with small energy differences suggest that a protein is a disordered system that may have glasslike properties at low temperatures (*18, 19*). We focus here on one striking property of spin glasses, namely, their ultrametricity (*15, 29, 30*). For a random spin system, it has been shown (*30*) that for a set of replica structures (each one is defined by a stable orientation of the spins), a similarity function can be defined such that the replicas fall into disjoint clusters, in which the overlap of all pairs is larger than a specified (but arbitrary) value; the size of the cluster depends on the choice of the overlap criterion. Such an ultrametric system is isomorphous with an evolutionary tree (*15, 29*). To apply this concept to the myoglobin simulation, we consider each of the minimized structures as a replica and use the rms difference between them as the overlap criterion. There is a rather sharp transition between the range (rms > 1.5 Å) when all structures are disjoint, and the range (0 < rms < 1 Å) when all the structures belong to the same cluster. In a very narrow region (1 < rms < 1.5 Å), there are sets of disjoint clusters with more than one structure per cluster; for rms = 1.2 Å, there are clusters of size 1 (several) and one each of 2, 4, 7, and 9. Since the rms overlap criterion is arbitrary, we have tried others (for example, distance matrices) and have obtained corresponding results.

It appears that ultrametricity is not a useful concept when applied to the myoglobin simulation. What this means for actual myoglobin molecules or other proteins is not clear, since the simulation is short and the structures form a sequence deviating in time. Also, it is not known whether glasses [in contrast to the special model for a spin glass considered in (*30*)] are ultrametric.

Myoglobin at normal room temperatures samples a very large number of different minima that arise from the inhomogeneity of the system. This is expected to have important consequences for the interpretation of myoglobin function and, more generally, for the functions of other proteins, including enzymes. There are solidlike microdomains (the helices), whose main-chain structure is relatively rigid, and liquidlike regions (the loops and the side-chain clusters at interhelix contacts) that readjust as the helices move from one minimum to another. Since the minima have similar energies, myoglobin is expected to be glasslike at low temperatures. Freezing in of the liquidlike regions could result in a transition to the glassy state.

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Construction of a Novel Oncogene Based on Synthetic Sequences Encoding Epidermal Growth Factor

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The autocrine model postulates that constitutive release of a mitogenic growth factor can lead to uncontrolled proliferation and cell transformation. A synthetic polynucleotide encoding epidermal growth factor conferred a tumorigenic phenotype on cells. These cells were transformed through the action of an autocrine circuit having an extracellular component.

NEOPLASTIC TRANSFORMATION OF cells by oncogenes and carcinogens results in the elaboration of growth factors (GF's). These include transforming growth factors (TGF's), which are able to induce some aspects of the transformed phenotype in nontransformed cells (*1*). Since such factors may affect the behavior of the cell that has released them, these findings suggested an autocrine mechanism of transformation whereby production of TGF's by a cell leads to uncontrolled stimulation of its own growth (*2*).

The role of TGF's in transformation has been difficult to assess because transformed cells produce a number of factors that interact in complex ways [for example, see (*3*)] and because the properties of TGF's have been studied primarily in culture. Furthermore, it remained unclear whether induction of transformation is a special property of TGF's, or whether constitutive production of any mitogen by cells that can respond to this mitogen is sufficient to induce transformation. To address these questions, we have used an expression vector to induce constitutive production and secretion of human epidermal growth factor (EGF). EGF was chosen for these studies because the factor and its receptor are well characterized (*4*) and because transformed cells frequently produce TGF-α, a factor that binds to the EGF receptor and has nearly identical physiological properties to those of EGF (*5*).

The EGF expression vector, designated pUCDS3 (*6*), consists of a murine leukemia virus long terminal repeat (LTR) that contains promoter/enhancer sequences, an SV40 polyadenylation site, sequences encoding a mouse immunoglobulin heavy-chain signal peptide (*7*) (to cause secretion of the translation product), and chemically synthesized human EGF-encoding sequences (originally designed for translation in *Escherichia coli*) (*8*) (Fig. 1). The immunoglobulin (Ig) signal peptide–encoding sequence was mutagenized to create an Eco RI cleavage site (Fig. 1C). To reconstruct the signal peptidase cleavage site, the 5′ end of a 175-bp synthetic gene encoding EGF

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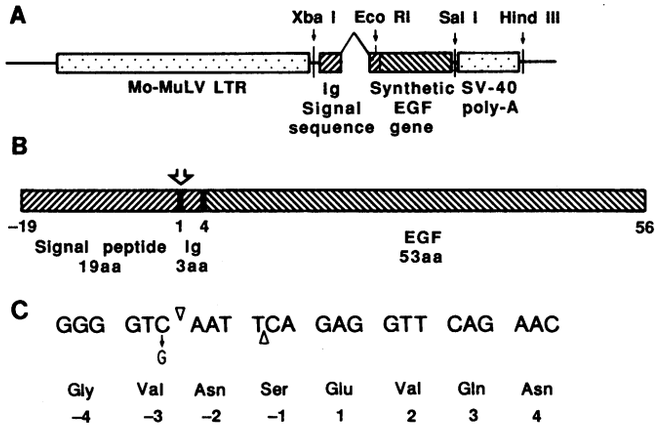


Fig. 1. IgEGF gene (*6*). (**A**) Functional domains. (**B**) Predicted translation product. Arrow indicates signal peptidase cleavage site. (**C**) Arrow indicates mutation introduced to create Eco RI cleavage site (triangles) in signal peptide-coding region.

was modified to include an Eco RI site and sequences encoding the first three amino acids of the immunoglobulin. The translation product encoded by these sequences should consist of a 19–amino acid signal peptide (that should be cleaved off) followed by three amino acids of immunoglobulin fused to the 53 amino acids of human EGF (Fig. 1B). The polypeptide specified by this artificial gene is substantially smaller than the authentic EGF propeptide (*9*).

This terminally modified form of EGF will be referred to as IgEGF. The modification of EGF should not greatly alter its properties because addition of up to 14 amino acids to the NH_2-terminus of human EGF has no effect on its activity (*10*). A control plasmid, pUCDS5, was constructed in which a 4-bp insertion causes a frameshift resulting in premature translation termination in the signal peptide (*6*).

Upon transfection into FR3T3 fibroblasts or Rat-1 fibroblasts (*11*), the IgEGF expression vector induced focus formation with the same efficiency as a plasmid carrying a Ha-*ras* oncogene (Fig. 2, B and C; approximately 400 foci per 2.5 μg of DNA per 10^6 cells). The control plasmid pUCDS5 did not induce focus formation (Fig. 2A), indicating that focus induction requires production of the complete translation product. Foci induced by the IgEGF gene were smaller than *ras*-induced foci and contained refractile cells that grew to high density (Fig. 3).

Clonal cell lines were derived after isolation of pUCDS3-induced foci (*12*). Some of these cell lines expressed high levels of IgEGF messenger RNA (mRNA) (Table 1) and Southern blot analysis showed that these same cell lines contained the highest copy number of transfected pUCDS3 DNA. To determine if these cell lines secreted IgEGF, medium conditioned by these cells was assayed for the presence of EGF-competing activity. Such an activity was detected only in medium conditioned by the cell lines expressing high levels of IgEGF RNA (Table 1), and therefore was correlated with expression of the transfected gene. The cell lines expressing low levels of IgEGF mRNA probably produce IgEGF at levels below the limit of detection in these competition assays (approximately 0.5 n*M*).

The EGF-competing activity of medium conditioned by one of the clonal cell lines (19-3) was blocked by a monoclonal anti-

Table 1. Properties of transfected FR3T3 cell lines. FR3T3 cells were transfected with pSV2-neo (*neo*; G418-resistance), pEJ6.6 (*ras*), pUCDS3 (IgEGF), or pSVc-myc-1 (*myc*) as described (*11*). EGF-competing activity (*26*) and growth in soft agar (*14*) were measured as described.

Cell line	Transfected gene(s)	IgEGF RNA*	EGF-competing activity (n*M*)	Saturation density†	Hexose uptake‡	Soft agar
FR3T3	None	None	<0.5	1.0	1.0	–
Neo-1	*neo*	None	<0.5	1.6	2.3	–
Ras-3	*neo/ras*	None	<0.5	9.3	24.8	+
2A20-2	IgEGF	None	<0.5	3.1	2.7	–
2B20-4	IgEGF	None	<0.5	1.6	1.9	–
2B20-5a	IgEGF	None	<0.5	1.1	1.6	–
2A20-7	IgEGF	Low	<0.5			–
2B20-6	IgEGF	Low	<0.5	1.6	4.7	–
3B20-5	IgEGF	High	3	4.2	4.1	–
3A5-4	IgEGF	High	4	4.4	10.7	±
2A20-3b	IgEGF	High	5	4.1	5.7	–
19-3	*neo*/IgEGF	High	3	9.4	10.1	±
26-17	*neo*/IgEGF/*myc*	High	14	4.5	11.0	±
62	*neo*/IgEGF	Low	<0.5			
21-21	*neo*/IgEGF	Low	<0.5			
20-6	*neo*/IgEGF	Low	<0.5			

*Estimated by visual inspection of Northern blots probed with the Eco RI–Sal I fragment of pUCDS3. †Cells were seeded in triplicate at 10^5 cells per 16-mm well in 24-well dishes, incubated in 2.0 ml of DMEM–10% CS-P/S without refeeding for 6 days, and counted. Experiments with representative cell lines showed that cells reached saturation densities within this time. The results are expressed relative to the number of FR3T3 cells (2.1×10^5 per well). ‡Performed as described (*27*) except that labeling was in phosphate-buffered saline (PBS) containing 1% dialyzed calf serum at 37°C and the Bradford protein assay (Bio-Rad) was used. Results are expressed as counts per minute per microgram of protein relative to the value obtained for FR3T3 cells.

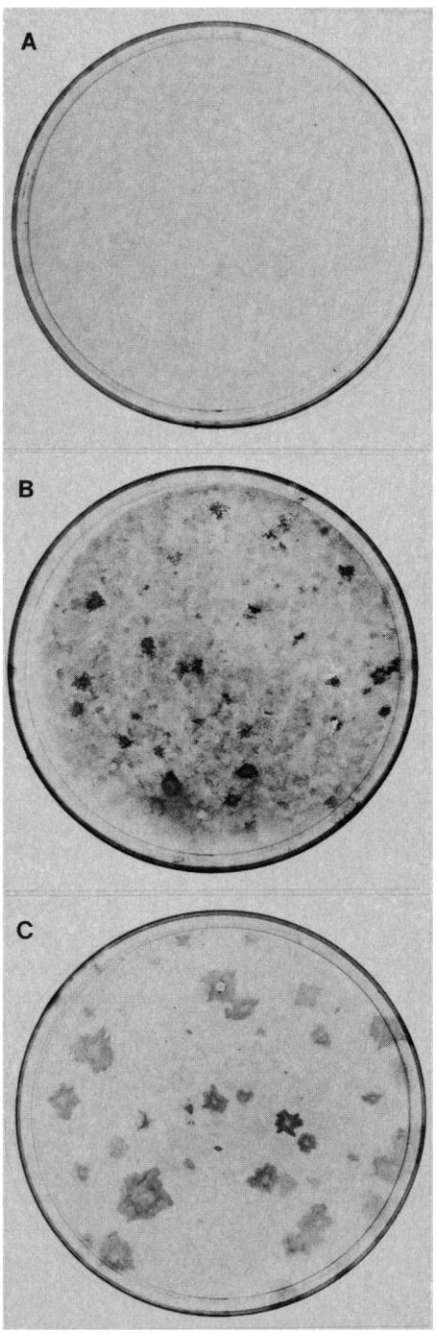


Fig. 2. Focus assays. Plasmids pUCDS5 (**A**), pUCDS3 (**B**), or pEJ6.6 or pEJ6.6 carrying an Ha-*ras* oncogene (**C**) were introduced by transfection into FR3T3 cells (*11*). Cells were split 1:20 30 hours after transfection. Cultures were stained after 3 weeks.

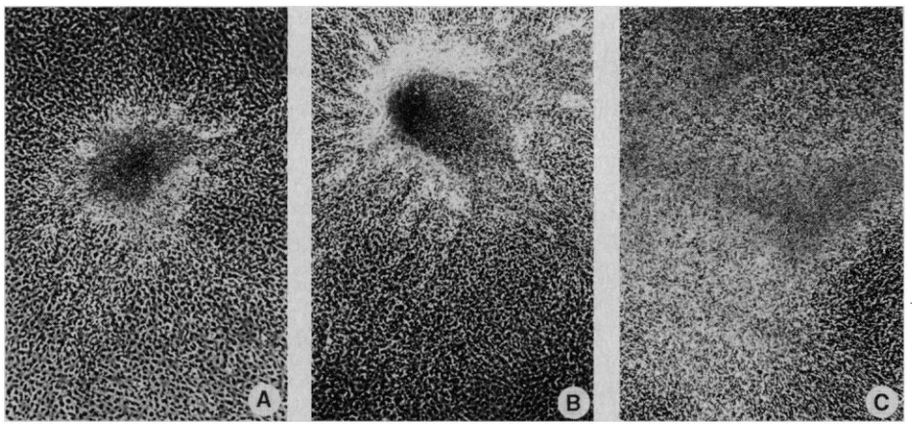


Fig. 3. Photomicrographs of foci induced by transfection of pUCDS3 (**A** and **B**) or pEJ6.6 (**C**) into FR3T3 cells.

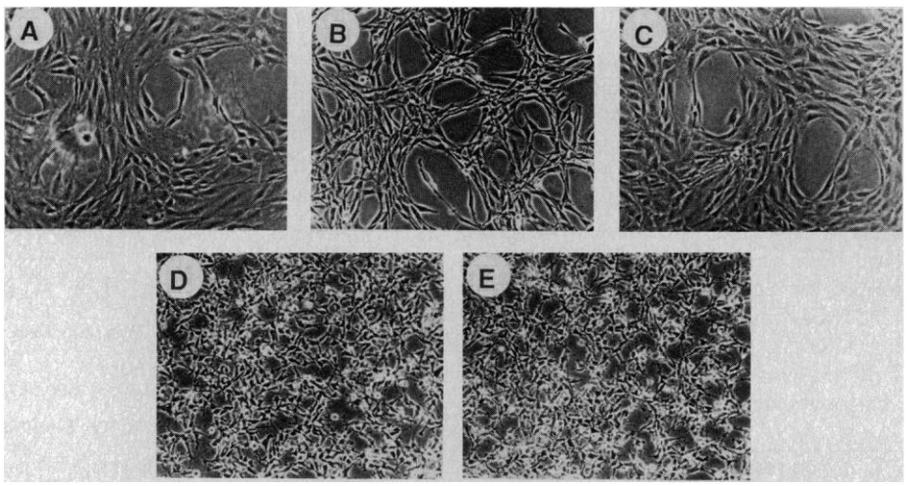
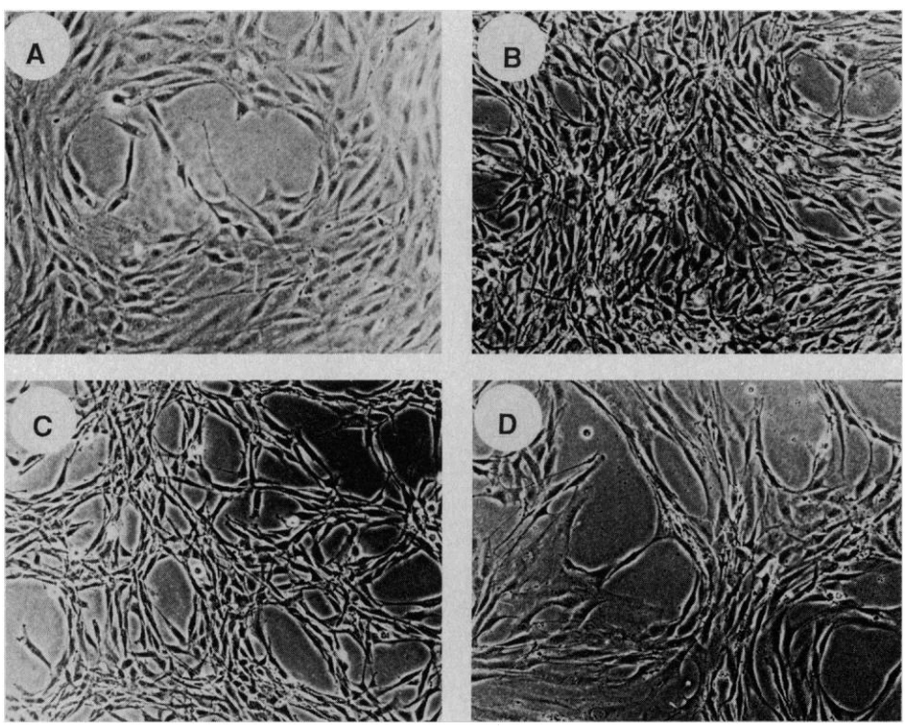


Fig. 4. Morphology of transfected cells. Cells were seeded in 60-mm culture dishes. The next day the medium was replaced and supplemented with 100 μl of hybridoma 5D12 (anti-EGF) ascites fluid (**C** and **E**). Cultures were photographed with Kodak Tri-X Pan film 4 days after seeding. (**A**) FR3T3 cells, (**B**) 19-3 cells, (**C**) 19-3 cells plus 5D12 ascites fluid, (**D**) Ras-3 cells, (**E**) Ras-3 cells plus 5D12 ascites.



body (5D12) to human EGF. This antibody prevents binding of human, but not murine, EGF to its receptor and does not recognize TGF-α (*13*). This indicated that the EGF-competing activity was specified by the introduced gene encoding human EGF.

The phenotypes of IgEGF-producing cell lines were examined to determine the consequences of chronic autocrine stimulation by IgEGF. One of the cell lines, 26-17, had been cotransfected with a *myc* oncogene, which could further influence the phenotype of these cells by altering their responsiveness to EGF (*14*). The cells that produced high levels of IgEGF were spindle-shaped and highly refractile (Figs. 4B and 5C) but were not as morphologically transformed as cells carrying the *ras* oncogene (Fig. 4D). Cells producing high levels of IgEGF were further distinguished from the parental cell line by growth to high saturation densities and rapid hexose uptake (Table 1). In contrast to *ras*-transformed Ras-3 cells, the IgEGF-producing cell lines grew poorly in soft agar. The higher saturation density, increased hexose uptake, and poor anchorage-independent growth of pUCDS3-transfected cells were similar to effects elicited by exogenously added EGF (*4, 14, 15*).

The IgEGF-producing cell lines induced tumors when injected into nude mice (Table 2). The tumors were well circumscribed, noninvasive fibrosarcomas. They generally appeared after 3 weeks and showed a longer latency than *ras*-induced tumors, which were visible as early as 8 days after inoculation. Two cell lines that expressed low levels of IgEGF mRNA (20-6 and 21-21) induced tumors with low frequency only after nearly 8 weeks (Table 2). This exceptionally long latency could have resulted from slow growth rate of these tumor cells, from selection of variants producing higher levels of EGF, or from outgrowth of subclones that acquired a second genetic alteration.

If the transformed phenotype of IgEGF-producing cells were due to the effects of secreted factor, it should be possible to cause a reversion of the phenotype by incubating these cells with antibody to EGF (anti-EGF), thereby interrupting the autocrine circuit. Indeed, ascites fluid containing monoclonal antibody 5D12 caused reversion of the refractile morphology of 19-3

Fig. 5. Transforming activity produced by 19-3 cells. Methods were as described in the legend to Fig. 4 except that 1 day after seeding the culture medium was replaced with 2.0 ml DMEM–10% CS-P/S plus 2.0 ml of conditioned medium (*26*). (**A**) FR3T3 cells in medium conditioned by FR3T3 cells; (**B**) FR3T3 cells in medium conditioned by 19-3 cells; (**C**) 19-3 cells; (**D**) FR3T3 cells in medium conditioned by 19-3 cells plus 100 μl 5D12 ascites.

Table 2. Tumorigenicity of transfected FR3T3 cell lines. Nude mice were irradiated (500 rads ^{137}Cs). Cells were washed and suspended in PBS, and 200 μl were injected subcutaneously. In experiment 1, NIH(S) nu mice (obtained from NIH), approximately 12 weeks old, received 0.4×10^6 or 2.0×10^6 cells per injection. In experiment 2, nu/nu female mice (Charles River Laboratories), 4 weeks old, received 10^6 cells per injection site. Tumors greater than 0.5 cm in diameter at the time indicated were scored positive. Results are expressed as the fraction number positive/number of sites injected.

Cell line	Experiment 1		Experiment 2
	4 weeks	8 weeks	4 weeks
FR3T3	0/5	0/5	
Neo-1	0/4	0/3	0/2
Ras-3	1/1		
2A20-2			0/5
2B20-4			0/2
2B20-5a			0/4
2B20-6			0/5
3B20-5			1/4
3A5-4			3/5
2A20-3b			4/5
19-3	4/5	5/5	10/12
26-17	3/4	4/4	2/2
62	0/4		
21-21	0/4	2/4	
20-6	0/4	1/4	

cells (Fig. 4C). This effect was specific—purified 5D12 IgG yielded the same result, a control hybridoma ascites fluid had no effect, and 5D12 ascites fluid did not alter the morphology of Ras-3 cells, which carry a Ha-*ras* oncogene (compare Fig. 4E to 4D). These results confirmed that the refractile morphology of 19-3 cells is a consequence of IgEGF production and demonstrated that the IgEGF autocrine circuit is completed outside the cell.

Medium conditioned by 19-3 cells induced a transformed morphology in nontransfected FR3T3 cells (Fig. 5) and therefore contains secreted transforming activity. This effect was blocked by 5D12 ascites (Fig. 5D) and purified 5D12 IgG but not control ascites, demonstrating that IgEGF is a necessary component of the transforming activity. Since EGF added to the medium elicits a similar transformed morphology, it is likely that IgEGF production is sufficient for the morphological transformation of these previously normal cells. It remains possible that cells transformed by pUCDS3 release factors other than IgEGF. However, such other factors apparently do not contribute greatly to the transformed phenotype induced by the conditioned medium.

Our results show that the mitogenic signals produced by the EGF receptor in response to its normal ligand are sufficient to transform fibroblasts. These results are consistent with a more general model in which constitutive production of normal growth stimulatory signals by a GF receptor leads to transformation. Such signals could be caused by constitutive elaboration of GF's or by a GF receptor that has been deregulated following alterations in its structure. Thus oncogenic mutations in genes encoding known (*erbB* and *fms*) or suspected (*ros* and *neu*) GF receptors may act by constitutively activating these receptors. The present results were complemented by the recent finding that expression of a transfected TGF-α gene also transforms fibroblasts (*20*). Since constitutive expression of the PDGF-like v-*sis* protein (*21*) and granulocyte-macrophage colony-stimulating factor (*22*) also results in neoplasia, it appears that any gene encoding a mitogenic growth factor may become an oncogene when expressed inappropriately by responsive cells. Others have succeeded in blocking autocrine circuits in transformed avian myeloblasts (*23*), *sis*-transformed cells (*24*), and human small cell lung carcinomas (*25*) with an approach similar to ours. Methods for blocking autocrine circuits may become clinically valuable in cases where GF oncogenes have been activated. The cell lines transformed by IgEGF will provide a useful model for comparing approaches to inhibiting autocrine transformation because anti-EGF sera, anti–EGF receptor sera, and EGF antagonists are available.

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6. Construction of pUCDS3. pFB1, which consists of a partial Sau 3A fragment containing the Moloney murine leukemia virus LTR cloned into the Bam HI site of pUC13, was constructed by J. Morgenstern and H. Land, Whitehead Institute. The single Eco RI site of pFB1 was destroyed by cleaving with Eco RI, filling in, and religating to produce pFB1ΔRI. The Nde I–Pst I fragment of immunoglobulin heavy chain complementary DNA clone 17.2.25 (*7*), which contains sequences encoding the signal peptide, was modified by oligonucleotide-directed mutagenesis [G. Dalbadie-McFarland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6409 (1982)] to create an Eco RI site (Fig. 1C). The fragment excised by cleavage at the Ava II site upstream from the coding sequences and at the newly created Eco RI site was joined to pFB1ΔRI cleaved with Sal I and Hind III by using Ava II–pseudo-Sal I and Eco RI–Hind III adapters to yield pUCDS2. A chemically synthesized gene encoding human EGF (*8*) was provided with an Eco RI site and upstream sequences encoding the first three amino acids of immunoglobulin 17.2.25 and subcloned into pUC8. The Hpa I–Bam HI fragment containing the SV40 polyadenylation site was provided with synthetic Hpa I–Sal I and Bam HI–Hind III adapters and cloned into pUC8 adjacent to the EGF gene. The Eco RI–Hind III fragment containing the EGF gene and polyadenylation site was then cloned into pUCDS2 to produce pUCDS3. pUCDS5 was produced by cleavage of pUCDS3 at the single Eco RI site, filling in with the large fragment of DNA polymerase I, and religating to yield a four nucleotide insertion. The structures of pUCDS3 and pUCDS5 were confirmed by DNA sequencing.
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13. The antibody 5D12 is a neutralizing monoclonal antibody to fMet-human EGF developed and characterized by Margery Nicolson, Amgen, Inc.
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26. To prepare conditioned media, dense cultures of cells were incubated in DMEM–10% CS-P/S containing 10 m*M* Hepes-NaOH, *p*H 7.3 (10 ml per 100 mm culture dish), for 2 to 4 days. Conditioned media were diluted 1:1 with binding buffer (bicarbonate-free DMEM–0.5% BSA–50 m*M*–Hepes-NaOH, *p*H 7.5) containing ^{125}I-labeled murine EGF (New England Nuclear; 2 ng/ml; 150 μCi/μg). Standard curves were prepared with nonlabeled fMet-human EGF (Amgen). Rat-1 cell monolayers were chilled, washed twice with binding buffer, incubated with duplicate portions of test samples for 4 hours on ice, washed three times rapidly with cold binding buffer, dried, and solubilized with 1*M* NaOH. Cell-associated radioactivity was determined and compared to standard curves.
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