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NOTES

Insulin Gene Expression in Nonexpressing Cells Appears To Be Regulated by Multiple Distinct Negative-Acting Control Elements

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Selective transcription of the insulin gene in pancreatic β cells is regulated by its enhancer, located between nucleotides −340 and −91 relative to the transcription start site. Transcription from the enhancer is controlled by both positive- and negative-acting cellular factors. Cell-type-specific expression is mediated principally by a single *cis*-acting enhancer element located between −100 and −91 in the rat insulin II gene (referred to as the insulin control element [ICE]), which is acted upon by both of these cellular activities. Analysis of the effect of 5′ deletions within the insulin enhancer has identified a region between nucleotides −217 and −197 that is also a site of negative control. Deletion of these sequences from the 5′ end of the enhancer leads to transcription of the enhancer in non-insulin-producing cells, even though the ICE is intact. Derepression of this ICE-mediated effect was shown to be due to the binding of a ubiquitously distributed cellular factor to a sequence element which resides just upstream of the ICE (i.e., between nucleotides −110 and −100). We discuss the possible relationship of these results to cell-type-specific regulation of the insulin gene.

Pancreatic β-cell-type-specific transcription of the insulin gene is due to the recognition, by specific cellular transcription factors, of its enhancer region (4, 8, 9, 11, 14, 30, 35). Expression mediated by this region, which is located between nucleotides −340 and −91 relative to the insulin gene transcription start site, appears to be regulated by both positive- and negative-acting cellular activities (4, 21, 22, 34, 35). There are a number of distinct DNA sequence elements within the enhancer that are important for expression in β cells (4, 8, 16, 35). The cell-type-specific expression pattern observed for this region can be attributed largely to the insulin control element (ICE; GCCATCTGC) (8, 15, 16, 17, 35), which is found 100 ± 14 bp upstream from the transcription initiation site of all characterized insulin genes (5). The ICE has been stringently conserved in vertebrate evolution, and mutations within this element drastically reduce enhancer activity in β cells (8, 16, 35). Recent studies suggest that positive control of the ICE is mediated through binding of a β-cell-specific transcription factor(s) (23, 33). The positive regulator of ICE element expression is a member of the basic helix-loop-helix family of transcription factors (7, 27, 31).

The ICE is also a site of negative transcriptional control in non-insulin-producing cells (34, 35). We previously have postulated that the absence of insulin gene transcription is due not only to the lack of a necessary positive regulator(s) but also to the presence of a negative regulator(s). Little is known, however, about the extent to which these regulators interact within the enhancer region and thereby influence the level of insulin gene transcription. In this report, we show that a *cis*-acting element, which is located just upstream of the ICE between nucleotides −110 and −100, markedly reduces the responsiveness of the ICE to negative regulation. This −110 to −100 element appears to be the site of action of a ubiquitously distributed positive effector. During the course of these studies, we also mapped a second negative regulatory element (at −217 to −197) within the insulin enhancer. This element acts to repress insulin enhancer-promoter activity in non-insulin-producing cells, even in the presence of the −110 to −100 element. These results suggest that a combination of positive and negative regulatory elements generates the correct, cell-type-specific expression of the insulin gene.

Identification of 5′-flanking sequences that regulate β-cell-specific expression. We previously observed that deleting the ICE from a rat insulin II gene expression plasmid containing enhancer sequences from −238 to −91 did not induce expression in non-insulin-producing cells (35). These results indicated that there were at least two independently acting enhancer elements, one internal to nucleotides −238 to −101 and the ICE, that are regulated by negative-acting cellular activities. To more precisely identify the additional site(s) of repression within residues −238 to −101, a series of insulin expression plasmids containing rat insulin II gene sequences spanning the enhancer region from −238 to −101 linked to the chloramphenicol acetyltransferase (CAT) gene were constructed by the polymerase chain reaction. Rat insulin II wild-type gene (−238 CAT; Fig. 1) and ICE deletion mutant −238del CAT; Fig. 1) expression plasmids were used as templates for these reactions. ICE plus and minus 5′-deletion mutants with endpoints at −238, −217, −196, −162, −135, and −120 were thus generated. The activities of 5′-flanking mutants were compared in insulin-producing (HIT T-15 2.2.2 [9]) and non-insulin-producing (HeLa) cells with the activity of the rat insulin II promoter expression plasmid, −90. The −90 mutant is constitutively active in

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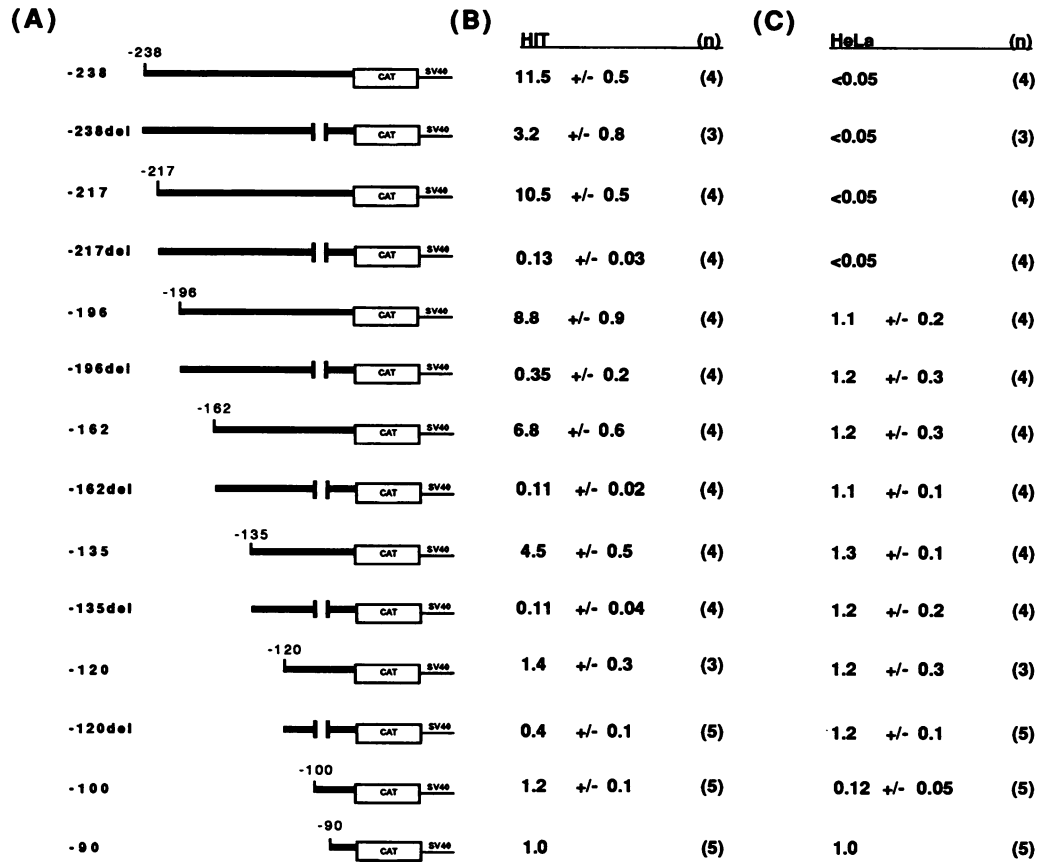

FIG. 1. (A) Map of rat insulin II gene 5′-flanking deletion mutants. Polymerase chain reaction-amplified rat insulin II gene sequences were fused to the CAT gene in pPLF CAT. Amplification from −238, a CAT expression plasmid containing rat insulin II gene sequences from −238 to +8, and −238del, the −238 expression with an ICE deletion (35), was performed by the method of Saiki et al. (25), using *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus). Reactions (100 μl) were run under conditions previously described (25) except that the DNA polymerase was added after the reaction mixture was heated to 94°C for 2 min and cooled to 72°C. Between 50 and 200 ng of plasmid DNA was used in each amplification reaction. Ten amplification cycles were performed in a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of a step program (94°C, 1 min; 37°C, 1 min; 72°C, 1 min). All clones have rat insulin II sequences with a 3′ end at +2 except the −238, −238del, and −100 clones, in which the 3′ end is at +8 (35). The sequences of all plasmid constructs were confirmed by DNA sequencing. Each mutant is named according to the 5′ endpoint of the deletion. All data are presented as means ± standard errors relative to −90 CAT activity. SV40, Simian virus 40. (B and C) Relative CAT enzymatic activities of the various 5′-flanking constructs in HIT (B) cells and HeLa (C) cells. All CAT constructs were introduced into HIT and HeLa cells as calcium phosphate coprecipitates made up of 10 μg of total DNA containing 5 μg of the insulin-CAT deletion mutants and 1 μg of RSV β-gal or carrier DNA (pUC19) under conditions described previously (35). Cells were harvested 40 to 48 h after transfection. The amount of extract from both the HIT and HeLa cells used in the CAT assay was normalized to β-galactosidase activity. CAT and β-galactosidase enzymatic assays were performed as described by Gorman et al. (13) and An et al. (1), respectively. (n) indicates the number of times each transfection was repeated.

both insulin- and non-insulin-producing cells (35). The insulin expression plasmids were introduced into cells by calcium phosphate coprecipitation with RSV β-gal, a Rous sarcoma virus enhancer-driven β-galactosidase expression plasmid. Cell extracts were prepared 40 to 48 h after transfection and assayed for both CAT and β-galactosidase enzyme levels. The amount of protein extract from both the HIT and HeLa cells used in the CAT assay was normalized to β-galactosidase activity.

All of the 5′-flanking mutant expression plasmids depicted in Fig. 1 were active in the insulin-producing HIT cell line. Maximal activity in HIT cells was obtained from the chimeric genes extending 238 nucleotides upstream of the transcription start site (Fig. 1B). Truncation of the 5′ sequence to −196 bp resulted in a slight decrease in CAT activity, while a truncation to −162 caused CAT activity to drop almost twofold. Activities of the 5′-flanking deletions extending to −135, −120, −100, and −90 were approximately 3-, 8-, 10-, and 11-fold, respectively, lower than that of the −238 construct. In contrast, the −238, −217, and −100 mutants were comparatively inactive in HeLa cells (Fig. 1C). However, CAT activity was detected in HeLa cells from the 5′-flanking enhancer deletion mutants extending to −196, −162, −135, and −120 at approximately the same level as the −90 promoter construct. Qualitatively similar results were obtained when a mouse pancreatic insulin-producing cell line (β TC-1 [10]) and other non-insulin-producing cell lines (XC and baby hamster kidney) were transfected with these insulin-CAT constructs (data not shown).

These results indicated that the sequences between −217 and −197 also functioned as a site of repression of rat insulin II enhancer expression. Moreover, since the −238 and −217 expression plasmids with internal ICE deletions were inac-

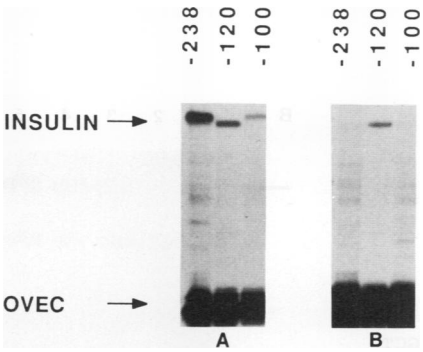

FIG. 2. Analyses of −120 to −101 element-driven activity in HIT (A) and HeLa (B) cells. HIT and HeLa cells were transfected as described in the legend to Fig. 1 except that 0.25 μg of OVEC-REF, a simian virus 40 enhancer-driven rabbit β-globin expression plasmid (32), was coprecipitated with the other DNAs. Total cellular RNA was isolated by the guanidinium isothiocyanate method (6). mRNA expression levels were measured from 30 μg of total cellular RNA by the RNase protection assay (35). Protected fragments 151, 280, and 286 nucleotides long are expected for correctly initiated transcripts generated from OVEC-REF, −120, −238, and −100 constructs, respectively.

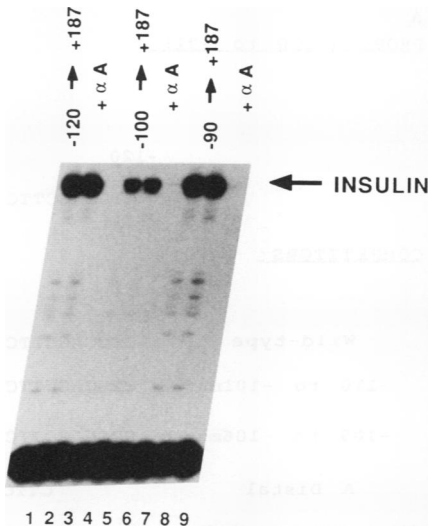

FIG. 3. Analyses of transcripts synthesized in vitro from various 5′-deletion mutants. Transcription reactions were conducted as described previously (35), using HeLa nuclear extracts prepared as described by Shapiro et al. (26). α-Amanitin at a final concentration of 1 μg/ml was included in the reactions indicated by αA. The rat insulin II gene sequences contained within the templates used in the transcription reactions were −120 to +187, −100 to +187, and −90 to +187. Specific transcription initiation was monitored by the primer extension method described previously (35). Insulin-specific transcription was analyzed with a primer complementary to bases +33 to +66 of the noncoding strand of the rat insulin II gene (35). The insulin-specific primer extension product is labeled insulin. The −120 to +187 (lanes 1 and 2), −100 to +187 (lanes 4 and 5), and −90 to +187 (lanes 7 and 8) templates were assayed in duplicate. A representative autoradiogram of the resulting analyses is shown.

tive in HeLa cells (Fig. 1C), it appears that the −217 to −197 sequences prevent transcription in non-insulin-producing cells by a mechanism independent of that operative through the −100 to −91 ICE.

Evidence for a regulatory element residing between sequences −120 and −101 that overcomes ICE-mediated repression. CAT activity from the insulin enhancer mutants depicted in Fig. 1 was detected in HeLa cells upon deletion of the −217 to −197 repressor element. Levels of expression of the −196, −162, −135, and −120 wild-type and ICE deletion mutant constructs were comparable in these cells. In contrast, there was much less CAT activity detected from the −100 construct in HeLa cells. A comparison of the mRNA levels from HIT and HeLa cells transfected with the −238, −120, and −100 chimeras is presented in Fig. 2. These plasmids were transfected along with an internal control plasmid, the simian virus 40 enhancer-driven rabbit β-globin expression plasmid OVEC-REF (32). The levels of the individual RNAs produced from the chimeric genes were quantitated by the RNase protection assay. The pattern of activity from these plasmids was similar to that shown in Fig. 1. The steady-state mRNA levels from the other transfected chimeras also mirrored the relative CAT enzyme levels shown in Fig. 1 (data not shown). These results suggest that a *cis*-acting element(s) within the −120 chimera is able to activate transcription in non-insulin-producing cells. Furthermore, this *cis*-acting element(s) appears to be dominant to ICE-mediated repression.

Expression from the −120 mutant is regulated in a HeLa-soluble transcription system. Previously we had shown that rat insulin II enhancer expression was negatively regulated in vitro (35). To determine whether expression from the −120 mutant was also regulated, we assayed the relative template activity of the −120, −100, and −90 5′-flanking deletion mutants in a HeLa-soluble in vitro transcription extract. Specific transcription products were measured by primer extension analyses. The −120 and −90 mutants were transcribed at comparable efficiencies and significantly more efficiently than the −100 mutant (Fig. 3). Transcription from these templates was mediated by RNA polymerase II, since the transcription signals were abolished by inclusion of α-amanitin (at 1 μg/ml) in these assays (Fig. 3). These results reaffirmed the in vivo observations indicating that the −120 construct contains a sequence element that interacts presumably with a positive-acting, ubiquitous factor(s) which can overcome ICE-directed repression.

A ubiquitously distributed cellular factor binds −110 to −100 insulin sequences. To ascertain whether factors could directly interact with the −120 to −100 regulatory region, gel shift assays were performed by using extracts prepared from HeLa cells and a ^{32}P-labeled oligonucleotide probe containing rat insulin II gene sequences from −120 to −91. Two protein-DNA complexes were detected in these binding experiments (Fig. 4B). The specificity of these interactions was determined by using competitor DNAs that would distinguish between specific (i.e., wild-type sequences) versus nonspecific (i.e., distal or mutated −120 to −91 sequences) protein-DNA complexes. The sequences of the competitor DNAs used for this experiment are shown in Fig. 4A. DNA containing the sequences spanning the −120 to −100 region competed with the wild-type probe for the more slowly migrating protein-DNA complex, whereas unrelated DNA and competitors containing mutations within the −110 to −101 region did not compete (Fig. 4B; compare lane 2 with lanes 3 to 5). The competition pattern of these various DNAs indicated that only the most slowly migrating complex represented a sequence-specific interaction between −120 to −91 sequences and a HeLa DNA-binding factor(s). The details of the interaction between this factor(s) and −110 to −100 sequences was probed by using the methylation

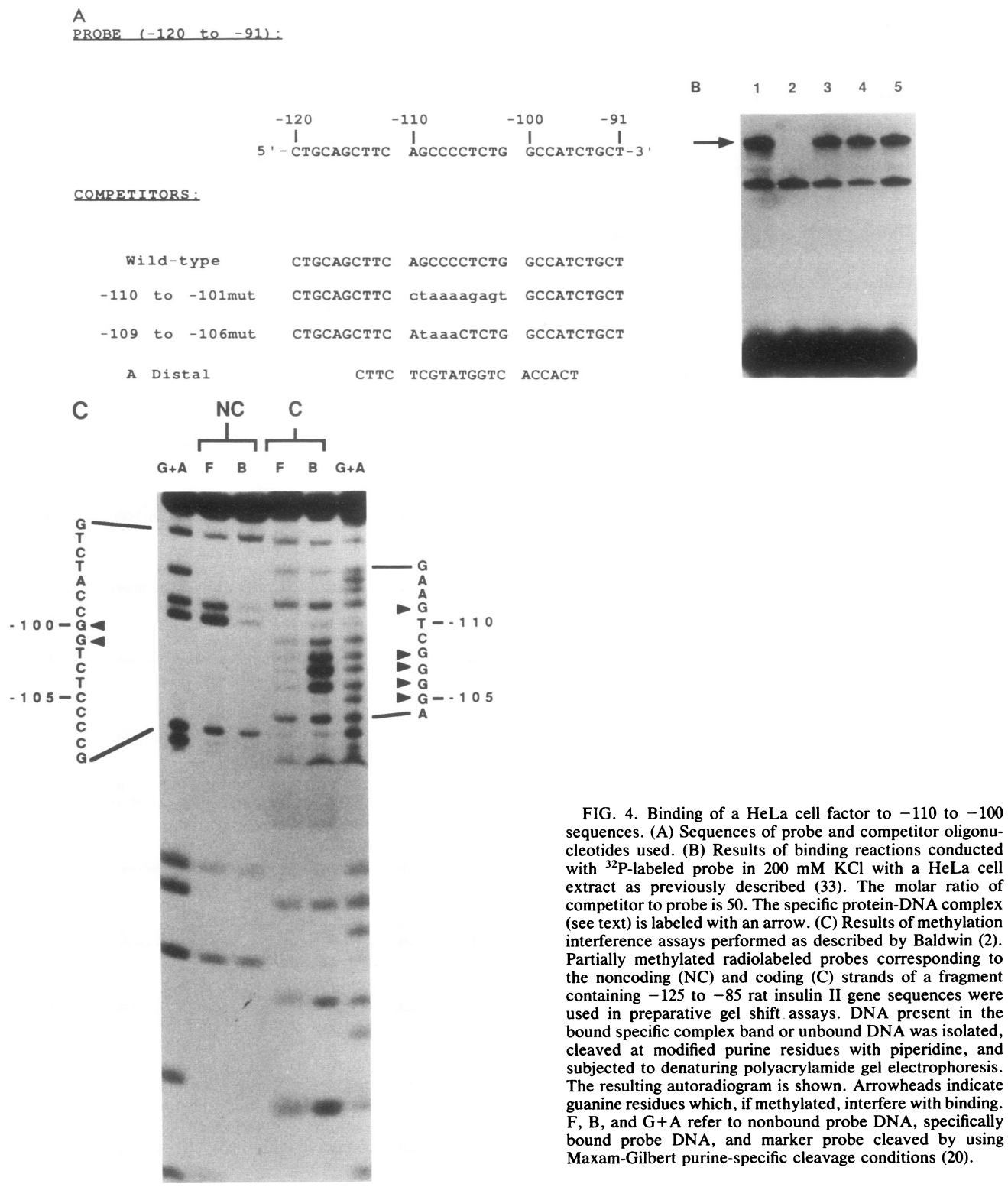

FIG. 4. Binding of a HeLa cell factor to −110 to −100 sequences. (A) Sequences of probe and competitor oligonucleotides used. (B) Results of binding reactions conducted with ^{32}P-labeled probe in 200 mM KCl with a HeLa cell extract as previously described (33). The molar ratio of competitor to probe is 50. The specific protein-DNA complex (see text) is labeled with an arrow. (C) Results of methylation interference assays performed as described by Baldwin (2). Partially methylated radiolabeled probes corresponding to the noncoding (NC) and coding (C) strands of a fragment containing −125 to −85 rat insulin II gene sequences were used in preparative gel shift assays. DNA present in the bound specific complex band or unbound DNA was isolated, cleaved at modified purine residues with piperidine, and subjected to denaturing polyacrylamide gel electrophoresis. The resulting autoradiogram is shown. Arrowheads indicate guanine residues which, if methylated, interfere with binding. F, B, and G+A refer to nonbound probe DNA, specifically bound probe DNA, and marker probe cleaved by using Maxam-Gilbert purine-specific cleavage conditions (20).

interference technique. The methylation interference pattern obtained with this complex was centered around the sequences between −110 and −100 (Fig. 4C). Additionally, a DNA probe containing a block mutation at residues −109 to −106 did not compete in a gel shift assay with the wild-type −120 to −91 probe (Fig. 4B). Similar results have been obtained in the gel mobility shift assay with the −120 to −91 probe, using extracts prepared from β-cell lines (data not shown). These results indicate that a ubiquitously distributed cellular factor(s) binds specifically within the −110 to

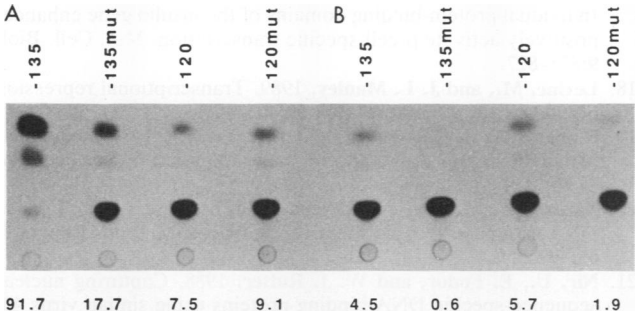

FIG. 5. Effect of mutagenesis of the −110 to −100 factor binding site on activity of −135 CAT and −120 CAT expression plasmids. The −135 CATmut and −120 CATmut expression plasmids were constructed as described in the legend to Fig. 1 from oligonucleotides containing the following mutations within sequences −109 to −106: −135, 5′-GCAAGTGTTTGGAAACTGCAGCTTCAtaaaCT CTGGCC-3′; and −120, 5′-CTGCAGCTTCAtaaaCTCTGGCCATG CTG-3′. Wild-type and mutant CAT constructs, in addition to an RSV β-gal internal standard, were transfected into HIT (A) and HeLa (B) cells as described in the legend to Fig. 1. Levels of CAT enzyme activity, expressed as the percentage of [^{14}C]chloramphenicol converted to acetylated products, are indicated below the lanes.

−100 region of the insulin enhancer. This region (−110 to −100) does not bear sequence homology with any heretofore characterized *cis* element. The identity of this factor remains unknown.

The −110 to −100 binding factor is a positive regulator of enhancer activity in vivo. The data from the preceding experiments indicated that sequences between −109 and −106 were involved in binding a cellular factor(s). To test whether this factor functioned as a positive regulator of insulin enhancer expression in vivo, we examined the effect on transcription of the −109 to −106 block mutation on the activity of the −135 CAT and −120 CAT expression plasmids. The CAT activity from the −109 to −106 block mutant expression plasmids was compared in HIT and HeLa cells with the activity of the wild-type expression plasmid, which was transfected in parallel (Fig. 5). This mutation caused a significant decrease in −135 CAT activity in HIT cells; however, it had little effect on expression of the −120 CAT plasmid. The activity of −135mut is comparable to that of the −100 construct in these cells (data not shown). The −135 CAT expression vectors are more active than the −120 CAT plasmids in HIT cells (Fig. 1 and 5A). The increased expression of the −135 expression plasmids may be a consequence of the positive-acting transcription factor described by Hwung et al. (15), which appears to interact with nucleotides that lie between −124 and −111 of the rat insulin II gene. In transfected HeLa cells, the block mutation caused a large decrease in the activity from the mutant expression plasmids. This finding suggests that the ICE-mediated negative control mechanisms are functional in these mutants.

Conclusions. Specific expression of the insulin gene enhancer in pancreatic β cells appears to be due in large part to the activity of a single distinct *cis*-acting element located between nucleotides −100 and −91 (i.e., ICE) in the rat II gene enhancer (8, 15, 16, 17, 35). Expression from this element is controlled by both positive- and negative-acting cellular activities (35). Mutagenesis of the ICE dramatically reduces insulin enhancer activity in β cells; however, the levels of enhancer repression from these mutants are similar to those found with the wild-type enhancer (35). These results indicated that there was another element(s) in the insulin enhancer, which functioned independently of the ICE, that was subject to cellular repression. Through an extensive mutagenesis analysis of the 5′-flanking region of the rat insulin II gene, we have identified a second region of the enhancer that is under negative control. The data presented herein indicate that this second regulatory element, which lies between nucleotides −217 and −197, is functionally distinct from the ICE. The significance of both of these negative regulatory elements in maintaining β-cell-specific expression is not yet fully understood. Evidence for the existence of *cis*-acting negative regulatory elements has also been described in a number of other eukaryotic genes that are expressed in a cell-type-specific manner (for reviews, see references 12, 18, and 19). However, it is still unclear from these studies how the negative- and positive-acting control elements interrelate to impart the cell-type-specific expression.

Sequential 5′ deletion of the negative control sequences led to constitutive expression of the insulin gene in non-insulin-producing cells to the level of the insulin promoter expression plasmid, −90 CAT. The β-cell-type-specific expression observed with wild-type −238 enhancer and ICE reporter expression plasmids was not detected from these 5′-enhancer mutants until nucleotides between −197 and −101 were deleted. The absence of ICE-mediated repression in these mutants was shown to be a consequence of a positive control element which appears to lie just upstream of the ICE between nucleotides −110 and −100. The sequences of both the −217 to −197 and −110 to −100 elements in the rat insulin II gene are very similar in both location and sequence in the other characterized mammalian insulin genes (3, 5, 28). Therefore, it is possible that the transcriptional mechanisms described for regulating −110 to −100 and −217 to −197 element activity in the rat insulin II gene could also be utilized to control expression of all mammalian insulin genes.

The positive-acting factor interacting with the −110 to −101 region appears to be present in both insulin-producing and non-insulin-producing cells. Mutational analysis of the factor-binding site has demonstrated that this factor functions in a positive fashion during expression of the insulin enhancer in β cells. Our results further indicate that the −110 to −100 element can override ICE-mediated repression in non-insulin-producing cells. It is unclear how the −110 to −100 element mediates this effect. The most straightforward interpretation of these results is that binding of the positive-acting factor to the −110 to −100 element prevents the negative-acting factor from interacting with the ICE, possibly because these two elements (i.e., the constitutive transcription element [−110 to −100] and the negative regulatory element [−100 to −91]) physically overlap. Spacing experiments in which these two elements are placed at various distances from one another could possibly address this point. The effect of the −110 to −100 element on ICE-mediated repression may indicate that the ICE is not a site of negative regulation. We cannot totally rule out this possibility; however, these observations could also be explained by postulating that the levels of the negative-acting regulatory factor(s) vary between cell types and under some circumstances favors ICE-mediated repression. The existence of such regulatory mechanisms has recently been described in other systems (24, 28). In the hopes of distinguishing between these models, we are now attempting to isolate the factor(s) involved in negative control. Our preliminary results indicate that the positive- and negative-

acting transcription factors that regulate ICE expression contain a basic helix-loop-helix structural motif (28a, 34).

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