

## Epidermal growth factor-nonresponsive 3T3 variants do not contain epidermal growth factor receptor-related antigens or mRNA

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**ABSTRACT** We have previously isolated three independent variants of Swiss 3T3 cells that are unable to generate a mitogenic response to epidermal growth factor (EGF). Each of the variants is unable to bind  $^{125}\text{I}$ -labeled EGF; each lacks a functional EGF receptor. We used an antiserum to murine EGF receptor to look for an EGF-receptor gene product in wild-type 3T3 cells and in the three EGF-nonresponsive variants. No cross-reactive material could be detected in any of the three variants, either in  $^{125}\text{I}$ -labeled cell extracts or in [ $^{35}\text{S}$ ]methionine metabolically labeled cells. 3T3 cells contained mRNA molecules homologous to a cDNA probe for the human EGF-receptor coding region. In contrast, no homologous RNA could be detected in any of the three variants. Analysis of genomic Southern blots of the DNA from 3T3 cells and the three EGF-nonresponsive variants indicated sequences from the EGF-receptor gene are present in the DNA of all four cell lines. These EGF-nonresponsive lines, which demonstrate proliferative responses to a variety of mitogens, will be ideal recipients for structure-function studies of the EGF receptor by transfection of the cloned gene.

The mechanism by which epidermal growth factor (EGF) stimulates cell proliferation is not well understood. To help elucidate this mitogenic response we have isolated three independent EGF-nonresponsive variants (3T3-NR6, 3T3-ENR7, and 3T3-TNR2) from the murine Swiss albino 3T3 cell line (1-3). All three variants retain a normal mitogenic response to serum and other polypeptide mitogens such as fibroblast growth factor (1-3). Despite widespread use of these variants (4-8), the biochemical nature of their EGF-nonresponsive phenotype has not been completely characterized. We have shown that each of the variants cannot bind  $^{125}\text{I}$ -labeled EGF, i.e., each is missing a functional EGF receptor (1-3). We have now used serologic procedures to examine our EGF-nonresponsive variants for the presence of EGF-receptor-related antigens, and a cDNA clone for the EGF-receptor coding region to analyze expression of EGF-receptor-related mRNA.

### MATERIALS AND METHODS

**Cell Culture.** Swiss albino 3T3 cells, the three variants (3T3-NR6, 3T3-TNR2, and 3T3-ENR7), and A431 cells were cultured in Dulbecco's modified Eagles' medium (GIBCO) in 10% (vol/vol) fetal calf serum (1-3).

**EGF Binding Assays.** Murine EGF was purified by using the procedure of Savage and Cohen (9). Binding assays were performed at 4°C, by using confluent cells in 35-mm plates. Details of EGF iodination and the binding assay have been described (1, 10).

**Iodination of Cell Extracts.** Cells were solubilized in RIPA buffer (11). The extracts were clarified by centrifugation

(100,000 × *g*, 60 min). Protein concentration was adjusted to about 0.7 mg/ml.  $^{125}\text{I}$ iodine (Amersham, 2.3 mCi per 0.5 ml extract; 1 Ci = 37 GBq) was added, and the iodination reaction was initiated by the addition of 60 μl of chloramine T (10 mg/ml). After 1 min at room temperature, the reaction was stopped with 30 μl of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (40 mg/ml) and 30 μl of KI (300 mg/ml). Free iodine was removed by sequential centrifugation through two desalting Sephadex G-25 columns (12).

**[ $^{35}\text{S}$ ]Methionine Labeling.** Cells were labeled for 6 hr in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) dialyzed fetal calf serum and 33 μCi of [ $^{35}\text{S}$ ]methionine/ml (Amersham). After three rinses with Ca<sup>2+</sup>/Mg<sup>2+</sup> free phosphate-buffered saline, the cells were solubilized by incubating in RIPA buffer for 1 hr on ice. Extracts were clarified by centrifugation (100,000 × *g*, 60 min).

**Immunoprecipitation of Radiolabeled EGF Receptors.** Rabbit polyclonal antiserum 286, prepared by immunization with murine EGF receptor purified by affinity chromatography on an EGF-Sepharose column (13), was a gift from Stanley Cohen. Cell extracts were preadsorbed with 5 μl of a control antiserum and 100 μl of Pansorbin (Calbiochem). The assay was initiated by addition of 5 μl of control serum or anti-receptor serum 286. After 30 min at 4°C, 100 μl of Pansorbin was added, and incubation was continued for 30 min. Pansorbin-bound immunoprecipitate was pelleted by centrifugation and washed four times. The immunoprecipitate was dissolved in 30 μl of 1% NaDodSO<sub>4</sub>, diluted 1:10 into a second immunoprecipitation buffer (14), and subjected to a second round of immunoprecipitation (14), by using the appropriate control or anti-receptor serum at 1:100 dilution. The final immunoprecipitate was solubilized (15) for electrophoresis on NaDodSO<sub>4</sub>/polyacrylamide gels (16). For autoradiography of iodinated preparations the dried gel was exposed at -70°C, using Kodak XAR-5 film. Gels containing [ $^{35}\text{S}$ ]methionine-labeled proteins were processed for fluorography (17).

**RNA Isolation.** Total RNA was isolated by a modification of the procedure of Chirgwin *et al.* (18). Monolayers were washed three times with PBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> (31) and harvested by scraping. Cell pellets were frozen in liquid N<sub>2</sub> and thawed in guanidinium thiocyanate solution (18). RNA was centrifuged through a CsCl cushion, resuspended in buffered guanidine hydrochloride (18), and ethanol precipitated. The pellet was resuspended in 10 mM Tris·HCl (pH 7.4), 5 mM EDTA, 1% NaDodSO<sub>4</sub>, extracted with CHCl<sub>3</sub>, and again precipitated with ethanol. RNA was resuspended in diethylpyrocarbonate-treated H<sub>2</sub>O, heated to 65°C for 5 min, cooled in ice water, and adjusted to 0.4 M NaCl, 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 0.3% NaDodSO<sub>4</sub>. RNA

Abbreviation: EGF, epidermal growth factor.

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was twice selected by oligo(dT)-cellulose chromatography to purify poly(A)<sup>+</sup> RNA.

**DNA Isolation and Restriction Endonuclease Digestion.** Nuclei were separated from cytosol by cell lysis in 0.5% Nonidet P-40, followed by a gentle centrifugation. DNA was then isolated as described by Gross-Bellard *et al.* (19). DNA was digested with *Eco*RI in 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl<sub>2</sub>.

**Electrophoresis, Transfer of RNA and DNA to Nitrocellulose, and Hybridization.** Electrophoresis of RNA in formaldehyde/agarose gels was performed as described by Rozek and Davidson (20). Electrophoresis of DNA is described in Maniatis *et al.* (21). RNA was transferred according to Thomas (22). DNA transfer and hybridization of both DNA and RNA blots was done according to Wahl *et al.* (23), with the following modifications: 4× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) was used instead of 5× SSC, 0.1% NaDodSO<sub>4</sub> was added for the RNA blots, hybridization was at 40°C for 12 hr, and blots were washed in 0.3× SSC with 0.1% NaDodSO<sub>4</sub> at 65°C. Hybridization probe, prepared as described by Lin *et al.* (24), was approximately 2 × 10<sup>8</sup> cpm/μg.

## RESULTS

**Binding of <sup>125</sup>I-Labeled EGF to 3T3 Cells and the EGF-Nonresponsive Variants.** In separate reports (1–3) we have described binding of <sup>125</sup>I-labeled EGF to 3T3 and the three independently isolated EGF-nonresponsive variants. As illustrated in Fig. 1, each of these variants is unable to bind <sup>125</sup>I-labeled EGF; each is missing a functional EGF receptor.

**Characterization of Anti-EGF-Receptor Serum 286.** A rabbit polyclonal antiserum (number 286) raised against purified mouse liver EGF receptor (13) was used to determine whether any of the three variants makes any nonfunctional but immunologically cross-reactive EGF-receptor gene product. We first showed that pretreatment of monolayer cultures of 3T3 cells with antiserum 286 could block <sup>125</sup>I-labeled EGF binding. A control serum had no effect (data not shown). We then showed that antiserum 286 could specifically precipitate the EGF receptor, a 170-kDa phosphoprotein, from extracts

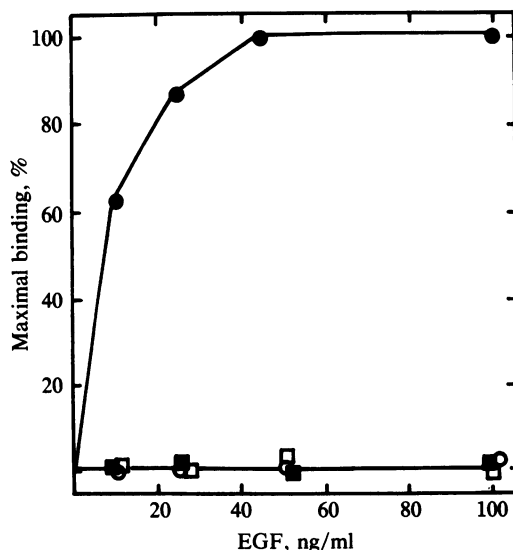


FIG. 1. Binding of <sup>125</sup>I-labeled EGF to 3T3 cells and the EGF-nonresponsive variants. These results are a composite of two separate experiments. Data are expressed as the percent binding relative to the saturating value observed for 3T3 cells and are normalized for cell number. 3T3 (●); 3T3-NR6 (○); 3T3-TNR2 (■); 3T3-ENR7 (□).

of *in vitro* phosphorylated (16) mouse liver microsomes. This 170-kDa phosphoprotein was more highly phosphorylated when microsomes were phosphorylated in the presence of EGF (data not shown). Since the EGF receptor possesses an EGF-enhanced autophosphorylating activity (13), our result indicated that antiserum 286 can precipitate the murine EGF receptor from a complex protein extract.

**Iodination and Immunoprecipitation of EGF-Receptor-Related Antigens from 3T3 Cells and the EGF-Nonresponsive Variants.** To look for EGF-receptor-related gene products in 3T3 cells and in each of the three variants, we first labeled solubilized cell extracts with <sup>125</sup>I, then subjected the iodinated extracts to immunoprecipitation with either antiserum 286 or a control antiserum. Anti-receptor antiserum precipitated a 170-kDa <sup>125</sup>I-labeled protein from the 3T3 cell extract, but not from the extracts of any of the three variants (Fig. 2*b*). Since anti-receptor antiserum did not precipitate from the extracts of the variants any protein that was not also present in precipitates with the control serum (Fig. 2*a*), there appear to be no truncated EGF-receptor molecules in the variants.

To evaluate the sensitivity of our immunoprecipitation assay, various proportions of <sup>125</sup>I-labeled 3T3-cell extract and <sup>125</sup>I-labeled extract from the 3T3-TNR2 variant were mixed together prior to immunoprecipitation. We could detect the presence of the 170-kDa EGF receptor in the immunoprecipitate even when the 3T3-cell extract constituted only 1.4% of the total radioactivity in the combined mixture (Fig. 2). The level of EGF-receptor cross-reacting material present in any of the variants could, therefore, not exceed 1% of that present in 3T3 cells.

**Metabolic Labeling and Immunoprecipitation of EGF-Receptor-Related Antigens from 3T3 Cells and the EGF-Nonresponsive Variants.** We next investigated whether the absence of EGF-receptor-related antigen in the variants is reflected by reduced EGF-receptor synthesis. Cells were incubated for 6 hr in medium containing [<sup>35</sup>S]methionine. The labeled cell extracts were subjected to immunoprecipitation (Fig. 3). A heavily-labeled EGF-receptor band was present in the immunoprecipitate from the 3T3-cell extract. In contrast,

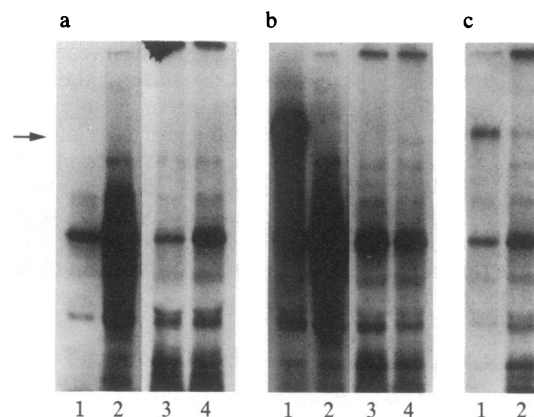


FIG. 2. Immunoprecipitation of <sup>125</sup>I-labeled EGF receptors from 3T3 cells and the EGF-nonresponsive variants. Approximately 7 × 10<sup>8</sup> cpm of <sup>125</sup>I-labeled extract from each cell type was used in each assay. A double-immunoprecipitable procedure was used to reduce nonspecific background. Immunoprecipitates using the control antiserum are shown in *a*; immunoprecipitates using anti-receptor antiserum 286 are in *b*. Arrows indicate the position of the EGF receptor. Lanes: 1, 3T3; 2, 3T3-TNR2; 3, 3T3-NR6; 4, 3T3-ENR7. To analyze the sensitivity of the immunoprecipitation assay <sup>125</sup>I-labeled 3T3 and 3T3-TNR2 extracts were mixed together in varying proportion, while keeping the total amount of radioactivity constant (*c*). The combined extracts were subjected to double immunoprecipitation. Immunoprecipitate from a 14:86 mixture of 3T3:3T3-TNR2 extracts is shown in lane 1; immunoprecipitate from a 1.4:98.6 mixture of 3T3:3T3-TNR2 extracts is shown in lane 2.

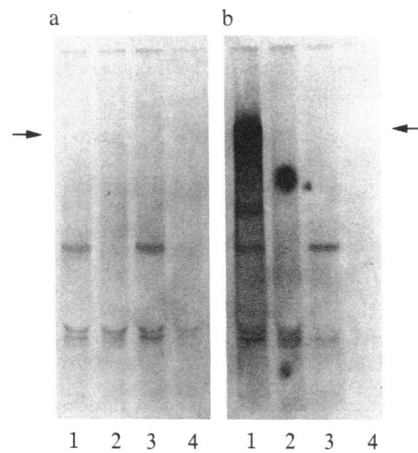


FIG. 3. Immunoprecipitates of [<sup>35</sup>S]methionine labeled cell extracts. Immunoprecipitates using control antiserum are shown in *a*; immunoprecipitates using antireceptor serum are in *b*. Arrows indicate the position of the EGF receptor. Lanes: 1, 3T3; 2, 3T3-TNR2; 3, 3T3-NR6; 4, 3T3-ENR7.

no immunologically cross-reactive material could be detected with the anti-receptor antiserum in extracts from any of the three variants. If EGF-receptor-related molecules were being synthesized at a normal rate in the variants, they would have to be degraded faster with a half-life much shorter than the normal half-life (10 hr) reported for the EGF receptor of human fibroblasts (25).

**RNA Blot Analysis of EGF-Receptor mRNA in 3T3 Cells and the EGF-Nonresponsive Variants.** We isolated poly(A)<sup>+</sup> RNA from 3T3 cells, the three EGF-nonproliferative variants, and A431 cells, a human cell line amplified for the EGF-receptor gene (24). Poly(A)<sup>+</sup> RNA was electrophoresed on a formaldehyde/agarose gel, blotted onto nitrocellulose and hybridized against a cDNA clone (pEGFR, the gift of M. G. Rosenfeld, University of California, San Diego) encompassing the complete coding region of the EGF receptor from A431 cells. mRNA homologous to this EGF-receptor cDNA is present in 3T3 cells (Fig. 4, lane 5). Although the EGF-receptor message from 3T3 cells differs in size distribution

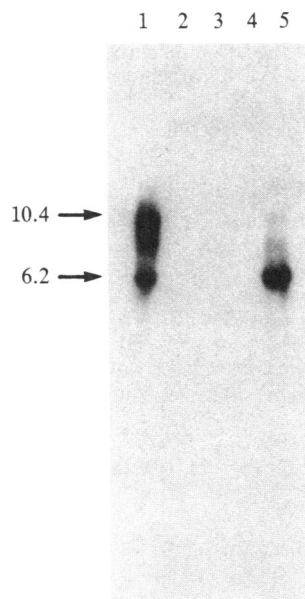


FIG. 4. RNA blot analysis of EGF-receptor mRNA. Each lane contains 10  $\mu$ g of poly(A)<sup>+</sup> RNA. Lanes: 1, A431; 2, 3T3-NR6; 3, 3T3-TNR2; 4, 3T3-ENR7; 5, 3T3. Lane 1 was autoradiographed for 2 hr, lanes 2-5 for 28 hr. The molecular size in kilobases is indicated.

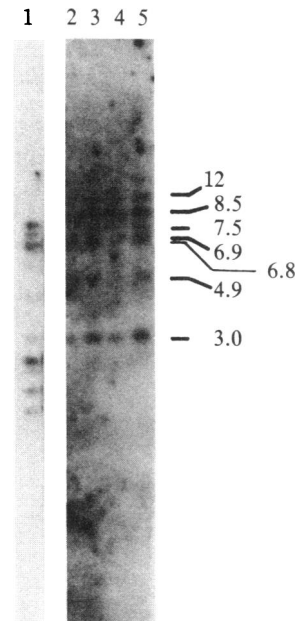


FIG. 5. Genomic Southern blot of the EGF-receptor gene. Each lane contains 10  $\mu$ g of *Eco*RI-digested DNA. Lanes: 1, A431; 2, 3T3-NR6; 3, 3T3-TNR2; 4, 3T3-ENR7; 5, 3T3. Lane 1 was autoradiographed for 2 hr, lanes 2-5 for 28 hr. The molecular size in kilobases is indicated.

from that of the human A431 cells, the high degree of stringency used in washing the blots indicates a strong homology between the mouse and human 6.2-kilobase RNAs. There is no homologous message detectable in any of the EGF-nonproliferative variants. Fig. 4 shows a 28-hr exposure. We were unable to detect any bands in these lanes even in a 1-week exposure. Either no mRNA coding for EGF receptor is transcribed in these variants, or it is very rapidly degraded.

**Southern Blot Analysis of the EGF-Receptor Gene in 3T3 Cells and the EGF-Nonresponsive Variants.** High molecular weight DNA was isolated from 3T3 cells, the three EGF-nonproliferative variants, and A431 cells. *Eco*RI digests were electrophoresed, blotted, and probed with the EGF-receptor cDNA clone. 3T3- and A431-restriction-fragment patterns for the EGF-receptor gene differ from one another, due no doubt to species differences in the structure of the gene. Unlike the case for RNA, however, restriction fragments diagnostic for the EGF-receptor gene are present in the DNA of the three EGF-nonproliferative variants (Fig. 5). No major qualitative differences occur in the restriction digest pattern for the EGF-nonproliferative variants when compared to the parental 3T3 cells. Thus at least the coding region of the EGF-receptor gene is present (although not necessarily unaltered) in all of the cell lines.

## DISCUSSION

We find it surprising that three independently isolated EGF-nonresponsive variants all fail to express any protein product from the EGF-receptor gene. We anticipated that serologically cross-reactive products unable to bind EGF, or unable to properly insert into the plasma membrane, might be made in some of the variants. However, utilizing a polyclonal antiserum and serological techniques that should be able to detect cross-reactive products present at a level less than 1% that of the wild-type receptor level, we found no evidence for receptor-related molecules in any of the variants. The absence of mRNA for the EGF receptor in all the variants

clearly demonstrates that the absence of receptor-related antigen is not due to mutations in the coding region of the EGF-receptor gene that render the resulting protein molecules serologically unrecognizable. The presence of restriction fragments diagnostic for the EGF-receptor gene eliminates the (unlikely) possibility that the entire EGF-receptor gene has been removed from the EGF-nonresponsive variants by chromosome loss or by massive deletion. The absence of EGF-receptor protein product and mRNA in the variants must, therefore, result from a defect either in messenger transcription or processing. Although this question could be approached by nuclear run-off transcription experiments, cloning of the EGF-receptor gene from wild-type and EGF-nonresponsive 3T3 cells would be necessary for a complete understanding of the differences in these cell lines.

The three EGF-nonresponsive variants do not all share a common phenotype. The 3T3-TNR2 variant expresses a dominant inability to bind EGF in somatic cell hybrids with parental 3T3 cells (26). The absence of EGF-receptor protein and mRNA in this variant and its hybrids suggests that a *trans*-acting repressor of transcription for this gene might be responsible for generating this dominant phenotype; 3T3-TNR2 cells may be constitutive for such a repressor. Indeed, such a *trans*-acting factor may be responsible for the lack of EGF-receptor expression in some normal tissues. In contrast, 3T3-NR6 and 3T3-ENR7 cells are recessive in hybrids with 3T3 cells. They do not complement one another in somatic cell hybrids (26). Alterations in a common, noncomplementing region are responsible for their inability to express EGF-receptor protein or mRNA. Inappropriate expression of an altered EGF-receptor gene is causal for tumor formation in two distinct situations; either as a consequence of viral insertion within the cellular EGF-receptor gene (27) or due to retroviral capture and transfer of a truncated portion of the gene (28). We have at least two genetically distinguishable variants that are altered in EGF-receptor expression, one dominant and at least one recessive. A complete understanding of the nature of these mutations should lead to valuable insights into the regulation of EGF-receptor expression in both normal and pathological situations.

The cloning of a complete cDNA for the EGF receptor (24, 29, 30) will permit the production of altered EGF receptors by site-directed mutagenesis in cDNA expression vectors. However, to carry out structure-function studies of the altered EGF receptors produced from these constructs, a recipient cell for transfection will require the following characteristics: (i) the cell line must demonstrate growth control and mitogen responsiveness in culture, (ii) it must be able to be transfected at a reasonable frequency, and (iii) it must not produce a functional product from the endogenous EGF-receptor gene. The 3T3-EGF-receptorless variants satisfy these criteria and, in fact, produce no EGF-receptor-related mRNA or protein. They should, therefore, be of great value in structure-function studies of the EGF receptor.

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