Multiple Promoter Elements Are Required for the Stimulatory Effect of Insulin on Human Collagenase-1 Gene Transcription

SELECTIVE EFFECTS ON ACTIVATOR PROTEIN-1 EXPRESSION MAY EXPLAIN THE QUANTITATIVE DIFFERENCE IN INSULIN AND PHORBOL ESTER ACTION

(Received for publication, November 18, 1998, and in revised form, April 7, 1999)

Stacey C. Chapman‡, Julio E. Ayala‡, Ryan S. Streeper‡, Ainsley A. Culbert§, Erin M. Eaton‡, Christina A. Svitak‡, Joshua K. Goldman‡, Jeremy M. Tavare§, and Richard M. O’Brien‡

From the ‡Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tennessee 37232 and the §Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom

Several of the complications seen in patients with both type I and type II diabetes mellitus are associated with alterations in the expression of matrix metalloproteinases. To identify the cis-acting elements that mediate the stimulatory effect of insulin on collagenase-1 (matrix metalloproteinase-1) gene transcription a series of collagenase-chloramphenicol acetyltransferase (CAT) fusion genes were transiently transfected into HeLa cells. Multiple promoter elements, including an Ets and activator protein-1 (AP-1) motif, were required for the effect of insulin. The AP-1 motif appears to be a target for insulin signaling because it is sufficient to mediate an effect of insulin on the expression of a heterologous fusion gene, whereas the data suggest that the Ets motif acts to enhance the effect of insulin mediated through the AP-1 motif. Multiple promoter elements were also required for the stimulatory effect of phorbol esters on collagenase-CAT gene transcription, and the AP-1 motif was also a target for phorbol ester signaling. However, the cis-acting elements required for the effects of insulin and phorbol esters were not identical. Moreover, phorbol esters were a much more potent inducer of collagenase-CAT gene transcription than insulin, a difference that may be explained by selective effects of insulin and phorbol esters on AP-1 expression.

The maintenance of the extracellular matrix is accomplished by a balance of synthesis and degradation, the latter being determined by the relative activities of a family of extracellular matrix metalloproteinases, the matrix metalloproteinases (MMPs), and the tissue inhibitors of MMPs (1–3). Tissue inhibitors of MMPs block MMP action by binding covalently to MMPs and preventing both activation of the MMP by enzymatic modification and the ability of MMPs to bind substrate (1–3).

* This research was supported by a grant from the Mark Collie Foundation and Grant RO1 DK52820 from the National Institutes of Health (to R. M. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† A British Diabetic Association Senior Research Fellow.

‡ To whom correspondence should be addressed: Dept. of Molecular Physiology and Biophysics, 761 MRB II, Vanderbilt University Medical School, Nashville, TN 37232-0615. Tel.: 615-936-1503; Fax: 615-322-7236.

1 The abbreviations used are: MMP, matrix metalloproteinase; PEPCK, phosphoenolpyruvate carboxykinase; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; ME, malic enzyme; IRS, insulin response sequence.
Insulin-regulated Collagenase Gene Transcription

The activator protein-1 (AP-1) motif, which binds members of the Fos and Jun transcription factor families (25) and, like the Ets motif and serum response element, mediates transcriptional changes in response to multiple ligands (26), may represent a fourth class of consensus IRS. Thus, Rutter et al. (12) have shown that in Chinese hamster ovary cells, mutation of the AP-1 motif in the collagenase promoter abolishes the stimulatory effect of insulin on the expression of a collagenase-luciferase fusion gene. Similarly, in rat H4IE hepatoma cells, an AP-1 motif is required for the stimulatory effect of insulin on the expression of a malic enzyme (ME)-chloramphenicol acetyltransferase (CAT) fusion gene (13). However, paradoxically, in HeLa cells insulin has almost no effect on ME gene transcription, whereas it markedly stimulates collagenase gene transcription (13). This observation raises the question as to why the AP-1 motif appears to only mediate an insulin-dependent activation of gene transcription in some cell contexts. To indirectly address this question, we have analyzed the promoter elements required for the stimulatory effect of insulin on collagenase gene transcription in HeLa cells. The results demonstrate that multiple promoter elements are required for the effect of insulin on collagenase gene transcription in addition to the AP-1 motif. However, the collagenase AP-1 motif appears to be a target of insulin signaling because it is sufficient to mediate an effect of insulin on the expression of a heterologous fusion gene. The additional collagenase promoter elements that are required for the full stimulatory effect of insulin may bind accessory factors that act to enhance the effect of insulin mediated through the AP-1 motif. The data also suggest that the mechanism of insulin and phorbol ester signaling through the collagenase-1 AP-1 motif are distinct.

EXPERIMENTAL PROCEDURES

Materials

[^32P]dATP (>3000 Ci/mmol) and [3H]acetic acid, sodium salt (>10 Ci/mmol) were purchased from Amersham Pharmacia Biotech and ICN, respectively. Insulin was obtained from Collaborative Bioproducts, and phorbol 12-myristate 13-acetate (PMA) and [α]-chymotrypsin were from Sigma. Specific antisera to c-Fos (sc-52), Fra-1 (sc-183), Fra-2 (sc-604), c-Jun (sc-45), Jun B (sc-46), and Jun D (sc-74) were all obtained from Santa Cruz Biotechnology, Inc.

Plasmid Construction

DNA manipulations were accomplished by standard techniques (27), and DNA sequencing was performed using the USB Sequenase kit. All plasmid constructs were purified by centrifugation twice through cesium chloride gradients (27).

A BglII-HindIII fragment of the human collagenase-1 promoter, spanning the sequence from −518 to +64, was isolated from the plasmid pCol-Luc (12) and ligated, in the same orientation as that of the endogenous gene, into the BglII-digested polylinker of the pCAT(An) expression vector, a generous gift from Dr. Howard Towle (28). The noncompatible HindIII-BglII junction was filled in using the Klenow fragment of Escherichia coli DNA polymerase I prior to blunt-end ligation. The pCAT(An) vector has polyadenylation signals located 5′ of the polylinker to prevent read-through transcription (28). Control experiments demonstrated that there was no basal CAT expression and no effect of insulin or PMA when the pCAT(An) vector, minus the collagenase promoter, was transiently transfected into HeLa cells (data not shown). A series of truncated collagenase-CAT fusion genes was then generated, with the 5′ end points shown in Fig. 4, using the −518/+64 pCAT(An) construct as a template, by either restriction enzyme digestion or polymerase chain reaction, using standard techniques (27). The 3′ polymerase chain reaction primers was designed to conserve the junction between the collagenase promoter and CAT reporter gene to be the same as that in all other collagenase-CAT fusion gene constructs. All promoter fragments generated by polymerase chain reaction were completely sequenced to ensure the absence of polymerase errors, whereas promoter fragments generated by restriction enzyme digestion were only sequenced to confirm the 5′ end points. Site-directed mutants of the collagenase Ets and AP-1 motifs in the context of the −97/+64 promoter fragment, as shown in Fig. 5, were generated by polymerase chain reaction in conjunction with 5′ primers that incorporated the mutated sequence (Table I).

A plasmid designated X(An) was generated by ligating a minimal Xenopus 68-kDa albumin promoter (29) with the following sequence: 5′-GGGTTTACCTTATCTCTCTGACAGGATTTATATAAGTTTAAACAGTTTGCTCTGG-3′ (HindIII-BglII restriction enzyme sites underlined; TATA box in italics) into HindIII-XhoI cleaved pCAT(An). The plasmid XMB was then generated by removing a BamHI site located just 5′ of the HindIII site by the use of mung bean nuclease. Double-stranded complementary oligonucleotides, representing various regions of the collagenase promoter (Table I), were synthesized with HindIII compatible ends and ligated into HindIII-XhoI cleaved XMB in multiple copies. The number of inserts was determined by restriction enzyme analysis and confirmed by DNA sequencing.

Cell Culture and Transient Transfection

Human HeLa cervical carcinoma cells were grown to 90% confluence in T150 flasks in DMEM containing 10% (v/v) calf serum and were replated the day before use into 55-cm² culture dishes (1 flask to 26 dishes). Attached cells were transfected by addition of 0.5 ml of a calcium phosphate-DNA co-precipitate (30), containing the reporter gene construct (15 μg), an expression vector for β-galactosidase (2.5 μg), and an expression vector encoding the insulin receptor (5 μg), courtesy of Dr. Jonathan Whittaker, to the 10 ml of culture medium. After an overnight incubation the next morning the cells were incubated in 10 ml PBS for 10 min at room temperature. The cells were then incubated for a further 8–24 h in 10 ml serum-free DMEM supplemented with or without various concentrations of PMA or insulin, as indicated in the Figure legends, prior to harvesting.

Hamster insulinoma tumor cells were grown to 70% confluence in T150 flasks in DMEM containing 2.5% (v/v) fetal bovine serum and 15% (v/v) horse serum and were replated the day before use into 55-cm² culture dishes (1 flask to 14 dishes). Attached cells were then co-transfected as described previously (31) by addition of 0.5 ml of a calcium phosphate-DNA co-precipitate containing 15 μg of reporter plasmid DNA and expression vectors encoding β-galactosidase (2.5 μg) and the insulin receptor (5 μg) to the 10 ml of culture medium. After incubation for between 4 and 6 h, the cells were treated for 2 min with 20% glycerol in serum-free DMEM (5 ml/dish). The cells were then rinsed for 5 min with serum-free DMEM (5 ml/dish) prior to incubation for 24 h in serum-free DMEM (10 ml/dish) supplemented with or without various concentrations of insulin, as indicated in the Figure legends.

Rat hepatoma H4IE cells were grown in 12-well plates in DMEM containing 10% (v/v) fetal bovine serum. Attached cells were transfected at ~60% confluence with the plasmid pCol-Luc (2 μg; Ref. 12) using 4 μl of the Tfx-50 transfection reagent (Promega) in a final volume of 400 μl serum-free DMEM, as described previously (32). After incubation for 2 h, the cells were then overlaid with 2 ml of serum-containing DMEM. After an overnight incubation, the cells were washed with serum-free DMEM for 2 h prior to incubation for 24 h in serum-free DMEM supplemented with or without various concentrations of insulin, as indicated in the Figure legends. Under these growth and transfection conditions 100 μl of Tris (pH 7.8) containing 2 μM phenylmethylsulfonyl fluoride, or for luciferase assays, cells were extracted by scraping into 2 ml of 250 mM Tris (pH 7.8) containing 2 mM phenylmethylsulfonyl fluoride, or for luciferase assays, cells were extracted by scraping into 2 ml of passive lysis buffer (Promega). CAT, β-galactosidase, and luciferase assays were performed exactly as described previously (20, 30, 32). To compare the relative basal CAT expression obtained with the various reporter gene constructs described, CAT activity in samples from control cells was corrected for the β-galactosidase activity in the same sample. Because phorbol esters and insulin affect Rous sarcoma virus-β galactosidase expression in HeLa cells (data not shown), CAT activity in insulin-stimulated cells was corrected for the protein content of the cell lysate, as measured by the Pierce BCA assay, and each plasmid construct was analyzed in duplicate in multiple transfections, as specified in the Figure legends.

 Gel Retardation Assay

Labeled Probes—Oligonucleotides representing the sense and antisense strands of the collagenase AP-1 and Ets motifs (Table I) were

synthesized with BamHI or HindIII compatible ends, respectively, gel purified, annealed, and then labeled with [α-32P]dATP using the Kle
now fragment of Escherichia coli DNA polymerase I to a specific activity of approximately 2.5 Ci/mmol.

**Nuclear Extract Preparation**—HeLa nuclear extracts were prepared exactly as described previously (13).

**AP-1 Binding Assay**—Labeled AP-1 oligonucleotide (7.5 fmol, ~30,000 cpm) was incubated with HeLa (3 μg) nuclear extract in a final reaction volume of 20 μl containing 20 mM HEPES, pH 7.8, 100 mM NaCl, 0.38 mM spermidine, 0.08 mM spermine, 0.1 mM EDTA, 1 mM EGTA, 2 mM dihydrothreitol, 12.5% glycerol (vol/vol), and 1 μg of poly(dI-dC)poly(dI-dC). After incubation for 10 min at room temperature, the reactants were loaded onto a 6% polyacrylamide gel and electrophoresed at room temperature for 90 min at 150 V in a buffer containing 25 mM Tris-HCl at pH 7.8, 190 mM glycine, and 1 mM EDTA. Following electrophoresis, the gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography.

**Ets Binding Assay**—When the Ets oligonucleotide was used as the labeled probe, the binding conditions were identical to those described for AP-1 except that the NaCl concentration was decreased to 50 mM and poly(dI-dC)poly(dI-dC) was reduced to 0.5 μg. In addition, visualization of specific Ets binding (see Fig. 8B) required precubation of HeLa nuclear extract with 10 ng of chymotrypsin for 2 min at room temperature prior to addition of the labeled probe and binding buffer and a further 10 min of incubation at room temperature. Binding was then analyzed by acrylamide gel electrophoresis as described above.

**Competition Experiments**—For competition experiments (see Fig. 8), the indicated unlabeled double-stranded oligonucleotides (100-fold mol
lar excess) were mixed with the labeled oligomer prior to addition of nuclear extract. Binding was then analyzed by acrylamide gel electrophoresis as described above.

**Gel Supershift**—Gel supershift assays (see Fig. 9) were carried out by incubating HeLa nuclear extract (3 μg) with the indicated antisera for 10 min at room temperature, prior to the addition of the labeled AP-1 oligonucleotide probe and binding buffer and incubation for an additional 10 min.

**RESULTS**

**Insulin Stimulates Collagenase-CAT Fusion Gene Transcription in HeLa Cells**—To begin to study the regulation of collagenase gene transcription by insulin, a collagenase-CAT fusion gene construct, containing collagenase promoter sequence from −518 to +64, relative to the transcription start site at +1 (33), was transiently transfected into HeLa cells (Fig. 1). An effect of insulin on reporter gene expression was only detected when the collagenase-CAT fusion gene was co-transfected with an expression vector encoding the insulin receptor (Fig. 1A). In the absence of insulin, co-transfection with the insulin receptor alone was insufficient to activate collagenase-CAT fusion gene expression, suggesting a low level of signaling through the basal receptor (Fig. 1B). Stanley (34) has previously shown that co-transfection with an insulin receptor expression vector is also required to observe an effect of insulin on the expression of a transiently transfected prolactin-CAT fusion gene in rat pituitary tumor GH4 cells. Interestingly, insulin stimulates the expression of the endogenous prolactin gene in these cells in the absence of receptor co-transfection (34). The maximal effect of insulin on collagenase-CAT gene expression was seen at 100 nM (Fig. 2A). This concentration of insulin is 10-fold higher than that required to see a maximal repression of glucocortico
coid-stimulated PEPCK-CAT fusion gene expression in rat H4IIE hepatoma cells (35, 36). This requirement for 100 nM insulin to manifest the maximal effect of the hormone is not explained by the degradation of insulin by HeLa cells because a similar EC_{50} for this effect is obtained following an 8 or 24 h incubation with insulin (Fig. 2B). In addition, the maximal effect of insulin on collagenase-CAT gene expression was also seen at 100 nM in hamster insulinoma tumor cells (Fig. 2C), H4IIE cells (Fig. 2D), and Chinese hamster ovary-T cells (data not shown). Although cell line-dependent variations in insulin sensitivity could arise due to differences in the expression of molecules in the insulin signaling pathway, our results suggest

![Fig. 1. Insulin stimulates collagenase-CAT fusion gene transcription in HeLa cells.](image)

**Insulin-regulated Collagenase Gene Transcription**

![Graph A: Fold Induction of CAT Activity by Insulin](image)

**Graph B: CAT/βGal. Expression in Control Cells**

(arbitrary units)

![Graph B: CAT/βGal. Expression in Control Cells](image)

**Multiple, Distinct cis-Acting Elements Are Required for the Actions of Insulin and Phorbol Esters on Collagenase Gene Transcription**—The result shown in Fig. 1 suggests that an IRS is present in the collagenase promoter between −518 and +64. Because the cis-acting elements that mediate the stimulatory effect of phorbol esters on collagenase gene transcription have been studied in detail (1, 2, 33, 37), and given the long running controversy concerning the potential role of protein kinase C in insulin action (38), it was of interest to determine whether insulin and phorbol esters both mediate their stimulatory action on collagenase gene transcription through the same elements. The phorbol ester PMA has a biphasic effect on collagenase gene transcription, with a maximal stimulation of 150-fold seen at 10 nM (Fig. 3). The reduction in PMA-stimulated CAT expression at higher PMA concentrations probably reflects the down-regulation of protein kinase C in insulin action (38), it was of interest to determine whether insulin and phorbol esters both mediate their stimulatory action on collagenase gene transcription through the same elements. The phorbol ester PMA has a biphasic effect on collagenase gene transcription, with a maximal stimulation of 150-fold seen at 10 nM (Fig. 3). The reduction in PMA-stimulated CAT expression at higher PMA concentrations probably reflects the down-regulation of protein kinase C (39). By contrast, the maximal stimulation obtained with insulin was 30-fold (Fig. 2A), and the stimulation of collagenase-CAT fusion gene expression by insulin and PMA was not additive (data not shown).

To delineate the collagenase IRS, a series of 5′ deletion mutations of the collagenase promoter was constructed in the
**Insulin-regulated Collagenase Gene Transcription**

**Fig. 2.** Concentration dependence of insulin-stimulated collagenase-CAT fusion gene transcription. HeLa cells were transiently co-transfected using calcium phosphate co-precipitation, as described under "Experimental Procedures," with a collagenase-CAT fusion gene (15 µg), containing promoter sequence from −518 to +64, and expression vectors encoding β-galactosidase (2.5 µg) and the insulin receptor (5 µg). HIT cells were transiently transfected using the Tfx-50 transfection reagent, as described under "Experimental Procedures," with a collagenase-luciferase fusion gene (2 µg), containing promoter sequence from −518 to +64. Following transfection, cells were incubated for either 24 h (A, C, and D) or 8 h (B) in serum-free medium in the presence or absence of various concentrations of insulin. The cells were then harvested and CAT or luciferase activity was assayed as described previously (20, 30). Results are presented as the ratio of CAT activity, corrected for protein concentration in the cell lysate, in PMA-treated versus control cells and are expressed as fold induction. Results represent the mean of ± S.E. of four experiments, in which each condition was assayed in duplicate.

**Fig. 3.** Concentration dependence of PMA-stimulated collagenase-CAT fusion gene transcription in HeLa cells. HeLa cells were transiently co-transfected, as described under "Experimental Procedures," with a collagenase-CAT fusion gene (15 µg), containing promoter sequence from −518 to +64, and expression vectors encoding β-galactosidase (2.5 µg) and the insulin receptor (5 µg). The insulin receptor expression vector was included to be consistent with all other transfection experiments. Following transfection, cells were incubated for 24 h in serum-free medium in the presence or absence of various concentrations of PMA. The cells were then harvested, and CAT activity was assayed as described previously (20, 30). Results are presented as the ratio of CAT activity, corrected for protein concentration in the cell lysate, in PMA-treated versus control cells and are expressed as fold induction. Results represent the mean of ± S.E. of four experiments, in which each condition was assayed in duplicate.

To directly assess the relative importance of the collagenase Ets and AP-1 motifs in mediating the induction of gene transcription by insulin and phorbol esters, these cis-acting elements were mutated within the context of the −97 to +64 promoter fragment (Fig. 5). Mutation of the Ets motif reduced the stimulatory effects of both insulin and phorbol esters on collagenase-CAT fusion gene transcription, whereas mutation of the AP-1 motif abolished the response to both agents (Fig. 5, A and B). Thus, an intact AP-1 motif is essential for the induction of collagenase-CAT gene expression by insulin and phorbol esters, whereas the Ets motif is not. The collagenase Ets and AP-1 motifs, particularly the latter, are also important for basal collagenase gene transcription (Fig. 5C). Thus, although mutation of the Ets motif reduces basal collagenase-CAT gene expression, mutation of the AP-1 motif reduces basal gene expression to barely detectable levels, despite the presence of an intact Ets motif (Fig. 5C). In addition, mutation of the AP-1 motif within the context of the −79 to +64 promoter fragment reduces basal fusion gene expression to the same level as that seen when the AP-1 motif is mutated in the context of the −97 to +64 promoter fragment, despite the presence of the intact Ets motif (Fig. 5C).

Mutation of the Ets motif within the context of the −97 to +64 promoter fragment has the same quantitative effect on both basal fusion gene expression and the response to insulin, as deletion of the −97 to −80 sequence (Fig. 5, A and C). By contrast, deletion of the −97 to −80 sequence is more deleterious to the phorbol ester response than a mutation of the Ets motif in the context of the −97 to +64 promoter fragment (Fig. 5B). This suggests that the transcription factor(s) binding the...
Ets motif mediates both basal gene expression and the response to insulin conferred through this region but that an additional element is important for the action of phorbol esters. Finally, mutation of the AP-1 motif in the context of the 279 to 164 promoter fragment almost completely abolishes the small effect of insulin and phorbol esters on the expression of this truncated fusion gene, just as it does in the context of the 297 to 164 promoter fragment (Fig. 5, A and B).

The Collagenase AP-1 and Ets Motifs Confer a Stimulatory Effect of Insulin and Phorbol Esters on the Expression of a Heterologous Promoter—The preceding experiments demonstrate that the AP-1 motif is required for the action of both insulin and phorbol esters on collagenase gene transcription and that the Ets motif enhances the action of both agents, although it is inactive in the absence of the AP-1 motif. However, these experiments do not prove that the transcription factors binding the AP-1 and/or Ets motifs are the targets of insulin and phorbol ester signaling, rather than acting as accessory factors to enhance the effects of these agents mediated through an unidentified response sequence. To determine whether the AP-1 and/or Ets motifs are the targets of insulin and phorbol ester signaling, multiple copies of an oligonucleotide representing the collagenase promoter sequence between

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**Fig. 4.** Progressive deletion of the collagenase promoter sequence between −518 and −80 reduces both basal collagenase-CAT fusion gene transcription and the stimulatory effects of insulin and PMA. HeLa cells were transiently co-transfected, as described under "Experimental Procedures," with a series of collagenase-CAT fusion genes, with 5′ deletion end points as shown on the abscissa, and expression vectors encoding β-galactosidase and the insulin receptor. Following transfection, cells were incubated for 24 h in serum-free medium in the presence or absence of 10 nM insulin or 100 nM PMA. The cells were then harvested, and both CAT and β-galactosidase activity were assayed as described previously (20, 30). Results are presented as the ratio of CAT activity, corrected for protein concentration in the cell lysate, in insulin-treated (A) or PMA-treated (B) versus control cells, and are expressed as fold induction. In C, results are presented as the ratio of CAT to β-galactosidase activity in control cells and are expressed as arbitrary units. Results represent the mean of ± S.E. of 4–13 experiments, in which each construct was assayed in duplicate.

**Fig. 5.** Site-directed mutation of the Ets and AP-1 motifs in the collagenase promoter reduces both basal collagenase-CAT fusion gene transcription and the stimulatory effects of insulin and PMA. HeLa cells were transiently co-transfected, as described under "Experimental Procedures," with a series of collagenase-CAT fusion genes, with 5′ deletion end points and mutations as shown on the abscissa, and expression vectors encoding β-galactosidase and the insulin receptor. Following transfection, cells were incubated for 24 h in serum-free medium in the presence or absence of 10 nM insulin or 100 nM PMA. The cells were then harvested, and both CAT and β-galactosidase activity were assayed as described previously (20, 30). Results are presented as the ratio of CAT activity, corrected for protein concentration in the cell lysate, in insulin-treated (A) or PMA-treated (B) versus control cells and are expressed as fold induction. In C, results are presented as the ratio of CAT to β-galactosidase activity in control cells and are expressed as arbitrary units. Results represent the mean of ± S.E. of 4–13 (insulin) or 3 (PMA) experiments, in which each construct was assayed in duplicate.
which a single copy of the basic XMB vector (Fig. 6) or with a fusion gene construct in insulin/PMA-induced CAT expression were detected with the into HeLa cells (Fig. 6). Neither basal CAT expression nor gene, and the resulting construct was transiently transfected 68-kDa albumin promoter (29) ligated to the CAT reporter vector, designated XMB, which contains a minimal (Table I), were ligated into the polylinker of a heterologous context of the XMB vector (Fig. 6). Mutation of the Ets motif (Table I), containing mutations in either the Ets or AP-1 motifs, to mediate an insulin or phorbol ester response in the tides (Table I), containing mutations in either the Ets or AP-1 motifs, to mediate an insulin or phorbol ester response in the context of the collagenase promoter markedly reduced the in- sion of the fusion gene, whereas the same mutation in the context of the XMB vector (Fig. 6). By contrast, the multimerized −97/−64 oligonucleotide was able to mediate a stimulatory effect of both insulin and phorbol esters on fusion gene expression, although the effect of insulin was small relative to that of phorbol esters (Fig. 6).

We next investigated the ability of multimerized oligonucleo- tides (Table I), containing mutations in either the Ets or AP-1 motifs, to mediate an insulin or phorbol ester response in the context of the XMB vector (Fig. 6). Mutation of the Ets motif reduced both basal fusion gene expression (Fig. 6C) and the stimulatory effect of phorbol esters (Fig. 6B), whereas mutation of the AP-1 motif abolished the stimulatory effect of phorbol esters (Fig. 6B) and greatly reduced basal gene expression, despite the presence of an intact Ets motif (Fig. 6C). The effects of these mutations on both basal fusion gene expression and the response to phorbol esters mimic the effects of these same mutations when made in the context of the endogenous collagenase promoter (Fig. 5, B and C). By contrast, mutation of the Ets motif in the heterologous context of the XMB vector had only a minor effect on the ability of insulin to stimulate expression of the fusion gene, whereas the same mutation in the context of the collagenase promoter markedly reduced the insulin response (Fig. 5A). The explanation for this discrepancy is unclear but may indicate that in this context, the presence of multiple AP-1 motifs reduces the requirement for the Ets motif with respect to insulin, although not phorbol ester signaling. This is consistent with the observation that the mechanisms of insulin and phorbol esters signaling through the AP-1 motif are distinct (see below). Mutation of the AP-1 motif in the hetero- logical context of the XMB vector abolished the effect of insulin

−97 and −64, which contains both the Ets and AP-1 motifs (Table I), were ligated into the polylinker of a heterologous vector, designated XMB, which contains a minimal Xenopus 68-kDa albumin promoter (29) ligated to the CAT reporter gene, and the resulting construct was transiently transfected into HeLa cells (Fig. 6). Neither basal CAT expression nor insulin/PMA-induced CAT expression were detected with the basic XMB vector (Fig. 6) or with a fusion gene construct in which a single copy of the −97/−64 oligonucleotide had been ligated into the polylinker of the XMB vector (data not shown). By contrast, the multimerized −97/−64 oligonucleotide was able to mediate a stimulatory effect of both insulin and phorbol esters on fusion gene expression, although the effect of insulin was small relative to that of phorbol esters (Fig. 6).

The preceding experiments demonstrate that the AP-1 motif is required for the action of both insulin and phorbol esters on collagenase gene transcription (Fig. 5) as well as for mediating an effect of these agents on the expression of a heterologous XMB fusion gene through the −97/−64 collagenase sequence (Fig. 6). In the collagenase promoter the Ets motif enhances the action of both agents, although it is inactive
insulin in the absence of the AP-1 motif (Fig. 5). However, it is unclear from these experiments whether the Ets motif-binding protein can also directly mediate an insulin/phorbol ester response or whether it just acts as an accessory factor to enhance the effects of these agents mediated through the AP-1 motif. To address this question, oligonucleotides representing just the AP-1 motif (Fig. 7). This suggests that the AP-1 motif, was used as the labeled probe (Fig. 8A). A 100-fold molar excess of the unlabeled –78 to –63 oligonucleotide competed effectively against the labeled probe for binding of the upper complex, indicating that this protein-DNA complex represents a specific interaction (Fig. 8A). By contrast, an oligonucleotide representing the same –78 to –63 collagenase sequence, but with a mutation in the AP-1 core sequence (Table I) that abolishes insulin and phorbol ester signaling through this element (Fig. 7), failed to compete for protein binding with the labeled probe, even in a 100-fold molar excess (Fig. 8A). Thus, the formation of this specific protein-DNA complex correlates with the effect of insulin and phorbol ester mediated through this sequence in the context of the collagenase promoter and the heterologous XMB vector. This specific protein-DNA complex detected in the gel retardation assay contains members of the AP-1 transcription factor family (Ref. 13 and see below).

We also analyzed protein binding to an oligonucleotide representing the wild-type collagenase Ets motif (Table I) but failed to detect a protein-DNA interaction, the formation of which was selectively competed for by an excess of the unlabeled wild-type oligonucleotide and not by an oligonucleotide containing the same mutated Ets sequence that abolished the effects of insulin and phorbol esters mediated through this element in the context of the collagenase promoter (Fig. 8B, left panel). This result contrasts with those of White et al. (42), who were able to detect specific binding to the collagenase Ets motif. We would speculate that this discrepancy may reflect a difference in the amount or nature of the Ets family transcription factors expressed in HeLa cell nuclear extracts, as used here, and fibroblast nuclear extracts, as used by White et al. (42).

A number of investigators have previously shown that DNA binding by Ets proteins is repressed by an intramolecular mechanism (43–45) that can be relieved through partial proteolysis (45). To determine whether a similar phenomenon was preventing the detection of specific protein binding to the wild-type Ets oligonucleotide, HeLa nuclear extract was preincubated with 10 ng of chymotrypsin prior to analysis of protein-DNA binding using the gel retardation assay. Using this strategy, a protein-DNA interaction was now detected, the formation of which was selectively competed for by an excess of the unlabeled wild-type oligonucleotide and not by an oligonucleotide containing the same mutated Ets sequence that abolished the effects of insulin and phorbol esters mediated through this element in the context of the collagenase promoter (Fig. 8B, right panel, arrow). Additional gel retardation experiments with an oligonucleotide containing both the collagenase AP-1 and Ets motifs, using HeLa nuclear extract without chymotrypsin treatment, failed to reveal the presence of a protein-DNA interaction that might be indicative of the formation of a ternary complex, although specific binding to the AP-1 motif was detected (data not shown). Thus, the presence of AP-1 did not appear to be sufficient to relieve the repression of Ets protein binding.

The Mechanisms of Insulin and Phorbol Ester Signaling through the Collagenase AP-1 Motif Are Distinct—The magnitude of insulin- and phorbol ester-stimulated collagenase-CAT fusion gene transcription are markedly different, with phorbol ester being the more potent activator (Figs. 2–5). This difference in potency was maintained when the effects of insulin and phorbol esters were compared on the expression of heterologous constructs containing multimerized oligonucleotides representing either the collagenase promoter sequence between –97 and –64, which contains both the Ets and AP-1 motifs (Fig. 6), or collagenase promoter sequence between –78 to –63, which contains just the AP-1 motif (Fig. 7). This suggests that the
difference in potency of insulin and phorbol esters on collagenase gene transcription can be at least partly ascribed to a difference in signaling through the AP-1 motif.

Because AP-1 is composed of homodimers and heterodimers of various members of the Fos and Jun transcription factor families (25), we hypothesized that the difference in potency of insulin and phorbol ester signaling might be due to differences in the ability of these factors to selectively induce the binding of specific members of the Fos/Jun families. To test this hypothesis, nuclear extracts were first prepared from HeLa cells incubated for 5 h in the presence or absence of insulin and phorbol esters, and then polyclonal antisera to specific Fos/Jun family members were assayed for their ability to supershift the specific protein-DNA complex detected using the collagenase AP-1 motif in gel retardation assays (Fig. 9). These experiments showed that although both insulin and phorbol esters induced AP-1 binding activity, several differences were apparent in the composition of the induced complex. Most notably, phorbol esters stimulated a marked increase in c-Fos binding and a slight increase in Fra-1 binding (Fig. 9B), whereas insulin was without effect on these proteins (Fig. 9A). By contrast, insulin induced the expression of Fra-2 and Jun D (Fig. 9A), whereas phorbol esters only had a minor effect on the binding of these factors (Fig. 9B). Because phosphatase inhibitors were not present during the preparation of nuclear extracts, it is likely that these changes in AP-1 binding reflect changes in AP-1 expression rather than phosphorylation. Although insulin induces c-Fos mRNA in multiple cell types, this effect is transient (13), which may thus explain the lack of insulin-stimulated c-Fos binding (Fig. 9A).

**DISCUSSION**

This analysis of the cis-acting elements that mediate the stimulatory effects of insulin and phorbol esters on collagenase gene transcription reveals that multiple elements are required for the action of both agents. The results obtained with respect to phorbol ester signaling are similar to those reported by Brinckerhoff and colleagues (reviews in Refs. 1 and 37) in their studies on the regulation of the rabbit collagenase −1 gene in fibroblasts. Although several of these elements are required for the effects of both insulin and phorbol esters, it is apparent that there are also unique features to their mechanisms of action. Thus, phorbol ester-stimulated collagenase-CAT gene transcription was reduced when the region of the promoter between −518 to −159 was deleted, whereas the effect of insulin was unchanged (Fig. 4). In addition, deletion of the −97 to −80 sequence was more deleterious to the phorbol ester response than a site-directed mutation of the Ets motif in the context of the −97 to +64 promoter fragment, whereas the insulin response was equally reduced by both manipulations (Fig. 5).

Perhaps the most striking difference, though, was the observation that the mechanisms of insulin and phorbol ester signaling through the AP-1 motif appear to be distinct (Fig. 9). Thus, these agents induce selective increases in the binding of specific members of the Fos/Jun transcription factor families; most notably, phorbol esters stimulated a marked increase in c-Fos binding (Fig. 9B), whereas insulin was without effect (Fig. 9A). We hypothesize that these selective effects on AP-1 binding may explain the greater potency of phorbol esters in stimulating collagenase gene transcription, although the existence of additional differences in insulin- and phorbol ester-stimulated changes in AP-1 phosphorylation cannot be excluded.

We first became interested in the regulation of collagenase gene transcription by insulin as a result of earlier studies on the regulation of ME gene transcription (13). Those experiments demonstrated that whereas insulin clearly stimulated ME-CAT gene transcription in H4IIE cells, it had little effect on ME-CAT gene transcription in HeLa cells (13). The action of insulin on ME gene transcription in H4IIE cells was shown to be mediated, in part, through an AP-1 motif in the ME promoter (13). Because insulin stimulates collagenase-CAT gene transcription in HeLa cells (13) (Fig. 1) and because the stimulatory effect of insulin on collagenase gene transcription requires an intact AP-1 motif in the collagenase promoter (12) (see Methods). Two questions arose, namely (i) why does insulin stimulate collagenase-CAT expression, but not ME-CAT expression, in HeLa cells given that both promoters contain AP-1 motifs and (ii) why does insulin stimulate ME-CAT gene transcription in H4IIE cells but not in HeLa cells? We postulated that the latter may reflect an inherent difference in insulin-signaling through AP-1 motifs in the H4IIE and HeLa cell lines, whereas the answer to the first question could reflect some inherent difference in the AP-1 motifs in the ME and collagenase promoter (13). Alternatively, we proposed that both observations might be explained by a difference in the promoter context in which the AP-1 motif was located; in other words, the nature of the transcription factors associated with the promoter other

![Image](https://example.com/image.png)
then prepared from HeLa cells incubated for 5 h in serum-free medium (48), and tissue inhibitor of MMP-1 (49) genes and the insulin mediated through a more proximal IRS (46). Interestingly, Ets and AP-1 motifs are found juxtaposed in several contexts (13). However, it is apparent that the marked stimulation of collagenase-CAT gene expression by phorbol esters is also dependent on multiple elements in addition to the AP-1 motif (Figs. 4 and 5).

Based on the data presented in Figs. 5 and 6, we propose that the transcription factor binding the collagenase Ets motif acts as accessory factor to enhance the effect of insulin mediated through the AP-1 motif. Such an arrangement would be similar to that found in the glucose-6-phosphatase promoter in which HNF-1 acts as an accessory factor to enhance the effect of insulin mediated through a more proximal IRS (46). Interestingly, Ets and AP-1 motifs are found juxtaposed in several promoters, including those encoding collagenase (41, 47), matrixins (48), and tissue inhibitor of MMP-1 (49) genes and the polyoma virus enhancer (50). In these genes, the Ets and AP-1 motifs mediate a synergistic activation of transcription in response to various agonists (for review, see Ref. 2). In addition, it has been shown that Ets proteins are capable of directly interacting with components of the AP-1 complex in vitro (49, 51, 52) and in vivo (51, 52), thus providing a molecular model to explain the ability of Ets motif-binding proteins to enhance signaling through AP-1 motifs.

Although the multimerized collagenase Ets motif fails to mediate an insulin response when ligated to the heterologous XMB vector (Fig. 7), the possibility that insulin directly signals through the Ets binding factor as well as through AP-1 cannot be entirely ruled out. Thus, Blackshear and colleagues (22) have demonstrated that at least in some cell lines, the stimulatory effect of insulin on c-fos gene transcription is mediated by MAP kinase through the phosphorylation of the Ets motif-binding protein, p62TCP, p62TCP only binds the c-fos serum response element as a ternary complex with p67SRF (23). By analogy, the possibility therefore exists that insulin does directly signal through the collagenase Ets motif-binding protein, but that this cannot be demonstrated in a heterologous context because AP-1 is required to stabilize binding of the Ets factor (22). MAP kinase also phosphorylates the Ets protein GABP, which mediates the stimulatory effect of insulin on prolactin gene transcription (53), although the significance of this phosphorylation is unclear because recent data has shown that PD98059 does not block this effect of insulin (54).

In summary, the experiments described in this report demonstrate that multiple promoter elements are required for the stimulatory effect of insulin on collagenase gene transcription in HeLa cells. The AP-1 motif in the collagenase promoter appears to be the target of both insulin and phorbol ester signaling; however, the mechanisms of insulin and phorbol ester action are distinct. Future studies will examine whether insulin also regulates collagenase gene expression in glomerula-derived cell lines. Although insulin receptor levels are low in the mesangial cells of the glomerulus (55), they increase in the diabetic state (56). Furthermore, it is interesting to note that in the streptozotocin rat model of type I diabetes, collagenase gene expression in the glomerulus decreases (10). This may indicate that the regulation of collagenase gene expression in this tissue by phorbol esters differs from that reported in HeLa cells because the hyperglycemia in these animals has been postulated to lead to activation of protein kinase C (57), which would be anticipated to lead to an increase, rather than a decrease, in collagenase gene expression.

Acknowledgments—We thank Bob Hall for useful suggestions during the course of this project and for insightful comments on the manuscript. We thank John Hassell and Howard Crawford for assistance with the analysis of Ets-binding proteins and Howard Towle and Jonathan Whittaker for the pCAT(An) and insulin receptor expression vector plasmids, respectively. We also thank Julia Breyer, Ray Harris, and R. Brooks Robey for interesting discussions on the potential role of
decreased collagenase-1 gene expression in the pathophysiology of glomerulonephritis. HeLa and hamster insulinoma tumor cells were kindly provided by Roland Stein and Eva Henderson. Data analysis was performed in part through the use of the Vanderbilt University Medical Center Imaging Resource (CA68485 and DK20093).

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