Accessory Elements, Flanking DNA Sequence, and Promoter Context Play Key Roles in Determining the Efficacy of Insulin and Phorbol Ester Signaling through the Malic Enzyme and Collagenase-1 AP-1 Motifs*

Insulin stimulates malic enzyme (ME)-chloramphenicol acetyltransferase (CAT) and collagenase-1-CAT fusion gene expression in H4IIIE cells through identical activator protein-1 (AP-1) motifs. In contrast, insulin and phorbol esters only stimulate collagenase-1-CAT and not ME-CAT fusion gene expression in HeLa cells. The experiments in this article were designed to explore the molecular basis for this differential cell type- and gene-specific regulation. The results highlight the influence of three variables, namely promoter context, AP-1 flanking sequence, and accessory elements that modulate insulin and phorbol ester signaling through the AP-1 motif. Thus, fusion gene transfection and proteolytic clipping gel retardation assays suggest that the AP-1 flanking sequence affects the conformation of AP-1 binding to the collagenase-1 and ME AP-1 motifs such that it selectively binds the latter in a fully activated state. However, this influence of ME AP-1 flanking sequence is dependent on promoter context. Thus, the ME AP-1 motif will mediate both an insulin and phorbol ester response in HeLa cells when introduced into either the collagenase-1 promoter or a specific heterologous promoter. But even in the context of the collagenase-1 promoter, the effects of both insulin and phorbol esters, mediated through the ME AP-1 motif are dependent on accessory factors.

Insulin regulates the transcription of more than 100 genes indicating that this represents a major action of this hormone (1, 2). The stimulatory and inhibitory effects of insulin on gene transcription are mediated through various cis-acting elements collectively referred to as insulin response sequences or elements (IRSs/IREs).1 Unlike cAMP, which regulates gene transcription predominantly through one cis-acting element (3), a single consensus IRS does not exist. Instead, six, distinct consensus IRSs have currently been well defined (2) in addition to several IRSs that appear to be unique to individual genes (1). Thus, this situation resembles that for phorbol esters, which are able to regulate gene transcription through at least eight distinct consensus sequences (4).

One of these consensus IRSs has the sequence T(G/A)TTT(T/G)(T/G) and mediates the inhibitory effect of insulin on phosphoenolpyruvate carboxykinase, insulin-like growth factor binding protein-1, apolipoprotein CIII, and glucose-6-phosphatase catalytic subunit gene transcription (1, 2). The transcription factor FKHR binds this IRS but whether it mediates the action of insulin through this motif is unclear (5–7). The other five consensus IRSs all mediate stimulatory effects of insulin on gene transcription. They are the activator protein-1 (AP-1) motif, the serum response element (SRE), the Ets motif, the thyroid transcription factor-2 motif, and the sterol response element-binding protein (SREBP) motif (1, 2). Multiple hormones other than insulin regulate gene expression through the AP-1 motif, the SRE, and the Ets motif (8–10). In contrast, the thyroid transcription factor-2 motif has currently only been shown to mediate effects of insulin, cAMP (11), and cytokines (12) on thyroid gene expression. Insulin and thyrotropin, the latter acting through cAMP, both stimulate the expression of thyroid transcription factor-2 (11), which contributes to the induction of thyroglobulin and thyroperoxidase gene transcription by these hormones (1, 2). Similarly, insulin and cAMP both regulate the expression of SREBP-1c, although in this case their effects are antagonistic (13).

The AP-1 motif binds members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) transcription factor families (8) and mediates the action of insulin on the expression of the hepatitis B virus X gene and the genes encoding collagenase-1 (henceforth referred to simply as collagenase) and malic enzyme (ME) (14–18). The mechanism of insulin signaling through the AP-1 motif is poorly understood but appears to involve effects of insulin on both the phosphorylation state and mass of the AP-1 complex (1, 2). Thus, the potential exists for insulin to have both short and long term effects on gene expression through the same element. However, the mechanism of insulin signaling appears to vary with the cell type studied (14, 16, 17, 19). For example, the stimulation of Fra-1 gene expression by insulin is seen in some (19, 20),

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AP-1, activator protein-1; SRE, serum response element; SREBP, sterol response element-binding protein; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase.
although not in all cell types (21) and the protein kinases JNK1 and JNK2, which phosphorylate and activate c-Jun (8), are only activated by insulin in some cell types (22) and not others (19).

We have previously shown that, whereas insulin stimulates collagenase-CAT and ME-CAT fusion gene expression in H41IE cells, insulin only stimulates collagenase-CAT and not ME-CAT fusion gene expression in HeLa cells (16, 17). In addition, phorbol esters stimulate collagenase-CAT but not ME-CAT fusion gene expression in HeLa cells (16). The experiments in this article were designed to explore the molecular basis for: (i) the differential regulation of collagenase and ME gene expression by insulin and phorbol esters in HeLa cells and (ii) the differential regulation of ME gene expression by insulin in H41IE and HeLa cells. The results highlight the influence of three additional variables, namely promoter context, AP-1 flanking sequence, and accessory elements that modulate insulin signaling through the AP-1 motif.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dATP (>3000 Ci mmol−1) and [3H]acetic acid, sodium salt (>10 Ci mmol−1), were obtained from Amersham Biosciences and ICN, respectively. Insulin was purchased from Collaborative Bio-products. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma. All oligonucleotides were synthesized by the Vanderbilt University Diabetes Core Laboratory.

Plasmid Construction—The construction of a collagenase-CAT plasmid, containing the wild-type human collagenase promoter sequence from −158 to +64, has been previously described (17). The TKCAT plasmid contains the herpes simplex virus-thymidine kinase (TK) promoter sequence from −105 to +51 ligated to the CAT reporter gene and has a unique BamHI site in the polylinker at −105 (23). The TKC-VI plasmid contains the herpes simplex virus-thymidine kinase–TK promoter sequence from −480 to +51 ligated to the CAT gene and has a unique BamHI linker between positions −40 and −35 (24). Various double-stranded complementary oligonucleotides, representing distinct regions of the ME or collagenase promoters (Table I), were synthesized with BamHI compatible ends and were ligated in multiple copies into BamHI-cleaved TKC-VI. Ligation of a single copy of the ME or collagenase AP-1 motifs into the BamHI site of TKC-VI fails to confer a phorbol ester response (data not shown). Plasmid XMB contains a minimal Xenopus 68-kDa albumin promoter ligated to the CAT reporter gene and has a unique HindIII site in the polylinker (17). Double-stranded complementary oligonucleotides, representing various regions of the ME or collagenase promoters (Table I), were synthesized with HindIII compatible ends and ligated into HindIII-cleaved XMB in multiple copies. The orientation and number of inserts in the TKCAT, TKC-VI, and XMB plasmid constructs were determined by restriction enzyme analysis and confirmed by DNA sequencing.

A previously described three-step PCR strategy (25, 26) was used to switch the collagenase AP-1 flanking sequence to that of the ME AP-1 motif in the context of the collagenase promoter. The resulting construct, designated Coll 158:ME (Fig. 8), was generated within the context of the −158 to +64 collagenase promoter fragment. Briefly, two complementary PCR primers were designed; the sequence of the sense strand oligonucleotide was as follows (mutated nucleotides are underlined): 5′-CAAGAGGAT-GTTATCCGCGGTGACTCGAGCCGACCTCTGGTCTTC-3′. The AP-1 core sequence was unchanged and is shown in italics. This sense strand oligonucleotide was used in conjunction with a 3′ PCR primer to generate the 3′-half of the collagenase promoter, whereas the complementary antisense strand oligonucleotide was used in conjunction with a 5′ PCR primer to generate the 5′-half of the collagenase promoter. These 5′ and 3′ primers were designed to maintain the 5′ and 3′ junctions of the collagenase promoter fragments to be the same as that in the wild-type −158 to +64 collagenase-CAT fusion gene construct. The PCR products from these reactions were then combined, and the total DNA used them as a primer and template in a second PCR reaction to generate a small amount of the full-length, mutated collagenase promoter fragment. Finally, the 5′ and 3′ PCR primers were then used to amplify this fragment. An identical strategy was used to generate a promoter fragment in which the orientation of the ME AP-1 motif in the context of the Coll 158:ME construct was switched by changing the core sequence from TGAATCA to TGACTCA. Truncated constructs, designated Coll 79 and Coll 79:ME (Fig. 8), were then generated using the same 3′ PCR primer described above and the following 5′ PCR primers, respectively: 5′-CCGGTCGAGAAGCCAT-GAGTCAGACG3′; 5′-CCGGTCGAGCCGGCGGTGACTCGAGCCGCTCGTTTTCTGG-3′ (AP-1 core sequences are in italics and XhoI cloning sites are underlined). All promoter fragments were completely sequenced to ensure the absence of polymerase errors and plasmid contamination by centrifugation through cesium chloride gradients (27).

Cell Culture and Transient Transfection—Rat H41IE hepatoma cells were grown to 40–70% confluence in T150 flasks in Dulbecco’s modified Eagle’s medium containing 2.5% (v/v) fetal calf serum and 2.5% (v/v) newborn calf serum and were transiently transfected using the calcium phosphate–DNA co-precipitation method as previously described (16). Transfected cells were incubated for 20 h in serum-free Dulbecco’s modified Eagle’s medium prior to harvesting.

Human HeLa cervical carcinoma cells were grown to 90% confluence in T150 flasks in Dulbecco’s modified Eagle’s medium containing 10% (v/v) calf serum and were replated the day before use into 55-cm² culture dishes. Attached cells were then transiently transfected using the calcium phosphate–DNA co-precipitation method as previously described (16). In some experiments (Figs. 6 and 8) the reporter gene construct (15 µg) and an expression vector for β-galactosidase (2.5 µg) were co-transfected with an expression vector encoding the insulin receptor (5 µg), courtesy of Dr. Jonathan Whittaker. After an overnight incubation the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium containing 2.5% (v/v) calf serum and 2.5% (v/v) newborn calf serum and were transiently transfected using the calcium phosphate–DNA co-precipitation method as previously described (16). Transfected cells were incubated for 20 h prior to harvesting. For the analysis of basal gene expression, three independent preparations of each plasmid construct were analyzed in duplicate.

CAT and β-Galactosidase Assays—Transfected HeLa and H41IE cells were harvested by trypsin digestion and then sonicated in 300 µl of 250 mM Tris (pH 7.8) containing 2 mM phenylmethylsulfonyl fluoride. The HeLa cell lysate was assayed for β-galactosidase activity as previously described (16). The remaining HeLa cell lysate and the H41IE lysate were heated for 10 min at 65 °C and cellular debris was removed by centrifugation. CAT assays were then performed on the supernatant as previously described (16). To correct for variations in HeLa cell transfection efficiency, the results were expressed as the ratio of CAT: β-galactosidase activity. In earlier studies we had found that phorbol esters and insulin stimulated Rous sarcoma virus-β-galactosidase expression in HeLa cells (16) but that was not apparent in this series of experiments. To correct for variations in H41IE cell transfection efficiency, CAT activity was corrected for the protein concentration in the cell lysate, as measured by the Pierce BCA assay.

Cell Retardation Assays—To study AP-1 binding, the preparation of HeLa cell nuclear extracts, the labeling of double-stranded oligonucleotide probes, and gel retardation assays were performed under conditions exactly as previously described (16, 28). Gel retardation competition experiments and partial proteolytic clipping bandshift assays were also performed as previously described (16).

RESULTS

The ME AP-1 Motif Markedly Enhances Basal Fusion Gene Transcription in Both HeLa and H41IE Cells—We have previously shown that insulin stimulates collagenase-CAT and ME-CAT fusion gene expression in H41IE cells but insulin only stimulates collagenase-CAT and not ME-CAT fusion gene expression in HeLa cells (16, 17). In addition, we found that phorbol esters stimulate collagenase-CAT but not ME-CAT fusion gene expression in HeLa cells (16). We previously suggested that these results may be explained, in part, by the observation that the ME and collagenase AP-1 motifs are functionally distinct (16). Thus, transient transfection experiments in HeLa cells using heterologous TKCAT fusion genes showed that AP-1 binds the ME AP-1 motif, but not the collagenase AP-1 motif, in an activated state. As a consequence, only the collagenase AP-1 motif confers an additional stimulatory effect on phorbol esters on the expression of a heterologous TKCAT fusion gene (16). In these experiments multiple copies of a double stranded oligonucleotide representing the ME AP-1 motif were ligated into the polylinker of a heterologous TKCAT fusion gene and this resulted in a marked increase in basal fusion gene expression, relative to that obtained with the basic TKCAT vector alone (16). This marked increase in basal fusion
gene expression was selectively seen with the ME AP-1 motif because ligation of multiple copies of a double stranded oligonucleotide representing the collagenase AP-1 motif into the TKCAT polylinker resulted in only a small increase in basal fusion gene expression (16). In contrast, when multiple copies of a double stranded oligonucleotide representing a mutated ME AP-1 motif, which fails to bind AP-1 in gel retardation assays, were ligated into the TKCAT polylinker there was no increase in basal fusion gene expression, relative to that obtained with the basic TKCAT vector alone (16).

To extend these observations, we first determined whether the marked stimulation of basal fusion gene expression by the ME AP-1 motif was specific to the context of the heterologous TKCAT vector. Therefore, experiments similar to those described above were repeated using a different heterologous promoter sequence, designated TKC-VI (24). The TKCAT vector contains the herpes simplex virus TK promoter sequence from position −105 to +51 ligated to the CAT reporter gene and has a unique BamHI site in the polylinker at −105 (23). By contrast, the TKC-VI plasmid contains the herpes simplex virus-TK promoter sequence from −480 to +51 ligated to the CAT gene and has a unique BamHI linker between positions −40 and −35 (24). Various double stranded oligonucleotides representing distinct regions of the ME or collagenase promoters (Table I) were synthesized with BamHI compatible ends and were ligated as single copies, in the same orientation as that found in the endogenous ME and collagenase promoters, into BamHI-cleaved TKC-VI. Typically, a maximal oligonucleotide size of 42 bp can be cloned into the BamHI site of the TKC-VI promoter in the same orientation as that in the native ME and collagenase promoters. Following transfection cells were incubated for 20 h in serum-free medium. Cells were then harvested and CAT activity, β-galactosidase activity, and protein concentration were assayed as previously described (16, 17). The results are expressed as the ratio of CAT:β-galactosidase activity, in HeLa cell transfections, or CAT:protein concentration, in H4IIE cell transfections. Results represent the mean ± S.E. of three experiments, using an independent preparation of each plasmid construct, in which each construct was assayed in duplicate.

| Construct | TKC-VI | ME WT | ME WT | ME WT | ME WT | Coll WT |
|-----------|--------|-------|-------|-------|-------|---------|
| CAT/βGal. Expression | | | | | | |
| (Fold Increase Relative to TKC-VI) | | | | | | |
| FIG. 1. The ME AP-1 motif markedly enhances basal TKC-VI gene transcription in both HeLa and H4IIE cells. HeLa (panel A) and H4IIE cells (panel B) were transiently transfected, as described under “Experimental Procedures,” with various TKC-VI fusion gene plasmids. In addition, HeLa cells were co-transfected with an expression vector encoding β-galactosidase. The fusion gene plasmids represented either the basic TKC-VI vector or constructs in which a single copy of oligonucleotides representing the indicated wild-type (WT) ME or collagenase (Coll) promoter sequences, as shown in Table I, had been ligated into the BamHI site of the TKC-VI promoter in the same orientation as that in the native ME and collagenase promoters. Following transfection cell lysates were incubated for 20 h in serum-free medium. Cells were then harvested and CAT activity, β-galactosidase activity, and protein concentration were assayed as previously described (16, 17). The results are expressed as the ratio of CAT:β-galactosidase activity, in HeLa cell transfections, or CAT:protein concentration, in H4IIE cell transfections. Results represent the mean ± S.E. of three experiments, using an independent preparation of each plasmid construct, in which each construct was assayed in duplicate.

| Table I Sequence of oligonucleotides used in these studies |
| All nucleotide positions are negative and are numbered relative to the transcription start site at +1. The consensus Sp1, Efr-1, and AP-1 motifs are boxed. Non-wild-type sequence is shown in lower case letters. WT, wild-type; MUT, mutant. |

| Oligonucleotide | ME WT | ME WT | ME WT | ME WT | Coll WT |
|----------------|-------|-------|-------|-------|---------|
| ME 138/101 WT | | | | | |
| ME 138/123 WT | CCGCGGCGACTCATGGCG |
| ME 138/123 MUT1 | CAGTGGTACTCATGGCG |
| ME 138/123 MUT2 | CCGCGGCGACTCATGGCG |
| ME 138/123 MUT3 | CCGCGGCGACTCATGat |
| Coll 63/78 WT | | | | | |
| Coll 63/78 MUT | | | | | |

| Oligonucleotide | ME WT | ME WT | ME WT | ME WT | Coll WT |
|----------------|-------|-------|-------|-------|---------|
| ME 138/123 WT | | | | | |
| ME 138/123 MUT1 | CaattGTACTCATGGCG |
| ME 138/123 MUT2 | CCGCGGCGACTCATGGCG |
| ME 138/123 MUT3 | CCGCGGCGACTCATGat |
| Coll 63/78 WT | | | | | |
| Coll 63/78 MUT | | | | | |

FIG. 1. The ME AP-1 motif markedly enhances basal TKC-VI gene transcription in both HeLa and H4IIE cells. HeLa (panel A) and H4IIE cells (panel B) were transiently transfected, as described under “Experimental Procedures,” with various TKC-VI fusion gene plasmids. In addition, HeLa cells were co-transfected with an expression vector encoding β-galactosidase. The fusion gene plasmids represented either the basic TKC-VI vector or constructs in which a single copy of oligonucleotides representing the indicated wild-type (WT) ME or collagenase (Coll) promoter sequences, as shown in Table I, had been ligated into the BamHI site of the TKC-VI promoter in the same orientation as that in the native ME and collagenase promoters. Following transfection cell lysates were incubated for 20 h in serum-free medium. Cells were then harvested and CAT activity, β-galactosidase activity, and protein concentration were assayed as previously described (16, 17). The results are expressed as the ratio of CAT:β-galactosidase activity, in HeLa cell transfections, or CAT:protein concentration, in H4IIE cell transfections. Results represent the mean ± S.E. of three experiments, using an independent preparation of each plasmid construct, in which each construct was assayed in duplicate.
relative to that obtained with the basic TKC-VI vector alone. Interestingly, an oligonucleotide representing the ME promoter sequence between -181 and -145, which contains overlapping Egr-1 and Sp protein-binding sites (Table I), only conferred an increase in basal fusion gene expression in HeLa and not H4IIE cells, relative to that obtained with the basic TKC-VI vector alone (Fig. 1). The potential significance of this observation to cell type-specific, insulin-stimulated ME gene expression is described under “Discussion.”

**Mutation of the Flanking Sequence Either 5’ or 3’ of the ME AP-1 Motif Markedly Reduces the Enhancement of Basal TKC-VI Gene Transcription in Both HeLa and H4IIE Cells without Affecting AP-1 Binding Affinity**—The selective activation of basal fusion gene expression by the ME and not the collagenase AP-1 motif was surprising because the AP-1 complex binding both the ME and collagenase AP-1 motifs in HeLa cells (16) and H4IIE cells (data not shown) is predominantly a heterodimer of Fra-2 and JunD. However, partial proteolytic clipping bandshift assays indicated that AP-1 binds the ME and collagenase AP-1 motifs in distinct conformations so we postulated that this could explain the distinct functional characteristics of the two AP-1 motifs (16). Surprisingly, the ME and collagenase AP-1 motifs both share an identical core consensus sequence, TGACTCA (Table I). However, the 5’ and 3’ sequences flanking the core are distinct (Table I). To determine whether the distinct AP-1-flanking sequence could explain the discrete functional characteristics of the ME and collagenase AP-1 motifs, the effect of mutating this flanking sequence was investigated.

Double stranded oligonucleotides representing the ME AP-1 motif but containing mutations of the 5’ (MUT1), core (MUT2), or 3’-flanking sequence (MUT3) (Table I) were synthesized with BamHI compatible ends and were ligated as single copies into BamHI-cleaved TKC-VI in either the same or the inverted orientation relative to that found in the endogenous ME promoter. The level of reporter gene expression directed by the resulting constructs was then analyzed by transient transfection of HeLa (Fig. 2, A and C) and H4IIE (Fig. 2B) cells. Similar results were obtained in both cell lines (compare Fig. 2, A and B) and with both orientations of the ME and collagenase AP-1 motifs (compare Fig. 2, A and C). The MUT2 oligonucleotide that contains a mutation of the core sequence of the ME AP-1 motif, which abolishes binding of AP-1 in gel retardation assays (16) (Fig. 3), failed to confer an increase in basal TKC-VI fusion gene expression (Fig. 2). Similarly, mutating the 3’-flanking sequence of the ME AP-1 motif resulted in markedly reduced basal TKC-VI fusion gene expression compared with that conferred by the wild-type ME AP-1 motif (Fig. 2). Similarly, mutation of the 5’-flanking sequence also reduced the activation of basal fusion gene expression, although it was less deleterious than the 3’-flanking sequence mutation (Fig. 2).

These results could potentially be explained by an effect of the flanking sequence mutations on the affinity of AP-1 binding. To investigate this possibility the oligonucleotides shown in Table I were used as competitors, at a 100-fold molar excess, in a gel retardation assay with the ME 138/123 WT oligonucleotide as the labeled probe (Fig. 3A). As expected, the wild-type collagenase AP-1 motif and the oligonucleotides containing the wild-type ME AP-1 motif, namely ME 161/123 WT, ME 138/101 WT, and ME 138/123 WT, all competed effectively against the labeled probe for formation of the AP-1 protein-DNA complex (Fig. 3A). In contrast, the ME 181/145 WT oligonucleotide that does not contain the ME AP-1 motif (Table I), and the ME 138/123 MUT2 oligonucleotide that contains a mutation of the AP-1 core sequence (Table I), did not compete for AP-1 binding (Fig. 3A). Importantly, the oligonucleotides containing the 5’ or 3’ ME AP-1 flanking sequence mutations, designated ME 138/123 MUT1 and MUT3, respectively, both competed effectively against the labeled probe for formation of the AP-1 protein-DNA complex when used at a 100-fold molar excess (Fig. 3A). Moreover, competition experiments in which the labeled ME 138/123 WT oligonucleotide was preincubated with various concentrations of the unlabeled ME 138/123 WT, MUT1, and MUT3 oligonucleotides indicated that these mutations do not markedly affect the affinity of AP-1 binding (Fig. 3B). Thus, all three oligonucleotides competed equally effectively for formation of the AP-1 complex (Fig. 3B).

**Mutation of the Flanking Sequence Either 5’ or 3’ of the ME AP-1 Motif Markedly Reduces the Enhancement of Basal TKCAT Gene Transcription and Restores Phorbol Ester Responsiveness**—To determine whether the effects of mutating the flanking sequence on either side of the ME AP-1 motif were specific to the context of the heterologous TKC-VI vector, experiments similar to those shown in Fig. 2 were repeated using the heterologous TKCAT vector. Multiple copies of the double stranded oligonucleotides containing the various mutations of the ME AP-1 motif described above were ligated into the polylinker of the TKCAT vector. The level of reporter gene expression directed by the resulting constructs in the presence and absence of a phorbol ester, PMA, was then analyzed by transient transfection of HeLa cells (Fig. 4).

Oligonucleotides representing the wild-type ME promoter sequence between -161 and -123 or between -138 and -123, both of which encompass the ME AP-1 motif, both conferred a marked activation of basal fusion gene expression (Fig. 4B) but neither oligonucleotide was able to confer a stimulatory effect of PMA on fusion gene expression beyond that seen with the basic TKCAT vector (Fig. 4A). With the multimerized ME 138/123 WT oligonucleotide the spacing between individual AP-1 motifs was similar to that obtained with the multimerized collagenase 63/78 WT oligonucleotide (Table I). The latter does confer a phorbol ester response (Fig. 4A) but not an activation of basal fusion gene expression (Fig. 4B). Thus, the inability of the longer ME 161/123 WT oligonucleotide (Table I) to confer a phorbol ester response was not indicative of an inability of the individual AP-1 motifs in the multimerized oligonucleotide to synergize because of increased spacing between individual AP-1 sites.

As seen in the context of the TKC-VI vector, mutation of the 5’- or 3’-flanking sequence of the ME AP-1 motif reduced the activation of basal TKCAT fusion gene expression, with mutation of the 3’-flanking sequence again being more deleterious (Fig. 4B). Importantly, in contrast to the wild-type ME AP-1 motif, these mutated ME AP-1 motifs were now able to confer a stimulatory effect of phorbol esters on fusion gene expression that was similar in magnitude, when the data was expressed as fold induction, to that obtained with the collagenase AP-1 motif (Fig. 4A). Fig. 4C shows that the wild-type ME and collagenase AP-1 motifs conferred a similar level of maximal, phorbol ester-stimulated fusion gene expression. Thus, the level of phorbol ester-stimulated collagenase AP-1 TKCAT fusion gene expression was similar to that of basal ME AP-1 TKCAT gene expression. Whereas the mutated ME AP-1 motifs were able to confer a stimulatory effect of phorbol esters on fusion gene expression (Fig. 4A) only the ME 5’-flanking mutant directs a maximal level of CAT expression similar to that obtained with the wild-type ME and collagenase AP-1 TKCAT fusion genes (Fig. 4C). This suggests that even phorbol ester treatment was unable to fully activate AP-1 bound to the ME AP-1 3’-flanking mutant. Unfortunately, in HeLa cells, unlike H4IIE cells (16), insulin markedly stimulates CAT expression directed by the control TKCAT fusion gene (data not shown). Therefore, it was not
possible to determine whether insulin can also selectively activate gene transcription through the collagenase but not the ME AP-1 motif, in the context of the TKCAT vector. Mutation of the Flanking Sequence of the ME AP-1 Motif Affects the Conformation of AP-1 Binding

The proteolytic band shift assay (29) was used to examine the possibility that the ME AP-1- and 3'-flanking mutations affected the conformation of AP-1 binding. As described above, we previously used this assay to demonstrate that AP-1 bound to the wild-type collagenase and ME AP-1 motifs have different surfaces exposed to proteolytic digestion indicative of a difference in binding conformation (16). This difference in binding conformation was hypothesized to be the basis for the selective activation of basal TKCAT fusion gene expression by the ME AP-1 motif (16).

To study the effect of partial protease digestion, HeLa cell nuclear extract was preincubated with the labeled collagenase 63/78 WT, ME 138/123 WT, ME 138/123 MUT1, or ME 138/123 MUT3 oligonucleotides (Table I) prior to the addition of various concentrations of chymotrypsin (Fig. 5). A distinct proteolytic product that selectively binds the collagenase 63/78 WT, ME

FIG. 2. Mutation of the flanking sequence either 5' or 3' of the ME AP-1 motif markedly reduces the enhancement of basal TKCAT gene transcription in both HeLa and H4IIE cells. HeLa (panels A and C) and H4IIE cells (panel B) were transiently transfected, as described under "Experimental Procedures," with various TKCAT fusion gene plasmids. In addition, HeLa cells were co-transfected with an expression vector encoding β-galactosidase. The fusion gene plasmids represented either the basic TKCAT vector or constructs in which oligonucleotides representing the indicated wild-type (WT) or mutated (MUT) ME or collagenase promoter sequences, as shown in Table I, had been ligated into the BamHI site of the TKCAT promoter in a single copy in either the same (correct; panels A and B) or inverted (panel C) orientation relative to that in the native ME and collagenase promoters. Following transfection cells were incubated for 20 h in serum-free medium. Cells were then harvested and CAT activity, β-galactosidase activity, and protein concentration were assayed as previously described (16, 17). The results are expressed as the ratio of CAT:β-galactosidase activity, in HeLa cell transfections, or CAT/protein concentration, in H4IIE cell transfections. Results represent the mean ± S.E. of three experiments, using an independent preparation of each plasmid construct, in which each construct was assayed in duplicate.

FIG. 3. Mutation of the flanking sequence 5' or 3' of the ME AP-1 motif does not affect the affinity of AP-1 binding. Panel A, the labeled ME 138/123 WT oligonucleotide probe was incubated in the absence (−) or presence of a 100-fold molar excess of the unlabeled oligonucleotide competitors shown (Table I) prior to addition of HeLa cell nuclear extract. Protein binding was then analyzed using the gel retardation assay as described under "Experimental Procedures." In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. A nonspecific (NS) protein-DNA interaction is indicated by an arrow as is the AP-1 complex. Panel B, the labeled ME 138/123 WT oligonucleotide probe was incubated in the absence (−) or presence of various concentrations of the unlabeled ME 138/123 WT (■), ME 138/123 MUT1 (▲), and ME 138/123 MUT3 (●) oligonucleotide competitors prior to addition of HeLa cell nuclear extract. Protein binding was then analyzed using the gel retardation assay as described under "Experimental Procedures." Protein binding was quantified by using a Packard Instant Imager to count 32P associated with retarded complexes. The data represents the mean ± S.D. of two experiments.

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138/123 MUT1, and ME 138/123 MUT3 oligonucleotide probes but not the ME 138/123 WT oligonucleotide probe was detected (Fig. 5, see arrow). This selectively bound product migrates faster than a nonspecific protein-DNA interaction detected in this assay (Fig. 5) so it was possible that this product was derived from proteolysis of the nonspecific protein-DNA interaction rather than the AP-1 complex. However, competition experiments revealed that the unlabeled wild-type collagenase 63/78 WT oligonucleotide competed effectively for the formation of this protein-DNA complex (data not shown), whereas it does not compete for formation of the nonspecific complex (Fig. 3A). This result demonstrates that the proteins bound to the wild-type collagenase AP-1 motif and the ME AP-1 5′/H11032- and 3′/H11032-flanking mutants have similar surfaces exposed to proteolytic digestion, indicative of similar binding conformations. Thus, the ME AP-1 5′/H11032- and 3′/H11032-flanking mutants bind AP-1 in a conformation more similar to that of AP-1 bound to the collagenase AP-1 motif rather than AP-1 bound to the wild-type ME AP-1 motif. These observations could explain why the oligonucleotides containing the ME AP-1 5′- or 3′-flanking mutations, just like the collagenase AP-1 motif, do not enhance basal TKC-VI (Fig. 2) or TKCAT (Fig. 4) fusion gene expression, but do mediate a phorbol ester response (Fig. 4).

The Wild-type ME AP-1 Motif Can Confer a Stimulatory Effect of Insulin and Phorbol Esters on the Expression of a Heterologous Xenopus Albumin-CAT Fusion Gene—The basic heterologous TKC-VI (Fig. 1) and TKCAT (Fig. 4) vectors both direct a high level of basal CAT expression, even without the ME AP-1 motif ligated into their respective polylinkers. In contrast, a heterologous Xenopus albumin-CAT fusion gene, designated XMB, has previously been shown to direct no basal CAT expression in HeLa cells (17). The ME and collagenase AP-1 motifs were therefore ligated into the polylinker of the XMB vector to determine whether, in this context, the functional characteristics of the two AP-1 motifs would be distinct. Fig. 6 shows that, in the context of the XMB vector, the ME AP-1 motif still confers a greater increase in basal fusion gene expression than the collagenase AP-1 motif.
expression than the collagenase AP-1 motif. However, in this context, both the ME and collagenase AP-1 motifs can mediate both a phorbol ester (Fig. 6A) and insulin (Fig. 6B) response in HeLa cells. When ligated into the XMB polylinker, oligonucleotides containing mutations of the ME or collagenase AP-1 motifs (Table I), which abolish AP-1 binding (16, 17), fail to confer basal reporter gene expression or an increase in expression in the presence of insulin or phorbol esters (Fig. 6). Thus, this result suggests that the inability of insulin to induce ME-CAT gene expression in HeLa cells, in contrast to H4IE cells, was because of some cell type-specific feature relating to the specific context of the ME promoter and not a difference in the insulin signaling pathway in these two cell lines. Similarly, this result further suggests that the inability of phorbol esters to induce ME-CAT gene expression in HeLa cells was also because of the same issue of ME promoter context. Indeed, both insulin and phorbol esters induce AP-1 binding to both the collagenase and ME AP-1 motifs in HeLa cells (Fig. 7). In summary, the data in Figs. 4 and 6 suggest that the functional characteristics of the ME and collagenase AP-1 motifs, with respect to basal activation and insulin/phorbol ester responsiveness, are determined by both flanking sequence and promoter context.

The ME AP-1 Motif Can Mediate an Insulin and Phorbol Ester Response in the Context of the Collagenase Promoter—Because the multimerized ME AP-1 motif was able to mediate both an insulin and phorbol ester response in the context of the heterologous XMB vector (Fig. 6) the molecular basis for the inability of insulin and phorbol esters to stimulate the activity of the native ME promoter in HeLa cells was further investigated. Fry and Farnham (30) recently reviewed various aspects of promoter context that are important in the regulation of gene transcription, one of which are the presence of accessory elements. We have previously shown that the stimulatory effects of insulin and phorbol esters on collagenase-CAT fusion gene expression are markedly enhanced by accessory elements in the collagenase promoter (17). Expression of a truncated collagenase fusion gene construct that contains the AP-1 motif but lacks these accessory elements was minimally induced by insulin and phorbol esters (17). We therefore speculated that perhaps the ME promoter lacks accessory elements that could enhance insulin and phorbol ester signaling through the ME AP-1 motif in HeLa cells.

To indirectly address this potential role for the absence of accessory elements in the ME promoter, a collagenase-CAT fusion gene was generated in which the flanking sequence of the collagenase AP-1 motif was replaced with that of the ME AP-1 motif in the context of a collagenase promoter fragment with a 5’ end point of −158. This fragment contains the accessory elements necessary for full induction of gene expression by insulin and phorbol esters (17). This strategy allowed us to ask the question as to whether the ME AP-1 motif could mediate an insulin and phorbol ester response if it were associated with accessory elements and located in the same context as the collagenase AP-1 motif. The effects of insulin and phorbol esters on the expression of this fusion gene, designated Coll 158;ME, were assessed by transient transfection of HeLa cells (Fig. 8).

One possible outcome of this experiment was that the ME AP-1 motif could have maximally activated basal collagenase-CAT fusion gene expression such that no effect of insulin and phorbol esters would be seen despite the presence of accessory elements. But in fact the data shows that the presence of the ME AP-1 motif in the collagenase promoter actually led to a decrease in basal fusion gene expression (Fig. 8A), and in this

![Fig. 6. The wild-type ME AP-1 motif can confer a stimulatory effect of insulin and phorbol esters on the expression of a heterologous Xenopus albumin-CAT fusion gene. HeLa cells were transiently co-transfected, as described under “Experimental Procedures,” with various XMB fusion gene plasmids and an expression vector encoding β-galactosidase. The fusion gene plasmids represented either the basic XMB vector or constructs in which oligonucleotides representing either the wild-type (WT) or mutated (MUT) ME or collagenase (Coll) promoter sequence from −138 to −123 and −63 to −78, respectively, as shown in Table I, had been ligated into the HindIII site of the Xenopus albumin promoter in multiple (4 to 5) copies. Following transfection cells were incubated for 20 h in serum-free medium in the presence of 100 nM PMA (P) or 100 nM insulin (I). Cells were then harvested and CAT and β-galactosidase activity were asayed as previously described (16, 17). The results are expressed as the ratio of CAT/β-galactosidase activity and represent the mean ± S.E. of six experiments, in which each construct was assayed in duplicate.

![Fig. 7. Insulin and phorbol esters stimulate protein binding to both the collagenase and ME AP-1 motifs. Nuclear extracts were prepared from HeLa cells incubated for 5 h in serum-free medium (C) or serum-free medium supplemented with either 100 nM insulin (I) or 100 nM PMA (P). Protein binding to the labeled ME 138/123 and collagenase (Coll) 63/78 oligonucleotide probes was then analyzed using the gel retardation assay, as described under “Experimental Procedures.” In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. A nonspecific (NS) protein-DNA interaction and the AP-1 complex are indicated by the arrows.](image-url)
The ME AP-1 motif can mediate an insulin and phorbol ester response in the context of the collagenase promoter. HeLa cells were transiently co-transfected, as described under “Experimental Procedures,” with an expression vector encoding β-galactosidase and either collagenase-CAT fusion genes with 5' deletion end points of −158 or −79, designated Coll 158 and Coll 79, respectively, or constructs, designated Coll 158:ME and Coll 79:ME, in which the collagenase AP-1 flanking sequence was replaced with that of the ME AP-1 motif within the context of the −158 or −79 end points, respectively. Following transfection cells were incubated for 20 h in serum-free medium in the absence or presence of 100 nM PMA or 100 nM insulin. Cells were then harvested and CAT and β-galactosidase activity were assayed as previously described (16, 17). The ratio of CAT/β-galactosidase activity in control cells (panel A) and the relative ratio of CAT/β-galactosidase activity in insulin-treated cells (panel B) or phorbol ester-treated cells (panel C) versus control cells were then calculated. The mean induction of Coll 158 expression by insulin and phorbol ester was ~14- and 43-fold, respectively. The results are presented as a percentage relative to the 158 Coll fusion gene and represent the mean ± S.E. of three to seven experiments, using several independent preparations of each plasmid construct, in which each construct was assayed in duplicate.

Discussion

The experiments in this article were designed to explore the molecular basis for: (i) the differential regulation of collagenase-1 and ME gene expression by insulin and phorbol esters in HeLa cells and (ii) the differential regulation of ME gene expression by insulin in H4IIE and HeLa cells. We hypothesize that the former is partly explained by the observation that the ME and collagenase AP-1 motifs are functionally distinct (16). Thus, AP-1 can bind the ME AP-1 motif, but not the collagenase AP-1 motif in an activated state and, in a heterologous context, this precludes further activation by phorbol esters. This observation was surprising because both motifs share an identical core consensus sequence (Table I) and predominantly bind a heterodimer of Fra-2 and JunD (16) with similar affinities (Fig. 3). We show here that this binding of AP-1 to the ME AP-1 motif in an activated state is determined by the specific sequence flanking the core AP-1 motif (Figs. 2 and 4). Phorbol ester-insensitive AP-1 motifs have also been identified in the stromelysin (31), JE (32), and glutathione S-transferase P1-1 promoters (33, 34) and it has also been previously shown that the sequence flanking the core AP-1 motif can influence phorbol ester responsiveness (35, 36). However, this has been attributed to changes in the affinity of AP-1 binding and/or the composition of the AP-1 complex (32, 37). The ME promoter is therefore distinct in that neither of the latter parameters differs in comparison with the phorbol ester-sensitive collagenase AP-1 motif (Fig. 3) (16). Instead, the flanking sequence of the ME AP-1 motif appears to affect phorbol ester responsiveness by altering the conformation of AP-1 binding (Fig. 5). Thus, these studies on the ME AP-1 motif are consistent with the emerging realization that hormone response elements are not inert but can act as allosteric regulators by affecting the conformation of the factors they bind (38).

The specific functional characteristics of the ME AP-1 motif are also affected by promoter context. Thus, when ligated to the heterologous XMB promoter, even though the ME AP-1 motif stimulates basal fusion gene expression, it does not do so sufficiently to prevent a further induction by phorbol esters and insulin (Fig. 6). Similarly, when switched with the collagenase AP-1 motif in the collagenase promoter, the ME AP-1 motif can again mediate both an insulin- and phorbol ester-dependent response in HeLa cells (Fig. 8). The specific context-dependent features of the XMB promoter that allow for insulin- and phorbol ester-dependent activation of the ME AP-1 motif are unclear. However, in the collagenase promoter, one critical context-dependent characteristic is the presence of accessory elements; the effects of insulin and phorbol esters are markedly reduced if these accessory elements are deleted (17) (Fig. 8). It is possible that the differential regulation of ME gene expression by insu-
lin in H4IIE and HeLa cells may also, in part, reflect the importance of an accessory element. We have previously shown that, in H4IIE cells, an accessory element, located between −180 and −152, enhances insulin signaling through the ME AP-1 motif (16). This region of the ME promoter contains overlapping binding sites for Egr-1 and Sp proteins (Table I), however, using nuclear extract prepared by the method of Shapiro et al. (28), only the insulin-induced binding of Egr-1 was detected; we therefore proposed that Egr-1 was the accessory factor binding this element (16). Using nuclear extract prepared by the method of Andrews and Faller (39), modified by the incorporation of Nonidet P-40 to lyse cells and isolate nuclei (40), the insulin-induced binding of both Egr-1 and Sp factors to this accessory element can be demonstrated. Gel retardation assays reveal an inverse relationship between the abundance of insulin-induced Egr-1 and Sp proteins in H4IIE and HeLa cells with Egr-1 more abundant than Sp proteins in the former. Therefore, we hypothesize that the selective regulation of ME gene expression in H4IIE and HeLa cells may be explained, at least in part, by the differential binding of these factors to the same accessory element in the ME promoter in the two cell types.

Competition between Egr-1 and Sp proteins for overlapping binding sites is known to be important in the regulated expression of other genes (41). Barroso and Santisteban (18) have shown that Egr-1 competes for Sp1 binding in the ME promoter but it remains to be determined whether Egr-1 or a specific Sp protein is the true accessory factor that enhances insulin signaling through AP-1. This is a complex question because interactions between the accessory proteins and AP-1, although the latter do have been reported to contribute to the functional specificity of wide variety of structurally unrelated transcription factors signaling through AP-1. This is a complex question because interactions between the accessory proteins and AP-1, although the latter do have been reported to contribute to the functional specificity of insulin action, might also activate SREBP-1a and SREBP-2 (53). SREBP-1c has also been implicated in the induction of pyruvate kinase and fatty acid synthase gene expression by glucose (13), although this may represent an indirect effect of SREBP-1c on glucose flux, resulting from its stimulation of gluconeogenic gene expression (54). This potential connection between SREBP-1c and glucose-regulated gene expression is interesting because there is some controversy as to the exact relationship between insulin and glucose in the stimulation of ME gene expression (55–57). One report suggests that insulin has little or no direct effect but has a permissive action on the response to glucose (55). By contrast, other investigators have reported that insulin has a direct effect in the absence of glucose (56, 57). Clearly, it will be of interest to delineate the relative contributions of insulin, glucose, AP-1, and SREBP in the regulation of ME gene expression in vivo.

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