A Consensus Insulin Response Element Is Activated by an ETS-related Transcription Factor*

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Insulin increases expression of somatostatin-chloramphenicol acetyltransferase (CAT) constructs 10-fold and thymidine kinase-CAT constructs 5-fold in GH4 cells. These responses are similar to our previously reported data on insulin-increased prolactin-CAT expression. They are also observed in HeLa cells and are thus not cell type specific. The evidence suggests that the insulin responsiveness of these genes is mediated by an Ets-related transcription factor. First, linker-scanning mutations and/or deletions of the prolactin, somatostatin, and thymidine kinase promoters suggest that their insulin responsiveness is mediated by the sequence CGGA. This sequence is identical with the response element of the Ets-related transcription factors. Second, CGGA-containing sequences placed at −88 in the ∆MTV-CAT reporter plasmid conferred insulin responsiveness to the mammary tumor virus promoter. Third, expression of the DNA-binding domain of c-Ets-2, which acts by blocking effects mediated by Ets-related transcription factors, inhibits the response of these promoters to insulin. Finally, the Ets-related proteins Sap and Elk-1 bind to the prolactin, somatostatin, and thymidine kinase insulin-response elements. An Ets-like element was found in all insulin-sensitive promoters examined and may serve a similar function in those promoters.

The mechanisms involved in the regulation of gene expression by hormones are not well characterized. Insulin-induced alterations in the steady-state levels of numerous mRNAs have been documented (1). For several genes including phosphoenolpyruvate carboxykinase (2), glyceraldehyde 3-phosphate dehydrogenase (3), growth hormone (4), and prolactin (5), it has been established that these alterations are due to effects of insulin on the rate of transcription and not to effects on mRNA half-life or processing (1). The effects of hormones on transcription are mediated through response elements in the promoters of genes. Several response elements may exist for a particular hormone that differs slightly in sequence or orientation, perhaps to allow for fine tuning of the hormonal response or for interaction with different tissue-specific factors. However, these elements are sufficiently similar to be recognized by the hormone-activated transcription factors and are thus said to form a consensus response element. The consensus hormone response element for cAMP-activated genes is the sequence TGACGTC and the sequence AGGTCA with varying numbers of intervening bases that determine hormone receptor specificity (7). A specific sequence comprising an insulin response element has been identified only for a small proportion of insulin-responsive genes. Comparison of these insulin response elements has not revealed sequence homologies that could constitute a consensus insulin response element (8).

The transcription factors that mediate responses to insulin have not been characterized. The putative insulin response element of the glyceraldehyde 3-phosphate dehydrogenase gene was identified by binding of an insulin-regulated protein to a specific sequence in the glyceraldehyde 3-phosphate dehydrogenase promoter. A protein that binds to this sequence has been cloned and is identical to the product of the testis determining gene, SRY (9). However, its role in the activation of glyceraldehyde 3-phosphate dehydrogenase gene expression by insulin was not further established.

We previously identified an insulin response element in the prolactin promoter (8). This insulin response element overlaps the cAMP response element of the prolactin gene TGACGGA. However, mutagenesis and deletional analysis revealed that the insulin response element was separable from the cAMP response element and consisted of a direct repeat of the sequence CGGAAA. This sequence is identical to sequences that bind the Ets family of transcription factors. These studies report the identification of insulin response elements in the somatostatin and herpes simplex virus thymidine kinase genes that, together with the previously identified insulin response element of the prolactin gene, define a consensus insulin response element. The consensus sequence is identical to known binding sites for Ets-related transcription factors, and an Ets factor inhibitor was found to inhibit insulin activation of all three promoters. The Ets-related proteins Sap-1 and Elk-1 specifically bind to these sequences.

EXPERIMENTAL PROCEDURES

Materials—[32P]dCTP, 3000 Ci/mmol, and [14C]chloramphenicol, 50 mCi/mmol, were obtained from ICN Biochemical Corp. Reagents and Taq polymerase for polymerase chain reaction were from Cetus. All other enzymes and linkers were obtained from either New England Biolabs or from Boehringer Mannheim and, unless otherwise indicated, were used under conditions recommended by the suppliers. Oligonucleotides were purchased from Operon. Duplex poly(dI-dC) was obtained from Pharmacia Biotech Inc. Reagents used for gel electrophoresis were purchased from Fisher. Acetyl-CoA and silica gel plates for thin layer chromatography were obtained from Sigma. Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (DMEM) was from Life Technologies, Inc., and iron-supplemented calf serum was obtained from Hydcono Laboratories. Triton X-100 and BCA reagent were from Pierce. All other reagents were of the highest purity available and were obtained from Sigma, Behring Diagnostics, Bio-Rad, Eastman, Fisher, or Boehringer Mannheim.

1 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; SRF, serum response factor; bp, base pair(s); TK, thymidine kinase; SS, somatostatin.

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Plasmids—The construction of pPr1-CAT plasmids containing 173/+75 of prolactin 5′-flanking DNA was described (5). The vector pBLCAT2 (10) was obtained from G. Schutz (Institut für Zell- und Tumobiologie, Heidelberg, Federal Republic of Germany). A plasmid containing the 5′-region of the prolactin gene from the Sprague-Dawley rat was the generous gift of Dr. J. Gorski (University of Wisconsin) (11). The rat somatostatin plasmid was the gift of Dr. D. Curran (Roche Institute, Nutley, NJ) (12). This plasmid contains three concatamered AP1 sites from the human metallothionein IIA gene fused to the SV40 early promoter. The (−846/+44)CAT was from Dr. J. L. Jameson (Case Western Reserve, Cleveland, OH) (13). This plasmid contains −846 bp of the human glycoprotein hormone α gene that contains two cAMP response elements (Pr65/57 CAT, containing cAMP-responsive sequences (−210′−142), was described previously (14). Deletion and linker-scanning mutants of the thymidine kinase promoter were a generous gift of Dr. S. L. McKnight (Tulurah, South San Francisco) (15). These were redone into pUC 8 as described (16). The plasmid TK(−95/+56)BamHI CAT was made by digesting TK(−95/+56)CAT with BamHI to linearize the plasmid at the 5′-end of the thymidine kinase promoter. This was then treated with mung bean nuclease to create flush ends, and the plasmid was recircularized by blunt end ligation. The plasmids TK(−385/+56)CAT, TK(−218/+56)CAT, and TK(−128/+56)CAT were constructed from TK(−128/+56)CAT and HindIII-NspI digestions of pBLCAT2. The plasmid TK(−385/+56)CAT was digested with BamHI, blunt ended with mung bean nuclease, and religated to form TK(−385/+56)CAT containing the 5′-flanking DNA was described (5). The vector pTK(−710)CAT and pSS(−48/+80)CAT were gifts of Dr. R. H. Goodman (Oregon Health Sciences University, Portland, OR) (6). SS(−71/−1)CAT and SS(−48/−1)CAT were prepared by polymerase chain reaction using the SS(−71/−80)CAT as a template. The universal primer served as the sense primer. The antisense primer used was (5′-GCTCTCGAGCTTCCGAGCGTGTCCTCC-3′). This adds an XhoI restriction site at −1 of the somatostatin promoter. The polymerase chain reaction product was digested with XbaI (there is an XbaI site in SS(−71/−80)CAT in the polylinker) and XhoI and then ligated into XbaI-XhoI digested and dephosphorylated pBLCAT2. The plasmid TK(−385/+56)CAT was made by ligating an 82-bp duplex oligonucleotide with G→C point mutations of each of the three potential Ets-response elements in the 5′-untranslated region of the somatostatin gene into the XhoI site of SS(−71/−1)CAT. These mutations converted three GGA motifs into GGCGGCTC, and the corresponding mutant sequence from the SS(−71/−80)CAT reporter plasmids. These were purified on polyacrylamide gels, annealed, and either end labeled with [3P]dCTP or filled in with unlabeled deoxyribonucleotides using the Klenow reaction. These labeled oligonucleotides were then used in gel mobility shift experiments performed as described (20). 2 μg of nuclear extract or 2 μl of in vitro translated and translated Sap-1 or Elk-1 were incubated at 25 °C for 30 min with 10,000 cpm (10−20 fmol) of the 32P-labeled oligonucleotide. The protein-DNA complexes were then analyzed by electrophoresis on a 5% polyacrylamide gel in Tris-acetate-EDTA buffer.

**RESULTS**

Insulin Increases Expression from the Somatostatin and the Thymidine Kinase Promoters—The observation that the insulin and cAMP response elements of the prolactin gene were composed of overlapping sequences (8) suggested that other cAMP-responsive genes might be insulin responsive. A comparison of such sequences might suggest a consensus insulin response element. We therefore tested a number of cAMP-responsive promoter-CAT constructs in GH4 cells to determine if these were also insulin responsive (Fig. 1). The Pit-1−738/−11)CAT, α(−846/+44)CAT, and (AP1)1CAT are not significantly affected by insulin incubation (Fig. 1). However, somatostatin(−71−80)CAT expression increased 8-fold in response to insulin, and TK(−95/+56)CAT expression increased 6-fold in response to insulin.
Insulin also activates expression of Prl(−173/+75)CAT, SS(−71/+80)CAT, and TK(−95/+56)CAT in HeLa cells (Fig. 2). Low levels of Prl(−173/+75)CAT expression are increased 16-fold in HeLa cells in response to insulin. SS(−71/+80)CAT is increased 10-fold and TK(−95/+56)CAT is increased 4-fold in HeLa cells in response to insulin. As in GH4 cells, the expression of CAT is not increased by insulin using plasmids containing the Pit-1 promoter, glycoprotein hormone α-subunit promoter, or the repeated AP1 element. These data indicate that the effects of insulin to increase transcription of these three genes is not unique to the GH cells.

Identification of the Insulin-responsive Sequence in Thymidine Kinase Constructs—A panel of deletion and linker-scanning mutants was used in GH4 cells to identify the insulin response element of the thymidine kinase promoter. Deletion of 5′ sequences to −46 (TK(−46/+56)CAT, Fig. 3) reduced the insulin-increased expression of CAT to 3-fold but did not eliminate it. The 3′-deletion plasmid TK(−725/−7)CAT (Fig. 3) was stimulated 6-fold by insulin, but the plasmid TK(−725/−16)CAT was not stimulated by insulin. Thus, it appeared that the insulin-responsive sequences were likely located between −46 and −7. However, linker-scanning mutants to this region (TK(−46/−36)CAT, TK(−42/−32)CAT, TK(−28/−18)CAT, TK(−21/−12)CAT, and TK(−16/−6)CAT) (Fig. 3) showed a loss of insulin-increased CAT expression only with the plasmid TK(−28/−18)CAT. This mutation deletes the TATA element and was reported to reduce basal expression from this promoter (35). Further, this deletion also eliminates the cAMP-mediated increase in thymidine kinase-CAT expression despite the absence of a cAMP response element. For example, the construct TK(−7/−3)CAT is stimulated 9.9 ± 2.3-fold by cAMP, while cAMP increases expression only 1.4 ± 0.29-fold using TK(−28/−18)CAT. Thus, this sequence does not likely represent the insulin-responsive element of the thymidine kinase gene. Comparison of the thymidine kinase gene with the insulin response element of the prolactin gene identified a sequence at −151/−146 that contained the sequence CCGAA. This sequence is present in all of the 3′-deletion mutants. The 5′-deletion mutants lack the putative wild type insulin response element, but they all have a BamHI restriction site containing the sequence CGGAA at their 5′-ends. The linker used to generate the linker-scanning mutants is also a BamHI restriction sequence that has been shown to act as a response element for the Ets-related transcription factors (24). If the BamHI site confers insulin responsiveness to the 5′-thymidine kinase-CAT deletion plasmids, then mutation of the BamHI site upstream of −95 in the plasmid TK(−95/+56)CAT should eliminate the insulin responsiveness of this plasmid. This BamHI site (CGGA) is removed in the plasmid TK(−95/+56ΔBamHI)CAT, and CAT expression is not increased by insulin using this reporter (Fig. 3). Thus, the insulin response element of the 5′-deletion mutants is the BamHI linker.

To further confirm that the BamHI linker sequence used to make the linker-scanning plasmids was sufficient to mediate the effects of insulin on thymidine kinase-CAT expression, the linker sequence from the plasmid TK(−21/−12)CAT between −22 and −2, containing the CCGAA motif, was cloned into ΔMTV-CAT in both the normal and inverted orientation to create ΔMTV(TKb)CAT and ΔMTV(TKa)CAT (Fig. 4, top). Insulin did not affect CAT expression from ΔMTV(TKa)CAT and ΔMTV(TKb)CAT that contain the −22/−2 sequence from the wild type thymidine kinase gene in both the normal and inverted orientation (Fig. 4, bottom). In contrast, insulin increases CAT expression 6-fold in ΔMTV(TKb)CAT and

**Fig. 2.** Effect of insulin on cAMP-responsive promoters in HeLa cells. HeLa cells were cotransfected with 10 μg of the CAT construct indicated in the figure and 5 μg of pRT3HIR-2. RSV-Pit-1 was also included in cotransfections with the prolactin-CAT reporter plasmid to achieve high level basal expression with this construct (30). Following a 24-h incubation without hormone, insulin was added at 1 μg/ml. After an additional 24 h of incubation, the cells were harvested, and CAT enzyme analysis was performed as in Fig. 1. Basal CAT expression/μg of protein was 0.022 ± 0.0058 for Pit-1(−738/+11)CAT, 0.27 ± 0.067% for α(−84/−44)CAT, 3.5 ± 0.023% for (AP1)3CAT, 1.1 ± 0.04% for Prl(−173/+75)CAT, 0.26 ± 0.016% for SS(−71/+80)CAT, and 0.23 ± 0.039% for TK(−95/+56)CAT.

**Fig. 3.** The effect of insulin on different thymidine kinase deletion and linker-scanning mutants in GH4 cells. GH4 cells were cotransfected with 10 μg of the thymidine kinase-CAT construct indicated in the figure and 5 μg of pRT3HIR-2. Following a 24-h incubation without hormone, insulin was added at 1 μg/ml. After an additional 24 h of incubation, the cells were harvested, and CAT enzyme analysis was performed as in Fig. 1. Basal CAT expression/μg of protein was 0.16 ± 0.05% for TK(−46/+56)CAT, 0.16 ± 0.028% for TK(−725/−7)CAT, 0.16 ± 0.04% for TK(−725/−16)CAT, 0.8 ± 0.071% for TK(−46/−36)CAT, 0.46 ± 0.15% for TK(−42/−32)CAT, 0.28 ± 0.08% for TK(−28/−18)CAT, 0.21 ± 0.04% for TK(−21/−12)CAT, 0.19 ± 0.025 for TK(−16/−6)CAT, and 0.057 ± 0.009% for TK(−95/+56ΔBamHI)CAT.
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Fig. 5. The effect of insulin on deletion mutants of the wild type thymidine kinase promoter in GH4 cells. GH4 cells were cotransfected with 10 μg of the thymidine kinase-CAT construct indicated in the figure and 5 μg of pRT3HR-2. Following a 24-h incubation without hormone, insulin was added at 1 μg/ml. After an additional 24 h of incubation, the cells were harvested, and CAT enzyme analysis was performed as in Fig. 1. Basal CAT expression/μg of protein was 0.19 ± 0.09% for TK(1s-5+/15)CAT, 0.42 ± 0.03% for TK(1s-5+/153BamH1)CAT, 1.97 ± 0.65% for TK(-385/+56)CAT, 0.19 ± 0.088% for TK(-218/+56)CAT, and 0.44 ± 0.083% for TK(-128/+56)CAT.

Fig. 4. The effect of insulin on △MTV(TK)CAT expression in GH4 cells. Top, the △MTV-CAT vector (7) was used to construct a hybrid promoter with sequences from the thymidine kinase promoter or from a linker-scanning mutant of the thymidine kinase promoter. △MTV-CAT contains 1200 bp of the mammary tumor virus long terminal repeat linked to the CAT structural gene terminated with an SV40 polyadenylation sequence. The glucocorticoid enhancer region, −190/−88, was deleted, and a HindIII restriction sequence was inserted at −88. Oligonucleotides were synthesized to the sequence −22/−2 of the wild type thymidine kinase promoter and to the same location from the linker-scanning mutant TK(1s−21/−12)CAT. These oligonucleotides were then ligated into HindIII-digested △MTV-CAT. The resulting plasmids were sequenced to confirm the presence of the proper sequence. The sequence of the final insert is given in the figure. Bottom, the response of the △MTV(TK)-CAT plasmids to insulin was determined as above. GH4 cells were transfected with 10 μg of the △MTV(TK)CAT plasmid indicated and 5 μg of pRT3HR-2. They were incubated with 1 μg/ml insulin as described in Fig. 1. The parental plasmid △MTV-CAT was unresponsive to insulin treatment (data not shown). Basal CAT expression/μg of protein was 0.56% for MTV(TK)CAT, 0.19% for MTV(TK)aCAT, 0.09% for MTV(TK)bCAT, and 0.083% for MTV(TK)cCAT.

△MTV(TKb)CAT that contain the CGGA sequence.

The deletion mutants shown in Fig. 5 address the possibility that the insulin response element of the native thymidine kinase promoter is the CGGAA sequence located at −151/−146. The linker-scanning plasmid TK(1s−5+/15)CAT was used to make 5′-deletions of the thymidine kinase promoter since the 3′-deletion plasmids all have a 3′-CGGA sequence as a result of their construction. First, the BamH1 site in the linker was removed with mung bean nuclease to create the plasmid TK(1s−5+/15ΔBamH1)CAT. The remaining plasmid contains the thymidine kinase promoter between −725 and −56. Insulin increases CAT expression 5-fold using this plasmid. Deletion to −385 (TK(−385/+56)CAT, Fig. 5) did not reduce the increase in CAT expression due to insulin. A further deletion to −218 (TK(−218+/15)CAT, Fig. 5) eliminates 5 potential Ets-binding sites including 2 CGGA sequences between −385 and −250, but this did not reduce the insulin-mediated increase in CAT production with this construct. Finally, a deletion to −128 (TK(−128/+56)CAT, Fig. 5), which eliminates the CGGAA at −151/−147, renders the thymidine kinase promoter insensitive to insulin.

Identification of the Insulin-responsive Sequence of the Somatostatin Promoter—Analysis of several deletion constructs suggests that the insulin response element of the somatostatin gene may be located in the 5′-untranslated region (Fig. 6A). Deletions of 5′ sequences to −48 were previously shown to reduce the cAMP responsiveness of this promoter by eliminating part of the cAMP response element (6). However, this deletion had no effect on insulin-mediated CAT activity (Fig. 6A, SS(1s−6/−80)CAT). The 5′-untranslated region of the somatostatin gene is a GA-rich area that contains a CGGA sequence at +43/+46 that was similar to the insulin response element of the prolactin gene and of the insulin-responsive 5′-deletion of the thymidine kinase promoter. A somatostatin-CAT construct lacking this region SS(−71−1)CAT was not stimulated by insulin (Fig. 6A). When the three potential Ets-binding sites were mutated in the plasmid SS(−71−1/80Ets-mut)CAT, the increase in CAT expression mediated by insulin was reduced by 75% (Fig. 6B). Deletion of the first two potential Ets-binding sites in the plasmid SS(−71−1/1+23/80)CAT did not further reduce the effect of insulin (Fig. 6B). This implies that the effect of insulin is predominantly mediated by the CGGA sequence at +43/+46. Finally, the putative insulin response region (+7/+47) of the somatostatin gene was added back to the non-insulin-responsive plasmid SS(−71−1)CAT to produce the plasmid SS(−71−1,+7/+47)CAT. This plasmid exhibits a 7-fold increase in response to insulin (Fig. 6B).

Insulin Stimulation of Gene Expression in GH Cells Is Ets Transcription Factor Mediated—The DNA-binding domain of the Ets-related transcription factors is highly conserved, and the Ets-related transcription factors bind to each other's recognition sequences with only slightly different affinities. Thus, overexpression of the DNA-binding domain of any Ets-related protein will function as a dominant negative inhibitor of all related transcription factors. The overexpression of the DNA binding domain of c-Ets-2 has been adapted to this purpose (25). Fig. 7 shows the effect of expression of the c-Ets-2 DNA-binding domain on insulin and cAMP stimulation of CAT expression from the prolactin, somatostatin, and thymidine kinase promoters. The insulin stimulation of each of these promoters is reduced by 60–75% in cells overexpressing the c-Ets-2 DNA-binding domain, while cAMP-mediated increases are not significantly different.

Analysis of the Binding of Ets-related Proteins to Insulin-responsive Sequences from the Prolactin, Somatostatin, and Thymidine Kinase Promoters—To determine if Ets-related pro-
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Effect of insulin on 5'- and 3'-deletion mutants of somatostatin (−71/+80)CAT. GH4 cells were cotransfected with 10 μg of the somatostatin-CAT construct indicated in the figure and 5 μg of pRT3H1R-2. Following a 24-h incubation without hormone, somatostatin was added at 1 μM. After an additional 24 h of incubation, the cells were harvested, and CAT enzyme analysis was performed as in Fig. 1. The results are from three separate experiments done in duplicate. A, somatostatin-CAT constructs. Basal CAT expression/μg of protein was 0.59 ± 0.012% for SS(−71/+80)CAT, 0.23 ± 0.09% for SS(−48/−80)CAT, 0.06% ± 0.022% for SS(−71/−1)CAT, and 0.04 ± 0.008% for SS(−48−1)CAT. B, mutated somatostatin-CAT constructs. Basal CAT expression/μg of protein was 0.18 ± 0.21% for SS(−71/+80)CAT, 0.24 ± 0.03% for SS(−71/−80)mutCAT, 0.152 ± 0.046% for SS(−71/−1, +23/+80 mut)CAT, and 0.14 ± 0.0023% for SS(−71/−1, +7/+47)CAT.

Fig. 6. Effect of insulin on 5'- and 3'-deletion mutants of somatostatin (−71/+80)CAT. GH4 cells were cotransfected with 10 μg of the somatostatin-CAT construct indicated in the figure and 5 μg of pRT3H1R-2. Following a 24-h incubation without hormone, somatostatin was added at 1 μM. After an additional 24 h of incubation, the cells were harvested, and CAT enzyme analysis was performed as in Fig. 1. The results are from three separate experiments done in duplicate. A, somatostatin-CAT constructs. Basal CAT expression/μg of protein was 0.59 ± 0.012% for SS(−71/+80)CAT, 0.23 ± 0.09% for SS(−48/−80)CAT, 0.06% ± 0.022% for SS(−71/−1)CAT, and 0.04 ± 0.008% for SS(−48−1)CAT. B, mutated somatostatin-CAT constructs. Basal CAT expression/μg of protein was 0.18 ± 0.21% for SS(−71/+80)CAT, 0.24 ± 0.03% for SS(−71/−80)mutCAT, 0.152 ± 0.046% for SS(−71/−1, +23/+80 mut)CAT, and 0.14 ± 0.0023% for SS(−71/−1, +7/+47)CAT.

Insulin is added with Elk-1 (lane 5) or Sap-1 (lane 6) slightly decreases the binding of the Ets-related member of the ternary complex. This may result from slight inhibition of binding by factors in the reticulocyte lysate. Addition of Sap-1 and Elk-1 together results in no enhancement of the shift produced by Sap-1 alone nor does it produce additional migrating forms.

Sap-1 incubation with an oligonucleotide to the somatostatin promoter (Fig. 8B) produces a more slowly migrating protein-DNA complex similar to that formed with Prl−106−87 (Fig. 8B, lane 2). An excess of non-radioactive somatostatin +71/−47 inhibits the formation of this complex (Fig. 8B, lane 8). Nuclear extract proteins also bind to the somatostatin promoter (Fig. 8B, lanes 1 and 7), but no specific interactions of comparable migration with the Sap1-DNA complex were seen even on longer exposure (Fig. 8B, lane 1 versus lane 2). No specific binding of Sap-1 was seen using an oligonucleotide whose Ets-binding sites were mutated by a G→C conversion (compare lane 5, Sap-1, with lane 6, unprogrammed lysate) (Fig. 8B).

Sap-1 binds to the thymidine kinase promoter only when it has been mutated to contain an Ets-binding site (Fig. 8C). The wild type thymidine kinase promoter (−22/−2) shows no retarded bands when labeled DNA is incubated with Sap-1 (Fig. 8C, lane 2) that are not also present with unprogrammed lysate (Fig. 8C, lane 3). The oligonucleotide TbK corresponds to the thymidine kinase promoter (−22/−2) that is found in the insulin-sensitive TbK(1−12)CAT and MTVtkb-CAT. Incubation of Sap-1 with 32P-labeled TbK results in two retarded complexes (Fig. 8C, lane 5). These bands are not seen with unprogrammed lysate (Fig. 8C, lane 6), and they are inhibited by an excess of unlabeled TbK (Fig. 8C, lane 8). Again, no specific bands corresponding to the Sap-1 shifted DNA are seen using nuclear extract (Fig. 8C, lane 4).

DISCUSSION

Multiple lines of evidence presented here and previously (8) indicate that the sequence CGGA is a consensus response element for insulin effects in GH and HeLa cells. First, deletion and linker-scanning mutants of the prolactin promoter identi-
fied the sequence CGGAAA as essential for the insulin effect on the prolactin promoter, and this sequence could confer insulin responsiveness to ΔMTV-CAT (8). Second, the expression of CAT from several CGGA containing linker-scanning and deletion mutants of the thymidine kinase promoter is increased by insulin. When this linker sequence is inactivated, as in the plasmid TK(−95/+56ΔBamHI)CAT, insulin responsiveness is lost. The CGGA-containing sequence from one of the linker-scanning mutant sequences was shown to confer insulin sensitivity when inserted into ΔMTV-CAT. Finally, CAT expression from a somatostatin promoter construct is also increased by insulin. Deletions that inactivate the cAMP response element of this gene have no effect on insulin regulation. However, deletion of sequences in the 5′-untranslated region of the gene, containing three Ets-binding motifs, eliminates the increased expression mediated by insulin. Point mutation of these motifs reduces the effect of insulin 75%, and a 24-bp deletion that removes the first 2 of these motifs completely eliminates the effect of insulin. When the three motifs are added back to the insulin-insensitive plasmid SS(−71/+1)CAT, the effect of insulin is restored. These effects are seen both in GH cells and in HeLa cells. Thus, the presence of one or more CGGA sites in the proximal promoter region confers insulin responsiveness in these cell lines.

The effect of insulin to increase gene expression can be mediated by one copy of the insulin response element. The constructs ΔMTV(Prl−106−/−77)CAT and ΔMTV(TKb)CAT are stimulated 4- and 6-fold by insulin, and they have only one copy of this sequence. The 5′-deletion mutants of the thymidine kinase promoter are also insulin responsive with only one copy of this sequence. However, the prolactin promoter has two Ets-related binding sequences, and the response of the prolactin promoter is approximately twice that of the thymidine kinase promoter constructs. Thus, multiple Ets motifs may mediate an increased response.

This is not true of all cell lines. Chinese hamster ovary cells transfected with the prolactin-CAT constructs and an expression vector for Pit-1 show low levels of prolactin-CAT expression. However, this activity is not inducible either by insulin or cAMP. Thus, it appears that Chinese hamster ovary cells lack transcription factors that are both important for high basal expression of this construct and its regulated expression. The sequence CGGGA is able to confer insulin sensitivity only in cells with a necessary complement of transcription factors.

The involvement of Ets-related proteins in insulin-increased gene transcription is suggested by the experiment with the dominant negative Ets plasmid and by the gel shift experiments. Cotransfection of cells with a plasmid that expresses the DNA-binding domain of c-Ets-2 causes a 75% reduction in the insulin sensitivity of the prolactin, somatostatin, and thymidine kinase promoters. The Ets-related proteins Elk-1 and Sap-1 were shown to bind sequences from these promoters that are 32P-labeled somatostatin + 7/−47, and lanes 4–6 are 32P-labeled somatostatin + 1/+80EtsMut with point mutations converting the three GGA sequences in the somatostatin promoter to GCA. These oligonucleotides were incubated with nuclear extract (lanes 1, 4, and 7), Sap-1 (lanes 2, 5, and 8), or unprogrammed lysate (lanes 3, 6, and 9). Lanes 7–9 also had a 100-fold excess of unlabeled TK-22/−2 mutant. These oligonucleotides were incubated with nuclear extract (lanes 1, 4, and 7), Sap-1 (lanes 2, 5, and 8), or unprogrammed lysate (lanes 3, 6, and 9).

Fig. 8. Ets-related proteins associate with insulin-sensitive sequences but not with insulin-insensitive sequences. Gel mobility shift experiments were conducted using 32P-labeled oligonucleotides to the prolactin (panel A), somatostatin (panel B), and thymidine kinase promoters (panel C). A, 32P-labeled prolactin −106/−87 containing a CGGAAA motif at −97/−92 was incubated with various proteins indicated and analyzed on a 5% polyacrylamide gel. Lane 1, GH4 cell nuclear extract; lane 2, Elk-1; lane 3, Sap-1; lane 4, SRF; lane 5, Elk-1 + SRF; lane 6, Sap-1 + SRF; lane 7, Elk-1 + Sap-1; lane 8, Elk-1 + Sap-1 + SRF; and lane 9, unprogrammed lysate. B, lanes 1–3 and 7–9 were 32P-labeled thymidine kinase −22/−2 sequence from the linker-scanning mutant TK(−22/−12)CAT. In the addition of a BamHI linker introduces a CGGA motif. Lanes 7–9 also had a 100-fold excess of unlabeled TK-22/−2 mutant. These oligonucleotides were incubated with nuclear extract (lanes 1, 4, and 7), Sap-1 (lanes 2, 5, and 8), or unprogrammed lysate (lanes 3, 6, and 9).
are insulin sensitive but not sequences that are insulin insensitive.

The location of this sequence may also be important for its ability to mediate responses to insulin. The three insulin-sensitive promoters that we have described have the insulin response element inserted close to the transcription start site, the farthest away being the putative insulin response element of the wild type thymidine kinase gene at –150. Since this sequence is not uncommon in the genome, it is likely that this sequence is only effective within the first few hundred base pairs of the transcription start site.

These data allowed us to establish several criteria for screening insulin-responsive genes for potential response elements. First, the sequence GGA is key to the insulin response element. Second, preference was given to sequences containing CGGA as the area of the gene known to be insulin sensitive does not correspond in all cases. For example, the negative insulin responsive promoter was mapped to the serum response element (27). This sequence (underlined). The insulin response of the c-fos promoter was mapped to the serum response element in the glucagon gene is apparently located at 27779 that contains a direct repeat of the insulin responsive sequence (28). Similarly, AGGA sequences reside outside of the insulin-responsive region of the amylase gene –167/137 (29).

In summary, we have defined a consensus insulin response element, CGGA, that can act in several different promoter contexts and in different cell types. The activity of this response element is most likely dependent on the presence of the proper insulin response pathway and insulin-responsive transcription factors in the cells.

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