nol series. They were exposed to x-ray film (XAR-5, Kodak Laboratories) with intensifying screens for 16 to 18 hours at −70°C to give a guide to intensity of hybridization. Slides were dipped in NTB-3 photoemulsion (Kodak Laboratories) diluted 1:1 with distilled water, air-dried, and exposed in sealed boxes for 5 to 21 days to 4°C. Photoemulsion was developed with D19 developer (Kodak), fixed, washed, air-dried, stained with hematoxylin and eosin, dehydrated in an ascending ethanol series and then xylene, and mounted and cover slips applied with Permount. The hybridization signal was visualized with the bright- and dark-field optics of a Leitz photomicroscope.

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A Chimeric, Ligand-Binding v-*erb*B/EGF Receptor Retains Transforming Potential

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Comparison of amino acid sequences from human epidermal growth factor (EGF) receptor and avian erythroblastosis virus *erb*B oncogene product suggests that v-*erb*B represents a truncated avian EGF receptor gene product. Although both proteins are transmembrane tyrosine kinases, the v-*erb*B protein lacks most of the extracellular ligand-binding domain and a 32–amino acid cytoplasmic sequence present in the human EGF receptor. To test the validity of the proposed origin of v-*erb*B and to investigate the functional significance of the deleted extracellular sequences, a chimeric gene encoding the extracellular and the transmembrane domain of the human EGF receptor joined to sequences coding for the cytoplasmic domain of the avian *erb*B oncogene product was constructed. When expressed in Rat1 fibroblasts, this reconstituted gene product (HER-*erb*B) was transported to the cell surface and bound EGF. Its autophosphorylation activity was stimulated by interaction with the ligand. Expression of the HER-*erb*B chimera led to anchorage-independent cell growth in soft agar and EGF-induced focus formation in Rat1 monolayers. Thus, it appears that v-*erb*B protein sequences in the chimeric receptor retain their transforming activity under the influence of the human extracellular EGF-binding domain.

UNTIL RECENTLY, THE NORMAL biological role of cellular homologs of transforming genes was poorly understood. Some of these genes have now been shown to encode growth factors or growth-factor receptors, which when constitutively expressed or structurally altered may result in loss of normal growth control and thus oncogenesis. Two members of the tyrosine kinase oncogene family, v-*erb*B and v-*fms*, are highly homologous to receptors for epidermal growth factor (EGF) (*1, 2*) and macrophage colony-stimulating factor CSF-1 (*3*), respectively. The chemically activated *neu* oncogene product has also been shown to be structurally similar to the epidermal growth factor (EGF) receptor and to v-*erb*B (*4–6*) and probably represents another cell surface receptor for an unknown ligand with unknown biological activity.

Amino acid sequence comparison suggested that v-*erb*B was derived from the avian EGF receptor gene by gene truncation as a consequence of the recombination event that created the avian erythroblastosis virus (*2*). Both molecules are transmembrane tyrosine kinases; when aligned with the human EGF receptor sequence, v-*erb*B lacks the signal peptide for membrane translocation (24 amino acids), most of the extracellular ligand-binding domain (amino acids 1–555), and 32 carboxyl-terminal amino acids. We have been interested in characterizing the structural alteration that led to the generation of an oncogene from a normal growth factor receptor gene. Therefore, we have replaced the EGF binding region missing from the v-*erb*B protein to determine whether the presence of this domain is sufficient to regenerate a normal, ligand-responsive receptor from a transforming protein. The polypeptide generated by this gene fusion consists of normal human extracellular ligand-binding and transmembrane domains linked to oncogenic avian cytoplasmic sequences.

Cloned complementary DNA (cDNA) sequences coding for the human EGF receptor (HER) ligand-binding and transmembrane domains (residues −24 to 646) (*2*) were fused in vitro without sequence alteration with cytoplasmic domain sequences of the v-*erb*B oncogene (AEV-H strain) (*7*). A 2-kb Sac I–Nar I restriction fragment coding for the complete extracellular and transmembrane domain of the EGF receptor was combined with a 1.7-kb Aha II–Stu I restriction fragment coding for the complete cytoplasmic portion of (AEV-H)-*erb*B and cloned into Sac I/Sma I double-digested pUC12 (*8*). A 3.7-kb Sac I–Xmn I restriction fragment from this subclone containing the entire coding region of the chimeric receptor was then introduced into an expression vector downstream of a 640-bp Sma I–Sac I restriction fragment with Harvey sarcoma virus long terminal repeat promoter sequences and upstream of 3′-untranslated sequences of the gene coding for the hepatitis B virus surface antigen (*9*) providing transcription stop and polyadenylation signals. The vector also contained a dihydrofolate reductase (DHFR) gene under SV40 early promoter control to permit gene amplification by methotrexate selection (*10*) in addition to a neomycin resistance gene (*11, 12*) for the initial selection of transfectants. Sequences of the *Escherichia coli* plasmid pBR322 allowed plasmid DNA replication in *E. coli*. Expression plasmids were purified from *E. coli* HB101 by the alkaline extraction procedure (*13*) followed by a gel filtration purification step. Normal Rat1 fibroblasts were transfected by the calcium phosphate precipitation method (*14*) and selected for neomycin and methotrexate resistance.

The ability of transfected cells to bind increased levels of ^{125}I-labeled EGF was used to determine whether mutant EGF receptors were properly synthesized and transported to the cell surface of Rat1 recipient cells. Two transfected cell lines used for subsequent experiments displayed dramatically increased ^{125}I-labeled EGF surface binding relative to control Rat1 cells (1×10^4 receptors per cell). The HER-*erb*B1 line expressed about 8×10^4 and HER-*erb*B2 about 6×10^5 chimeric molecules per cell, which compares with

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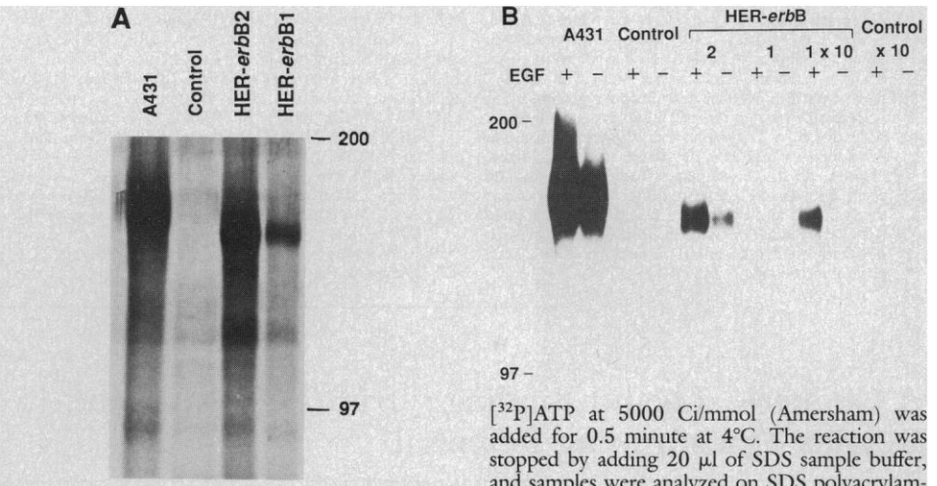

Fig. 1. (**A**) Immunoprecipitation of [^{35}S]methionine–labeled HER-*erb*B chimera. Stable transfected Rat1 cell lines were selected through the use of media supplemented with 400 μg of Geneticin (Gibco Laboratories) per milliliter starting 2 days after transfection. Medium supplemented with appropriate concentrations of methotrexate (Gibco Laboratories) containing 7% dialyzed fetal bovine serum was used for stepwise selection of amplified cDNA expression in neomycin-resistant cell lines. Human epidermoid carcinoma cells A431 were cultured in Dulbecco's minimum essential medium (DMEM) containing 4.5 mg of glucose per liter. Confluent cell monolayers in 8-cm culture dishes were washed twice with phosphate-buffered saline (PBS), 5 ml of methionine-free medium prepared from a minimum essential medium select amine kit (Gibco Laboratories) was added, and the incubation continued for 17 hours with 300 μCi of L-[^{35}S]methionine at 1000 Ci/mmol (Amersham). Cells were washed twice with PBS before being lysed and immunoprecipitated as described (*22*) using human EGF receptor-specific MabRI (*23*). Half the number of A431 cells were used as compared to the Rat1 cell lines. Size markers are indicated in kilodaltons. (**B**) EGF-stimulated autophosphorylation of HER-*erb*B receptors. Detergent cell lysates diluted to 0.5% TX-100 in 0.4 ml were incubated with (+) or without (−) 5 μg of EGF (Collaborative Research) per milliliter for 15 minutes at 4°C. Antibody (MabRI) (*23*) that had been bound for 30 minutes to protein A–Sepharose (Pharmacia) was then added, and the incubation continued for 15 minutes at 4°C. The washed immunoprecipitates (volume of 30 μl) were adjusted to 5m*M* $MnCl_2$, and 15 μCi of γ-[^{32}P]ATP at 5000 Ci/mmol (Amersham) was added for 0.5 minute at 4°C. The reaction was stopped by adding 20 μl of SDS sample buffer, and samples were analyzed on SDS polyacrylamide gels (*24*). The HER-*erb*B1 expression product is shown at levels comparable to those of the other constructs and in addition at a tenfold higher concentration (×10). The monoclonal antibody used (RI) does not recognize the endogenous Rat1 cell EGF receptor. Five times as many Rat1 cells (nontransfected as a control) as A431 cells were used per experiment. Size markers are indicated in kilodaltons.

1.8×10^6 EGF receptor molecules on the surface of A431 human epidermoid carcinoma cells. The ^{125}I-labeled EGF binding was specific in that it was abolished in the presence of excess unlabeled growth factor. It also correlated with receptor levels detected by immunoprecipitation with a monoclonal antibody specific for human EGF receptor (Fig. 1A). Metabolically labeled [^{35}S]methionine and immunoprecipitated HER-*erb*B protein migrated slightly faster than the human EGF receptor glycoprotein of A431 cells on SDS gels, probably because of structural differences at the carboxyl terminus of the two molecules (Fig. 1A). Thus the in vitro constructed hybrid gene directs expression of a protein that is faithfully transported to the cell surface, is able to bind EGF, and therefore constitutes a functional chimeric receptor.

The ability of cells expressing the chimeric receptor to bind increased levels of EGF demonstrated the formation and maintenance of a functional extracellular domain. We tested the functional integrity of the intracellular domain by analyzing the autophosphorylation activity of the *erbB* portion of the chimeric receptor. Immunoprecipitates of the chimeric protein were incubated with [^{32}P]γ-ATP and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Like the EGF receptor from A431 cells, the HER-*erb*B chimeric receptor demonstrated autophosphorylation activity in immunoprecipitates (Fig. 1B). When compared with a similar amount of EGF receptor from A431 cells, the HER-*erb*B chimera displayed weaker specific autophosphorylation activity (Fig. 1B).

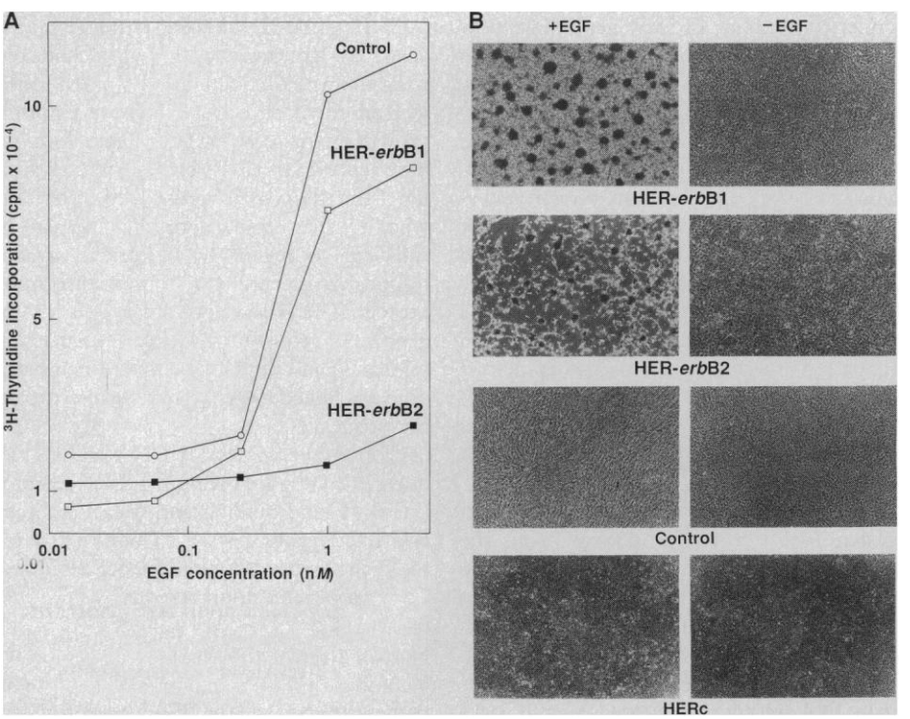

Fig. 2. (**A**) Mitogenic response of stably expressing Rat1 fibroblasts to EGF. Confluent monolayers of stably expressing Rat1 fibroblasts in 2.2-cm, 12-well culture dishes were grown to quiescence in medium containing 0.5% fetal bovine serum for 48 hours. DNA synthesis was stimulated by adding various amounts of mouse EGF (Collaborative Research) and was monitored 18 hours later by a 4-hour pulse-labeling with 0.5 μCi of [*methyl*-^{3}H]thymidine at 339 μCi/mg and 84 Ci/mmol (Amersham). Cells were washed three times with cold PBS, and soluble radioactivity was extracted with 1 ml of 10% trichloroacetic acid for 1 hour at 4°C followed by one wash with the same solution at 4°C. Cells were solubilized in 0.5 ml of 0.1*N* NaOH and 1% SDS for 30 minutes at 37°C, and the lysate was brought to neutral *p*H by adding 0.5 ml of 2*M* tris buffer at *p*H 6.8. The incorporated radioactivity was determined by using 10 ml of aqueous scintillation cocktail (Aquasol, New England Nuclear). The average of two measurements is shown for each data point (5% error). Normal Rat1 cells were used as a control. (**B**) EGF-induced cell-focus formation. Normal and transfected (HER-*erb*B1, HER-*erb*B2, and HERc; see also legend to Fig. 3) Rat1 cells were plated at 20% confluency and grown in normal culture medium containing 10% fetal bovine serum in the presence (+) or absence (−) of 100 ng of EGF per milliliter for 1 week. The width of the panels corresponds to 3.3 mm.

A fully functional HER-*erb*B chimeric receptor should display signal transfer between extracellular and intracellular domains, that is, EGF-stimulable autophosphorylation activity. EGF induced autophosphorylation of the chimeric receptors three- to fivefold, similar to the levels observed for the normal receptor, when examined after a 30-second incubation (Fig. 1B). Thus, ligand-responsiveness of the v-*erb*B cytoplasmic domain was restored in the chimeric protein.

To test if this restoration of ligand-responsiveness was sufficient to convert the oncogenic v-*erb*B domain into a domain capable of transmitting a normal, EGF-stimulated mitogenic signal, we analyzed the proliferative response of stably transfected Rat1 fibroblast lines HER-*erb*B1 and HER-*erb*B2 to EGF exposure. The ability of EGF to stimulate quiescent cells to synthesize DNA was determined by measuring [^{3}H]thymidine incorporation over a 4-hour period after serum starvation. Control Rat1 fibroblasts displayed EGF-dependent [^{3}H]thymidine incorporation mediated by their endogenous rat EGF receptors (Fig. 2A). In contrast, Rat1 cells expressing the chimeric HER-*erb*B receptor exhibited a decreased mitogenic response to EGF. This inhibitory effect on the endogenous EGF response was more pronounced in the high-expression line HER-*erb*B2, where it dramatically reduced sensitivity to EGF.

Long-term exposure of HER-*erb*B–expressing Rat1 cells to 100 ng of EGF per milliliter resulted in severe inhibition of growth, whereas growth of control Rat1 cells was not affected by this dose of the growth factor. The effect was strongest for HER-*erb*B2 and weaker for HER-*erb*B1 with lower expression levels. Therefore, HER-*erb*B expression interfered with the normally EGF receptor–mediated mitogenic response of Rat1 cells in a dose-dependent manner.

Further evidence that restoration of ligand-responsiveness did not convert v-*erb*B to a normal protein was the finding that transforming activity was not abolished in the chimera. The remaining transforming activity, however, was now inducible by EGF. When nearly confluent monolayers of stably transfected Rat1 fibroblasts were exposed to EGF at 100 ng/ml for several days, formation of cell foci was observed for cells expressing the HER-*erb*B fusion product (Fig. 2B). Focus formation in HER-*erb*B Rat1 cells was more pronounced at lower levels of expression (HER-*erb*B1) than at higher levels (HER-*erb*B2), probably because of the strong growth inhibition suffered by cells expressing HER-*erb*B2. No cell foci were observed without the addition of EGF, or under any conditions for control Rat1 or A431 cells. Furthermore, Rat1 cells expressing normal human EGF receptor (HERc, 10^5 receptors per cell) at levels comparable to HER-*erb*B1 and under SV40 early promoter control generated no foci in the presence or absence of EGF (Fig. 2B).

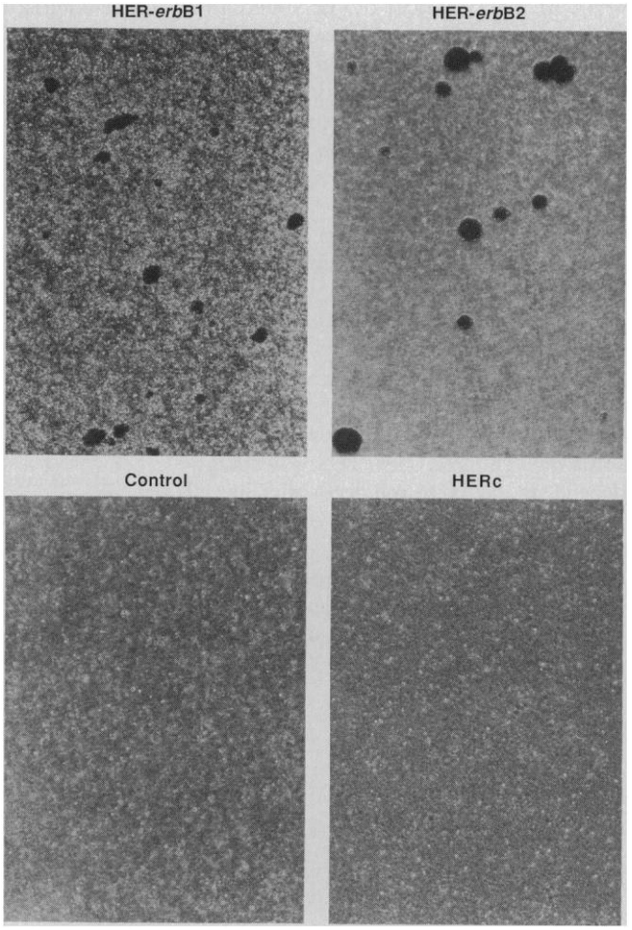

Fig. 3. Anchorage-independent soft agar growth. Normal and stably expressing (HER-*erb*B1, HER-*erb*B2, and HERc) Rat1 cells were plated in 5 ml of DMEM containing 10% fetal bovine serum and 0.25% agar (Difco, agar noble) on a bottom layer of 0.5% agar in 5 ml of medium (10^5 cells per 6-cm culture dish). Colonies were scored after 2 weeks. The complete human EGF receptor was expressed from cDNA under the control of early SV40 transcriptional elements in the plasmid also used for HER-*erb*B expression. Cell surface receptor numbers had been determined to be 1×10^5 per cell by ^{125}I[EGF] binding. The height of the panels corresponds to 3.3 mm.

The transforming activity of the HER-*erb*B chimera was also demonstrated by the ability of transfected Rat1 cells to form colonies in soft agar in the absence of EGF (Fig. 3). Once again, this property was not observed for Rat1 cells overexpressing HERc. We were not able to test possible EGF stimulation of this transforming activity since EGF induced soft agar colony formation of the control Rat1 fibroblasts, as others have observed for NRK cells (*15*).

The experiments reported here demonstrate that attachment of the complete extracellular ligand-binding domain of the human EGF receptor restored ligand inducibility of the intracellular v-*erb*B tyrosine kinase. Although the v-*erb*B tyrosine kinase regained its ligand responsiveness in the chimera, mere restoration of the EGF receptor extracellular domain did not regenerate a normal, nontransforming protein. A basal level of transforming activity was still detectable as colony forming ability in soft agar in the absence of EGF. This weak, unstimulated activity appeared to be enhanced by overexpression of the chimeric protein (*16*). Furthermore, the residual transforming potential of the chimeric receptor could actually be stimulated by EGF as determined by ligand-induced focus formation in cell monolayers. This finding strongly suggests that the transforming potential resides in intracellular features unique to v-*erb*B sequences.

The carboxyl-terminal deletion in the chimera (and v-*erb*B) is likely to be predominantly responsible for the transforming potential of this protein. This sequence deletion in a domain that is thought to be important in receptor-specific signal generation removes the major autophosphorylated tyrosine residue in the human receptor homolog and is also a structural characteristic of other tyrosine kinase oncogene–proto-oncogene pairs, such as c-*fms*/v-*fms* (*17*), c-*src*/v-*src* (*18*), and the close relatives insulin receptor/v-*ros* (*19, 20*). We do not yet know if the tyrosine residue deletion is the key to transformation or if the total deleted portion is responsible for this effect. Since regulated phosphorylation of cytoplasmic components is thought to be a key event in the cellular signaling process stimulated by growth fac-

tors, one must consider the possibility that the structural differences between *erb*B and the EGF receptor may lead to a constitutive, basal level of ligand-independent substrate phosphorylation activity. Alternatively, the specificity of phosphorylation may be altered. The chimeric HER-*erb*B molecule represents a homolog of the v-*fms* oncogene that carries an apparently intact ligand-binding domain. It offers the possibility of characterizing in detail enzymatic parameters of the receptor kinase and represents a model for studying ligand-stimulated transformation in an autocrine or paracrine scenario.

We observed inhibition of EGF-stimulated mitogenesis in Rat1 cells expressing the HER-*erb*B chimera. This effect was dosage-dependent and led to cell death after several days of EGF exposure, which is reminiscent of A431 cells that overexpress normal and altered human EGF receptors (*2, 21*). Whether this phenomenon can also be attributed to the carboxyl-terminal deletion or other criteria, such as receptor overexpression, structural differences between v-*erb*B and c-*erb*B sequences, or incompatibility of avian receptor signaling functions with Rat1 cell signal transduction pathways, is currently unclear.

The hybrid receptor approach used in these experiments may provide a powerful means of identifying growth factor receptor domains that are crucial in the initiation of the cascade of events that leads to normal cell division or oncogenesis.

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Extended Culture of Mouse Embryo Cells Without Senescence: Inhibition by Serum

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Mouse embryo cells cultured in vitro in serum-supplemented media undergo growth crisis, resulting in the loss of genomically normal cells prior to the appearance of established, aneuploid cell lines. Mouse embryo cells established and maintained for multiple passages in the absence of serum did not exhibit growth crisis or gross chromosomal aberration. Cells cultured under these conditions were dependent on epidermal growth factor for survival. Proliferation was reversibly inhibited by serum or platelet-free plasma, suggesting that mouse embryo cultures maintained by conventional procedures are under the influence of inhibitory factors.

MOUSE EMBRYO CELLS CULTURED in conventional serum-supplemented media rapidly lose proliferative potential, leading to growth crisis or senescence followed by the appearance of genomically altered immortalized (established) cell lines. Swiss, BALB, and NIH lines (3T3, 3T6, and 3T12) as well as C3H 10T1/2, which are commonly used in studies of carcinogenesis and growth control, were derived in this way and exhibit near-tetraploid karyotypes with major chromosomal abnormalities (*1–4*). Because extended culture of some cell types has been achieved by replacing serum in the culture medium (*5–11*), we developed a serum-free approach to the culture of mouse embryo cells. These procedures allowed extended cell proliferation in the absence of detectable crisis or gross genomic alterations. Growth of cultures derived under these conditions was markedly inhibited by both serum and plasma, suggesting the presence of circulating inhibitors distinct from platelet-associated factors.

Cells from 16-day-old mouse embryos were cultured in nutrient medium supplemented with 10% calf serum (*1–4*) or in medium in which serum was replaced with insulin, transferrin, epidermal growth factor (EGF), high-density lipoprotein (HDL), and fibronectin. This medium was developed originally for the growth of the serum-derived, established C3H 10T1/2 and Swiss NIH 3T3 mouse embryo cell lines (*12, 13*) and will also support the growth, at least in the short term, of early passage mouse embryo cells cultured initially in serum. Growth of serum-derived cultures in this medium is enhanced by the addition of platelet-derived growth factor (PDGF). The medium formulation is related in nutritional and hormonal composition to other media developed for the growth of established lines of mouse embryo cells (*14, 15*).

Cultures carried in serum-containing media, in the presence or absence of additional supplementation with the above factors, underwent a well-defined crisis (Fig. 1). A single batch of unheated serum was used for the serum-containing cultures for the duration of the experiment. Similar results were obtained when the experiment was repeated with a different batch of serum or with heat-inactivated serum (56°C, 30 minutes). Cultures maintained in serum-free media grew exponentially without a significant time lag and represented a major portion of the population of embryo cells initially plated. Cell number in primary cultures 6 days after plating was three times higher in serum-free medium than in serum-containing medium. Cells of serum-free cultures appeared morphologically homogeneous upon light microscopic examination by the second or third passage and remained so upon long-term culture.

Cultures were derived in serum-free medium from both BALB/c and Swiss embryos. Initiation and long-term growth in the absence of serum was carried out with three independent Swiss mouse embryo cultures; similar results were observed in all cases.

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