 5′ end of the cloned BCGF gene was utilized with mRNA from unactivated human T cells and human T cells activated with PHA for 20 hours. There was no significant extension of the Pst I–Acc I fragment with either mRNA population and this suggests an intact 5′ end for the cloned gene. We next did experiments with an Acc I–Sau 3A′ fragment (140 bp) from the middle region of the BCGF cDNA, again by using mRNA from unactivated and PHA-activated T cells. There was significant primer extension of the 140-bp fragment when reannealed to activated T-cell mRNA with major bands around 260 bp and 400 bp. Several minor components were visualized in the >440-bp region of the gel. Although these results suggest that the entire coding region for the BCGF gene is present in the cDNA, the minor components visualized in the >440-bp region may also indicate that the entire 5′ end of the gene is not fully represented. The possibility should be explored that the cDNA isolated represents a component of a larger gene fragment that encodes a higher molecular weight BCGF.

The predicted amino acid sequence suggests the presence of a relative hydrophobic region of 18 amino acids at the NH_2 terminal of the polypeptide. There are three cysteine residues located at positions 30, 69, and 122 of the deduced amino acid sequence. No polyadenylation signal sequence was identified in the noncoding 3′ end region. Other important features include presence of the repeat sequences *AAAA-TATTCTGCA* and *TGGCCCAG* at positions 44 (133) and at 322 (545), respectively, of the nucleotide sequence.

Nucleotide sequence homology was analyzed by means of the ALIGN program of Intelligenetics, Inc. Human IL-2 (*15*) and human granulocyte-macrophage colony-stimulating factor (GM-CSF) (*16*) noncoding regions showed 56 and 53% homology, respectively, with the human BCGF sequences encompassing nucleotides 81–101 (IL-2) and 107–267 (GM-CSF). Alignment of the BCGF cDNA sequence with the human consensus Alu repeat unit (*17*) and the human IL-1β gene (nucleotides 463–657) showed 87 and 46% homology, respectively (Fig. 3). The sequence homology with the IL-1β gene (*18*) resides within the coding region of both genes. In addition, 46% homology with the IL-1β gene lies within the BCGF Alu insert and its immediate 5′ flanking region, suggesting the presence of the Alu-like sequences in the human IL-1β gene. The recently cloned murine BSF-1 gene (*7*) sequence and its human homolog did not show significant homolo-

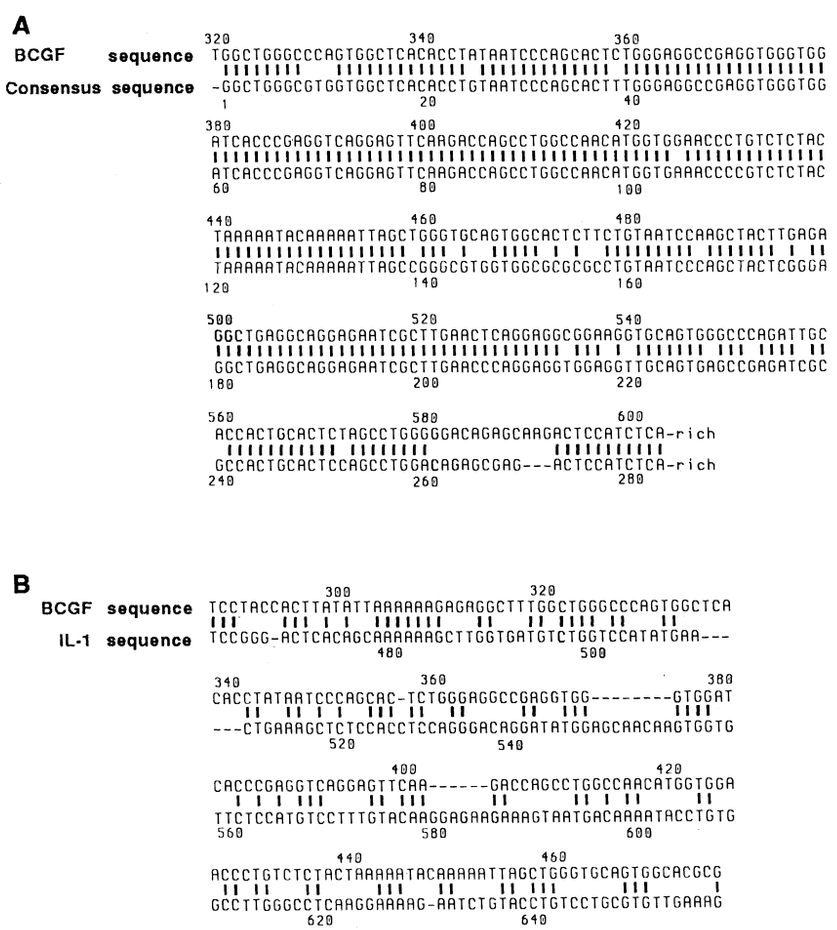

Fig. 3. Analysis of homology (**A**) between BCGF cDNA 3′ end containing the Alu repeat sequences (nucleotides 319–620) and a single unit of the Alu repeat family and (**B**) between the BCGF 3′ end region (nucleotides 287–477) and IL-1β coding sequences (nucleotides 463–657). The ALIGN program and gene bank of Intelligenetics, Inc., was used.

gy at either the nucleotide or amino acid level with 12-kD BCGF.

The 3′ end of the cDNA molecule that shares homology with the consensus Alu sequence contributes 32 amino acids to the BCGF protein. A potential *N*-glycosylation site is also located within this amino acid cluster (residues 94–96). Alu sequences are often flanked by direct repeats (*19*). In the nucleotide sequence presented here we are unable to define the terminal repeats as the 3′ end of the cDNA does not extend beyond the A-rich region. However, a perfect direct repeat *TGGGCCCAG* was found within the cDNA Alu region at positions 322 and 545 of the nucleotide sequence. The sequence containing direct repeat *TGGGCCCAG* represents the RNA polymerase III promoter site normally found in the left monomeric unit of the Alu repeat.

We can rule out the possibility that the nucleotides homologous to Alu sequences in the 3′ end of the BCGF gene represents an unspliced intron. First, no potential splice sites, 3′ consensus signal $^{tttttt}_{ccccccc}$ cag/G and 5′ consensus signal $^{C}_{A}$AG/gt$^{a}_{g}$agt (*20*), are found flanking the BCGF Alu insert. Second, the 5′ end of the Alu repeat within the BCGF cDNA gives rise to an open reading frame through 100 bp. Alu sequences have been found to contain numerous stop codons (*19*). Third, no canonical polyadenylation signal is found within the Alu repeat. If it were the case, then the transcription process would be terminated within the introns of many genes. Therefore, we presume that the polyadenylation signal is likely to be located downstream from the poly(A)$^+$ tail of the Alu repeat, and the Alu poly(A)$^+$ tail was utilized during cDNA construction.

The size of the biologically active hybrid protein specified by pARJ43 was approximately 15 kD (Fig. 4A). The fractions from plasmid pARJ43 that had BCGF activity on long-term cultured B cells also exhibited activity in anti-μ–activated peripheral blood β cells (Fig. 4B). No activity was detected in the fractions derived from pUC9. Sephadex G-50 fractions exhibiting BCGF activity were also shown to stimulate proliferation of anti-μ–activated tonsillar B cells (*5*). These results indicate that recombinant BCGF is capable of exhibiting the same biological activities as natural BCGF produced by human lymphocytes.

To demonstrate the specific hybridization of BCGF mRNA as compared to other moieties, we used the Pst I–Acc I fragment (Fig. 2), which corresponds to the Alu-free region of the cDNA, as a probe in a Northern blot analysis of total RNA from unactivated and PHA-activated peripheral blood lymphocytes. The Alu-free region of the DNA (Fig. 2; Pst I–Acc I fragment) minimally hybridized to mRNA of approximately 1.7 kb in unactivated peripheral blood lymphocytes (Fig. 5). However, after PHA activation (16 hours), the Alu-free cDNA probe also hybridized to an approximate 1.0-kb mRNA band. The amount of hybridizable mRNA, in particular the 1.7-kb RNA, was reduced when lymphocytes were activated for 30 or more hours. The induction of the 1.0-kb mRNA agrees with the fact that 12-kD BCGF is detected in supernatants only after activation of T lymphocytes. The human BCGF probe hybridized with 1.75-kb and 0.65-kb mRNA in PMA-activated EL-4 cells.

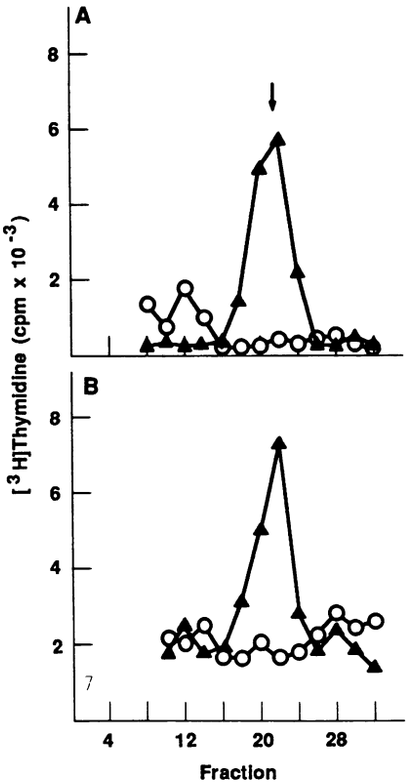

Fig. 4. Biological activity of recombinant BCGF. (**A**). Specificity on long-term cultured, factor-responsive B cells. *Escherichia coli* (100 ml) containing BCGF expression clones pARJ43 and control vector pUC9 were grown to optical density of 1.0 at 600 mn in the presence of isopropyl-β-D-thiogalactopyranoside (1 m*M*). The bacteria were pelleted and washed once with hypotonic buffer containing 20 m*M* Hepes (*p*H 7.5), 1.5 m*M* magnesium acetate, and 2 m*M* 2-mercaptoethanol. Cell pellets were resuspended in 5 ml of hypotonic buffer and sonicated three times at 30-second intervals by immersing the tubes in ice-chilled water. Cell extracts (supernatants) were obtained by centrifugation at 25,000 rpm for 30 minutes at 4°C and were immediately dialyzed overnight against 20 m*M* $NaPO_4$ (*p*H 7.5) buffer containing 0.2 m*M* phenylmethylsulfonyl fluoride. Dialized cell extracts were loaded onto a Sephadex G-50 gel-filtration column. Proteins were eluted with 20 m*M* phosphate buffer (*p*H 7.0), and fractions were assayed for the BCGF activity with long-term cultured, factor-responsive B cells. Results are represented in terms of [^{3}H]thymidine incorporation of a representative microtiter culture at 5% final concentration of each fraction. Symbols represent cell extract from pUC9 (○) and pARJ43 (▲). The arrow shows sedimentation of myoglobin. (**B**). Specificity on freshly prepared anti-μ–activated peripheral venous blood B cells. Fractions (1.2% final concentration) were assayed essentially as described in (A), except incubation in the microtiter assay continued for 72 hours before addition of [^{3}H]thymidine; pUC9 (○); pARJ43 (▲).

The detection of high molecular weight mRNA (1.7 kb) in T cells is noteworthy. Recently, a high molecular weight BCGF moiety of 60 kD has been reported both as a secreted molecule and as a putative intracellular precursor for the mature BCGF molecule (*3, 5*). If the 1.7-kb mRNA represents the gene for the high molecular weight BCGF, it is reasonable to presume that the BCGF gene belongs either to a multigene

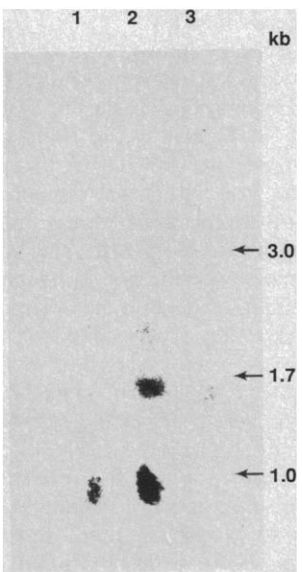

Fig. 5. Expression of BCGF mRNA in lectin-stimulated human T lymphocytes. Northern blot analysis was carried out by electrophoresis of 30 μg of total RNA from peripheral blood lymphocytes that were unactivated (lane 3), activated for 16 hours (lane 2), and peripheral blood lymphocytes activated for 30 hours (lane 1) on a 1% glyoxal-agarose gel. RNA samples were denatured with glyoxal for 1 hour at 50°C before gel electrophoresis. After electrophoresis, the RNA was transferred to nitrocellulose paper, and the blots were baked for 3 hours at 80°C. The blots were prehybridized and hybridized with ^{32}P-labeled BCGF cDNA (Alu-free cDNA from pARJ43) probe in the presence of 5× SSC (standard saline citrate). The hybridization and washing were carried out at 66°C. *Escherichia coli* ribosomal RNA (23*S* and 16*S*) was run in a parallel lane and visualized by ethidium bromide staining. The ^{32}P-labeled IL-2 probe was used to standardize the mRNA size in the 10*S* to 12*S* region.

family or to a differentially spliced single gene, and that there is a functional and structural relation between the low and high molecular weight BCGF moieties.

Note added in proof: Recent results from Caras *et al.* (*21*) indicate the presence of Alu-like sequences within the coding region of the nucleotide sequence for one form of decay-accelerating factor.

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9. Relative size determinations for BCGF mRNA were made by sizing total poly(A)$^+$ mRNA in association with in vitro translation. Normal human T cells (500 × 10^6), in the presence of monocytes (5%), were stimulated with PHA (0.75%) for 18 hours at a density of 2 × 10^6 cells per milliliter. Total RNA was isolated by CsCl gradient centrifugation in the presence of guanidinium isothiocyanate. Poly(A)$^+$ mRNA was isolated by elution of total RNA through an oligo-dT cellulose column. Total RNA (300 μg) routinely contributed 10 μg of poly(A)$^+$ RNA. Poly(A)$^+$ mRNA (60 μg) was size-fractionated on a 1% low-melting agarose gel in the presence of 10 m*M* methylmercuric hydroxide. The RNA was eluted from each sliced gel fraction and injected into *Xenopus laevis* oocytes. Supernatants conditioned by the injected oocytes were assayed for BCGF and TCGF (T-cell growth factor) activity at multiple dilutions.
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Elevated Levels of Glucose Transport and Transporter Messenger RNA Are Induced by *ras* or *src* Oncogenes

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An accelerated rate of glucose transport is among the most characteristic biochemical markers of cellular transformation. To study the molecular mechanism by which transporter activity is altered, cultured rodent fibroblasts transfected with activated *myc*, *ras*, or *src* oncogenes were used. In *myc*-transfected cells, the rate of 2-deoxy-D-glucose uptake was unchanged. However, in cells transfected with activated *ras* and *src* oncogenes, the rate of glucose uptake was markedly increased. The increased transport rate in *ras*- and *src*-transfected cells was paralleled by a marked increase in the amount of glucose transporter protein, as assessed by immunoblots, as well as by a markedly increased abundance of glucose transporter messenger RNA. Exposure of control cells to the tumor-promoting phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) for 18 hours had a similar effect of increasing the rate of glucose transport and the abundance of transporter messenger RNA. For *ras*, *src*, and TPA, the predominant mechanism responsible for activation of the transport system is increased expression of the structural gene encoding the glucose transport protein.

SINCE THE EARLY OBSERVATIONS OF Warburg (*1*), it has been widely appreciated that most tumor cells display increased rates of respiration, glucose uptake, and glucose metabolism as compared to untransformed cells. An accelerated rate of glucose transport is among the most characteristic biochemical markers of the transformed phenotype (*2, 3*). The molecular mechanisms responsible for the accelerated rate of glucose transport in transformed cells are unknown, but have been the subject of considerable inquiry.

When chicken embryo cells are infected with the Rous sarcoma virus (RSV), there is a marked increase both in the rate of glucose transport (*4, 5*) and in the abundance of glucose transport proteins in the plasma membrane (*6*). Substantial evidence indicates that these changes in glucose transport are independent of growth rate and are transformation specific (*5*). Although these changes are dependent on the activity of the viral transforming protein $pp60^{v\text{-}src}$ (*4, 7*), the molecular mechanism through which this transforming protein influences the number or activity (or both) of functional glucose transporters is unknown. In contrast to these findings with RSV, evidence obtained with another transforming virus, simian virus 40 (SV40), indicates that the increased transport rate may be caused, not by a change in the number of transporters, but by a functional change in transporters, including a redistribution to the plasma membrane (*8*).

Glucose transport in both normal and transformed fibroblasts is a facilitated diffusion, Na^+-independent process (*9*). These characteristics are similar to those of the erythrocyte glucose transporter (*9*); we have recently cloned the complementary DNA (cDNA) encoding this transporter (*10*) and have demonstrated its expression in many tissues and lines of cultured cells (*11*).

To explore the relation of transformation and glucose transport, we have examined

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