Journal of Cellular Biochemistry 44:61–68 (1990)

Control of Expression and Maturation of Epidermal Growth Factor Receptor

Manjusri Das, Prabakaran Kesavan, and Jayant Khire

Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6059

Epidermal growth factor (EGF)-receptor is a 170 kDa transmembrane glycoprotein that contains a ligand-binding external domain, and a catalytically active cytoplasmic region. The expression of this receptor as well as a 100 kDa homolog that contains only the external domain is enhanced by the ligand EGF. EGF acts at transcriptional and post-transcriptional levels. To dissociate these pre-translational effects and the effects of EGF on receptor polypeptide synthesis from these on receptor export, pulse-chase experiments were conducted. These studies indicate that EGF stimulates post-translational transport and processing of the receptor, and this stimulation can occur in the absence of new protein synthesis. Other studies show that EGF accelerates at least two slow events in receptor maturation—the deoxynojirimycin sensitive processing in endoplasmic reticulum (ER) and the swainsonine sensitive processing in Golgi. The results suggest that EGF may influence one or more of the rate determining steps that control receptor export from ER, and this may represent yet another aspect of EGF action that contributes to the positive regulation of EGF-receptor protein expression.

Key words: transmembrane glycoprotein, 100 kDa homolog, polypeptide synthesis, receptor export, pulse chase experiments

The epidermal growth factor (EGF)-receptor belongs to a group of membrane-bound tyrosine kinases that includes as its members the *neu* protein and the receptors for insulin and insulin-like growth factors [1]. The external domains of these proteins contain two structural features—the cysteine-rich regions and the sites for N-linked glycosylation—that are important from the viewpoint of post-translational maturation and achievement of active conformation. It is known that N-linked glycosylation is obligatory for the acquisition of binding and kinase activities by these receptors [1]. In contrast, the PDGF-receptor, a member of the tyrosine-kinase family that lacks cysteine-rich regions, shows a different characteristic. In this case N-linked glycosylation occurs, but is not obligatory for the acquisition of binding or kinase functions [2].

Abbreviations used: EGF, epidermal growth factor; DME-medium, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serine albumin; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Received April 16, 1990; accepted May 30, 1990.

The studies described here were initiated with the objective of better understanding the regulation of biosynthetic maturation and export of EGF-receptor and closely related proteins using the isolated extracellular domain—a secreted 100 kDa EGF-receptor—as a model. This receptor appears to be identical to the external domain of the transmembrane EGF-receptor in its polypeptide structure, glycosylation state, and EGF-binding active conformation [3–6], but because it lacks a membrane anchor, it is unable to undergo endocytosis. Thus studies on the effect of EGF on its maturation are not complicated by any possibility of ligand-mediated down-regulation.

The studies described here were conducted with human A431 cells—a cell type that expresses both the kinase active (i.e., EGF-responsive) transmembrane 170 kDa receptor and the kinase domain deficient secreted 100 kDa homolog. The results indicate that EGF influences EGF-receptor expression at transcriptional, post-transcriptional, and post-translational levels. The post-translational effects occur independent of new protein synthesis and can be dissociated from the effects on receptor polypeptide synthesis.

METHODS

Northern Analysis

A431 cells in 6 cm dishes were treated as described in Figures 2 and 4. Cellular RNA was isolated using the guanidium isothiocyanate/cesium chloride procedure [7] and subjected to Northern analysis using ^{32}P-labelled EGF-receptor specific cDNA probe [4] or β_2 microglobulin probe [8] as described [9].

Assay of Transcription Activity

A431 cells grown in 15 cm dishes were treated as described in Figure 3 and then subjected to nuclear run-on analysis [10] using EGF-receptor cDNA [4] or β_2 microglobulin cDNA [8] as described [9].

Immunoprecipitation Analysis

^{35}S-labelled A431 cellular media were collected from 16 mm dishes as described in legends for Figures 5–7. Mouse monoclonal anti-EGF-receptor antibody (~ 6 μg) was pre-bound to 1 mg of IgGSorb (The Enzyme Center, MA) and then added to an aliquot of the labelled medium [11]. After stirring at 4°C for 1.5 h, the IgGSorb was washed and the bound radioactivity subjected to SDS-PAGE and fluorography as described [11].

RESULTS

Regulation of Expression of the Truncated Receptor Gene

In preliminary studies the amount of 100 kDa receptor in A431 cellular medium was quantified by Western blotting using an EGF-receptor specific rabbit antibody [12]. The results in Figure 1 show that EGF brings about an increase in the amount of immunoreactive receptor in the medium. To dissociate any effect of EGF on receptor export from its other possible effects on receptor mRNA and protein expression, Northern analyses were conducted to quantify the 2.6 kb mRNA that encodes for the 100 kDa EGF-receptor. The results in Figure 2 show that EGF increases the

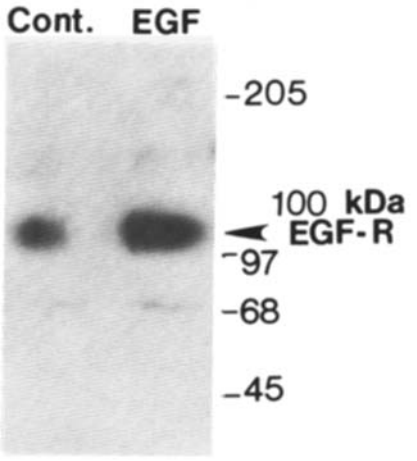
steady-state amount of the 2.6 kb receptor mRNA. EGF also increases the 5.6 kb and 9.5 kb mRNAs that encode for the intact 170 kDa EGF-receptor. To analyze the basis for this increase, we studied receptor gene transcription. The cDNA used in our studies is directed towards the region of transcript that specifies the entire externally located (N-terminal) domain of the EGF-receptor [4,9] and is thus capable of hybridizing with all species of transcripts of EGF-receptor gene in A431 cells. The results in Figure 3 show that EGF specifically increases the rate of synthesis of EGF-receptor gene transcripts by more than 2-fold. This increase occurs over the general increase in transcription that is induced by EGF. Next we tested whether EGF also stabilizes the synthesized mRNA. The results in Figure 4 show that in untreated cells, the half-lives of the 2.6 kb and 5.6 kb mRNAs are 2.6 h and 1.2 h, respectively. In the presence of EGF the half-life of the 5.6 kb mRNA is increased to 2.4 h, but there is no significant change in the half-life of the 2.6 kb mRNA. Overall the results in Figures 2–4 indicate that EGF induces an increase in the 2.6 kb mRNA that encodes for the 100 kDa receptor, but the increase is due to stimulation of receptor gene transcription and not due to stabilization of this mRNA entity.

EGF Stimulates Maturation and Export of the Truncated Receptor

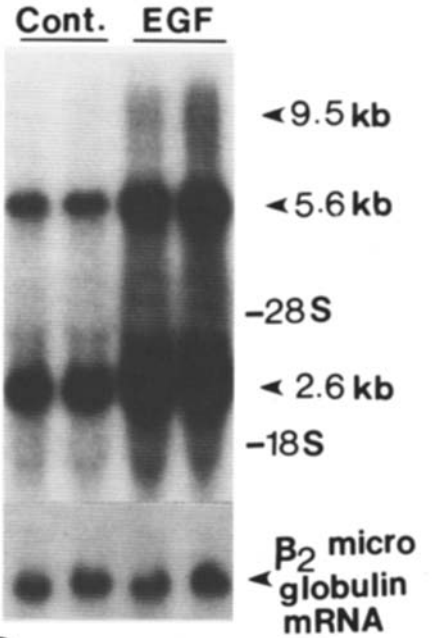
To dissociate the effects of EGF upon receptor mRNA and protein synthesis from its effects upon maturation/export, pulse-chase studies were conducted. Cells were labelled with ^{35}S-methionine for 1 h, and then chased with cycloheximide in the presence and absence of EGF (Fig. 5). The release of pre-synthesized immunoreactive receptor into the medium was slow ($t_{1/2} \sim 4$ h), but was enhanced in the presence of EGF ($t_{1/2} \sim 2$ h). The EGF-induced enhancement of release was specific and not seen for general pre-labelled proteins (data not shown) Cycloheximide, which had been added during chase to ensure release of only presynthesized receptors, had no effect per se on receptor release, i.e., the amount of labelled receptor released in the presence of cycloheximide was not different from that released in its absence (data not shown).

Our next objective was to test the effect of EGF on post-translational events. In preliminary studies we tested for receptor secretion in the presence of the following glycosylation inhibitors [13]: a) tunicamycin, an inhibitor of core N-linked glycosylation that occurs within endoplasmic reticulum (ER); b) deoxynojirimycin, an inhibitor of ER-located glucosidases; and c) swainsonine, an inhibitor of Golgi-located mannosidase II. The appearance of labelled receptor in the medium was inhibited when tunicamycin was added during cell labelling (data not shown), but not when it was added during chase (Fig. 6). Similar experiments with deoxynojirimycin showed that it inhibited labelled receptor export irrespective of whether it was present during labelling (data not shown) or during chase (Fig. 6). In contrast swainsonine did not affect receptor export, only the radioactive product released during cell labelling or chase was smaller than the fully processed glycoprotein (Fig. 6). Overall these results indicate that core glycosylation of the receptor occurs soon after its entry into the lumen of ER, but the subsequent modifications (including the deoxynojirimycin sensitive removal of glucose) which lead to its export from ER to Golgi are slow, and rate determining from the viewpoint of secretion.

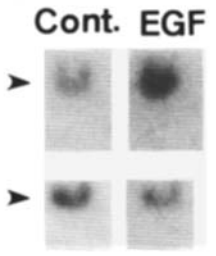
We tested whether EGF accelerates these rate-determining events. In the first experiment the deoxynojirimycin sensitive event was studied (Fig. 7). Cells were


Cont.
EGF
-205
100 kDa
EGF-R
-97
-68
-45

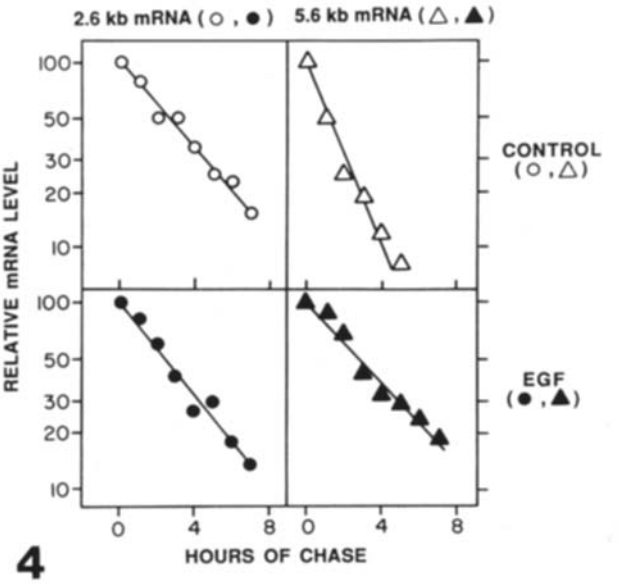
1


Cont.
EGF
9.5 kb
5.6 kb
-28S
2.6 kb
-18S
β2 micro globulin mRNA

2


Cont.
EGF

3


2.6 kb mRNA (○, ●)
5.6 kb mRNA (△, ▲)
RELATIVE mRNA LEVEL
100
50
30
20
10
CONTROL (○, △)
EGF (●, ▲)
0
4
8
HOURS OF CHASE

4

Figs. 1–4

labelled with ^{35}S-methionine, then chased with unlabelled medium containing EGF or no EGF with deoxynojirimycin added at various time points during the chase. Since receptors that do not undergo the deoxynojirimycin sensitive processing are not exported, the experiment outlined in Figure 7 provides a means for studying the time-course of this processing. The results in Figure 7 show that this step is slow, requiring about 3 h for completion. EGF accelerates it by 3–4-fold.

Similar analysis was conducted for the swainsonine sensitive event. As mentioned earlier, swainsonine does not prevent secretion, but blocks the conversion of the immature small receptor form to the mature fully formed 100 kDa receptor (see Fig. 6). We find that the event occurs during 1 and 4 h of chase and is completed by about 4 h; however, in the presence of EGF, the process is accelerated and completed within 2 h of chase (data not shown).

DISCUSSION

In earlier studies conducted with KB cells, an EGF-induced increase in the mRNA for intact EGF-receptor was found to be due to stabilization of the mRNA [14]. In other studies conducted with NRK cells, no such stabilization was seen; rather, the EGF-induced increase in intact receptor mRNA occurred as a consequence of a 2-fold increase in receptor gene transcription [15]. Our studies with A431 cells suggest that the intact receptor mRNA is controlled at levels of both synthesis and stability; however, the truncated receptor mRNA (whose intrinsic stability is higher than that of the intact mRNA) is controlled only at the level of synthesis. The sequence of this smaller RNA is identical (up to nucleotide 2079) to that of the normal intact EGF-receptor mRNA, but it diverges downstream in the 3′ region, and lacks the regions encoding for transmembrane spanning and cytoplasmic kinase domains [4]. It

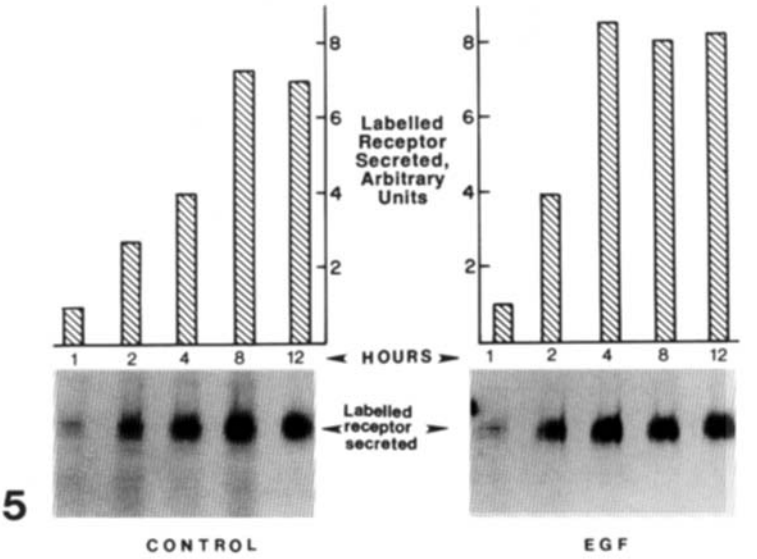
---

Fig. 1. EGF increases the steady-state level of 100 kDa receptor in the medium. A431 cells in 6 cm dishes were washed with serum-free DME-medium and then incubated at 37°C for 6 h with 3 ml of the same medium containing 20 nM EGF, or no addition (Cont.). At the end of incubation, 2.5 ml aliquot of the medium was added to 0.3 ml of 100% trichloroacetic acid and 20 μl of 10 mg/ml insulin (as carrier protein). The precipitated proteins were subjected to Western blotting using an EGF-receptor specific rabbit polyclonal antibody and ^{125}I-labelled protein A as described [12].

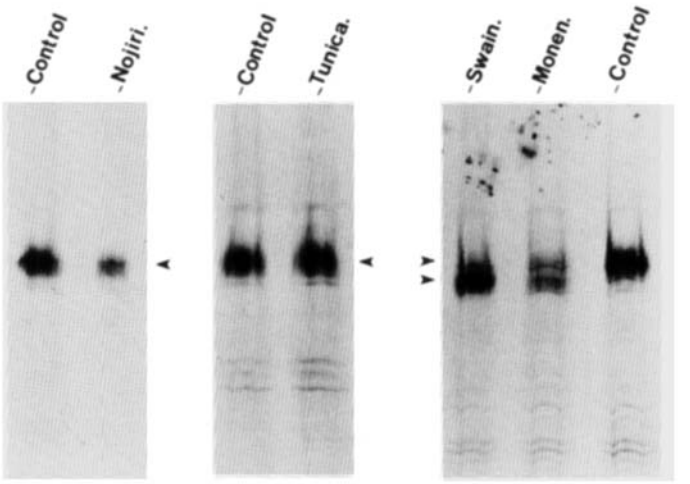
Fig. 2. EGF-induced increase in the 2.6 kb mRNA that encodes for the 100 kDa EGF-receptor. A431 cells were treated at 37°C for 4 h with 20 nM EGF or no EGF (Cont.) in DME-medium containing 1% FBS and then subjected to Northern analysis using EGF-receptor cDNA (**top panel**) or β_2 microglobulin cDNA (**bottom panel**). The arrow-marked bands at 2.6 kb, 5.6 kb, and 9.5 kb represent the three different forms of EGF-receptor mRNA.

Fig. 3. EGF-receptor gene transcription. A431 cells were treated at 37°C for 4 h with 20 nM EGF or no EGF (Cont.) in DME-1% FBS medium. Nuclear run-on assays were conducted as described in Methods, using EGF-receptor specific cDNA (**top panel**) or β_2 microglobulin cDNA (**bottom panel**).

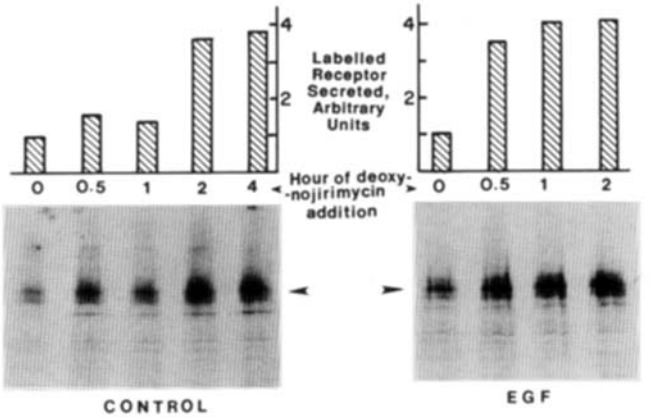
Fig. 4. The 2.6 kb EGF-receptor mRNA is not stabilized by EGF. A431 cells were incubated at 37°C with or without 20 nM EGF in DME-1% FBS medium. Gene transcription was blocked after 2 h by the addition of actinomycin D (10 μg/ml). Cellular RNA was collected at various time points after this addition and subjected to Northern blot analysis. The 2.6 kb and 5.6 kb EGF-receptor mRNAs were quantified by densitometry after appropriate autoradiographic exposure periods. Semi-log plots of the densitometric data are depicted.


Labelled Receptor Secreted, Arbitrary Units
HOURS
Labelled receptor secreted
CONTROL
EGF

5


-Control
-Nojiri.
-Control
-Tunica.
-Swain.
-Monen.
-Control

6


Labelled Receptor Secreted, Arbitrary Units
Hour of deoxy-nojirimycin addition
CONTROL
EGF

7

Figs. 5–7

remains to be seen how these structural divergencies are responsible for the differences in stability characteristics.

Previous studies on glycosylation of the intact 170 kDa EGF receptor had indicated that both tunicamycin and deoxynojirimycin inhibit acquisition of binding and kinase activities in parallel with their effects on the exit of the protein from the ER [1]. Thus core glycosylation and the removal of glucose are essential for the acquisition of proper conformation. The results described here for the soluble receptor (in Fig. 6) suggest that the folding pathway of the isolated external domain may not differ much from that of the multi-domained intact receptor, despite the fact that the intact receptor is membrane-anchored and the other (the 100 kDa form) is free-floating in the lumen of ER.

In the present study the isolated external domain was used as a model to examine the effects of EGF upon receptor maturation and export. The results obtained here can be summarized as follows: a) Treatment with EGF results in an increase in the steady-state level of 100 kDa receptor in the medium. b) Pulse-chase studies show that EGF exerts a post-translational stimulatory effect on receptor maturation and export that occurs independent of any effect on receptor polypeptide synthesis. c) EGF accelerates the slow deoxynojirimycin sensitive processing of receptors within ER, as well as a subsequent event within golgi that is sensitive to swainsonine. d) Thus EGF appears to stimulate a rate-determining step that occurs in

---

Fig. 5. Effect of EGF on the time-course of secretion of pre-synthesized 100 kDa receptor. A431 cells in 16 mm dishes were washed with 4 μM methionine containing 2% dialized FBS and ^{35}S-methionine (100 μCi/ml). Following this incubation, the cells were washed with unlabelled DME-medium and chased at 37°C for the indicated times with 20 nM EGF or no EGF (control) in 0.3 ml of DME-0.1% BSA medium. Cycloheximide (10 μg/ml) was present during the chase. Cellular media were subjected to immunoprecipitation as described in Methods. Radiolabelled receptor was quantified by densitometric analysis of the autoradiograms. **Bottom panel:** Autoradiographic depiction of results. **Top panel:** Plot of the densitometric data. The results shown here are representative of at least three separate experiments. The results of two additional experiments are as follows (the amounts of labelled receptor secreted are given in arbitrary units as in the figure). Experiment 1: 1 h (cont.), 1; 1 h (EGF), 1; 2 h (cont.), 2.1; 2 h (EGF), 4; 4 h (cont.), 3.5; 4 h (EGF), 7.5; 8 h (cont.), 6.4; 8 h (EGF), 8; 12 h (cont.), 7.1; 12 h (EGF), 8.2. Experiment 2: 1 h (cont.), 1; 1 h (EGF), 1; 2 h (cont.), 2.5; 2 h (EGF), 3.4; 4 h (cont.), 3.1; 4 h (EGF), 8.2; 8 h (cont.), 7.8; 8 h (EGF), 8.5; 12 h (cont.), 7.1; 12 h (EGF), 8.

Fig. 6. Effect of glycosylation inhibitors on the secretion of pre-synthesized 100 kDa receptors. Cells in 16 mm dishes were labelled with ^{35}S-methionine for 4 h, then washed with unlabelled DME-medium and incubated at 37°C for 11 h in 0.3 ml of DME-0.1% BSA medium containing 4 mM deoxynojirimycin (Nojiri.), 1 μg/ml tunicamycin (Tunica.), 1.5 μg/ml swainsonine (Swain.), 10 μM monensin (Monen.) or no addition (control). Cellular media were subjected to immunoprecipitation as described in Methods.

Fig. 7. EGF stimulates the deoxynojirimycin sensitive processing step in biosynthetic transport of the EGF-receptor. Cells in 16 mm dishes were labelled with ^{35}S-methionine for 2 h, then washed with unlabelled DME-medium and incubated at 37°C for 12 h with 20 nM EGF or no EGF (Control) in 0.3 ml of DME-0.1% BSA. Deoxynojirimycin (4 mM) was added at the indicated time points of chase. All cellular media were collected at 12 h and subjected to immunoprecipitation as described in Methods. **Bottom panel:** Autoradiographic depiction of results. **Top panel:** Plot of the densitometric data. The results here are representative of at least three separate experiments. The results of two additional experiments are as follows (the amounts of labelled receptor secreted are given in arbitrary units as in the figure). Experiment 1: 0 h (cont.), 1; 0 h (EGF), 1; 0.5 h (cont.), 0.9; 0.5 h (EGF), 2.8; 1 h (cont.), 1.7; 1 h (EGF), 3.5; 2 h (cont.), 3.1; 2 h (EGF), 4; 4 h (cont.), 3.5. Experiment 2: 0 h (cont.), 1; 0 h (EGF), 1; 0.5 h (cont.), 1.2; 0.5 h (EGF), 3.1; 1 h (cont.), 1.8; 1 h (EGF), 4.2; 2 h (cont.), 3.6; 2 h (EGF), 3.8; 4 h (cont.), 3.8.

ER after the tunicamycin sensitive acquisition of core oligosaccharides but perhaps before the occurrence of the deoxynojirimycin sensitive modification.

The studies described here demonstrate that in addition to its effects on receptor mRNA synthesis and stability EGF exerts receptor regulation at yet another level—the level of post-translational folding, maturation, and export. With respect to the pathway of EGF action, we considered the possibility of C-kinase which is known to be involved in EGF-induced receptor expression at pre-translational levels [14,16]. However, this kinase appears to have no direct involvement in post-translational receptor expression as seen by the inability of TPA to modulate this process (unpublished data). On the other hand, the lack of inhibition by cycloheximide indicates that the EGF effect requires no new protein synthesis. Thus it remains to be seen what EGF-induced cellular signalling pathways are involved in this receptor expression and export system.

ACKNOWLEDGMENTS

We thank Hae Kyeung Park and Jia Li for assistance with cell culture. This work was supported by NIH grants CA-43787 and CA-15822 (to M.D.).

REFERENCES

1. Carpenter G: Annu Rev Biochem 56:881–914, 1987.
2. Keating MT, Harryman CC, Williams LT: J Biol Chem 264:9129–9132, 1989.
3. Weber W, Gill GN, Spiess J: Science 224:294–297, 1984.
4. Merlino GT, Ishii S, Whang-Peng J, Knusten T, Xu YH, Clark AJL, Stratton RA, Wilson RK, Ma DP, Roe BA, Hunts JH, Shimizu N, Pastan I: Mol Cell Biol 5:1722–1734, 1985.
5. Basu A, Raghuanth M, Bishayee S, Das M: Mol Cell Biol 9:671–677, 1989.
6. Basu M, Sen-Majumdar A, Basu M, Murthy U, Das M: J Biol Chem 262:12879–12882, 1986.
7. Maniatis T, Fritsch EF, Sambrook J: "Molecular Cloning: A Laboratory Manual." New York: Cold Spring Harbor Laboratories, 1982.
8. Suggs SV, Wallace RB, Hirose T, Kawashima EH, Itakura K: Proc Natl Acad Sci USA 78:6613–6617, 1981.
9. Kesavan P, Das P, Kern J, Das M: Oncogene 5:483–488, 1990.
10. Greenburg ME, Ziff EB: Nature 311:433–438, 1984.
11. Depalo L, Das M: Cancer Res 48:1105–1109, 1988.
12. Murthy U, Basu M, Sen-Majumder M, Das M: J Cell Biol 103:333–342, 1986.
13. Elbein AD: Annu Rev Biochem 56:497–534, 1987.
14. Jinno Y, Merlino GT, Pastan I: Nucleic Acids Res 16:4957–4966, 1988.
15. Thompson JL, Rosner MR: J Biol Chem 264:3230–3234, 1989.
16. Bjorge D Jr, Paterson AJ, Kudlow JE: J Biol Chem 264:4021–4027, 1989.