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Differential Regulation of EGF-like Growth Factor Genes in Human Keratinocytes

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ErbB signaling increases protein levels of multiple EGF-like growth factors in epithelial cells. To better understand this process, we examined the effects of EGF receptor stimulation on the transcription and mRNA stability of TGF-α, amphiregulin (AR), and heparin-binding EGF-like growth factor (HB-EGF) in human keratinocytes. EGF stimulation increased transcription of TGF-α, AR, and HB-EGF by 3- to 4-fold within 1 to 2 h. However, AR and HB-EGF mRNA levels peaked at 2 h and then rapidly declined, whereas TGF-α transcripts remained elevated for at least 6 h. Actinomycin D decay experiments yielded the rank order of transcript stability TGF-α > AR > HB-EGF. Interestingly, ligand treatment appeared to stabilize TGF-α and AR mRNAs, whereas HB-EGF transcripts were destabilized. These data demonstrate that gene-specific alterations in gene transcription and mRNA stability play important roles in the temporal regulation of EGF-like growth factor gene expression. © 1999 Academic Press

EGF-like growth factors are known to exert their biological functions via the ErbB family of transmembrane receptor tyrosine kinases (1). In keratinocytes and other epithelial cells, stimulation of this receptor system has both mitogenic and anti-apoptotic effects (2, 3). A significant feature of this ligand-receptor system in epithelial cells is its autocrine nature. Thus, addition of TGF-α or EGF to a variety of epithelial cells stimulates the expression of the ErbB ligands TGF-α, amphiregulin (AR), and heparin-binding EGF-like growth factor (HB-EGF). This phenomenon is known as autoinduction (4–7). Recent evidence indicates that autoinduction is an important mechanism for amplification of wound healing responses in the skin (8). An accumulating body of evidence also indicates that EGF receptor signaling plays a critical role in epithelial carcinogenesis (9–12).

The mechanisms by which autoinduction takes place have previously been studied for TGF-α in normal human keratinocytes (NHK) and LIM1215, a colon cancer cell line (13). Those studies found that mRNA stabilization plays an important role in the autoinduction of TGF-α in NHK, but not in LM1215 cells, suggesting a fundamental difference between normal and malignant epithelial cells. Focusing on keratinocytes, in this report we confirm earlier findings regarding TGF-α and extend these studies to AR and HB-EGF. Our findings demonstrate that these three EGF-like genes differ significantly in their utilization of transcriptional vs post-transcriptional control mechanisms in normal and immortalized keratinocytes.

MATERIALS AND METHODS

Materials. The EGFR tyrosine kinase inhibitor PD153035 was a generous gift of Drs. D. Fry and W. Leopold (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). Actinomycin D and α-amanitin were obtained from Sigma (St. Louis, MO). Human recombinant TGF-α was purchased from R&D Systems (Minneapolis, MN), mouse EGF was obtained from Upstate Biochemicals, Inc. (Lake Placid, NY) or Sigma (St. Louis, MO). All other materials were of reagent grade.

Cell culture. Normal adult human keratinocytes (NHK) were grown in modified (2) MCDB 153 medium (KGM, Clonetics, San Diego, CA or Medium 154, Cascade Biologics, Portland, OR). HaCaT cells (14) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco/BRL, Gaithersburg, MA). At 40–50% confluence, both cell types were rendered quiescent by incubation in modified MCDB153 medium without growth factors for 48 h as described (5, 7). Growth factor-depleted cells were incubated with 3.3 nM TGF-α or EGF for various times in the presence or absence of various inhibitors prior to assay of nuclear runoff transcription or mRNA stability (see below).

Nuclear runoff transcription. Nuclear runoff transcription assays were performed using HaCaT cells as previously described (15). The ^{32}P-labeled nuclear transcripts were hybridized to Zeta-Probe nylon filters (Bio-Rad, Richmond, CA) dot-blotted with 5 μg of various denatured plasmid DNAs. The HB-EGF (1.9 kb), AR (0.9 kb) and S100A2 (0.5 kb) cDNAs were isolated from a human keratinocyte

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cDNA library and cloned into pUC19 (15). The plasmids containing TGF-α (1.4 kb), 36B4 (0.75 kb) and cyclophilin (1 kb) cDNAs have been previously described (16–18). The plasmid pSP65 (Promega, Madison, WI) was used as a control for nonspecific hybridization. Detection and quantitation by autoradiography and densitometry have been described (15).

mRNA stability assay. After 48 h of growth factor depletion, HaCaT cells or NHK were treated with EGF or TGF-α (3.3 nM). After 2 h 10 μg/ml actinomycin D was added, and dishes were harvested at two-hour intervals up until 8 h after ligand addition. RNA was isolated using RNAzol (Cinna BioTecx, Friendswood, TX) according to the manufacturer's instructions, and analyzed by quantitative Northern blotting as described below. Results were expressed as a percentage of the normalized steady-state mRNA level present at the onset of actinomycin D treatment.

Northern blotting. Equal amounts (20 to 40 μg) of purified total RNA were fractionated on 1% formaldehyde-agarose gels, blotted to Zeta-probe nylon membranes, hybridized against random-primed ^{32}P-labeled cDNA inserts, autoradiographed, quantitated by phosphorimager or densitometer (Molecular Dynamics), normalized to the control gene 36B4 (17), and stripped for reprobing, all as previously described (19, 20).

RESULTS

Steady-state mRNA levels. Treatment of NHK (Fig. 1a) and HaCaT cells (Fig. 1b) with EGF or TGF-α markedly increased the expression of AR, HB-EGF and TGF-α mRNAs. TGF-α consistently induced higher levels of all three transcripts in NHK compared to equimolar quantities of EGF (Fig. 1a). While the time courses of the responses were similar in both cell types, they differed significantly depending on the gene involved. Thus, in both cell lines, AR and HB-EGF mRNA levels peaked at 2 h post growth factor-treatment, then declined. In contrast, TGF-α transcripts were also increased by 2 h, but remained elevated for at least 6 h after growth factor treatment.

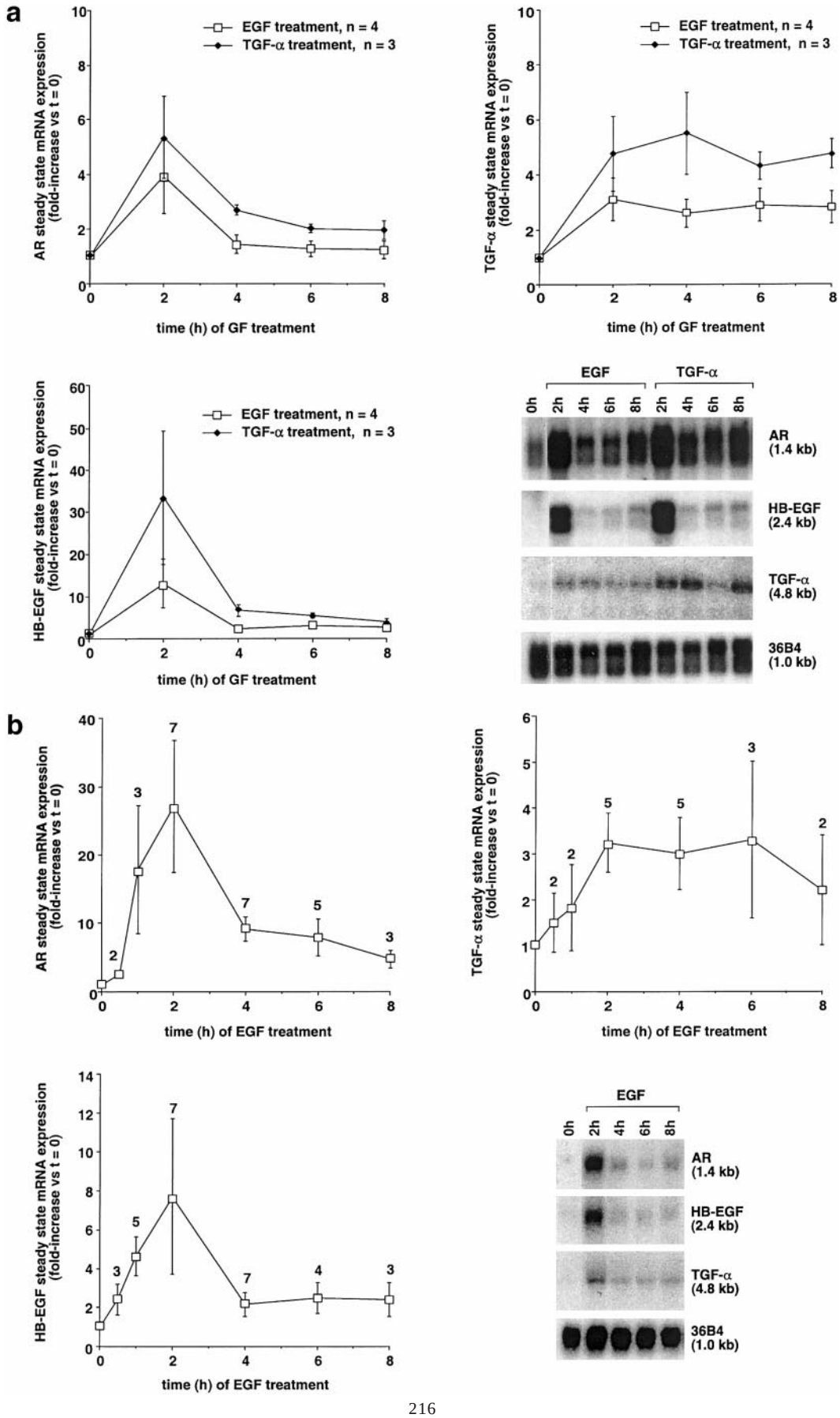
Nuclear runoff transcription assay. Figure 2a shows the results of a representative experiment demonstrating the effects of EGF stimulation on nuclear runoff transcription in HaCaT cells. The quantitated results of multiple experiments are shown in Fig. 2b. EGF treatment led to a general increase in transcription that was reflected in modest increases in ^{32}P-UTP incorporation as well as in mRNA levels of the control gene 36B4. The highest levels of 36B4 transcription and total UTP incorporation (approx. 2- to 3-fold) were observed after 4 h of EGF stimulation. In contrast, transcription of AR and HB-EGF peaked by 1 h post-stimulation, at a level 3 to 4-fold higher than basal transcription (Fig. 2b). Transcriptional induction of TGF-α was somewhat more delayed, peaking at 2 h after EGF treatment. Pretreatment of intact HaCaT cells with the EGFR tyrosine kinase inhibitor PD153035 (21) abrogated EGF-induced transcription, but had only a slight effect on transcription of the control gene 36B4 (Fig. 2c). Pretreatment of isolated HaCaT nuclei with 2 μg/ml of α-amanitin strongly reduced transcription of all genes tested, verifying that transcription was mediated by RNA polymerase II (Fig. 2d).

mRNA stability assay. Growth factor-deprived and EGF-stimulated cells were treated with actinomycin D, and assayed by quantitative Northern blotting. Results obtained for NHK are shown in Fig. 3, and those obtained for HaCaT cells are shown in Fig. 4. Of the three mRNAs, TGF-α was the most stable in the absence of ligand in NHK. Ligand stimulation of NHK led to marked stabilization of TGF-α mRNA (Fig. 3a), and modest stabilization of AR mRNA (Fig. 3b). In contrast, HB-EGF transcripts were actually more unstable after EGF- or TGF-α-treatment (Fig. 3c). In HaCaT cells, the rank order of stability was the same as that observed in ligand-treated NHK, as assessed by the actinomycin D decay curves under conditions of ligand stimulation. (TGF-α > AR > HB-EGF) (Fig. 4a). It was difficult to assess the ligand dependence of AR and HB-EGF mRNA stability in HaCaT cells, due to low baseline mRNA levels under conditions of ligand deprivation (Fig. 1b). TGF-α was the only mRNA detectable under ligand-deprived conditions (and only in one of three experiments). In this experiment, EGF treatment did appear to stabilize TGF-α mRNA (Fig. 4b).

DISCUSSION

Autocrine activation of the ErbB receptor system plays a central role in the control of keratinocyte growth and differentiation (8, 22, 23). This receptor system can be activated by multiple ligands, at least three of which (TGF-α, AR, and HB-EGF) are expressed at substantial levels by keratinocytes (4). Moreover, levels of all three mRNAs, and levels of TGF-α and AR proteins, are increased in response to EGF or EGF-like growth factor treatment (4, 6). At present, it is not completely clear why the ErbB system utilizes multiple ligands, several of which are induced in response to ErbB activation. It is known that TGF-α differs from AR and HB-EGF in terms of heparin-

FIG. 1. EGF and TGF-α increase EGF-like growth factor steady-state mRNA levels in normal and immortalized human keratinocytes. Normal human keratinocytes (a) and HaCaT cells (b) and were grown until 40–50% confluent, then switched to growth factor-free keratinocyte basal medium for 48 h. Cells were then incubated with or without EGF or TGF-α for the indicated times. Zero-time controls were harvested at the time of growth factor treatment. Total RNA was isolated and analyzed by quantitative Northern blotting (see Materials and Methods), using ^{32}P-labeled TGF-α, AR and HB-EGF probes. Data are expressed as fold-change relative to untreated control samples; error bars represent standard error of the mean (SEM). In b the number of observations are indicated above each data point.


a
AR steady state mRNA expression (fold-increase vs t = 0)
EGF treatment, n = 4
TGF-α treatment, n = 3
time (h) of GF treatment
TGF-α steady state mRNA expression (fold-increase vs t = 0)
HB-EGF steady state mRNA expression (fold-increase vs t = 0)
EGF
TGF-α
0h 2h 4h 6h 8h 2h 4h 6h 8h
AR (1.4 kb)
HB-EGF (2.4 kb)
TGF-α (4.8 kb)
36B4 (1.0 kb)
b
AR steady state mRNA expression (fold-increase vs t = 0)
time (h) of EGF treatment
TGF-α steady state mRNA expression (fold-increase vs t = 0)
HB-EGF steady state mRNA expression (fold-increase vs t = 0)
EGF
0h 2h 4h 6h 8h
AR (1.4 kb)
HB-EGF (2.4 kb)
TGF-α (4.8 kb)
36B4 (1.0 kb)

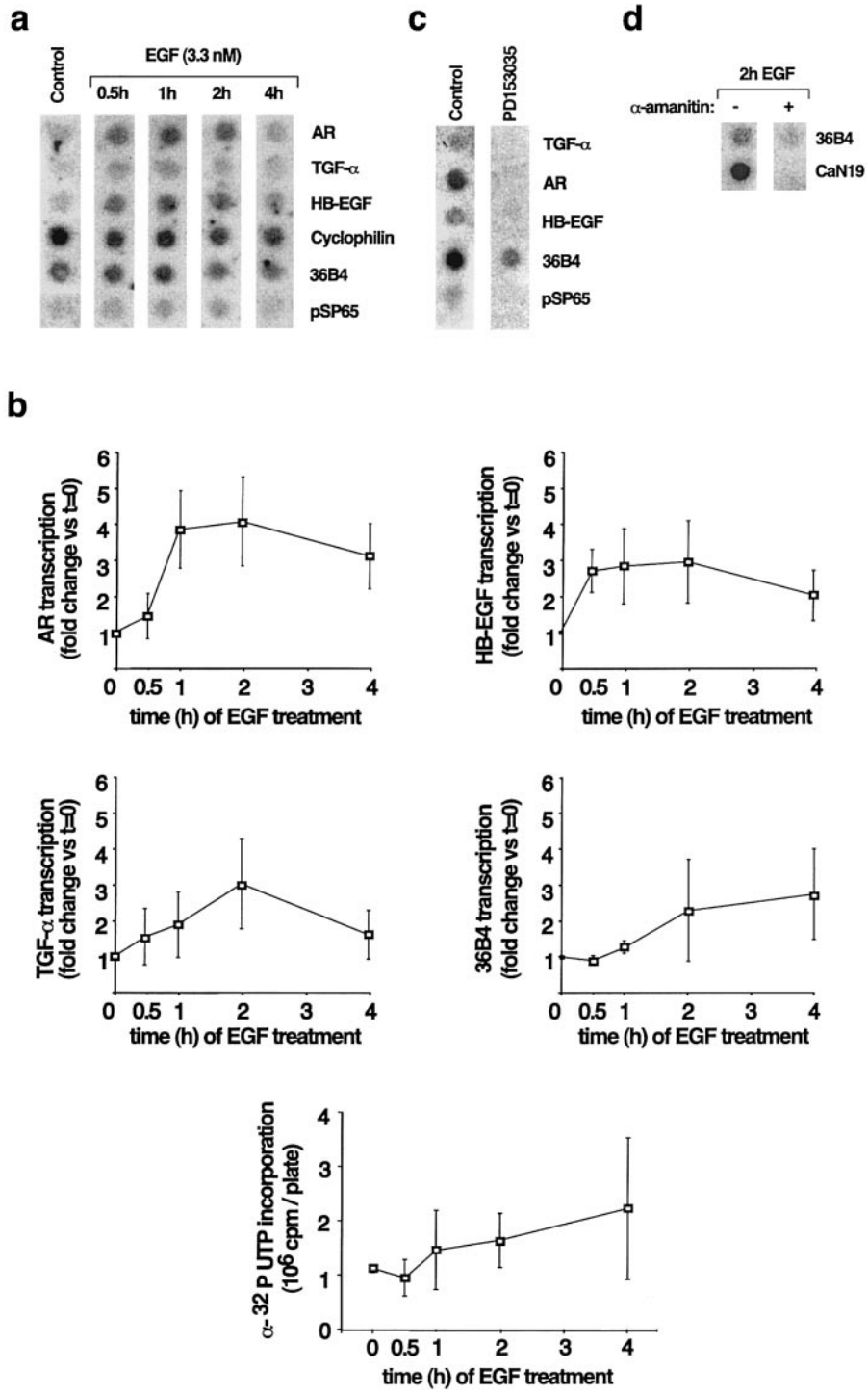


FIG. 2. EGF treatment of HaCaT cells leads to an increase of EGF-like growth factor gene transcription. Nuclear runoff transcripts from growth factor-depleted HaCaT-cells were isolated and analyzed as described under Materials and Methods. Zero-time controls were harvested at the time of EGF stimulation. (a) Autoradiograms from a single representative experiment are shown. (b) Quantitation of time course of transcription. Data are expressed as fold induction versus unstimulated cells (t = 0), error bars represent mean ± SEM (n = 3). The data for [α-^{32}P]UTP incorporation are expressed as total counts per plate of HaCaT cells. (c) Effects of the ErbB tyrosine kinase inhibitor PD153035. Growth factor-depleted cells were incubated with 100 nM PD153035 for 30 min prior to EGF stimulation for 2 h. (d) Effects of α-amanitin. Nuclei isolated from HaCaT cells treated for 2 h with 20 ng/ml EGF were incubated with 2 μg/ml of α-amanitin for 15 min at 4°C prior to the nuclear runoff transcription reaction. Results for 36B4 and CaN19 (S100A2) are shown to illustrate the effects of α-amanitin on EGF-inducible vs constitutive gene transcription; these results have been reported previously (15).

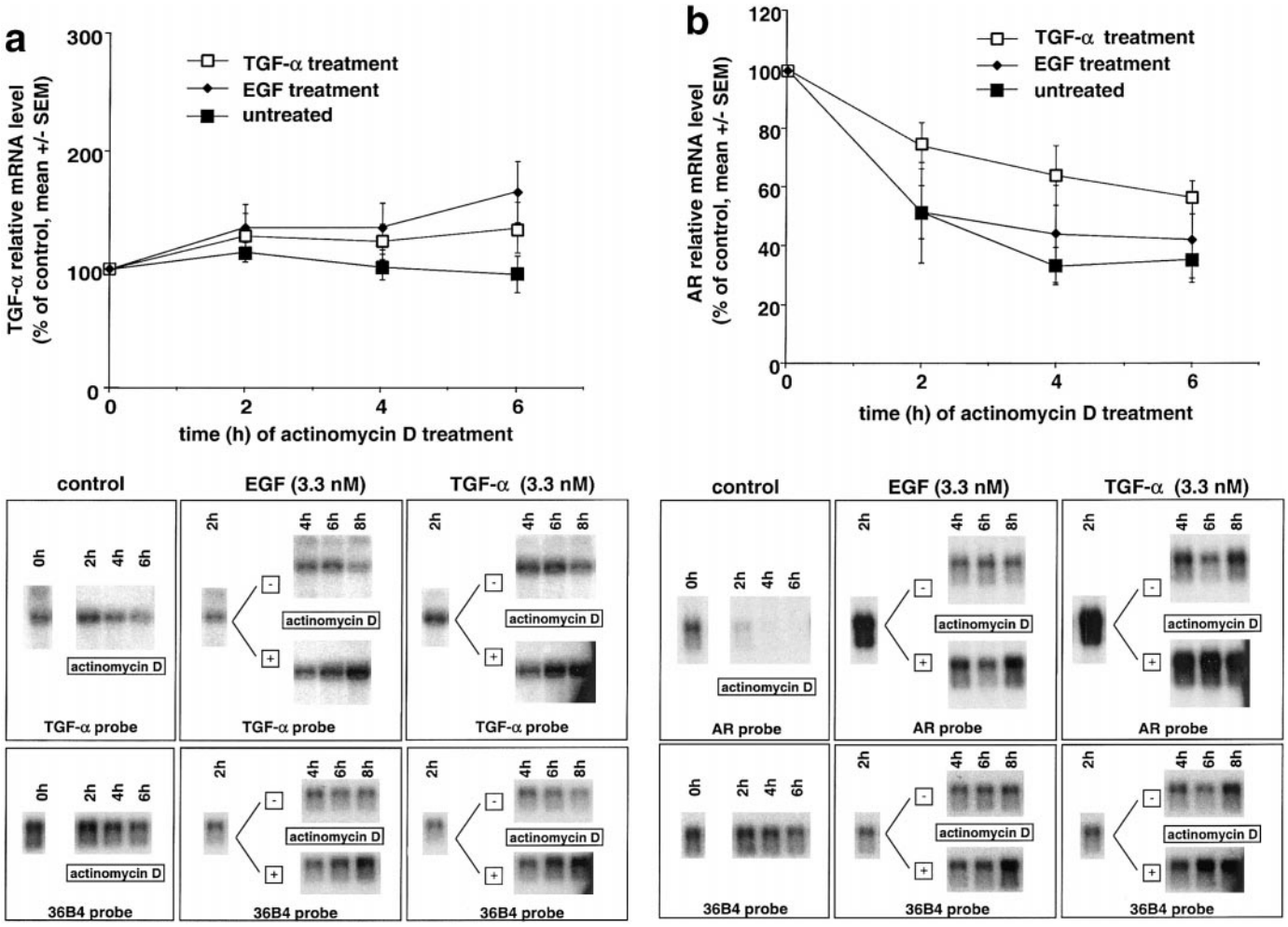


FIG. 3. Gene-specific effects of EGF and TGF-α treatment on the stability of AR, HB-EGF, and TGF-α mRNAs in NHK. (a) TGF-α; (b) AR; (c) HB-EGF. Growth factor-depleted NHK were incubated with or without EGF or TGF-α as described under Materials and Methods. After 2 h one plate per GF treatment was harvested and the remaining cultures were either incubated with actinomycin D (10 μg/ml) or left untreated. Cells were harvested at 2-h intervals thereafter and mRNA levels were assessed by quantitative Northern blotting. The quantitative data are expressed as a percentage of the signal obtained for cells treated with ligand for 2 h. The panels underneath the graphs show autoradiographs of representative hybridizations. Note that the 4-, 6-, and 8-h actinomycin D-untreated controls shown in the lower panels have not been quantitated here; these quantitations are included in Fig. 1a. Error bars represent standard error of the mean (SEM); n = 4 for EGF and untreated cells, n = 3 for TGF-α treatment.

binding capacity, and there is some evidence that EGF and TGF-α differ in their pathways of post-receptor ligand-receptor processing (24). Another possible reason for the existence of multiple ligands would be to provide for differential regulation in response to ErbB receptor activation. This could allow keratinocytes the flexibility to respond differentially to ErbB signaling in different environments (such as normal homeostasis vs wound healing). Previous studies in NHK have measured the steady-state levels of these mRNAs in response to ErbB activation (4), and the relative roles of gene transcription vs mRNA stability have been addressed in the case of TGF-α autoinduction (13). The objective of these studies was to determine, in a parallel fashion, the relative contributions of gene transcription and mRNA stability to the ligand-inducible expression of all three genes.

While others have reported successful nuclear runoff experiments in NHK (25–27), in our hands nuclear runoff assays were repeatedly unsuccessful due to extensive clumping of NHK nuclei during the isolation procedure. As an alternative to NHK, we utilized HaCaT cells, a differentiation-competent but immortalized keratinocyte cell line, which consistently yielded excellent nuclear preparations (data not shown). By comparing the nuclear runoff results obtained in HaCaT cells to the kinetics of mRNA accumulation and decay in both NHK and HaCaT cells, a consistent picture of gene-specific differential utilization of transcription vs mRNA stabilization has emerged.

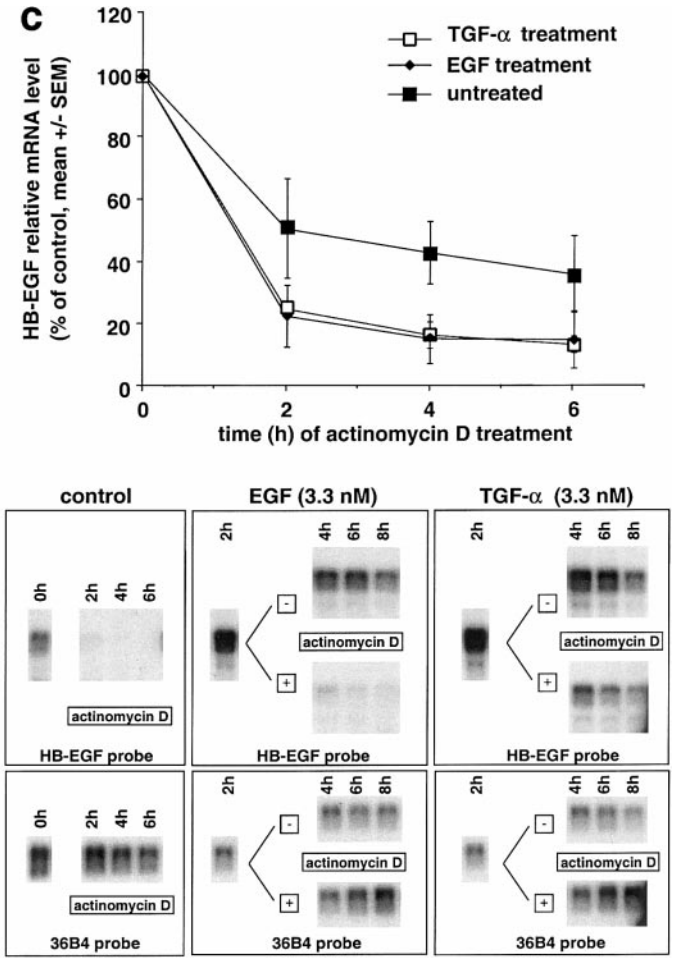


FIG. 3—*Continued*

As previously reported (4), ligand stimulation of NHK produced a rapid increase in HB-EGF and AR mRNA levels, whereas the increase in TGF-α mRNA was more gradual and prolonged (Fig. 1). Subsequent experiments demonstrated for the first time that these differences could be related to gene-specific differences in the transcription rate and in mRNA stability (see below). Consistent with previous reports (24), TGF-α was more potent than EGF in inducing all three mRNAs. These differences appear to be due to greater ligand recycling and reduced receptor downregulation by TGF-α compared to EGF (24).

Coffey and co-workers were unable to detect a transcriptional effect of TGF-α on the expression of the TGF-α gene in NHK, whether by nuclear runoff transcription or reporter gene assay (13). Our results are somewhat at variance with these findings, as we found a modest induction of TGF-α transcription in response to EGF (Fig. 2). However, the time course and extent of TGF-α transcription resembled the increase in global transcription found to occur under these experimental conditions, whether assayed by UTP incorporation or transcription of the "control gene" 36B4 (15). Based on our results and on those of Coffey and co-workers (13), we conclude that ligand-induced transcriptional activation of the TGF-α gene in human keratinocytes is modest at best.

By contrast, the AR and HB-EGF genes displayed a more rapid and robust transcriptional response to EGF (Fig. 2), reaching 3 to 4 times the basal level after 1 hour of ligand stimulation. However, EGF-inducible transcription of these genes was less pronounced than we have previously observed for S100A2 (greater than 15 times the basal level) (15) (compare Figs. 2a and 2d). For all three messages, the time course of transcription observed in HaCaT cells correlated very well with the observed patterns of steady state mRNA accumulation in both NHK and HaCaT, with transcription increasing prior to mRNA accumulation. Taken together, these data strongly suggest that the kinetics of TGF-α, AR and HB-EGF transcription are gene-specific, but similar in normal (NHK) and immortalized (HaCaT) keratinocytes.

Our results confirm the finding of Coffey and co-workers (13) that ErbB ligand treatment causes stabilization of TGF-α mRNA in NHK (Fig. 3a). Our results indicate that AR mRNA is also stabilized by EGF or TGF-α treatment, albeit to a lesser extent than is TGF-α (Fig. 3b). In contrast, HB-EGF mRNA actually appeared to be destabilized in response to ligand treatment (Fig. 3c). Although we were unable to quantify ligand effects on AR and HB-EGF mRNA stability in HaCaT cells due to low basal levels of expression, we did observe clear ligand-dependent stabilization of TGF-α mRNA in one experiment (Fig. 4b). Moreover, the actinomycin D decay curves obtained for the three mRNAs indicated the same rank order of mRNA stability in ligand-stimulated NHK and HaCaT cells (TGF-α > AR > HB-EGF). Finally, the time courses of steady-state mRNA levels in both NHK and HaCaT cells are consistent with the decay rates found for each transcript. Taken together, these data demonstrate very similar gene-specific profiles of mRNA stability in both NHK and HaCaT cells, and suggest that these differences in stability are accentuated by ligand activation of ErbB signaling.

We and others (13) have observed a paradoxical increase in TGF-α mRNA levels after actinomycin D treatment of EGF-stimulated NHK. This effect was also observed for AR and the "control gene" 36B4, albeit to lesser extents (Figs. 3 and 4). Several examples of actinomycin D-dependent mRNA stabilization have appeared in the literature [cf. (28)]. Recently, studies of transferrin receptor mRNA stability in the presence of α-amanitin have suggested an important role for RNA polymerase III transcripts in the control of mRNA stability. Thus, high concentrations of α-amanitin (200 μg/ml), which inhibit Pol III directly (29), stabilize transferrin mRNA, whereas low concentrations (10 μg/ml), which do not inhibit Pol III, have no effect (28).

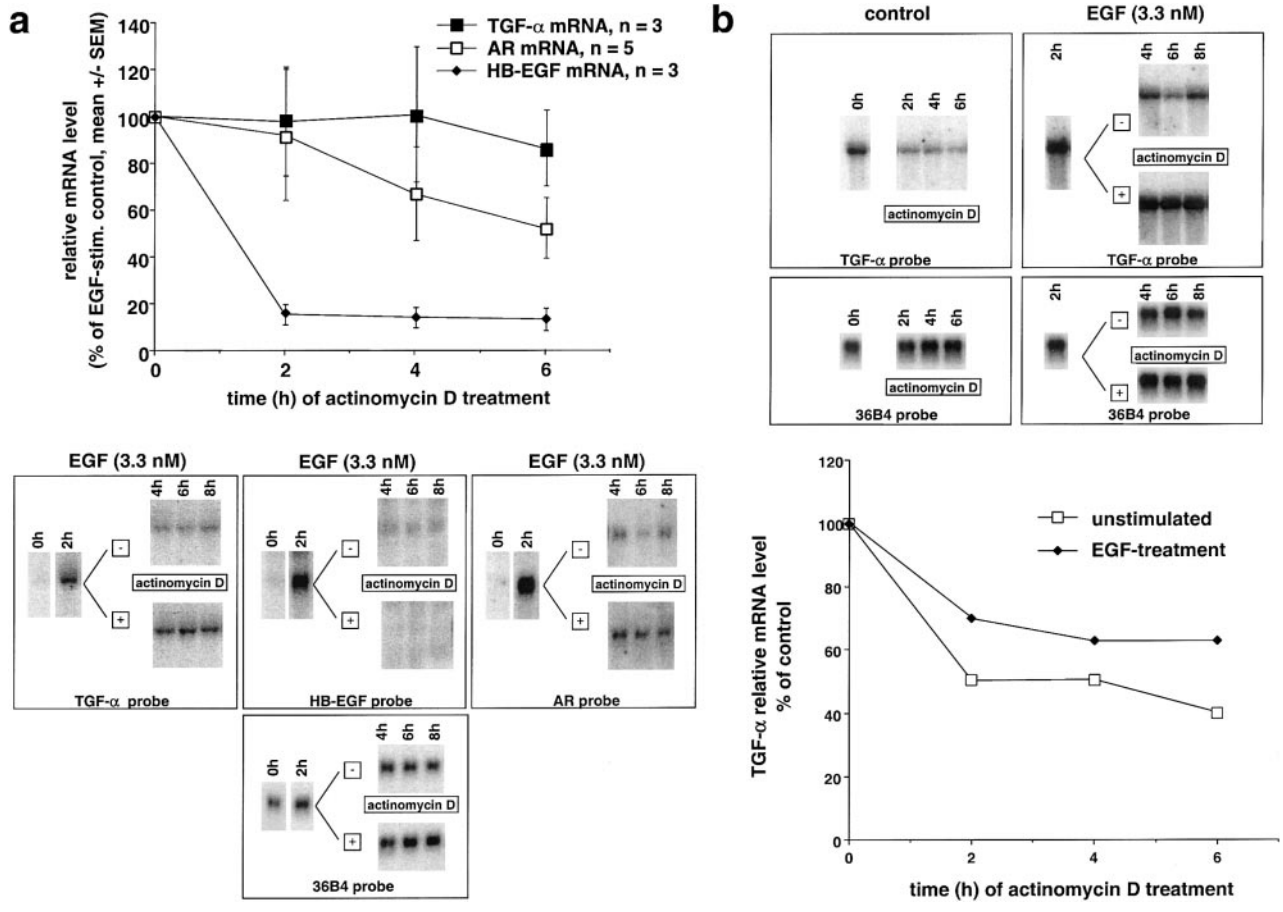


FIG. 4. Gene-specific effects of EGF treatment on AR, HB-EGF, and TGF-α mRNA stability in HaCaT cells. (a) Actinomycin D decay curves. Representative autoradiographs are shown below the graph. Due to undetectable baseline mRNA levels of TGF-α (2 of 3 experiments), AR (5 of 5 experiments), and HB-EGF (3 of 3 experiments), the decay curves for cells not treated with EGF are not shown. Note that the 4-, 6-, and 8-h actinomycin D-untreated controls shown in the lower panels have not been quantitated here; these quantitations are included in Fig. 1b. (b) The autoradiographs shown are from the experiment in which TGF-α mRNA was detectable in ligand-untreated cells. The graph underneath the autoradiograms shows the quantitated results of this experiment.

This finding could explain our results, as Pol III is inhibited by the concentrations of actinomycin D used in our experiments (10 μg/ml) (29). However, in our experiments, actinomycin D did not increase TGF-α or AR mRNA levels in cells maintained under conditions of growth factor deprivation, suggesting that this effect of actinomycin D requires ErbB activation.

It has been appreciated for some time that EGF can promote mRNA stabilization in epithelial cells (30). Several lines of evidence suggest that this phenomenon may relate to the control of translation (31). PHAS-1, an inhibitor of translation, is inactivated by mitogen activated protein kinase (MAPK)-dependent phosphorylation in response to treatment with EGF, insulin or other growth factors (32, 33). MAPK activation also increases the activity of ribosomal S6 kinase and glycogen synthase kinase-3, which enhance polysome formation and the availability of initiator tRNAs, respectively (34). It is attractive to speculate that ErbB $\rightarrow$ MAPK signaling might be responsible for the beneficial effects of EGF/TGF-α on mRNA stability by increasing the rate of translation, possibly at several points in the translational control pathway. If this is the case, then it is possible that dysregulation of these mechanisms may be an important component of the multistep process of carcinogenesis.

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