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31. We thank A. Wlodawer and his colleagues at the National Cancer Institute for making available to us their crystallographic coordinates of the RSV protease, synthetic HIV-1 protease, and synthetic HIV-1 protease–MVT-101 complex before publication; without their generosity this study could not have been completed. We also thank H. Stein for performing the renin assay, E. Devine for performing the stability studies, and A. Craig-Kennard for technical assistance. We are grateful to J. Greer and C. Abad-Zapatero for critically reading the manuscript. Supported by NIH grant AI 27220 to J.E.

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Identification of a Sequence in the PEPCK Gene That Mediates a Negative Effect of Insulin on Transcription

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Phosphoenolpyruvate carboxykinase (PEPCK) governs the rate-limiting step in gluconeogenesis. Glucocorticoids and adenosine 3′,5′-monophosphate (cAMP) increase PEPCK gene transcription and gluconeogenesis, whereas insulin has the opposite effect. Insulin is dominant, since it prevents cAMP and glucocorticoid-stimulated transcription. Glucocorticoid and cAMP response elements have been located in the PEPCK gene and now a 15–base pair insulin-responsive sequence (IRS) is described. Evidence for a binding activity that recognizes this sequence is presented.

INSULIN STIMULATES THE EXPRESSION of several genes including those encoding glyceraldehyde-3-phosphate dehydrogenase, c-Fos, glucokinase, gene 33 product, and α-amylase (*1, 2*). In contrast, insulin inhibits the expression of the PEPCK, adipsin, and growth hormone genes (*3*). Whereas cAMP and glucocorticoids increase PEPCK gene transcription (*4–6*), insulin inhibits transcription of this gene in rat liver and the H4IIE rat hepatoma cell line. Inhibition by insulin predominates in the presence of cAMP and glucocorticoids. The basal promoter elements, the cAMP-responsive element (CRE), and the glucocorticoid-responsive elements (GREs) in the PEPCK gene have been mapped in an effort to understand this complex interplay (*7*). The DNA sequences responsible for the dominant, negative effect of insulin on PEPCK have remained elusive, as have the sequences responsible for the regulation of transcription of other genes by insulin. Our goal was to identify such an element.

A fusion gene (PEPCK-CAT), containing the PEPCK promoter sequence between −600 and +69, relative to the transcription start site, ligated to the chloramphenicol acetyltransferase (CAT) reporter gene, is responsive to insulin when transiently expressed in H4IIE cells. Insulin inhibited basal PEPCK-CAT expression and suppressed the stimulatory effects of cAMP and dexamethasone (dex) (*8*). Attempts to define an IRS by the use of PEPCK-CAT fusion genes containing progressively shorter PEPCK promoter segments yielded inconsistent results in the transient expression system (*9*). Because the presence of the normal chromatin environment might favorably influence the function of one or more IRSs in the intact promoter, we isolated a series of H4IIE cell lines stably transfected with various PEPCK-CAT fusion genes. Results obtained from stable cell lines made using four such constructs are shown in Fig. 1A. Although insulin generally inhibited basal expression of the chimeric gene in each case, CAT expression was stimulated with the use of dex and cAMP in order to emphasize the magnitude of the insulin effect. Insulin almost completely suppressed cAMP- plus dex-stimulated CAT expression in the HL9, HL32, and FBG32 clones, just as it inhibits transcription of the endogenous gene (*6*). However, in the HL45 clones, which contain the region between −271 and +69 of the PEPCK promoter, the effect of insulin, though still present, was clearly reduced. Two other HL45 clones showed the same partial effect of insulin (*9*). The CAT assay results were confirmed by primer extension analysis in which insulin caused 86 and 39% reductions of CAT mRNA in HL9 and HL45 cells, respectively (Fig. 1B). These results suggest that the PEPCK promoter contains at least two IRSs, one located between −468 and −271, and another or others between −271 and +69.

To further delineate the distal IRS, we made use of a vector containing the herpes simplex virus thymidine kinase (TK) promoter. A series of overlapping, double-stranded oligomers spanning the region between −453 and −271 of the PEPCK promoter were synthesized and each was ligated into the Bam HI site of TKC-VI (Fig. 2A), a vector similar to that previously used to identify the inhibitory sterol-responsive element in the low-density lipoprotein receptor gene promoter (*10*). Each oligomer contained 38 bp of PEPCK sequence with Bam HI–compatible sticky ends (GATC) at each end. Previous studies show that the insertion of a 42-bp random DNA sequence into the Bam HI linker in this TK promoter has little effect on transcription (*11*). The effect of insulin on CAT expression by these constructs was initially analyzed by the use of transient expression of the chimeric DNA in H4IIE cells (Fig. 2B). The vector lacking an insert, and most of the constructs spanning the region from position −403 to −270, showed a slight stimulation of CAT expression in response to insulin. Constructs containing either the PEPCK sequence between −453 and −415 or that between −433 and −396 showed an insulin-responsive inhibition of CAT expression (Fig. 2B). Since the −433 to −396 construct gave the insulin effect of greatest magnitude, and worked well in both orientations, the search for an IRS was directed to this region of the PEPCK gene.

H4IIE cells stably transfected with either the insulin-responsive −433 to −396 construct or, as a negative control, the −403 to −366 construct were used to confirm that this effect was not simply a function of the transient transfection system. Two cell lines containing each construct were obtained. Both −433 to −396–containing clones (O and P) showed a concentration-dependent inhibition of CAT expression in response to insulin; the median effective concentration (EC_{50}) was about 0.1 nM and maximal inhibition was obtained with 10 nM insulin (Fig. 3), concentrations that provide similar

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degrees of inhibition of the endogenous gene (*6*). In contrast, the −403 to −366–containing clones (G and D) were either inactive or showed a slight increase of CAT expression in response to insulin, a characteristic shared by the native vector. In contrast to the inhibitory effect, which reached a plateau within the physiologic range, this stimulatory effect was still increasing at 100 nM insulin (Fig. 3).

Having shown, using both transient and stable transfection assays, that the −433 to −396 PEPCK sequence contains an IRS, we sought to further delineate the boundaries of this element. Three separate 5-bp mutations within the −433 to −396 region were constructed by altering the sequences with the end points −425 to −421, −411 to −407, and −401 to −397; these were designated M1, M2, and M3, respectively. These constructs were inserted into the TKC-VI vector and the transient expression of CAT activity was analyzed in H4IIE cells. Though plasmids containing both orientations of the M1 and M3 mutations still responded to insulin, the M2 mutation abolished the insulin effect (Fig. 4A). Another mutation, designated M4/5, was then constructed by altering the 5-bp sequences between −416 to −412 and −406 to −402 on either side of the wild-type −411 to −407 sequence. This mutation failed to give an insulin-dependent inhibition of CAT expression in either orientation (Fig. 4A).

A smaller construct (PC425) consisting of the wild-type PEPCK sequence between −416 and −402 was used to further define the region involved in the insulin response. This 15-bp core sequence, which spans the regions covered by the M2 and M4/5 mutations, showed insulin-dependent, orientation-independent inhibition of CAT expression in the transient transfection assay. Mutations equivalent to M2 and M4/5 within PC425, designated PC4M5 and PCM2M, respectively, abolished the inhibitory effect of insulin, as did identical mutations in the full-length oligomer (Fig. 4A). The CAT assay results were confirmed by primer extension analysis in which insulin caused 64 and 5% reductions of CAT mRNA in cells transfected with the PC425 and PC4M5 constructs, respectively (Fig. 4B). The 15-bp sequence −416 to −402 is therefore a functional IRS.

Specific binding of nuclear proteins to the IRS was analyzed with the gel retardation assay (Fig. 5). Several protein-DNA complexes indicative of specific interactions were detected when the wild-type −433 to −396

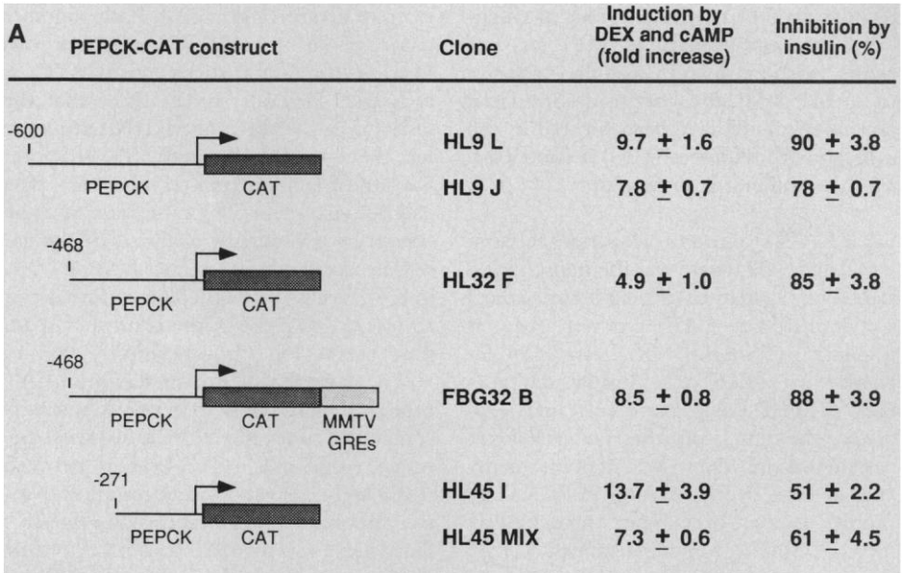

Fig. 1. (**A**) Hormonal regulation of PEPCK-CAT fusion genes. Cultures of stable transfectants (*15*) were incubated in serum-free medium with various combinations of insulin (10 nM), dex (500 nM), and 8-(4-chlorophenylthio)-cAMP (0.1 mM) as indicated. This cAMP analog is abbreviated to cAMP in the text to reflect the active moiety. Cells were harvested after 18 hours, and CAT activity was assayed by the method of Nordeen *et al.* (*17*). CAT activity was corrected for the protein concentration in the cell lysate. Background activity from a lysate-free blank was subtracted from all assays, and results are expressed relative to CAT activity in untreated cells. Results represent the mean ± SEM of three to six separate experiments. (**B**) Primer extension analysis of the multihormonal regulation of CAT expression in the HL9L (−600 to +69) and HL45E (−271 to +69) stable transfectants. Messenger RNA was isolated from cells exposed for 4 hours to dex (500 nM) plus 8-(4-chlorophenylthio)-cAMP (0.1 mM), with or without insulin (10 nM), and a primer extension assay was performed (*18*). The positions of the CAT and calmodulin-extended products (CAM) are indicated by the arrows and were of the expected size. The percent inhibition by insulin was calculated by densitometric scanning and corrected for small variations in the loading of RNA in each lane by using calmodulin as the hormonally unresponsive internal control. The results shown are representative of experiments performed on two different series of cells.

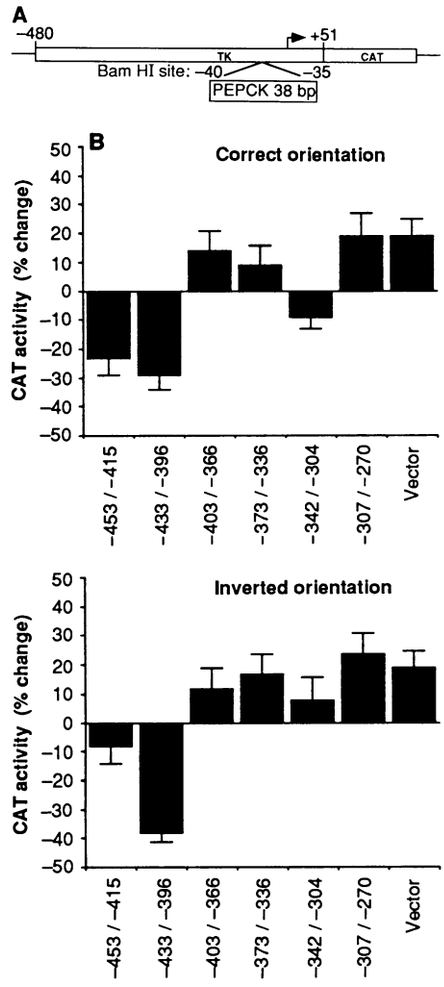

Fig. 2. Use of the herpes simplex virus thymidine kinase (TK) promoter to identify a PEPCK IRS. A schematic representation of the TKC-VI vector (obtained from T. Sudhof, which contains TK promoter sequence from −480 to +51, similar to that described (*10*), is shown in (**A**). A series of 42-nucleotide oligomers containing 38 bp of PEPCK sequence with GATC ends was inserted into the Bam HI site of the TKCAT vector. The analysis of PEPCK-TKCAT constructs by transient transfection (*21*) is illustrated in (**B**). Results are the ratio of CAT activity in insulin-treated versus control cells (expressed as percent change) and represent the mean ± SEM of nine separate transfections. Cloned PEPCK sequences were analyzed when inserted into TKCAT in the correct (**upper panel**) or inverted (**lower panel**) orientation.

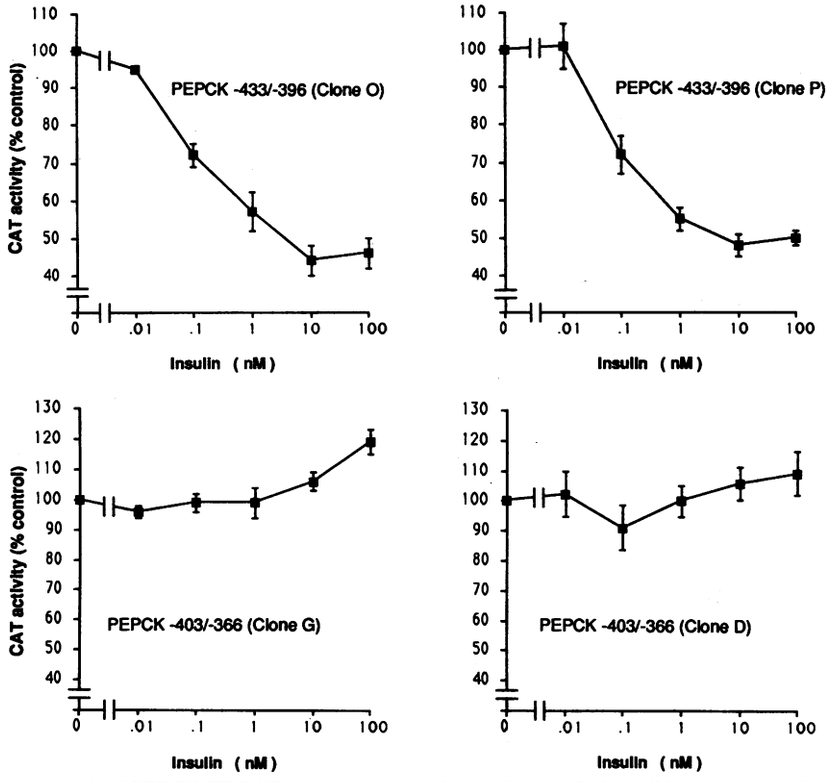

Fig. 3. Analysis of PEPCK-TKCAT constructs by stable transfection. Stable cell lines were established, using the −433 to −396 (inverted orientation) and −403 to −366 (correct orientation) PEPCK-TKCAT constructs (*15*). Two cell lines were obtained for each construct. Cultures of individual transfectants were incubated in serum-free medium for 18 hours with the concentrations of insulin indicated on the abscissa. Cells were harvested and CAT activity was assayed as described in Fig. 1. Results are expressed as a percentage of the CAT activity in control cells and represent the mean ± SEM of six separate experiments.

sequence was used as the labeled probe. Nuclear extracts prepared from rat liver or H4IIE cells gave qualitatively identical patterns. Competition experiments, in which an excess of unlabeled DNA was included with the labeled probe in the gel retardation assay, were used to correlate protein binding with the insulin response. Sequences that conferred a response to insulin, including −433 to −396, M1, M3, and the 15-bp −416 to −402 sequence (PC425), effectively competed for binding with the labeled probe. In contrast, a 100-fold excess of sequences that did not respond to insulin, that is, the M2, M4/5, PC4M5, and PCM2M mutations, failed to reduce the intensity of any of the bands (Fig. 5A). Thus, the DNA-protein interactions represented by all the bands correlate with the insulin response. The gel retardation pattern differed somewhat when the −416 to −402 sequence (PC425) was used as the labeled probe (Fig. 5B). Fewer specific bands were detected, but the pattern of competition was identical to that observed when the −433 to −396 probe was used. The DNA-protein interactions again correlated with the insulin response, as judged by the competition assays.

The hormone response element paradigm implies that discrete segments of DNA should confer the response of a hormone in a heterologous system, and that this DNA

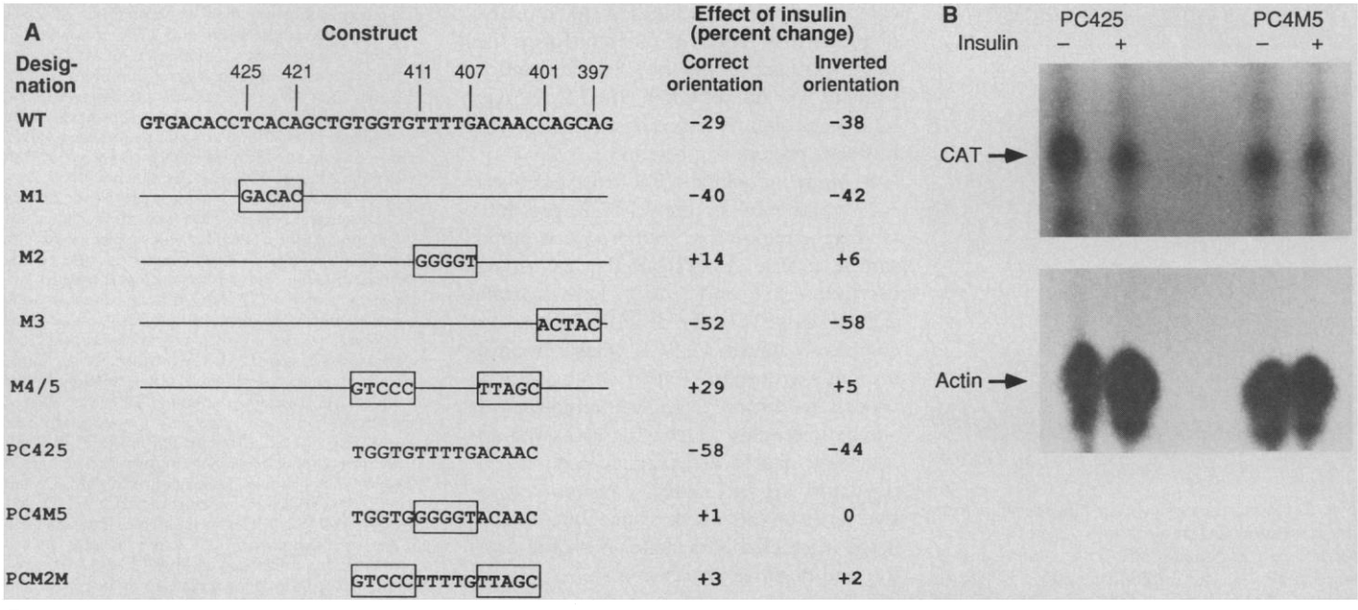

Fig. 4. (**A**) Localization of the PEPCK IRS in the sequence −433 to −396. Various mutants of the PEPCK sequence between −433 and −396 were synthesized with Bam HI (GATC) ends and cloned into the TKCAT vector in both orientations. The effect of insulin on CAT expression was analyzed by transient transfection as described (*21*). Results are the ratio of CAT activity in insulin-treated versus control cells (expressed as percent change) and represent the mean of 6 to 14 separate transfections for each construct. Maximum error (±SEM) was 7%. The boxed areas contain the mutant sequences. (**B**) Primer extension analysis of the insulin regulation of CAT expression in the PC425 and PC4M5 PEPCK-TKCAT plasmids. H4IIE cells were transiently transfected (*21*) with PEPCK-TKCAT plasmids containing either the PC425 or PC4M5 15-bp inserts and treated with or without insulin for 4 hours. Messenger RNA was isolated, and a primer extension assay was performed (*18*), with β-actin end-labeled with [γ-^{32}P]ATP as the hormonally unresponsive internal control. The CAT mRNA initiated from the correct site. After correction for the variation in the loading of RNA in each lane, the percent inhibition by insulin, calculated by densitometric scanning, was 64% for PC425 and 5% for PC4M5. The results shown are representative of two separate experiments.

sequence should interact specifically with one or more proteins that are involved in regulating transcription. We have located a functional insulin-responsive sequence, or IRS, from the PEPCK promoter between −416 and −402. This IRS functions as a silencing element when attached to a heterologous promoter and it works in an orientation-independent fashion. Two assays provided evidence to suggest that an activity exists in extracts from liver that recognizes the IRS. A deoxyribonuclease (DNase) I footprint has been identified in this region (*12*), and binding was detected by gel mobility shift analysis (Fig. 5).

The gel retardation pattern was not different when nuclear extracts prepared from the livers of rats made diabetic by treatment with streptozotocin were used. Also the pattern did not differ when nuclear extracts were prepared from H4IIE cells treated with or without insulin (*9*). This suggests that the binding capacity of the insulin-responsive protein or proteins is not under insulin control. The effect of insulin on PEPCK transcription is rapid and quickly reversed (*6*), which is consistent with an action through a protein modification mechanism such as phosphorylation or dephosphorylation (*13*). However, since changes in the phosphorylation state of the IRS binding proteins may not be maintained during the isolation of nuclear extracts (*14*), the lack of change in the gel retardation pattern in response to insulin is not surprising.

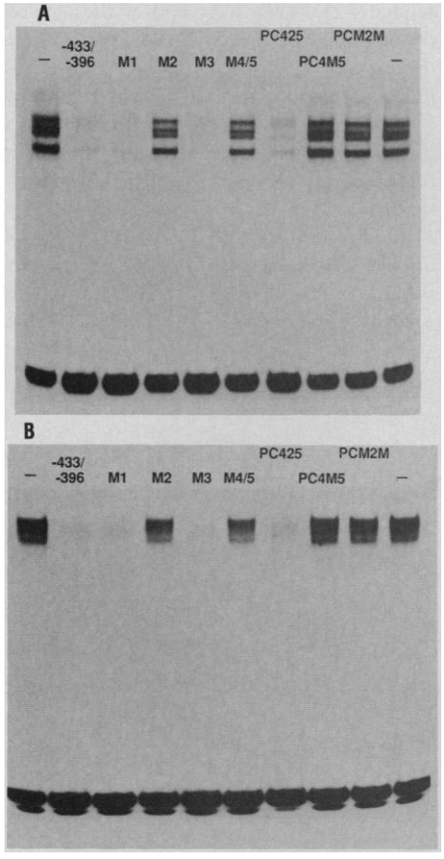

Fig. 5. Correlation of protein binding with insulin response. Gel retardation assays were performed as described (*22*). Two labeled probes were analyzed, the −433 to −396 PEPCK sequence (**A**) and the −416 to −402 PEPCK sequence (PC425) (**B**). When the −433 to −396 PEPCK sequence was used as the probe, four major bands were detected. The PC425 probe is considerably shorter (15 bp) than the −433 to −396 probe (38 bp) and thus migrates faster. Running the gel further when PC425 was used as the labeled probe revealed the presence of four bands (data not shown). Competitor DNAs (see Fig. 4A for construct) were added in 100-fold molar excess.

How might this IRS function in the context of the PEPCK promoter to inhibit basal, glucocorticoid- and cAMP-induced transcription? Recent studies show that the PEPCK glucocorticoid response unit is a complex structure consisting of two accessory factor binding sites (elements AF1 and AF2) and two glucocorticoid receptor binding sites (GR1 and GR2). The entire complex is necessary for full glucocorticoid-mediated induction of PEPCK transcription (*12*). The AF2 element is located in the same area of the PEPCK regulatory region (located between −420 to −403) as the IRS, as seen by DNase I footprinting and gel mobility shift assays. Thus, insulin could inhibit the glucocorticoid induction of PEPCK transcription by disabling the binding or function of an AF2 binding protein. The complex pattern of binding to the IRS oligomer may represent different forms of a single protein or the binding of multiple proteins. We do not know whether the AF2 binding protein is equivalent to the insulin response protein or proteins.

A more proximal IRS exists between −271 and +69 of the PEPCK promoter (Fig. 1), a region that contains no sequence similar to the distal IRS that is located between −416 and −402. This proximal element alone, like the distal IRS, does not completely inhibit PEPCK transcription in response to insulin. Rather, both must be present to achieve complete inhibition. It will be interesting to see whether a comparison of the distal and proximal IRSs with the regulatory regions of other insulin-responsive genes reveals a consensus sequence and, if so, whether this mediates both the positive and negative effects of insulin.

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15. H4IIE cells, adapted to Dulbecco's modified Eagle's medium containing final concentrations of 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum, were grown to confluence, diluted the day before transfection and replated in 75-cm^2 culture dishes (2×10^6 to 3×10^6 cells per dish). Attached cells were transfected, after removal of medium, by incubation for 30 min at room temperature with 2 ml of a calcium phosphate:DNA coprecipitate containing 20 μg of PEPCK-CAT plasmid DNA, 2 μg of SV2NEO selection marker (*16*), and 20 μM chloroquine. This solution was then diluted by addition of 8 ml of medium and supplemented with 1/20 of the volume of calcium chloride and Hepes-buffered saline (pH 7.10), which was used to prepare precipitates (*7*). After 6 hours, the cells were treated with medium containing 20% (v/v) dimethyl sulfoxide for 5 min, then washed once, and fresh medium was added. After incubation for 48 hours, fresh medium containing G418 (500 μg/ml) was added. The medium containing G418 was replaced every 48 hours and, after 2 to 3 weeks, individual foci were isolated. Construction of the PEPCK 5′-end promoter deletion mutations has been described (*7, 8*). The plasmids designated pPL9, pPL32, and pPL45 were used to construct the HL9, HL32, and HL45 stable cell lines, respectively. Individual cell lines are designated by a letter, whereas pooled colonies are designated as "mix." FBG32 was constructed by ligating a synthetic 46-bp insert containing two mouse mammary tumor virus GREs (obtained from K. Yamamoto) into a Bam HI site located immediately 3′ of the CAT reporter gene in pPL32.
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isolated on oligo(dT) cellulose columns (*19*). Oligonucleotides complementary to PEPCK and calmodulin were end-labeled with [γ-^{32}P]ATP, whereas an oligonucleotide complementary to CAT was internally labeled with [α-^{32}P]dATP (deoxyadenosine triphosphate) and [α-^{32}P]dCTP (deoxycytidine triphosphate). Labeled primers were added to 10 μg of poly(A)$^+$ RNA and extended with reverse transcriptase (*20*). Reaction products were separated on a denaturing polyacylamide-urea gel and analyzed by autoradiography.

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21. Each PEPCK-TKCAT construct (30 μg) was transfected in duplicate into H4IIE cells at 40 to 70% confluency in 75-cm^2 culture dishes as described (*15*). Six hours after transfection the cells were treated with medium containing 20% (v/v) dimethyl sulfoxide for 5 min, washed, and then incubated for 18 hours in the presence or absence of insulin (10 nM). Cells were harvested and CAT activity was assayed as in Fig. 1.
22. DNA fragments, labeled with [α-^{32}P]dATP and the Klenow fragment of *Escherichia coli* DNA polymerase I, were incubated with 10 μg of rat liver nuclear extract, at room temperature for 15 min in the presence of 10 mM Hepes at pH 7.8, 1 mM spermidine, 10 mM dithiothreitol, 50 mM NaCl, 2 μg of poly(dI/dC), 10% glycerol (v/v), and 0.1% NP-40 (v/v). Unlabeled competitor DNA fragments (a 100-fold molar excess) were added for competition analyses. Samples were loaded onto a 6% polyacrylamide gel and separated by electrophoresis (150 V) at 4°C for 2 hours in a buffer containing 25 mM tris, 190 mM glycine, and 1 mM EDTA (*23*). Gels were dried, and binding was analyzed by autoradiography.
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Protection Against Mucoid *Pseudomonas aeruginosa* in Rodent Models of Endobronchial Infections

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Chronic endobronchial infection with mucoid *Pseudomonas aeruginosa* accounts for much of the morbidity and mortality in patients with cystic fibrosis (CF). Reduced morbidity is observed when infection is absent. Clinical investigations have implicated opsonizing antibody specific for the mucoid exopolysaccharide (MEP) surrounding these bacteria as a potential immunologic protective mechanism, whereas nonopsonizing antibody to MEP is not protective. Mice and rats immunized with doses of MEP that elicited opsonizing antibody had reduced levels of infection compared with nonimmune controls after intratracheal challenge with mucoid *P. aeruginosa* enmeshed in agar beads. Doses of MEP that elicited nonopsonizing antibody were not protective. Parallel experiments in which passive transfer of polyclonal and monoclonal opsonizing and nonopsonizing antibody were used yielded similar results. These data indicate that MEP-specific opsonizing antibody can protect against chronic *P. aeruginosa* infection in this model of disease.

THE ABILITY OF MICROORGANISMS to establish chronic infections in immunocompetent individuals is an enigma of host-parasite interactions. Such microorganisms must somehow subvert or escape the immune defenses for long periods. In some instances, such as the acquired immunodeficiency syndrome (AIDS), the human immunodeficiency virus directly attacks the cells of the immune system (*1*). Alternatively, organisms may hide inside cells and thus avoid host defenses (*2*) or may change their antigenic structures and produce new antigens that do not react with antibody produced against previously expressed antigens (*3*). However, none of these mechanisms has been identified as critical to the maintenance of chronic lung infections in patients with CF. These infections, mostly caused by mucoid strains of *P. aeruginosa*, cause the vast majority of the morbidity and ultimate mortality early in life (*4*). Eighty-seven percent of CF patients develop respiratory tract colonization with *P. aeruginosa* by their 15th birthday (*5*). However, before the appearance of *P. aeruginosa* in sputum cultures of young patients, and among those older CF patients without *P. aeruginosa* colonization, there is greatly reduced morbidity (*6*). Most CF patients respond immunologically to their *P. aeruginosa* infection by producing antibodies against a large number of bacterial antigens (*7*). Among these antibodies are those directed against the outer polysaccharide coat, known both as alginate and as mucoid exopolysaccharide (MEP) (*8*). Chronically colonized CF patients produce antibodies to MEP that are not opsonic, that is, they are incapable of mediating phagocytosis and of killing bacteria in the presence of human leukocytes and complement (*9*). In contrast, 14 of 16 older (>12 years) CF patients who had managed to escape chronic *P. aeruginosa* infection had MEP-specific antibodies that were capable of mediating bacterial killing via opsonization (*9*).

Although no animal model of CF yet exists, chronic lung infections can be established in laboratory animals by administering bacteria enmeshed in agar beads (*10*). When purified MEP [prepared as described in (*11*)] was used to immunize mice and rats, opsonizing antibody was elicited after two doses of 1 to 10 μg per animal. This activity can be quantitated as an opsonic-killing index, which represents the percentage of bacteria killed in the presence of the opsonizing antibody compared to normal serum. Indices of >45% usually represented statistically significant ($P > 0.05$) killing and are considered positive. In contrast, doses of $\geq$40 μg per animal elicited only nonopsonizing antibody (<45% killing), and these animals did not produce opsonizing antibody when boosted from 1 to 8 weeks later with 1-μg doses (*11*). An exception was when only the highest molecular-sized [estimate of the distribution coefficient (K_{av}) = 0.05 on a Sepharose CL 4B column] polymers of MEP were used as immunogens. These polymers elicited opsonizing antibody in mice at doses of 50 μg (*11*). Thus, we were able to selectively induce either nonopsonizing or opsonizing antibody in animals and to study their effect on protective immunity.

We initially immunized adult, female rats with two doses 7 days apart of either 10 or 100 μg of MEP, which induced opsonizing and nonopsonizing antibodies, respectively. Five days after the second dose, two challenge strains of mucoid *P. aeruginosa* enmeshed in agar beads by the method of Cash *et al.* (*10*) were used to infect the rats: strain 2192, from which the MEP was isolated, and strain 258, which consistently produces chronic infections in these animals (*12*). After 21 days the animals were killed and their lungs were removed for quantitative and qualitative bacterial enumeration (*13*). Compared with saline controls, the rats immunized with doses of MEP that elicited high ($\geq$91%) mean opsonic-killing indices in their pooled serum had significantly ($P <$ 0.02) fewer lungs that yielded growth of

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