Regulation of epidermal growth factor expression in mammary epithelial cells by a Yin-Yang-1-like element

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ABSTRACT

The hormone combination of insulin, dexamethasone and prolactin induced accumulation of preproepidermal growth factor (EGF) mRNA in HC11 mouse mammary epithelial cells 16–24 h after the hormones were added to the cultures. Individual hormones or combinations of two of the hormones had no effect on EGF mRNA concentrations. The same hormone combination was capable of inducing expression of a reporter gene construct containing –888 to +25 bp of the EGF gene fused with luciferase. Deletions of the promoter between –888 and –271 bp had no detectable effect on basal or hormone-induced reporter gene expression. However, further deletion from –270 to –74 bp increased baseline to approximately

equal hormone-induced reporter gene expression. This deletion also abolished the hormone-induced increase in reporter gene expression. Sequence analysis suggested that this region contained a binding site for Yin-Yang-1 (YY1), which was confirmed by gelshift analysis. Mutation of the YY1 binding site increased baseline reporter gene expression to the same level as induced by insulin, dexamethasone and prolactin in the wild-type promoter. These results indicate that expression of the EGF gene in mammary epithelium is repressed by the YY1 site, and that removal of repression may play a part in regulating EGF gene expression in lactating mammary tissue.

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INTRODUCTION

Epidermal growth factor (EGF) is a 53 amino acid peptide present in a variety of tissues and fluids, including milk (Carpenter 1980, Beardmore & Richards 1983, Mroczkowski & Reich 1993). It is synthesized as a 1217 amino acid glycoprotein (preproEGF), which contains the authentic EGF peptide and seven other EGF-like repeats (Gray *et al.* 1983, Scott *et al.* 1983). PreproEGF is processed via kallikrein-like enzymes to mature EGF and possibly a variety of other molecules also (Jahnke *et al.* 1994). In the lactating mammary gland, EGF appears to be apically localized (Brown *et al.* 1989).

EGF mRNA has been detected in mammary tissue (Brown *et al.* 1989) and appears to be more abundant in lactating tissue. EGF mRNA appears to localize to epithelial cells in the mammary gland (Snedeker *et al.* 1991). The lactogenic hormones insulin, hydrocortisone and prolactin are capable of inducing EGF mRNA accumulation in mouse mammary organ cultures (Fenton & Sheffield 1991).

Furthermore, EGF mRNA has been reported in mammary epithelial cell lines and found to be increased by prolactin (Fenton & Sheffield 1994). *In vivo*, insulin has been shown to regulate the EGF content of rat milk (Thulesen *et al.* 1993). These relationships appear different than those in the salivary gland and kidney (both of which are major sites of EGF expression; Fisher & Lakshmanan 1990), in which androgens and thyroid hormones are major regulators of EGF expression (Gubits *et al.* 1986, Kasayama *et al.* 1989, Pascall *et al.* 1989).

Pascall & Brown (1988) cloned the 5' flanking region of EGF and have subsequently shown that a 5.5 kb fragment of the 5' flank resulted in tissue-specific and hormone-responsive expression of the SV40 T antigen in transgenic mice (Pascall *et al.* 1994). Price *et al.* (1995) found that a 1010 bp fragment of the rat EGF promoter responded to renal ischemia in a manner similar to that of the endogenous EGF gene. Fenton *et al.* (1996) reported the ability of an approximately 2 kb clone of the EGF 5' flank to drive EGF expression in cell

lines. None of these studies have yet identified the specific DNA sequences responsible for hormonal regulation of EGF transcription. Also, little is known about the regulation of EGF production by mammary tissue at a molecular level. Therefore, the objective of the present study was to define further the molecular control of EGF expression in mammary epithelium.

MATERIALS AND METHODS

Cell culture and treatment

HC11 mouse mammary epithelial cells (Ball *et al.* 1988) were obtained via Dr J Rosen with permission of the line's originator, Dr B Groner (Institute for Experimental Cancer, Freiburg, Germany). Cells were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum, 5 µg/ml insulin and 10 ng/ml EGF (growth medium). Culture was at 37 °C in a humidified atmosphere of 95% air–5% CO₂. For lactogenic hormone treatment, cells were allowed to grow to confluence and remain confluent for 1 day, after which the medium was changed to RPMI 1640 supplemented with 10% gelding serum, 1 µg/ml insulin, 0.1 µM dexamethasone and 100 ng/ml ovine prolactin. Standard lactogenic treatment was for 24 h. However, for specific studies, other times and other hormone combinations were also used.

mRNA determination

To prepare an EGF RNA probe, a 361 bp DNA fragment corresponding to 3489–3850 bp of pre-proEGF cDNA was subcloned from pmegf1 (ATCC, Rockville, MD, USA) into pGEM-7Zf (Promega, Madison, WI, USA). The plasmid was then grown in *E. coli* DH5α and purified using Clear-Cut mini-prep kit (Stratagene, La Jolla, CA, USA). The resulting plasmid was linearized with XbaI and digitonin-labeled RNA synthesized from the SP6 promoter to give an antisense RNA. The reaction mixture was 20 µl and contained 1 µg linearized plasmid, 1 mM each ATP, CTP and GTP, 0.65 mM UTP, 0.35 mM digitonin-UTP, 40 mM Tris, pH 8.0, 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 2 mM spermidine, 10 mM NaCl, 0.05 U RNase inhibitor and 40 U SP6 RNA polymerase. Reactions were continued at 37 °C for 2 h, followed by addition of DNase I for 15 min and final addition of 2 µl of a 200 mM EDTA solution.

Total cytoplasmic RNA was isolated from HC11 cells grown in 60 mm dishes (approximately 5 × 10⁶ cells per dish) as previously described (Chomczynski & Sacchi 1987). RNA (10 µg) was separated

on a 1% agarose gel containing 3% formaldehyde and transferred to nylon membranes (Boehringer Mannheim Co., Indianapolis, IN, USA) by capillary transfer in 10 × SSC (1.5 M NaCl, 150 mM sodium citrate). Membranes were u.v. crosslinked and probed using the digitonin-labeled RNA probe and the Genius detection system (Boehringer Mannheim). Briefly, blots were incubated for 2 h at 68 °C in hybridization buffer containing 50% formamide, 5 × SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 2% blocking reagent (Boehringer Mannheim). Digitonin-labeled EGF probe (40 ng/ml) was added and hybridization continued for 18 h. The membrane was washed twice for 5 min each with 2 × SSC containing 0.1% SDS at room temperature and twice for 15 min each with 0.5% SSC containing 0.1% SDS at 72 °C. The membrane was then incubated with alkaline phosphatase conjugated anti-digitonin, washed twice and detection of reactive bands performed by chemiluminescence (CDP-Star, Boehrer Mannheim). Computer assisted densitometry was used to determine relative amounts of EGF mRNA, which was adjusted for equal amounts of 18S rRNA, determined by ethidium bromide fluorescence (Collage, Fotodyne, New Berlin, WI, USA).

Reporter gene constructs

Various lengths of the EGF 5' flank were subcloned from p5'EGF (Pascall & Brown 1988) by PCR using primers with HindIII and XhoI tails to facilitate cloning. These fragments were subcloned into the HindIII–XhoI site of pGL2basic (Promega) and amplified in *E. coli* DH5α. In addition, pGL2 basic with no promoter was used as a negative control and pGL2Control (Promega), which contains luciferase driven by an SV40 promoter/enhancer sequence, was used as a positive control.

To prepare mutations in the Yin-Yang-1 (YY1) site in the EGF gene, the wild-type construct consisting of –238 to +25 bp was prepared as described above. A 2 bp mutation (the wild AACCAT was mutated to AACTAGT) was inserted by polymerase chain reaction using a mutagenic 5' primer (Kadowaki *et al.* 1989).

Transfection studies

HC11 cells were grown as described above. Approximately 10⁷ cells were suspended in 0.5 ml Hanks's balanced salt solution (HBSS), together with 10 µg purified plasmid DNA in an electroporation cuvet with 2 mm electrode space. Cells were transfected by electroporation with a 2 kV/cm field strength, incubated on ice in HBSS for

5 min and plated in culture media at a density of $5 \times 10^5/\text{cm}^2$. Cells were attached and grown for 24 h in growth medium, then subjected to lactogenic hormone treatments as described above. Luciferase expression was assessed using a commercial luciferase assay system (Promega).

Gelshift analysis

Nuclear extracts of control or lactogenic hormone-treated HC11 cells were prepared as previously described (Ye *et al.* 1994) and protein determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Double-stranded DNA sequences corresponding to -250 to -215 bp of the EGF 5' flank sequence were synthesized (Gibco/BRL, Gaithersburg, MD, USA), labeled with polynucleotide kinase (Promega) and incubated at room temperature with nuclear extracts in 20 μl of an assay mixture consisting of 6 μg nuclear extract protein, 1 μg poly d(I-C), 3 μg BSA, 4% Ficoll, 20 mM Tris, pH 7.4, 60 mM KCl, 2 mM EDTA, 0.5 mM DTT and 10^6 d.p.m. of ^{32}P -labeled oligonucleotide probe. After 25 min, samples were separated by 6% PAGE electrophoresis and X-ray film (Fuji Photofilm Co., Japan) was exposed to the gels. In addition, oligonucleotides containing a 2 bp mutation (AACCATT sequence mutated to AACTAGT) were used as negative controls. Also, samples were incubated with anti-YY1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) before gelshift, to verify the identity of YY1.

Statistical analysis

Data were analyzed by analysis of variance as a randomized complete block design (replicate=block). Means were compared with controls by Dunnett's test with the significance level set at $P < 0.05$ (Steel & Torrie 1980).

RESULTS

Initial studies, in which cells were treated with combinations of insulin, dexamethasone and prolactin, revealed that only the combination of all three hormones was capable of increasing preproEGF mRNA accumulation in HC11 cells. In the absence of all three hormones, very little preproEGF mRNA was detected (Fig. 1). Time-course studies indicated that there was no detectable change in preproEGF mRNA 12 h after the hormones were added. PreproEGF mRNA content of cells was near maximum 16 h after the hormone combination was added and remained increased after 24 h (Fig. 1).

FIGURE 1. Accumulation of EGF mRNA in HC11 cells after treatment with combinations of insulin (I, 1 $\mu\text{g}/\text{ml}$), dexamethasone (D, 0.1 μM) and prolactin (P, 100 ng/ml) for various times. Top: representative northern blot and ethidium bromide-stained RNA gel showing EGF mRNA 24 h after the addition of treatments. Graph: mean densitometry estimates of EGF mRNA expression, relative to 0 h control. $n=3$. S.E.M. averaged 12% and is not shown, for simplicity. *Significantly greater than 0 h control, $P < 0.05$.

Initial dose-response studies (not shown) indicated that 100 ng/ml prolactin gave the greatest response. A lower concentration (10 ng/ml in the presence of dexamethasone and insulin) resulted in little stimulation of preproEGF mRNA accumulation, and 1 $\mu\text{g}/\text{ml}$ resulted in no greater a response than was obtained with 100 ng/ml. An insulin concentration of 100 ng/ml was ineffective in supporting EGF production in the presence of 100 ng/ml prolactin and 0.1 μM dexamethasone, but 1 or 10 $\mu\text{g}/\text{ml}$ gave maximum induction of preproEGF mRNA. Dexamethasone concentrations of either 0.1 or 1 μM resulted in lower EGF mRNA accumulation in the presence of 100 ng/ml prolactin and 1 $\mu\text{g}/\text{ml}$ insulin than was achieved with 0.1 μM dexamethasone.

A DNA sequence consisting of -888 to +25 bp of the EGF 5' flanking region was also hormonally responsive upon transient transfection into HC11 cells (Fig. 2). Plasmids lacking the promoter region did not express detectable luciferase (not shown).

FIGURE 2. Luciferase activity in HC11 cells transiently transfected with luciferase driven by -888 to $+25$ bp of the EGF gene and treated with combinations of insulin (I, $1 \mu\text{g/ml}$), dexamethasone (D, $0.1 \mu\text{M}$) and prolactin (P, 100 ng/ml) for various times. Mean luciferase activity, relative to 0 h control. $n=3$. S.E.M. averaged 9% , and is not shown, for simplicity. *Significantly greater than 0 h control, $P<0.05$.

Luciferase expression driven by an SV40 promoter and enhancer was readily detected (about three- to fivefold that of the baseline of the EGF promoter), but was not affected by hormone treatment (not shown). The hormone requirement for induction of luciferase expression was similar to that of the endogenous gene, in that all three hormones were required for maximum induction of luciferase expression. The time course of induction of expression of luciferase by insulin, dexamethasone and prolactin was similar to that observed for the endogenous EGF gene.

Deletion analysis (Fig. 3) indicated that deletions between -888 and -271 bp had no effect on either baseline or hormone-induced reporter gene expression. However, when the region between -270 and -74 bp was further deleted, a major change in reporter expression was observed. Baseline expression of luciferase driven by the $-73/+25$ bp promoter construct was increased over that observed with the baseline of the $-270/+25$ bp construct, and was equivalent to the hormone-induced levels observed with longer constructs. The combination of insulin, dexamethasone and prolactin did not affect expression of luciferase driven by -73 to $+25$ bp of the EGF gene.

The sequence of the -270 to -74 region of the EGF gene indicated the presence of a likely binding site for YY1 (Fig. 4). Gelshift analysis (Fig. 5) indicated that this site was indeed capable of binding YY1. Nuclear extracts of HC11 cells were capable of inducing a strong gelshift, which was

FIGURE 3. Luciferase activity in HC11 cells transiently transfected with luciferase driven by indicated fragments of the 5' flank of the EGF gene and treated with no hormones (solid columns) or with insulin ($1 \mu\text{g/ml}$), dexamethasone ($0.1 \mu\text{M}$) and prolactin (100 ng/ml) (shaded columns) for 24 h . Mean luciferase activity, relative to no hormone, $-888/+25$ promoter $\pm$ S.E.M. of densitometry estimates of EGF mRNA expression. $n=3$. *Significantly greater than the no hormone, $-888/+25$ promoter, $P<0.05$.

inhibited by anti-YY1 or excess unlabeled DNA, but not by an oligonucleotide containing a 2 bp mutation. Anti-YY1 blocked the gelshift induced by nuclear proteins from HC11 cells. Interestingly, little difference in YY1-DNA interaction was observed between control and hormone-treated nuclear extracts. In addition to the YY1 complex (identified by antibody inhibition studies), these studies also demonstrated two additional mobility shifts. One, of less mobility than the YY1 complex, was inhibited by 50-fold excess unlabeled oligonucleotide, but not by anti-YY1, and appeared to be independent of hormone treatment. The second complex, of greater mobility (lower on gel) than the YY1 complex, was inhibited by excess wild-type oligonucleotide, but not by excess mutated

FIGURE 4. Sequence analysis showing the presence of a YY1-like element in the 5' flank of the EGF gene. Sequence as reported by Pascall & Brown (1988) and Fenton *et al.* (1996). Wild sequence is the actual DNA sequence, with solid underline indicating the putative YY1 binding site and dashed underline indicating cryptic glucocorticoid receptor binding half-sites. Mutated sequence indicates the mutation induced in the putative YY1 binding site in subsequent gelshift and reporter gene analysis (mutated bases indicated by outline).

FIGURE 5. Gelshift analysis showing YY1 binding to the putative YY1 element in the EGF gene. Nuclear extracts from control (C) and hormone-treated (T) HC11 cells were prepared and gelshifts performed as described in Materials and Methods, using labeled wild-type oligonucleotides as a probe. In some studies, anti-YY1 was also added. In other studies, 50-fold excess unlabeled wild type oligonucleotide (W) or oligonucleotides mutated to remove YY1 binding (M) were also added. Representatives of three experiments.

oligonucleotide, suggesting that the complex may involve the YY1 sequence or an overlapping sequence. However, formation of this complex was not inhibited by anti-YY1. Interestingly, the formation of this complex appeared to be inhibited in hormone-treated cells.

In order to test more definitively whether or not the YY1 binding site has a role in regulating EGF expression, the YY1 site was mutated using the same 2 bp mutation that had been shown in gelshift analysis not to interact with YY1. The wild-type construct consisting of -238 to +25 bp of the EGF gene exhibited hormone-responsive expression similar to the -888 to +25 bp construct in previous studies. Mutation of the YY1 site resulted in a reporter gene expression pattern similar to the -73 to +25 bp construct: the baseline was increased to approximately equal that of the hormone-induced wild-type construct, and hormone responsiveness was abolished (Fig. 6).

DISCUSSION

Results of these studies indicate that HC11 cells express EGF, and that EGF expression in this cell line is responsive to the lactogenic complex of insulin, dexamethasone and prolactin. These results are similar to those from previous reports showing

that a similar hormone mixture induced EGF mRNA accumulation in mammary organ cultures (Fenton & Sheffield 1991). Furthermore, these results suggest that this expression is at least partly under the control of 5' regulatory sequences in the EGF gene. Previously, Fenton *et al.* (1996) observed that 5' flanking sequences of EGF could drive reporter gene expression in various cell lines. They also reported that the effect of deletion of the EGF 5' flank was highly variable among cell lines, suggesting differential mechanisms of regulation in diverse cells. This observation is consistent with other studies showing substantial variation in the hormonal regulation of EGF expression among tissues (Gubits *et al.* 1986, Pascall *et al.* 1989).

The pattern of EGF expression in HC11 cells was reminiscent of milk protein expression, at least in terms of the hormonal requirements. The combination of insulin, a glucocorticoid and prolactin are well established as minimum requirements for milk protein synthesis (Topper & Freeman 1980). However, the time course of EGF expression (both the endogenous gene and the reporter construct) was somewhat longer than that usually reported for milk proteins such as β -casein. Whether this represents a fundamental difference in the signaling pathways used in the induction of β -casein and EGF genes is unclear. However, the fact that the 5' flank of EGF does not appear to contain any

FIGURE 6. Effect of mutation of the putative YY1 site in the EGF gene on luciferase activity in HC11 cells transfected with wild-type (solid columns) or mutated (shaded columns) constructs and treated with no hormones (C) or insulin, dexamethasone and prolactin (DIP) as described in Materials and Methods. Mean $\pm$ S.E.M. of three experiments. *Significantly greater than wild-type transfection, no hormone treatment, $P < 0.05$.

element resembling a STAT5 binding sequence suggests that the genes may be regulated by different mechanisms. STAT5 phosphorylation and DNA binding (in response to JAK2 activation by prolactin receptor) is believed to be a critical signal by which prolactin activates β -casein transcription (Horseman & Yu-Lee 1994, Hynes *et al.* 1997). The findings of this study would suggest that STAT5 does not directly alter EGF transcription. However, the results do not rule out the possibility of a STAT5-responsive gene being involved in regulating EGF transcription.

The present studies indicate that a YY1 binding site in the 5' flank of the EGF gene is critical for hormonal regulation of the gene, at least in the context of the mammary gland. YY1 (Yin-Yang-1, also known as δ factor or NF-E1) is a multifunctional nuclear factor that acts as a transcription initiator or promotion factor for several genes, including immunoglobulin heavy chain, *c-myc*, rpl30, rpl32 and dihydrofolate reductase (Shrivastava & Calame 1994). However, YY1 has also been shown to inhibit transcription of other genes, including *c-fos*, immunoglobulin κ chain, α -actin, ϵ -globulin, serum amyloid and β -casein (Shrivastava & Calame 1994). The finding that YY1 may play a part in regulating EGF expression in the mammary gland shows an obvious parallel with the β -casein gene, in which a YY1 site has also been proposed (Meier & Groner 1994). Unlike others who found that in the β -casein gene there is a STAT5 binding site present adjacent to the

YY1 site that may remove YY1 inhibition by competition (Meier & Groner 1994), we have not found a definitive binding site for another transcription factor in such close proximity to the YY1 site. However, this lack of identification of a positive regulator does not prove that it is absent.

In addition to prolactin, the expression of EGF, in common with that of β -casein, appeared to require the presence of glucocorticoids. The β -casein gene appears to respond to glucocorticoids through a well-recognized glucocorticoid response element (Doppler *et al.* 1989). No consensus glucocorticoid response element was identified on the EGF 5' flank, but possible cryptic half-sites (GGTCCC beginning at -244 and TGTTC, beginning at -225) were present flanking the YY1 sequence. Although we have not been able definitively to show glucocorticoid receptor binding to these sites, the possibility of their involvement in regulating YY1 interaction with the EGF 5' flank remains.

Although the mutagenesis studies indicated that the YY1 site on the EGF 5' flanking sequence (or a binding site overlapping the YY1 site) has a critical role in regulating EGF transcription, the exact identity of the protein interacting with the sequence is equivocal. Our results indicate that YY1 is capable of interacting with the site, but an additional protein, probably not YY1, also appears to interact with the sequence, or an overlapping sequence. Gelshift and western blot analysis indicates that YY1 content and DNA binding activity are unaffected by hormone treatment, as was observed in HC11 cells by Meier & Groner (1994). Raught *et al.* (1994) observed increased YY1-DNA interaction during lactation *in vivo*. However, comparisons of these results should be made with caution. HC11 cells show heterogeneity in their accumulation of β -casein in response to lactogenic hormones, with only a relatively small percentage expressing immunologically detectable β -casein 24 h after hormone treatment (Ball *et al.* 1988). Thus, it is possible that YY1 changes in a relatively small subset of HC11 cells that account for the hormone responsiveness, and that this change might not be detected by the methods used. In addition, the gelshift analysis from these experiments suggests the possibility that a factor other than YY1, which appears to be decreased in content or DNA binding activity in response to insulin, glucocorticoids and prolactin, can bind at or near the YY1 binding site.

The observation of a factor apparently distinct from YY1 that appears to interact at or near the YY1 site may indicate an important regulatory mechanism at this site. The fact that this protein, or

its interaction with the YY1 site, is hormonally regulated is of considerable interest, and could indicate that hormonal removal of transcriptional repression at the YY1 site is via removal of this protein, rather than of authentic YY1. At present, the identity of this protein remains unknown. However, identification of the protein and its regulation may provide important insights into the regulation of EGF transcription.

The present results indicate that a combination of three distinct hormones, insulin, prolactin and dexamethasone, is required to increase transcription of the EGF gene. Such results have been shown previously for other milk protein genes, such as caseins (Topper & Freeman 1980). However, this observation poses interesting mechanistic questions, particularly in light of the present findings that removal of YY1 repression appears to be a key feature in regulating EGF gene expression. How three distinct hormones might affect such a site remains uncertain. Importantly, the time-course studies suggest that regulation of EGF expression requires several hours, which suggests the possibility that regulation of EGF transcription may be downstream of other genes, although this has not been shown definitively. Thus the three distinct hormones may not actually be acting at a single site on the EGF gene, but could be affecting that site via distinct sites on other genes. In addition, the possibility that these hormones affect EGF transcription in ways undetected in these studies (for example, by influencing sites located outside the regions studied) must also be considered.

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