Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide

(peptide growth factor/specific gene transcription/actin mRNA/cell proliferation)

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ABSTRACT Stimulation of quiescent AKR-2B mouse embryo cells with epidermal growth factor (EGF) results in a rapid and specific induction of actin mRNA sequences. These mRNAs include those coding for both β - and γ -cytoskeletal, but not α -skeletal muscle, actin isoforms. Elongation of nascent RNA chains in isolated nuclei (run-off transcription) demonstrates that the mRNA accumulation is preceded by an increase in actin gene transcription. This increase is transient, however, and is followed by a rapid attenuation of transcriptional activity. An inhibitor of protein synthesis, cycloheximide, was also found to induce β - and γ -actin mRNA accumulation. Furthermore, the simultaneous addition of EGF and cycloheximide produced a synergistic effect on actin sequences in both steady-state nuclear and polysomal RNA. Run-off transcription experiments demonstrate that this synergistic effect results from an increase in the magnitude and duration of actin gene transcription. It is also specific in that α -tubulin gene transcription is not similarly affected. These data suggest the existence of a specific labile repressor of actin gene transcription.

The binding of epidermal growth factor (EGF) to specific receptors in the membranes of quiescent cells initiates a variety of biochemical events that can culminate in the initiation of DNA synthesis and cell division (reviewed in ref. 1). Inhibitors of RNA synthesis can block this process, implying that the transcription of certain genes is required for a quiescent cell to reenter the cell cycle (2). The molecular mechanisms that regulate these genes are poorly understood but do not appear to involve the nuclear translocation of the hormone-receptor complex. Indeed, the complex itself is rapidly internalized and degraded several hours prior to the initiation of DNA synthesis (3, 4). This observation has led to speculation that a second messenger(s) of hormone action must be responsible for initiating the expression of specific genes required for cell proliferation (1).

A major constraint on any proposed mechanism of EGF or second messenger action is the requirement for a high degree of specificity. This consideration arises from several studies that have shown that peptide growth factors regulate a very limited domain of specific genes (5-8). This specificity could be achieved by a specific DNA binding protein acting either as a positive or negative regulator of gene transcription. Although theoretical constraints have been imposed on specific protein-DNA interactions in the context of a mammalian nucleus, these constraints are not absolute and a variety of compensating strategies are available to a eukaryotic cell (9). Therefore, it may be noteworthy that recent studies have shown that inhibitors of protein synthesis can potentiate the induction of specific mRNA sequences by platelet-derived growth factor (7, 10). Although this observation is consistent with a protein repressor of growth-factor-regulated genes, a

direct effect on gene transcription rates was not shown.

Prompted by a report that actin protein synthesis was specifically increased in serum-stimulated 3T3 cells (11), we have investigated the regulation of actin mRNA levels after stimulation of quiescent AKR-2B cells with purified EGF. We find that these levels rapidly increase within 1-2 hr after hormone binding. Our data further suggest that this induction is modulated, in part, by a specific repressor of actin gene transcription.

METHODS

Cell Culture. Mouse embryo-derived AKR-2B cells were cultured as described (12). Quiescent cultures were obtained by incubating cells for 48 hr in serum-free medium or in medium containing 0.5% fetal bovine serum. Cells were stimulated either with EGF (10 ng/ml)/insulin (500 ng/ml) or with EGF alone as indicated. The rate of [3 H]thymidine incorporation was determined both before and 20 hr after stimulation as described (5). Cell fractionation and purification of RNA have been described (5).

Blot Analysis of RNA. Electrophoresis of RNA in 1% agarose gels containing formaldehyde and formamide, transfer to nitrocellulose, and hybridization to 32 P-labeled cloned probes were performed exactly as described (12).

In Vitro Transcription. Nuclear run-off transcription assays were performed by a modification of the technique of Stallcup *et al.* (13); 5 $\times$ 100 mm dishes of cells were rinsed two times with phosphate-buffered saline at 4°C. Nuclei were isolated by scraping cells in the presence of a hypotonic buffer (TKM) plus detergents (10 mM Tris-HCl, pH 7.4/3 mM MgCl₂/15 mM KCl/0.75% Triton X-100) and rinsing several times using a short Pasteur pipette. The lysed cells were pelleted over a pad (1 M sucrose in TKM) and the nuclear pellets were resuspended in 20% (vol/vol) glycerol/50 mM Na Hepes, pH 8.0/5 mM Mg acetate/0.5 mM dithiothreitol/1% bovine serum albumin to a concentration of 2-3 mg per ml of DNA. *In vitro* transcription reactions (0.1 ml) contained DNA (0.9 mg/ml)/50 mM Na Hepes, pH 8.0/150 mM NH₄Cl/5 mM Mg acetate/0.5 mM MnCl₂/2.0 mM dithiothreitol/1 mM GTP/1 mM CTP/1 mM ATP/10 μ M UTP/[32 P]UTP (1.5 mCi/ml) (3000 Ci/mmol; 1 Ci = 37 GBq)/10% glycerol/1% bovine serum albumin and were incubated 60 min at 25°C. Reactions were digested with DNase I (50 μ g/ml), diluted to 0.3% NaDodSO₄ with buffer A (0.1 M NaCl/10 mM Tris-HCl, pH 7.4/1 mM EDTA/0.5% NaDodSO₄), digested with proteinase K (0.5 mg/ml), extracted one time with an equal volume phenol:SEVAG in buffer A, and ETOH-precipitated with 2% KOAc in ETOH. 32 P-labeled RNA was resuspended in 20 mM Tris-HCl, pH 8.0/10 mM MgCl₂/2 mM CaCl₂, and the DNase I digestion, proteinase K digestion, phenol:SEVAG extraction, and ETOH pre-

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Abbreviation: EGF, epidermal growth factor.

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cipitation were repeated a second time. Unincorporated [32 P]UTP was removed by passing [32 P]RNA over Sephadex G-50 in a buffer A column and the excluded fractions were ETOH-precipitated as before. Ten micrograms of α -actin and 10 μ g of α -tubulin subcloned plasmid DNAs were denatured and dotted onto nitrocellulose filters. The respective cloning vector DNAs were also included as controls. These yielded no detectable hybrids under the conditions used. The filters were vacuum dried at 80°C for 2 hr. Prehybridization and hybridization conditions were as follows: 50% deionized formamide/1 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/poly(A) (10 μ g/ml)/herring sperm DNA (50 μ g/ml)/0.1% NaDodSO₄/3 $\times$ NaCl/Cit (1 $\times$ NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate) at 50°C. After hybridization and washing, filters were exposed to x-ray film and the resulting dots were quantitated by microdensitometry.

RESULTS

Induction of Actin mRNA Sequences After EGF Stimulation. The addition of either serum or purified peptide growth factors to quiescent AKR-2B cells results in the initiation of DNA synthesis 12–14 hr later (5, 14). Entry into S phase is preceded by increased hnRNA synthesis, mRNA accumulation, and protein synthesis (14, 15).

Fig. 1 illustrates a time course of actin mRNA accumulation after the addition of EGF to quiescent cultures of AKR-2B cells. The probe used in this experiment was pAM91, a cDNA clone containing coding sequences derived from an α -skeletal muscle actin mRNA (16). The major actin-specific mRNA detected, however, exhibited an apparent chain length of 2.2 kilobases corresponding to β - and/or γ -cytoskeletal mRNAs (17). These mRNAs encode actin isoforms that are closely related in amino acid sequence to the muscle-specific actin group (18). A minor band with an apparent chain length of 1.6 kilobases was also detected, perhaps corresponding to either α -skeletal or α -cardiac muscle actin mRNA (17). Although the induction of actin mRNA is rapid, reaching maximal levels 2–4 hr after stimulation, it also appears transitory, declining as cells approach and enter S phase.

Effect of Cycloheximide on Actin RNA Accumulation. It was important to determine whether the EGF-mediated increase in actin mRNA levels was a primary genetic response or required the prior synthesis of other gene products. The effect of cycloheximide, an inhibitor of protein synthesis, on EGF-induced actin mRNA levels was determined. Quies-

FIG. 1. Kinetics of actin mRNA accumulation after EGF stimulation. Quiescent AKR-2B cells in 0.5% fetal bovine serum-containing medium were stimulated by the addition of fresh medium containing 10 ng of EGF per ml. Cells were harvested at the indicated hourly intervals and polysomal poly(A)⁺ RNA was prepared. Actin-specific sequences were determined by agarose gel electrophoresis and RNA transfer analysis using a 32 P-labeled α -actin cDNA clone (pAM91). Each lane contained 2 μ g of poly(A)⁺ RNA. Chain lengths were estimated with reference to 16S, 18S, 23S, and 28S ribosomal RNA markers co-electrophoresed in a parallel lane. kb, Kilobases.

cent cells were treated for 4 hr with either EGF, cycloheximide, or a combination of EGF and cycloheximide. The concentration of cycloheximide used (1 μ g/ml) was low but sufficient to inhibit the synthesis of proteins required for the initiation of DNA synthesis. Total polysomal and total nuclear RNAs were prepared, spotted onto nitrocellulose paper, and hybridized to the labeled pAM91 probe. Fig. 2 illustrates that cycloheximide was not an inhibitor but, in fact, was an effective inducer of polysome-associated actin mRNA sequences. When given in combination with EGF, cycloheximide was found to potentiate the action of the hormone. Interestingly, cycloheximide, either alone or in concert with EGF, was also found to result in the accumulation of actin-specific sequences in steady-state nuclear RNA.

Increased Actin Gene Transcription Precedes Actin mRNA Accumulation. To determine whether the EGF-dependent increase in actin mRNA sequences was mediated by an increase in actin gene transcription, nuclear run-off transcription assays were performed. Nascent RNA chains in nuclei isolated from quiescent cells and from cells stimulated for various times with EGF were allowed to elongate in the presence of [32 P]UTP and other nucleotide triphosphates (13). Numerous studies have shown that the extent of nascent RNA chain elongation in isolated nuclei is an accurate approximation of the number of active transcription complexes existing *in vivo* (13, 19–22). The RNA was then purified and hybridized to a vast excess of cloned α -actin cDNA, α -tubulin cDNA (23), and the respective cloning vector DNAs immobilized on nitrocellulose paper in the form of dots. Fig. 3 illustrates microdensitometer tracings of the resulting autoradiographic exposures. As expected, α -tubulin gene transcription remained relatively unchanged during 4 hr of EGF treatment. Previous results had shown that α -tubulin mRNA levels were not affected by EGF stimulation (not shown). Although actin transcription exhibited unexpectedly high basal levels in quiescent cell nuclei, a 2- to 3-fold increase was observed using nuclei isolated from cells stimulated with EGF for 30 min. This increase was transient, returning to basal levels within $\approx$ 1 hr. No hybridization to cloning vector DNA was detected and the vast majority of hybridizable RNA sequences were sensitive to the inclusion of 1 μ g of α -amanitin per ml in the *in vitro* reaction mix. Thus, these RNAs represented specific RNA polymerase II transcription products. These data demonstrated that the increase and

FIG. 2. Effects of cycloheximide and EGF on steady-state nuclear and polysomal RNA levels of actin-specific RNA. Quiescent cells in serum-free medium were treated for 4 hr with either cycloheximide (1 μ g/ml), EGF (10 ng/ml), or a combination of the two. Nuclei and polysomes were prepared, and the RNA was extracted and analyzed by RNA dot hybridization using a 32 P-labeled α -actin cDNA probe (pAM91). Each dot contained 4 μ g of either total polysomal or total nuclear RNA. Lane 1, no treatment; lane 2, 4 hr with cycloheximide; lane 3, 4 hr with EGF; lane 4, 4 hr with EGF and cycloheximide.

FIG. 3. Actin and α -tubulin gene transcription in nuclei isolated from EGF-stimulated AKR-2B cells. Quiescent cells in serum-free medium were stimulated with EGF (10 ng/ml) and insulin (500 ng/ml) in the presence or absence of cycloheximide (CH) (1 μ g/ml). Although insulin potentiates the mitogenic effect of EGF in serum-free medium (14), it has no effect on actin mRNA accumulation (not shown). Nuclei were isolated at the indicated times and used in run-off transcription assays in the presence or absence of α -amanitin (1 μ g/ml). 32 P-labeled transcripts were hybridized to 10 μ g of α -actin (Ac) DNA, α -tubulin (Tu) DNA, and the respective cloning vector DNAs were immobilized on filters. Hybridization was quantitated by autoradiography and microdensitometry. Solid lines represent the absorbance trace, and dotted lines delineate the integration limits for the individual peaks.

subsequent decrease in actin mRNA levels was preceded by a similar pattern of actin gene transcription. This type of transcriptional response has been observed previously for prolactin genes after EGF stimulation of cultured rat cells (22) and has been termed "burst-attenuation." The observed kinetics closely resemble those measured for the formation and subsequent degradation of the EGF-receptor complex (1, 3, 4).

Fig. 3 also suggested a differential effect of cycloheximide on actin and α -tubulin gene transcription. α -Tubulin gene transcription appeared to be reduced in cells treated for 4 hr with a combination of EGF and cycloheximide, while actin transcription appeared to remain at near maximal levels. These results suggested that the synergistic effect of cycloheximide on actin mRNA levels might be due to an inhibitory effect on transcriptional attenuation. Alternatively, EGF and cycloheximide could be separately regulating different cytoskeletal actin genes. The data of Figs. 1 and 2 do not allow a distinction between β - and γ -actin mRNA accumulation, because these two mRNAs comigrate in the denaturing gel system utilized (17) and the α coding sequence-derived cDNA probe does not distinguish between the various actin isotypes (16).

Isotype Specificity of EGF-Dependent Actin Gene Regulation. Recently, Ponte *et al.* (17) described the derivation of isotype-specific cDNA clones from the 3' untranslated regions of human α -skeletal, α -cardiac, β -cytoskeletal, and γ -cytoskeletal actin mRNAs. Moreover, the 3' untranslated sequences contained in these clones were shown to be highly conserved in both chickens and mice. We have used these clones to examine in greater detail the isotype specificity of EGF- and cycloheximide-induced actin mRNA accumulation.

Fig. 4 illustrates that both β - and γ - but not α -skeletal mRNA species are induced by both EGF and cycloheximide treatment. Moreover, the synergistic effect is clearly evident for both β and γ species, but it is especially pronounced for the γ species. One qualification is appropriate at this point. Recent studies have shown that several β - and γ -related sequences are present in the mouse genome (16, 17). Most of these appear to be pseudogenes, but the presence of more than one functional β and γ gene cannot be ruled out on the basis of existing data.

A comparison between lanes 3 and 5 of Fig. 4 also illustrates that α -amanitin applied to intact cells at the time of EGF stimulation diminishes but does not eliminate the induction of β and γ mRNA. Although we have previously shown that α -amanitin effectively inhibits mRNA synthesis in intact AKR-2B cells (2), the time required for effective uptake (1–2 hr) exceeds the time required for maximal stimulation of actin gene transcription (≤ 30 min). This may account for the lack of complete inhibition of actin mRNA accumulation. Alternatively, a contribution from post-transcriptional mechanisms cannot be ruled out.

The Synergistic Effect of EGF and Cycloheximide on Actin Gene Expression Is Specific and Observable at the Level of Transcription. Fig. 5 illustrates in greater detail a specific synergistic effect of EGF and cycloheximide on actin gene transcription in isolated nuclei. Quiescent cells were treated for the indicated times with either EGF, cycloheximide, or a combination of the two. Nuclei were isolated and used for run-off transcription assays as before. In agreement with the previous data, a 2- to 3-fold increase in actin gene transcription was observed by 30 min after EGF stimulation. A quantitatively similar increase was observed after cycloheximide treatment, although a considerably longer lag period was required (2–4 hr). Although these increases appear modest, they are specific because α -tubulin gene transcription in the same nuclei was again unstimulated. It is also quite possible that the maximum increase in EGF-induced actin gene transcription occurs earlier than 30 min and that the present data underestimate the magnitude of this response.

In contrast to the modest increases induced by EGF and cycloheximide separately, the combination of the two pro-

FIG. 4. Isotype specificity of EGF- and cycloheximide-induced actin mRNA accumulation. Quiescent cells in serum-free medium were treated for 4 hr with either cycloheximide (1 μ g/ml), EGF (10 ng/ml), EGF and cycloheximide, or EGF and α -amanitin (10 μ g/ml). All cultures also contained 500 ng of insulin per ml. Polysomal RNAs were prepared and analyzed for isotype-specific actin mRNA sequences by RNA blot hybridization using the indicated β -, γ -, and α -skeletal muscle-specific 3' untranslated region (3'-UT) probes described by Ponte *et al.* (17). Lane 1, no treatment; lane 2, cycloheximide alone; lane 3, EGF alone; lane 4, EGF and cycloheximide; lane 5, EGF and α -amanitin.

FIG. 5. Synergistic effect of EGF and cycloheximide (CH) on specific actin gene transcription in isolated nuclei. Quiescent AKR-2B cells in serum-free medium were treated for the indicated times with either EGF, cycloheximide, or a combination of the two. Insulin was not included. Nuclei were isolated and used for run-off transcription. The resulting 32 P-labeled RNA was hybridized to duplicate DNA dots containing 10 μ g of immobilized α -actin and α -tubulin cloned cDNA. Arrows indicate times of maximal stimulation.

duced a much larger transcriptional response (7- to 10-fold). This activity appeared maximal at 60 min, but remained elevated for at least 2 hr. Again, this synergistic effect was specific for actin genes, as evidenced by the complete lack of a stimulatory effect on α -tubulin gene transcription. Some variability in the duration of the cycloheximide effect was also observed, as the 4-hr levels of actin gene transcription in this particular series had returned to basal levels. This variability may result from the relatively low doses of cycloheximide used (1 μ g/ml). Nevertheless, these data demonstrate that the magnitude and the duration of actin gene transcription in nuclei isolated from EGF-stimulated cells are enhanced by simultaneous treatment with cycloheximide.

Pretreatment with Cycloheximide Does Not Diminish EGF-Stimulated Actin Gene Transcription. In an effort to determine whether the synergistic effect of EGF and cycloheximide is mediated through a common target molecule—i.e., a labile constitutively synthesized repressor of actin gene transcription—the effects of pretreatment with cycloheximide were examined. This experiment was predicated on the assumption that prolonged treatment with cycloheximide should deplete the intracellular levels of a labile repressor. If EGF binding simply results in the inactivation of this repressor, the ability of EGF to potentiate actin gene transcription should be diminished. The data shown in Fig. 6 illustrate that this is not the case. Although cycloheximide treatment resulted in increased actin gene transcription for at least 6–8 hr, the subsequent addition of EGF produced a strong synergistic effect within 30 min. Within the limits imposed by the time frame of this experiment, we conclude that prior treatment with cycloheximide does not diminish the ability of EGF to stimulate actin gene transcription.

DISCUSSION

Caution must be exercised in the interpretation of experiments involving the use of metabolic inhibitors. Often these inhibitors produce side effects that, if undetected, can lead to erroneous conclusions. Cycloheximide, in particular, is known to produce a nonspecific stabilizing effect on polyosomal RNA (24). However, this effect is relatively weak and is not known to encompass RNA species that are not associated with polyribosomes. While we cannot discount a nuclear artifact of cycloheximide action, this artifact would have to specifically stimulate actin and not tubulin gene transcription rates. We consider this highly unlikely but cannot formally eliminate the possibility.

These data offer strong, albeit circumstantial, evidence for the existence of a labile negative regulator of specific actin gene transcription. The exceedingly long half-lives of the major actin proteins make it unlikely that this negative tran-

scription factor is actin itself. A more likely candidate would be a separate DNA binding protein with high affinity for specific regulatory sequences. In this regard, it may be noteworthy that specific 5' genomic sequences have been recently shown to be required for the EGF-regulated expression of a rat prolactin-growth hormone fusion gene (25).

The apparent inability of cycloheximide pretreatment to diminish the synergistic effect of EGF on actin gene transcription argues that the inactivation of this putative negative transcription factor is not the mechanism by which EGF stimulates actin gene transcription in these cells. An alternative possibility is the induction of a transient positive acting factor that acts as an antagonist to the negative factor. The maximum permissible transcription rate could be primarily determined by the properties of the negative transcription factor and its interaction with DNA. This interpretation, although speculative, is consistent with the ability of cycloheximide to alter the relative EGF-induced amounts of β - and γ -actin mRNAs as illustrated in Fig. 4. Microdensitometry scans of these and similar data indicate that the ratio of β to γ mRNA is ≈ 5 in both quiescent and EGF-stimulated cells but is reduced to ≈ 2 in cells stimulated with both EGF and cycloheximide. This decrease is due to a preferential synergistic effect of cycloheximide on γ -actin mRNA accumulation and suggests that the steady-state ratio of β - to γ -actin

FIG. 6. Effects of cycloheximide (CH) pretreatment on EGF-induced actin gene transcription. Quiescent cells in serum-free medium were treated with 1 μ g of cycloheximide per ml for the indicated times. After 6 hr of cycloheximide treatment, a duplicate culture was stimulated for 30 min by the addition of 10 ng of EGF per ml. Nuclei were isolated and used for run-off transcription to determine the effects on actin (Ac) and α -tubulin (Tu) transcription rates.

may be largely determined by the negative control of gene transcription. This may have important biological significance, because the ratio of β - to γ -actin is a highly regulated parameter in a wide variety of mammalian cells and tissues. Reported values are independent of species and almost invariably range between 2 and 5 (18, 26, 27).

The demonstration that cytoskeletal actin genes are specifically regulated by EGF further implicates actin or its higher-ordered derivatives in the processes that regulate cell growth and development. β - and γ -actin isotypes are the principal components of microfilaments, structures believed to be involved in cellular morphogenesis, motility, and mitosis. Recent experimental evidence also suggests that microfilaments may play a necessary role in the initiation of DNA synthesis in response to EGF (28), and an altered microfilament organization has long been recognized in cells transformed by diverse biological and chemical agents (29, 30). The enhanced expression of cytoskeletal actin genes has been shown to be a phylogenetically conserved feature of late cleavage stage embryogenesis, occurring in echinoderms (31), amphibians (32), insects (33), and mammals (34). Thus, the regulated expression of these genes is an essential feature of early embryonic development. Studies by Farmer *et al.* (35) and Spiegelman and Farmer (36) have also shown that mammalian actin mRNA levels are modulated in response to changes in cell shape and during cell differentiation *in vitro*.

The recent demonstration that the oncogene of the Gardner-Rasheed feline sarcoma virus, *v-fgr*, is derived in part from a cytoskeletal actin gene (37) may add significance to the fact that actin genes, in similar fashion to the *c-myc* and *c-ras*^k proto-oncogenes (10, 38), are specifically regulated following peptide growth factor stimulation. In addition, *c-myc* and actin expression may share another common feature, the involvement of a negative regulator of gene expression. This possibility is suggested by the fact that the induction of *c-myc* mRNA by platelet-derived growth factor is also potentiated by cycloheximide and other inhibitors of protein synthesis (10). Whether this represents a truly common mechanism or simply a similar manifestation of unrelated effects remains to be established. Nevertheless, the accumulating examples of the association between proto-oncogenes and cell cycle regulation emphasize the importance of understanding the molecular mechanisms involved.

After submission of this manuscript, a paper by Ringold *et al.* (39) appeared that described a stimulatory effect of cycloheximide on the transcription of a transfected human β -interferon gene in Chinese hamster ovary cells. As controls, these authors also examined both β - and γ -actin gene transcription and α -tubulin gene transcription. In agreement with our results, they found that cycloheximide alone induced a 2.5-fold increase in actin gene transcription and a slight decrease in tubulin gene transcription.

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