Regulation of Epidermal Growth Factor Receptor Gene Expression in Murine Embryonal Carcinoma Cells

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Abstract
The protooncogene c-erbB1 [epidermal growth factor receptor (EGF-R)] is expressed in a wide variety of cell types and in most adult tissues. The precise roles of the EGF-R in vivo are largely unknown, especially their role in growth and development of embryonic tissues. We reported earlier that EGF-Rs are not expressed on the cell surface of undifferentiated embryonal carcinoma (EC) cells, but intracellular receptor protein is detectable (A. Weller, J. Meek, and E. D. Adamson, Development, 100: 351–363, 1987). We document here that in embryonal carcinoma cells, low levels of both receptor mRNA and protein are observed, but after 4 days of retinoic acid-induced differentiation, large increases are seen. Most notable is the 35–70-fold rise in the levels of EGF-R transcripts during the differentiation of P19 embryonal carcinoma cells to neural and glial cells, and this is paralleled by a 10-fold rise in protein. Measurements of the degradation rates of EGF-R mRNA and receptor protein show that both are rather stable and may partially explain the steady-state increases during differentiation. Run-on transcription assays of the EGF-R gene show very low rates of transcriptional activity at all stages: about 2-fold changes in transcription rate can be detected. It is concluded that transcriptional mechanisms may also partially account for increased levels of gene products. We hypothesize that the appearance of EGF-Rs at the cell surface leads to the slow induction of further receptor levels by EGF transforming growth factor α stimulation, and this contributes to the driving force of differentiation and to the stability of the differentiated state.

Introduction
Oncogenes play a crucial role in growth and differentiation. The EGF-R2 (c-erbB1) is a protooncogene that is involved in both of these processes. Elevated expression of the EGF-R gene is frequently found in breast, prostate, glioma, and other tumor cell types. The multistage process of cellular transformation involves the activation of several growth-regulatory genes, and the identification of the initial aberrant events in this process is an important goal for diagnosis and prevention. The EGF-R gene is activated at a very early stage in embryonic development (1), and its expression is developmentally regulated (2). Most adult tissues contain cell types that express EGF-Rs, but their precise role is unknown. In those tissues that proliferate or regenerate, such as skin, gut epithelium, and liver, EGF-Rs are modulated in response to appropriate signals and appear to play roles in normal physiological function.

In order to identify the mechanisms that underlie both normal and abnormal growth and development and the role of EGF-Rs in these processes, we have analyzed the regulation of EGF-Rs during differentiation. Murine EC cell lines are excellent models of developmental processes, since they differentiate along well-defined pathways to single or multiple identified cell products. The undifferentiated or stem cell (EC) derives from and mimics early embryonic ectoderm cells from the blastocyst or early egg cylinder stage (day 3.5 to 5.5 of mouse gestation). EC cells inoculated into adult syngeneic hosts rapidly form partially differentiated tumors that kill the host in 5–14 days. They can therefore be used as models for tumor production. On the other hand, EC cells grow as undifferentiated stem cells under appropriate conditions in vitro as well as differentiate in response to certain environmental signals. For EC cells, the two chief signals for differentiation in vitro are close cell contact during aggregation and compaction and/or appropriate concentrations of RA or DMSO.

Different EC cell lines mimic different developmental events. We have studied three such lines in order to study how each expresses and regulates the EGF-R gene. We have previously reported that the OC15 cell line does not express cell surface EGF-binding activity and have documented that cell surface receptors appear gradually during differentiation induced by RA (3). In this cell line, however, we were able to detect intracellular receptor protein that was responsive to EGF by the stimulation of receptor autophosphokinase activity and therefore suspected that the EGF-R gene is transcribed and translated well before overt differentiation. In the case of F9 EC cells, a low level cell surface EGF-binding activity is detectable, and this level increases in a stage-specific manner during differentiation. We concluded that the F9 cell line is partially differentiated based on this observation and the presence of other markers (4). In the case of P19 cells, no EGF binding is detectable. It has been documented by others that EGF-Rs appear during RA-induced differentiation of P19 monolayer cells to cell types that were identified as endoderm, mesoderm, and ectoderm cell lines (5, 6). P19 cells are the most pluripotent of the three lines studied here. When
Table 1  Summary of differentiation of EC cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>OC15 cells</td>
<td>RA + monolayers</td>
<td>OC15 (endoderm-like); cell surface EGF-Rs</td>
</tr>
<tr>
<td>F9 EC cells</td>
<td>RA + aggregates</td>
<td>Visceral endoderm + modified EC; cell surface EGF-Rs</td>
</tr>
<tr>
<td>P19 EC cells</td>
<td>RA + cyclic AMP monolayers</td>
<td>Parietal endoderm + modified EC; few cell surface EGF-Rs</td>
</tr>
<tr>
<td>P19 EC cells</td>
<td>10^{-4} M RA or 0.5% DMSO</td>
<td>Cardiac muscle + visceral cells; EGF-R protein synthesis*</td>
</tr>
<tr>
<td>P19 EC cells</td>
<td>10^{-6} M RA or 1% DMSO</td>
<td>Skeletal muscle + other cells; EGF-R protein synthesis*</td>
</tr>
<tr>
<td>P19 EC cells</td>
<td>5 x 10^{-7} M RA</td>
<td>Neurons + glial cells; EGF-R protein synthesis*</td>
</tr>
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* See text.

the stem cells are aggregated by culture in Petri dishes and treated with specified concentrations of retinoic acid or DMSO, they differentiate into neural tissue (neurons and glial cells), into skeletal muscle, and into predominantly cardiac muscle and visceral endoderm (7–10; see Table 1). All of these tissues express some EGF-binding activity, but how EGF-R gene modulation is achieved is unclear.

Until recently, the EGF-R genes in only a few species (human, Drosophila) were cloned. Petch et al. (11) have derived cDNA clones from rat liver, and a mouse genomic clone was reported by Hung et al. (12). Recently, the cDNA encoding the extracellular portion of the mouse receptor gene was cloned and sequenced (13). We also have isolated mouse cDNA fragments and constructed probes that detect EGF-R gene expression in differentiating EC cells. The present paper describes for the first time the varying levels of EGF-R transcripts in EC cell differentiation and the mechanisms of these changes. The predominant finding is that although EGF-R transcripts reach up to 70-fold greater levels in differentiated cells, only part of this increase is achieved by transcriptional regulation. Regulation may occur at several levels, including transcription rate regulation, transcript degradation rate, and EGF-R protein location. This paper identifies some of the stages at which regulation occurs and forms a basis for understanding the roles of EGF-Rs in embryonic development.

Results

EC Cells as Models of Differentiation. Different EC cell lines may be used to study different types of differentiation. In general, EC cells do not express cell surface EGF-R protein, but a low level of intracellular protein can be detected in all three EC lines studied here. OC15 monolayers are a good model for differentiation, leading to 100% homogeneous endoderm-like cells (14, 15); F9 cells are models for the two diverging pathways of primitive endoderm differentiation that are important to early embryonic development (16, 17); P19 cells are pluripotent and give rise to a variety of cell types when first cultured as aggregates with particular concentrations of inducing agents (see "Materials and Methods" for details). Table 1 summarizes those model systems and their level of expression of EGF-binding activity so far reported. No EGF binding can be detected on OC15 and P19 EC cells, and very low levels have been detected on F9 EC cells, but binding levels rise gradually starting soon after induction with retinoic acid (3, 4, 18).

Immunohistochemical Detection of EGF-R. A rabbit polyclonal antibody (3) detects EGF-R protein in differentiated tissues such as liver (Fig. 1D) and cells produced after P19 differentiation. In Fig. 1A, ABC peroxidase staining demonstrated widely distributed receptor protein after RA-induced differentiation of P19 into cells that

![Fig. 1. Immunoperoxidase staining for EGF-R protein. A–C, P19 cells were cultured on plastic before being fixed in methanol for immunoperoxidase staining (black), as described in "Materials and Methods." D, section of adult mouse liver similarly stained. A and C, P19 cells treated for 3 days in aggregate culture with 5 x 10^{-7} M RA followed by 3 days outgrowth on tissue culture plastic. A, predominantly glial-type cells; B, P19 differentiated cells after 4 days in aggregate culture with 0.5% DMSO and 4 days on tissue culture plastic. Bar = 20 μm. C, neurons and axons (arrowheads) on a layer of glial-type cells.](image-url)
Expression of growth factor receptors during P19 differentiation

A. Anti-EGF-R

B. Anti-PDG-F-R

Expression of EGF-R in differentiating P19 cells

Days in RA/DMSO

Relative Amount

Fig. 2. Immunoprecipitation of EGF-R protein in differentiating teratocarcinoma cells. EC and differentiating cells at different stages were metabolically labeled for 2 h with [35S]methionine. Cells were collected, washed, and lysed before dividing into aliquots containing equal amounts of total radioactive protein. One aliquot of each culture was immunoprecipitated with anti-EGF-R antiserum or preimmune serum (control), and, in some cases, with an antipeptide antibody to the β form of PDGF-R to demonstrate the specificities of the antibodies (8). A. P19 cell cultures were analyzed over the time course of differentiation with RA (Lanes 2–5) and DMSO (Lanes 6–10). Radioactive EGF-R was seen at 170 (mature) and 150 kDa (degradation product); PDGF-β-R was seen at 190 (mature) and 170 kDa (precursor). Although EGF-R protein is present in P19 EC cells (A, Lane 1), no PDGF-R is detectable (B), thus serving as a negative control for these cells. C. Densitometric scan of the gel shown in A. D. Lanes 1 and 5, F9 undifferentiated; Lanes 2 and 6, F9 cells differentiated by exposure to RA plus cyclic AMP for 5 days; Lanes 3 and 7, OC15 undifferentiated cells; Lanes 4 and 8, OC15 7-day RA-differentiated cells.

are either glial cells or fibroblasts. In Fig. 1C, a group of neurons and their axons also stain strongly, in agreement with reports that both neurons and glial cells express EGF-Rs (19–21). Differentiation induced with DMSO (Fig. 18) often results in patterns of stained cells among groups of nonstained cells. The latter are probably nonresponding undifferentiated EC cells, which stain poorly. The heterogeneous population of cells includes cardiac muscle, visceral endoderm, smooth muscle, and other unidentified cell types.

EGF-R Synthesis in Differentiating P19 Cells. EGF-R synthesis in three lines of EC cells stimulated to differentiate was examined by metabolic labeling with [35S]methionine. In P19 cells differentiating in two directions of differentiation, synthesis of EGF-R protein initially remains very low but rises to become readily detectable in fully differentiated cells. Like OC15 cells (3), P19 EC cells synthesize low levels of a 170 kDa polypeptide that immunoprecipitates with polyclonal rabbit anti-mouse EGF-R (Fig. 2A, Lane 1). In contrast, as a control, no PDGF-β-R synthesis is detectable (Fig. 2B, Lane 1). As an additional control, precipitation with a preimmune serum fails to detect a 170 kDa polypeptide (data not shown). From 1 to 2 days of induction with RA or DMSO, the rates of synthesis fall but then rise (Fig. 2A, Lanes 4–10). PDGF-β-R synthesis also becomes detectable after 4 days of induction (Fig. 2B, Lanes 4–10) and rises to very high levels in differentiated cells. The increase in EGF and PDGF-R expression in this process mimics the developing embryo (2, 22). Densitometric scanning (Fig. 2C) of the autofluorographs indicates that receptor protein levels reach at least 10-fold higher in differentiated P19 cells compared to stem cells, with levels rising more rapidly in RA-differentiated cells. Fig. 2D demonstrates a similar induction of EGF-R protein synthesis in metabolically labeled F9 and OC15 undifferentiated and differ-
entiated cells. In addition, the generality of the increased production of EGF-R after differentiation of EC cells is observed.

**EGF-R Protein Turnover in P19 Cells.** We used pulse chase experiments to determine the stability of the EGF-R protein in P19 cells. The rate of turnover could indicate the responsiveness of the receptor to growth factor signals in the serum-containing culture medium. EGF-R protein in several types of cells is quite stable when not undergoing endocytosis after binding to ligand (23-25). Fig. 3 (upper panel) shows that EGF-Rs in RA-differentiated P19 cells are expressed as two main types in these mixed cell type cultures. About half of the receptors are short lived, with a half-life of 1 h, whereas the other population is very stable. In neural tissues, therefore, a portion of the EGF-Rs appear to be actively metabolized. The EGF-Rs in DMSO-differentiated P19 cells are moderately stable with a half-life of 10 h (Fig. 3, lower panel). These parameters are similar to those observed for other cell types (24). In contrast, PDGF-B-R protein in both RA- and DMSO-differentiated cells has a half-life of 2 h.

**Measurement of Mouse EGF-R mRNA Levels.** In order to study the regulation of the EGF-R gene, cDNA probes pmER-1 and pmER-2 (Fig. 4) were isolated from a mouse liver library and used to detect EGF-R transcripts in mouse tissues and cultured cells. Partial sequencing of pmER-1 showed 93% homology with rat and 85% with human cDNA sequences (11, 26). The pmER-2 probe was 88% homologous with the human cDNA oligonucleotide sequence, thus identifying these probes as coding for the mouse EGF-R.

Northern blotting confirmed that liver contains the highest level of EGF-R mRNA and that there are three sizes of transcripts, as reported in other species; these are approximately 10, 7, and 3 kb (Fig. 5A). Liver from males and females was not significantly different in expression, although the 3-kb transcript was present in the highest proportions in liver from females compared to the other transcript sizes. The 3-kb transcript is not large enough to code for the full length EGF-R polypeptide, but similar sizes have been reported in human and rat tissues also. The significance of the presence of two transcripts of 10 and 7 kb is unknown, but both can code for receptor protein (27). Other tissues, such as kidney, express intermediate levels of mRNA, whereas in lung and brain, the levels are extremely low.

A similar analysis was made of the RNA present in untreated and differentiated EC cells. Surprisingly, the lowest levels of EGF-R transcripts were in F9 cells (which do express some cell surface EGF-Rs), whereas OC15 expressed the highest levels. This cell line and P19 showed significantly increased levels of expression during the process of differentiation (Fig. 5B). The same pattern of three transcript sizes was again seen, with marked levels of the 3-kb transcript in OC15 EC cells. Scanning densitometric measurements of the autoradiographs showed that the transcript levels increased 4-6-fold in OC15 cells during differentiation. For P19, the increases were 6-fold (3 kb), 22-fold (7 kb), and 26-fold (10 kb) during differentiation with DMSO. To examine further the kinetics of the regulation, a more sensitive method was needed to assess the low abundance messages, and this was provided by RNase protection assays.

**RNase Protection Assays of EGF-R Transcript Levels.** In order to detect truncated EGF-R transcripts, as reported by Petch et al. (11), as well as full length transcripts, two probes were constructed that could be used in RNase protection assays. The 5' probe protects transcripts of 199 bp of extracellular coding sequences (LBD) and detects all expected transcript sizes. The positions of this probe and the 3' probe are shown in Fig. 4 in
Fig. 5. Northern blot analysis of total RNA probed for EGF-R. A, total RNA (20 μg) was isolated from various mouse tissues and electrophoresed and blotted as described in “Materials and Methods.” The nitrocellulose papers were probed with 32P-labeled pmER-1 and exposed to film for 2 days. Upper panel, autoradiograph of radiolabeled mRNA products detected; lower panel, ethidium bromide-stained agarose gel to show equal loading of RNA samples. RNA from male (M), female (F), and 6-day-old neonatal tissues are shown in reference to 28S and 185 ribosomal RNA. Transcripts were detected at ca. 10, 7, and 3 kb. B, analysis of 20 μg total RNA from teratocarcinoma cell lines. From left to right, F9 undifferentiated EC; F9 + RA at 10 nm for 8 days to form embryoid bodies; F9 + RA + dibutyryl cyclic AMP + IMX (isobutyl methylxanthine) 6 days to form parietal endoderm; OC15 undifferentiated EC; OC15 + RA 7 days of differentiation; P19 undifferentiated EC; P19 differentiated with DMSO for 4 + 6 days. Upper panel, blot hybridized with 32P-labeled EGF-R probe (derived from the ethidium bromide-stained agarose gel below.

The levels of EGF-R transcripts differed between the three EC lines and appeared to show little correlation to the levels of protein detected in these cells (Fig. 6A, left). However, during differentiation of P19 cells, transcript levels followed a similar pattern to protein synthetic levels seen in Fig. 2. Transcription levels decreased to the lowest level on day 2 of differentiation, followed by gradually increasing levels thereafter. Three different pathways of differentiation were assayed. Differentiation to neural tissue gave the highest final levels of transcripts, reaching 70-fold higher than undifferentiated stem cells. Both intracellular (3’ or ICD) probes and extracellular (5’ or LBD) probes gave similar patterns. In general, the patterns of expression were similar to those detected by Northern analysis (Fig. 4B). In Fig. 6C, the levels detected by ICD appear higher than those detected by LBD, but this was an artifact because each determination was expressed relative to the very low (and hence less reliable) measurements in EC cell monolayers (day 0). Levels in untreated aggregate EC cultures were always slightly lower than in monolayer cultures. Transcript levels increased rapidly only after treated aggregate cultures were seeded on tissue culture plates, i.e., at 3 or 4 days for RA and DMSO-treated aggregates, respectively.

A similar analysis of differentiating OC15 EC cells showed a much higher initial level of EGF-R transcripts than P19 and F9 cells. EGF-R transcript levels rose more modestly with differentiation after an initial low point 2 days after commencing RA treatment (Fig. 7, A and B). Since other workers have reported rapid changes in transcript levels, we also assayed cultures after shorter periods of induction (Fig. 7, C and D). Here, the two probes detected differences. Transcripts detected by the 5’ probe (LBD) increased slightly up to 4 h after RA addition, before falling. Transcripts measured by the 3’ probe (ICD) decreased in level after short-term RA treatment. This could mean that there might be an excess of truncated, extracellular EGF-R domain protein produced by short-term stimulation of OC15 EC cells. This was assayed by immunoprecipitation of [35S]methionine-labeled culture media, but none could be detected (data not shown).

Rates of EGF-R mRNA Synthesis. Using run-on transcription assays, two cell lines were examined for rates of EGF-R gene transcription. We expected to find a large increase in transcription rate in differentiated cells to account for the large increase in steady-state levels, but this was not the case. The rate of transcription of EGF-R is barely detectable in undifferentiated cells, but it is usually less than 2-fold higher in differentiated cells. Fig. 8 shows one such result after a 2-day RA treatment of OC15 cells (the low point in steady-state levels of transcripts), compared with other gene transcription rates (Egr-1, c-fos, c-jun, and junB transcription factors). All assays gave barely visible signals in photographs, but densitometric scans were evaluated by reference to the signal for the ribosomal protein gene, L32. In two experiments, EGF-R transcription rates in OC15 cells varied during differentiation from 0.10 to 0.19 (at day 7) of that of L32. Similarly, in four experiments with P19 cells, the rate of transcription of the EGF-R gene in EC cells was 0.14–0.27 of that of the L32 gene, fell to 0.08–0.14 after RA treatment for 2–3 days, and regained the original
range of rate on day 4 or 5 onward. We were careful to harvest cells at least 16 h after a medium change, so that serum-stimulated transcription (28–30) did not obscure the rate typical of differentiating cells. The largest single increase in EGF-R transcription rate measured by densitometric scanning was observed between days 3 and 4 during P19 differentiation (2.3-fold). Similarly, an increase of 2.2-fold in the rate of EGF-R transcription after the addition of 20% serum or 12-O-tetradecanoylphorbol-13-acetate to quiescent NIH3T3 cells indicated that the EGF-R gene is poorly inducible even in these cells (data not shown). We were able to detect increases in the transcription rate of other genes because, in parallel experiments, we detected a 3.5-fold increase in the transcription rate of the transcription factor Egr-1 during the course of differentiation with RA (31).

**Stability of the EGF-R Transcripts.** Since the rate of transcription of the EGF-R gene increases modestly during differentiation over 9 days, one must conclude that posttranscriptional regulation predominates during normal differentiation and development. This question was approached by analyzing the stability of the mRNA in EC and differentiating cells. Transcript levels were measured in cells exposed to actinomycin D (a polymerase II inhibitor) for different periods of time in order to determine the rate of degradation of mRNA encoding EGF-R. Allowing 30 min for the drug to completely inhibit transcription, the cells were harvested during a time course of up to 7 h later. RNA was isolated and assayed by RNase protection.

In undifferentiated OC15 cells, the level of EGF-R transcripts is higher than in P19 cells, and it was possible to measure the degradation rate of EGF-R mRNA. Whereas transcripts from the ribosomal gene barely changed over this period (Fig. 9A), EGF-R mRNA levels noticeably declined, and although the degradation rate in differentiated cells appeared to be faster, the 95% confidence limits for both cell types overlapped because of the variations encountered. Similarly, there was great variation in measurements of mRNA stability in P19 cells. It was not possible to measure the degradation rate of transcripts in P19 EC cells because the initial levels were too low. But two types of differentiated cultures were examined. The averages of four experiments are shown in Fig. 9C. RA-differentiated cultures, which have initially usually at least 5-fold higher levels of transcripts than DMSO-differentiated cells, have EGF-R decay rates not significantly different. After prolonged exposure to actinomycin D, RA-differentiated cells appeared to be more sensitive to the drug than DMSO-differentiated cells.
Thus, EGF-R mRNAs appear to have similar stabilities in differentiated cells. The apparently more stable forms of EGF-R mRNA were found in those cells where levels were low or where the receptor protein was not on the cell surface, suggesting that mRNA decays down to a low level that remains stable. One interpretation is that a small proportion of EGF-R mRNA is retained in all of these cell types in an inactive, stable form, whereas a different population of transcripts is turned over in correspondence with the demands for more protein as receptors are internalized and degraded.

Discussion
Knowledge of the levels at which the expression of the gene is regulated is necessary before manipulating the gene to over- and underexpress for studies of the role of the gene. We have documented here the rise in EGF-R gene activity during the differentiation of embryonic cells and measured the rates of synthesis and degradation of EGF-R mRNA and protein. The main finding is that during differentiation of EC cells, both mRNA and EGF-R protein levels rise to very high levels during the differentiation process. Most notably, in P19 cells, although the level of EGF-R transcripts rises up to 70-fold, the initial level in stem cells after differentiation, only small changes in transcriptional rate can be detected during the differentiation period (Fig. 8). However, the rates are low and difficult to quantify. Thus, these small increases in rate during the 9-day process of differentiation must account for the accumulated mRNA levels because the stability of the mRNA does not change significantly (Fig. 9). We used the RNA polymerase II inhibitor actinomycin D to determine the rates of degradation of EGF-R transcripts shown in Fig. 9. In some studies, we used α-amanitin to inhibit RNA synthesis but found that transcript levels rose to higher levels for 4 h in the presence of this drug. We conclude that measurements of the degradation rate of a long-lived transcript such as EGF-R using either of these drugs may not give an accurate measure of the event in the intact cell.

In the case of P19, the differentiated cell products are highly heterogeneous with respect to EGF-R expression (see Fig. 1B); in all pathways, multiple mixed types of tissues are produced, and only the average of their individual characteristics can be measured. This heterogeneity further obscures the analysis. We did find, however, that steady-state levels correlated with receptor protein synthetic levels, and also that both the mRNA and protein are relatively stable in undifferentiated and differentiated cells. We conclude that the EGF-R gene must also be regulated by another posttranscriptional mechanism such as by changing the rate of initiation or elongation of transcription. Further studies are needed to test these hypotheses.

The cause of the initial appearance of surface EGF-Rs some time later than 2 days after RA addition is still conjecture, since RA appears to be inhibitory to EGF-R gene expression. During the first 2 days after RA or DMSO addition, decreases in EGF-R mRNA and protein levels appeared to occur, in support of the data of Hudson et al. (32). In transient assays in HeLa cells, Hudson et al. also found that RA inhibited the activity of an EGF-R promoter. In contrast, in fetal rat lung cells, RA
stimulates EGF-R expression within 6 h of addition to the culture medium, although by an indirect mechanism that depends on protein synthesis (33). In the case of EC cells, RA has multiple effects; for example, the cytoskeletal genes are induced as the transcription factor complex AP-1 appears (34). This may explain why the cryptic receptors of the EC cell change to exposed receptors after 2–3 days of differentiation. Slight decreases in EGF-R gene expression are also seen upon aggregation of EC cells in anchorage-independent cultures (Fig. 6A), even without drug induction, again implicating a role for the cytoskeleton.

The long-term stimulatory action of RA or DMSO on EC cell differentiation is clearly an indirect effect of the inducing drugs and occurs after the induced cells have been brought back to anchorage-dependent culture, when both EGF-R and PDGF-R proteins appear on the cell surface. There may be a cell substrate or cell cytoskeletal regulatory mechanism involved here, also, either in transcript stabilization, transport, translation, or protein transport to the cell surface. Once at the surface, receptors can respond to EGF or PDGF binding by further increasing the rate of transcription of the gene via the serum response factor (35).

An important finding by den Hertog et al. (36) is that P19 cells overexpressing human EGF-Rs differentiate into neurons when stimulated with EGF, without the addition of RA. This strongly indicates a positive feedback mechanism whereby, once EGF-Rs are present at the surface, EGF can stimulate their further expression to levels expressed by neuronal cells. Indeed, such expression could be the major driving force to the differentiation of P19 cells.

In immortal fibroblast cell lines, the addition of RA causes an increase in EGF-R numbers starting 5 h later and maximizing at 5 days (37). In BALB/c 3T6 cells, 6-fold enhancement of 125I-EGF binding occurs, although concomitant growth rates are reduced to half in RA-treated cells (38). The basis for this increased expression of EGF-R numbers has not been reported but is unlikely to occur by the same mechanisms as in differentiating EC cells, because for fibroblasts the increased expression has different kinetics and also is reversible upon removal of RA. A rat liver cell line, however, responds to RA in a number of ways. Although EGF-R affinity falls and EGF-binding activity decreases, the steady-state level of EGF-R mRNA and protein increases (39). A variety of phenotypic responses to RA have been observed, even in closely related liver cell colonies (40). It is likely that individual cell types respond differently depending on which and how many receptors are induced. This, in turn, depends on combinations of modulatory transcription factors such as Sp1, AP-1, and retinoic acid receptors and on the relative levels in each cell type (41). Furthermore, even if growth factor receptors are expressed on cells, their response to ligand binding varies from proliferation to inhibition or differentiation, probably depending on the nature of the signal-transducing mechanisms and components that are present.
Accumulated evidence suggests that the initial appearance of receptors at the cell surface is an important self-perpetuating event that could be an important driving force to the continuation of differentiation. For instance, a cellular architectural change caused by stimulus of cytokines could bring EGFRI to the surface. Once there, EGF and transforming growth factor α in the culture medium could stimulate further receptor gene induction, and so on. The induction of EGF receptors in differentiating cells now can stimulate increased differentiated expression by increasing the levels of components involved in gene switches, such as the transcription factors c-fos, c-jun, and Egr-1. We have shown that all of these factors increase during P19 differentiation (31). Although the mechanism of EGF-R gene activation is not yet known, we suggest that it is an important element in the molecular processes that lead to differentiation and its irreversibility.

Materials and Methods

Cell Culture. F9, OC15, and P19 cells were cultured as described earlier (3, 7, 42) in α modification of minimal essential medium with 10% fetal bovine serum. For differentiation, all-trans-retinoic acid and DMSO (Sigma Chemical Co.) were used. In brief, P19 cells were cultured with RA at 0.5 μM concentration for 3 days with daily feeding and renewal of RA, or with 0.5% or 1% DMSO for 4 days with daily renewal of medium and drugs, as aggregate cultures in untreated plastic Petri dishes (Fisher Scientific Co.), to which they were nonadhesive. The aggregates were then seeded in tissue culture plastic for a further 4–9 days in medium without drugs to allow outgrowth and differentiation.

Immunohistochemistry. Indirect immunoperoxidase reactions using the three-layer ABC peroxidase method (Vector Laboratories) followed the recommended procedures. Cells were fixed in 3% formaldehyde buffered with phosphate, pH 7.5, and rabbit antibody (2 μg/ml) was incubated with cells overnight. Adsorbed antibody was revealed with hydrogen peroxide and diaminobenzidine (a brown stain that appears black in Fig. 1). Tissue sections were made on a cryostat at 6-μm thickness, and sections were mounted on gelatinized slides fixed in formaldehyde before staining as described. The antibodies have been described earlier (3) and shown to react specifically with EGF-Rs in mouse, rat, and dog cells (43).

Immunoprecipitations. Cells were cultured in 35-mm dishes. At each stage of differentiation, preincubation in methionine-free medium for 60 min preceded the addition of 200 μCi/ml [35S]methionine in methionine-free medium containing dialyzed fetal bovine serum. Labeling was carried out for 2–3 h after lysing in radiolimmunoprecipitation assay buffer (1% sodium deoxycholate-1% Nonidet P-40-0.1% sodium dodecyl sulfate-0.1 M Tris, pH 7.5-0.15 NaCl-5 mM EDTA-0.02% sodium azide). Vortexed lysates were precleared by the addition of 2 μl preimmune serum and 50 μl fixed Staphylococcus aureus and centrifuged for 10 min at 15,000 rpm in a Microfuge. Aliquots were sampled for total radioactive protein by trichloroacetic acid precipitation, and equal amounts of acid-precipitable radioactive proteins were compared in subsequent immunoprecipitation reactions. In some cases, the medium was also analyzed for EGF-R-related proteins. Antibodies to mouse EGF-R (3) or to mouse PDGF-R (44) were added for overnight reaction, and lysates were exposed to Staphylococcus A for 30–60 min, before thorough washing by resuspension and centrifugation. The immunoprecipitates were analyzed by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and gels were stained and soaked in Autoradiol (West Chem) before being dried and exposed to flashed film for 16 h to 1 month.

For receptor protein degradation studies, P19 aggregates were seeded in 24-well plates after 3–4 days of RA or DMSO treatment and allowed to differentiate for 4 or 6 more days. [35S]Methionine (1 mCi/ml) was added for 2 h and then removed. Fresh medium containing cold methionine was added, and wells were lysed at different time intervals before immunoprecipitation as described above.

Mouse EGF-R cDNA Probes. A λgt11 cDNA library from adult male BALB/c mouse liver (Clontech) was screened with a random oligonucleotide-primed 32P-labeled 0.6-kb EcoRI/XbaI fragment of rat EGF-R cloned in pEMBL 18 (kindly given to us by S. Earp, University of North Carolina, Chapel Hill, NC). Positive clones were subcloned via their EcoRI inserts into the Bluescript vector (Stratagene). Two mouse cDNA clones were isolated as shown in Fig. 3: pmER-1 and pmER-2 containing 2.3 kb and 776 bp, respectively. These were partially sequenced by the dideoxynucleotide chain termination method (45) using a Sequenase kit (United States Biochemical Corp.).

Two riboprobes were prepared in order to assay for the ICD and the LBD. Both probes were prepared using Exonuclease III and SI nuclease as described by Henikoff (46). Fragments were cloned into Bluescript: the ICD fragment (199 bp corresponding to nucleotides 2516–2318 of human EGF-R cDNA) was linearized with Hind III, and cRNA was synthesized by T7 polymerase; the LBD fragment (193 bp, corresponding to nucleotides 1345–1537 of human EGF-R (26)), was linearized with XbaI, and cRNA was synthesized by T3 polymerase.

mRNA Extraction and Measurement. RNA was extracted by the guanidinium thiocyanate procedure essentially as described by Maniatis et al. (47) or by Chomczynski and Sacchi (48). Northern analysis was performed as described (47) by transfer of formaldehyde-denatured electrophoresed samples to nitrocellulose. The 2.3-kb fragment of mouse EGF-R was labeled with the Klenow fragment of DNA polymerase.

RNase protection assays were performed essentially as described by Melton et al. (49). As an invariant control for standardization of signals, the ribosomal protein gene L32, cloned into pGEM4 (50), gives a protected fragment of 410 bp and was used simultaneously with each of the LBD and ICD EGF-R probes described above. Fifty μg of RNA were analyzed as described earlier (31). Dried gels were exposed to X-ray film for 2–10 days. The levels of protected radioactive RNA on gels were quantified by scanning the films after suitable times of exposure using an LKB laser densitometer.

To measure the degradation rates of mRNA, cells were pretreated for 30 min with actinomycin D (5 μg/ml) and then harvested at different time intervals. In some experiments, α-amanitin was used to inhibit RNA synthesis. The remaining level of transcript was assayed in total RNA as described above, quantified, and expressed as a percentage of the initial time (100%).
For analyses of RNA by Northern blotting, 20 μg total RNA from each sample were analyzed as described by Maniatis et al. (47). The probe was pMIR, labeled with \(^{32}P\) as described by Feinberg and Vogelstein (51).

**Transcription Run-on Assays.** Procedures were slightly modified from those of Greenberg and Ziff (52) and Groudine et al. (53). Nuclei were prepared from four 100-mm dishes of P19 or OC15 EC cells or from differentiating cells that had been treated with 0.5–1.0 μM RA or 0.5% or 1% DMSO as indicated in the figure legends. \(^{3}P\)UTP-labeled (800 Ci/mol) RNA transcripts were purified by ethanol precipitation, and 10\(^\circ\) cpm were used in each incubation mixture. Nitrocellulose strips were loaded with various DNAs (2 μg) and, after baking, were prehydrated for at least 2 h. This step was found to lower the background and increase signal intensity after hybridization in 5X saline-sodium phosphate-EDTA-50% formamide at 2–4 days at 42°C. Nitrocellulose strips were washed and treated with RNase to remove background as described (54). DNA samples were 2.3 kb pmER-1, L32, for standardization of the levels of synthesis at each stage. For positive controls, full length Egr-1 cDNA (OC 3.1 (55)), a full length c-fos plasmid (56), a cDNA encoding c-jun [RSV-c] (57), and one encoding JunB [p46.5.20 (58)] were used. PGEM plasmid DNA was the control for nonspecific hybridization. After densitometry of the autoradiograph produced from film exposed to the gel for 1–2 weeks, the result was expressed relative to the signal given by L32.

A second type of run-on transcription study was performed to determine the rate of transcription in NIH3T3 fibroblasts after stimulation with the phorbol ester tumor promoter, 12-O-tetradecanoylphorbol-13-acetate for 2 h. To determine whether serum stimulates EGF-R transcription, NIH3T3 as well as P19 cells were made quiescent by growth for 24 h in 1% serum followed by serum addition for 2 h before nuclear isolation and run-on transcription assays.

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**References**


