Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8819–8823, December 1987
Biochemistry

A mutational analysis of the insulin gene transcription control region: Expression in beta cells is dependent on two related sequences within the enhancer

(cell specificity/pancreas/promoter)

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Contributed by William J. Rutter, August 21, 1987

ABSTRACT **Cell-specific expression of the insulin gene is controlled by cis-acting DNA sequences located within ≈350 base pairs of the 5′ flanking DNA immediately upstream from the transcription start site. Using synthetic oligonucleotides, we have constructed a systematic series of block replacement mutants spanning this region. No single sequence appears to be absolutely required for expression. However, three of the mutants exhibit 5–10 times less activity and several others show 2–3 times less. Simultaneous mutation of two of the most mutationally sensitive regions leads to virtual abolition of activity. These two elements are structurally related and presumably represent key components of the machinery determining the cell-specific expression of the insulin gene.**

The development of complex multicellular organisms requires selective spatial and temporal regulation of the genetic repertoire. Although cell-specific gene expression in higher eukaryotes can be controlled at multiple levels (1), a primary determinant is the efficiency of initiation of new transcripts (2). Specific transcription is apparently controlled by the interaction of cellular factors with certain cis-acting DNA sequences in the vicinity of the gene.

Cis-acting elements involved in controlling cell-specific expression have been identified in several cellular genes. In the insulin gene, two distinct positive control elements have been identified in the 5′ flanking DNA (3): a cell-specific enhancer and a cell-specific promoter (4). Additional negatively acting cis elements have also been detected in this region (5). In immunoglobulin genes, cell specificity is also attributable to cell-specific enhancers (6–9) and cell-specific promoters (10–12). A further measure of control may be exerted in this system at the post-transcriptional level (1, 12).

In vitro binding studies of transcriptional control regions reveal multiple sites for binding of sequence-specific protein factors (13). Cell-specific transcription may be the consequence of the interaction of specific DNA-binding protein factors, which are present at elevated levels in cells actively expressing the specific gene (14–16).

To more completely define the elements of the insulin gene that control transcription *in vivo* and to correlate *in vitro* protein binding to transcriptional activity, we have constructed a systematic series of block replacement mutants spanning the proximal 345 base pairs (bp) of flanking DNA by using an oligonucleotide-directed approach that permits mutation of a region without changing distances or the stereospecific alignment between transcriptionally important elements. Each mutant contained a block of noncomplementary transversions (i.e., A ↔ C, G ↔ T; see ref. 17) spanning ≈10 bp. None of these mutations leads to complete loss of activity. However, several produce dramatic reductions (by a factor of 5–10): the "TATA" region of the promoter and two short sequences within the enhancer, which are similar to each other and are also related to the transcriptional control regions of certain other genes. Simultaneous mutation of these two sequences virtually abolishes activity.

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MATERIALS AND METHODS

Enzymes. All the enzymes used were purchased from Boehringer Mannheim.

Plasmid Constructions and Systematic Mutagenesis. The vector pOK1 (Fig. 1*A*) used for mutagenesis was derived from pUC18 (18) by inserting a 2-kilobase (kb) *Bam*HI fragment containing 410 bp of rat insulin I gene 5′ flanking DNA (3, 19), the entire chloramphenicol acetyltransferase (CAT) coding sequence (20), and simian virus 40 splice sites and polyadenylylation signals into the polylinker region modified to remove all sites between *Hin*dIII and *Sal* I. To construct mutants between −160 and −1, pOK1 was digested at the unique *Pst* I site (−160) and the unique *Hin*dIII site (+1) and dephosphorylated. For mutants between −345 and −161, the *Xba* I (−410) and *Pst* I sites were used. To facilitate subsequent subcloning steps, two additional sites were introduced into this region, *Xho* I at −114 and *Bam*HI at −85. Creation of the new sites required minimal alteration of wild-type sequences and did not affect the wild-type expression (data not shown). Mutants with block replacements in the +1 to −112 region contain the *Xho* I site and those with replacements within the −85 to −345 region contain the *Bam*HI site.

Assembly of Oligonucleotides and Sequence Analysis of Plasmid DNA. Oligonucleotides (20–25 bp) were designed with overlaps of 8–12 bp (Fig. 1*B*). Thirty picomoles of each synthetic oligonucleotide was mixed and phosphorylated using T4 DNA kinase in a single reaction. The oligonucleotides were purified by isopropanol precipitation and ligated together using T4 DNA ligase. Multimers were cleaved by addition of the appropriate restriction enzymes and the assembled oligonucleotides were inserted directly into the vector (without prior gel purification of the appropriately sized fragment) by incubation of oligonucleotides with dephosphorylated vector in the presence of T4 DNA ligase. The ligation mixture was used directly to transform *Escherichia coli* strain HB101. Plasmid DNA from 2-ml cultures was

Abbreviations: CAT, chloramphenicol acetyltransferase; TK, thymidine kinase.

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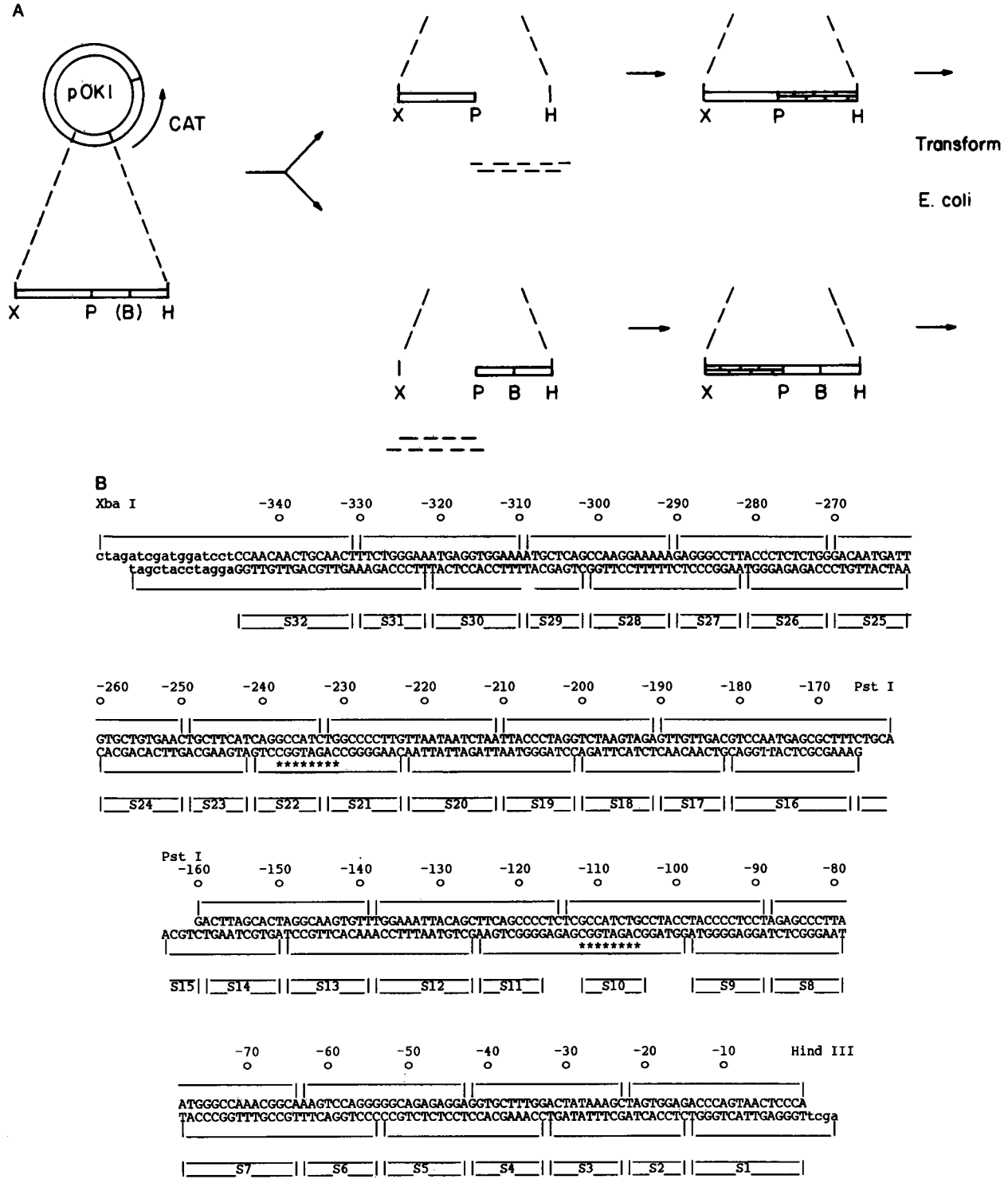

FIG. 1. (*A*) Strategy for systematic mutagenesis of insulin 5′ flanking DNA. Oligonucleotides with wild-type or mutant sequences were ligated into the test vector (pOK1) upstream of the CAT structural gene. Sequences from +1 to −160 were inserted between the restriction endonuclease sites *Hin*dIII (H) and *Pst* I (P) (upper portion of figure). Sequences between −161 to −345 were inserted between *Pst* I and *Xba* I (X) (lower portion of figure). Inserted regions were confirmed by DNA sequencing following transformation of *E. coli* and isolation of plasmid DNA. The *Bam*HI site (B) was used for subsequent subcloning of mutant sequences. (*B*) Sequence of the wild-type insulin 5′ flanking DNA showing the location of oligonucleotides for both strands and the coordinates of the block mutations. The block mutations are designated S1–S32. The sequences shown in lowercase letters are restriction enzyme recognition sequences used for insertion of the synthetic portion. Asterisks indicate the conserved mutationally sensitive regions discussed in the text.

isolated (21) and the desired recombinants were identified by restriction enzyme analysis. Plasmid DNA preparations were further purified by successive treatment with RNase A and phenol/chloroform followed by two successive isopropanol precipitations. Supercoiled plasmid DNA was denatured in alkali and sequenced by using a modification of the method of Chen and Seeburg (22) with either a synthetic universal primer or a synthetic CAT primer (3). The sequencing protocol was modified to improve the readability of the sequence. (*i*) Hybridization of the primer to the template was carried out for 30 min at 60°C. (*ii*) The amount of *E. coli* DNA polymerase I, Klenow fragment, was reduced to 0.2 unit per

reaction. (*iii*) The elongation reaction was carried out at 42°C. (*iv*) Samples were heated at 65°C rather than 95°C prior to loading on the gel to reduce degradation of DNA.

Verified mutants were resequenced by using DNA isolated after two cycles of equilibrium sedimentation on CsCl gradients. All transfections were performed with DNA prepared in this way. A more detailed description of the mutagenesis procedure will be presented elsewhere.

Cell Culture, Transfection, and Analysis of Gene Expression. HIT-T15 M.2.2.2 (4, 23) and BHK 21 cells were grown as monolayer cultures as described (4). The mutant or wild-type CAT test plasmids were cotransfected with the internal control plasmid pRSVβ-Gal (4) by using the calcium phosphate coprecipitation technique (24). Cell extracts were prepared 48 hr after transfection. CAT and β-galactosidase activities were determined and the values for β-galactosidase were used to normalize CAT activity measurements as described (4). Total RNA was isolated from cultures transfected with CAT test plasmids together with the internal control plasmid pMSV.TK.CAT (4). Primer extension reactions were performed using a synthetic CAT primer as described (4).

RESULTS

Strategy for Systematic Mutagenesis. To precisely identify cis-acting DNA sequences, we elected to systematically mutate short blocks of sequences throughout the region by using an approach based on assembly of overlapping synthetic oligonucleotides. A similar methodology has been used for systematic mutagenesis of the simian virus 40 enhancer region (17, 25). Our method offers the additional advantage of direct cloning into the plasmid expression vector, thus eliminating the need to subclone from an M13 vector to a plasmid vector.

Effects of Mutations. The CAT activities produced in HIT cells by plasmids containing mutant control regions are shown in Fig. 2. The majority of the mutations cause little or no reduction (<2 times) in CAT activity. However, six block replacements led to large (>3 times) reductions in activity (S3, S10, S20–22, and S29). Mutation of the TATA region leads to lowered expression but does not abolish it. Mutation of the sequences −104 to −112 (GCCATCTGC), the most conserved sequence among the known mammalian insulin 5′ flanking regions (26, 27), reduces expression to 14% of wild type. The third region that is sensitive to mutation spans three consecutive 10-bp segments (−211 to −241). The most dramatically affected block in this region encompasses −233 to −241 (16% of wild-type activity). Two other mutant blocks (covering −211 to −222 and −223 to −232) also affect the expression level significantly (33% and 36% activity, respectively). The fourth mutationally sensitive region (spanning bases −302 to −309) is located immediately downstream of a conserved "enhancer core" (between −310 and −316; see ref. 28). All the mutant plasmids were also tested in a cell that does not produce insulin (BHK cells). No significant increase in expression over the natural insulin flank was observed. Therefore, none of these mutants blocks the negative regulatory cis-acting element detected by Nir *et al.* (5).

To confirm that the observed effects are attributable to changes in RNA levels, we performed primer-extension analysis on RNA preparations isolated from HIT cells transfected with mutant plasmids. Fig. 3 shows primer-extension analysis of RNA obtained after transfection of those mutants determined by CAT activity measurements to have significantly reduced activity. In the case of these mutants (Fig. 3) and the remaining mutants (data not shown), there was a close correlation between primer-extension data and CAT enzymatic activities.

The −104/−112 Region Is an Integral Part of the Insulin Enhancer. To test whether the −104 to −112 sequence functions as part of the enhancer element, we analyzed whether mutation of these sequences influenced enhancer activity operating on the herpes simplex virus thymidine kinase (TK) promoter or the insulin promoter. Synthetic DNA fragments spanning insulin sequences −85 to −345, containing either the mutant or the wild-type −104/−112 region, were inserted in both orientations upstream of the TK promoter or the insulin promoter (+1 to −85). With either promoter, the presence of the mutation leads to a substantial reduction in enhancement (Table 1). Thus, the −104/−112 region contributes to the enhancer action. In its position relative to the insulin TATA box, the element corresponds to the "second distal element" (29, 30) of other promoters. It remains to be seen whether the element can also function as an upstream promoter element. The sequences located be-

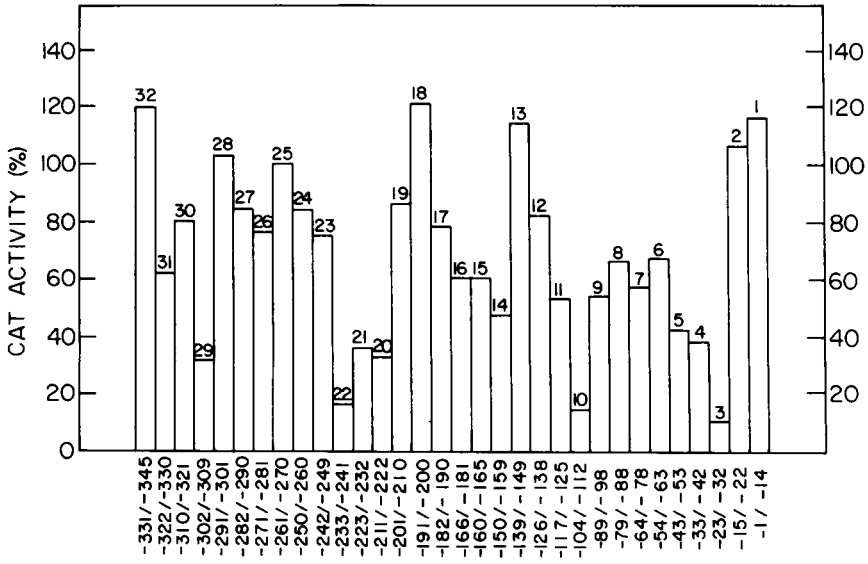

FIG. 2. Histogram showing CAT activities directed by mutant plasmids. Plasmids bearing mutations in the insulin 5′ flanking DNA were transfected into HIT cells and CAT activity was measured. Activities are expressed relative to that directed by the wild-type plasmid. Each bar is labeled 1–32 corresponding to the designation S1–S32 as shown in Fig. 1*B*. Each data point represents the mean of at least four independent DNA transfection experiments. Standard deviations were generally ≈15% and ranged from 3% to 23%.

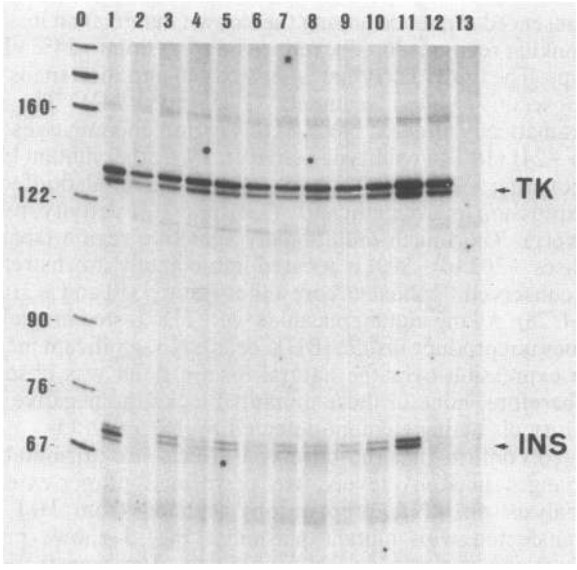

FIG. 3. Analysis by primer extension of CAT transcripts produced by wild-type or mutant plasmids after transfection of HIT cells. Lanes: 1, wild type; 2, mutant S3; 3, mutant S9; 4, mutant S10; 5, mutant S11; 6, mutant S20; 7, mutant S21; 8, mutant S22; 9, mutant S29; 10, mutant S30; 11, mutant S31; 12, double mutant S10/S22; 13, mock transfection. The location of mutations in these plasmids is shown in Fig. 1*B*. All constructions were cotransfected with plasmid pMSV.TK.CAT.pUC as an internal control. Arrows indicate the position of bands corresponding to RNA initiating at the rat insulin I gene cap site (INS) and at the TK cap site (TK). Lane 0 contains radioactively labeled size markers. The predicted insulin flank-derived transcripts contain 71 nucleotides of CAT sequence. The predicted MSV.TK-derived transcripts contain, in addition, 63 nucleotides derived from the *TK* gene (combined length, 134). In the case of the TK promoter, an additional band (129 nucleotides) is observed, which results from a reverse transcriptase pause site. The CAT primer used has been described (3). Numbers on left represent nucleotides.

tween the TATA region (−30) and the −104/−112 region apparently do not contribute significantly to the enhancer activity since enhancement of the TK promoter by the −30 to −410 and −103 to −410 fragments is identical (data not shown). Thus, it appears that the −104/−112 region is the most proximal component of the enhancer.

Transcription of the Insulin Gene Is Dependent on Two Short Homologous Regions in the Enhancer Element. To further clarify the role of mutationally sensitive regions in the overall transcriptional activity of the insulin 5′ flanking DNA, we constructed a series of double mutants in these regions (Table 2). The double mutant (−104 to −112 and −233 to −241) exhibited extremely low activity (0.3% of wild type), underscoring the essential role of these sequences in transcription of the gene. In contrast to the dramatic loss of activity shown by this double mutant (compare observed values with those predicted based on multiplicative effects; Table 2), all other combinations tested showed roughly a multiplicative reduction in activity compared to the respective single mutants, suggesting that they interact independently. As a control, several other double mutants were constructed: these showed an activity similar to wild type or the corresponding affected mutant (Table 2).

Table 1. Effect of mutation on enhancer activity

Construction	Orientation	Replacement	Promoter	Activity, % TK	Activity, % Ins
1	Normal	None	TK	100	
2	Normal	−104/−112	TK	13	
3	Inverted	None	TK	43	
4	Inverted	−104/−112	TK	9	
5	—		TK	3	
6	Normal	None	Ins		100
7	Inverted	None	Ins		43
8	Inverted	−104/−112	Ins		4.5

Wild-type or mutated insulin flanking sequences were positioned in either orientation upstream of either the TK promoter (+49 to −109) or the insulin (Ins) promoter (+1 to −85). The plasmids were introduced into HIT cells and CAT activities were measured. Activities of mutants are expressed relative to that of the wild-type sequence controlling the TK or insulin promoter.

Table 2. Activity of insulin enhancer double mutants

Mutant	Sequences mutated	CAT activity, % Predicted	CAT activity, % Observed
S10/19	−104 to −112/−201 to −210	12.0	4.6
S10/20	−104 to −112/−211 to −222	4.6	3.5
S10/21	−104 to −112/−223 to −232	5.0	4.5
S10/22	−104 to −112/−233 to −241	2.2	0.3
S10/29	−104 to −112/−302 to −309	4.5	3.0
S22/20	−233 to −241/−211 to −222	5.2	2.5
S22/11	−233 to −241/−117 to −125	8.5	17.0
S18/11	−191 to −200/−117 to −125	64.0	115.0

Double mutants of insulin 5′ flanking DNA sequences were assembled by using appropriate combinations of oligonucleotides. The mutant plasmids were introduced into HIT cells and CAT activity was measured as described. Activities are expressed relative to that of the wild-type flank. The column designated "predicted" shows the values calculated for each double mutant, assuming a simple multiplicative reduction in activity.

DISCUSSION

Our previous analysis revealed that 5′ flanking sequences of the insulin gene determine selective expression in insulin-producing cells (3). This conclusion has been confirmed by studies in alternate cultured cells (31) and in transgenic mice (32). We have also demonstrated that at least two specific control elements, the enhancer and the promoter, are involved (4). Since deletions within the region eliminate activity, presumably these act as positive control elements. *In vivo* competition experiments (5) suggest that negative control elements play a role in depressing expression of this gene in cells that do not normally express insulin. Negative control factors have also been implicated in control of other mammalian genes (33–35).

To further characterize this complex regulatory region, we have carried out a systematic mutational analysis. This has been accomplished by assembling a series of overlapping 20-bp oligonucleotides, constructed in such a way that 8- to 12-bp segments within the flanking DNA can be selectively altered without changing the distances between elements. The method permits appropriate mutant sequences to be generated directly and rapidly. The mutant sequences are tested for transcriptional activity using an internally controlled transient transfection protocol (4).

In spite of the overall conservation of sequence in the 5′ flanking DNA region (26, 27), a large number of blocks of sequence in the insulin 5′ flanking DNA can be mutated without significantly affecting the overall activity: no single block mutation entirely eliminated activity. Only six of the block mutants displayed a strong reduction in expression (Fig. 2). These blocks define four distinct regions within the 5′ flank: the TATA box (−23 to −32) and sequences −104 to −112, −211 to −241, and −302 to −309. The structure of the TATA region and its location are similar to those found in many eukaryotic genes; the TATA region presumably inter-

acts with constitutive components of the transcription system.

The −104 to −112 region is the most highly conserved region of the flanking DNA (26, 27). Its location correlates with the distal promoter element of the *TK* gene and mouse and human β-globin genes (29, 30). However, in the insulin gene the element behaves as an integral component of the enhancer, since it exerts its effects on promoters independently of orientation or precise positioning.

The −211 to −241 region appears to be complex and probably contains more than one element. Within this region the most severely compromised block mutant is −233 to −241. Interestingly, the 8 bases between −231 and −238 are identical to −105 to −112 (Fig. 1*B*). Whereas block mutations between −233 to −241 or −104 to −112 lead to 5–10 times less activity, mutant plasmids containing block mutations in both regions display almost undetectable activity (reduced by a factor of >300; Table 2). This result suggests that these regions act synergistically to activate transcription. This 8-bp homology GCCATCTG resembles the sequence GCCAAT, which is a high-affinity binding site for CCAAT-binding transcription factor or nuclear factor I (36). Upon introduction of an additional adenine into the sequence at the −104/−112 region to form GCCAAT, the mutant sequence displayed markedly reduced activity (≈12%; data not shown). This implies that factors exhibit very stringent nucleotide-binding specificities. Conceivably, different binding specificities have evolved from an ancestral binding domain, presumably in concert with the complementary evolution of a set of binding proteins.

The sequence −211 to −232 plays a significant role in transcription but is apparently not as important for transcription as the −104 to −112 and −233 to −241 sequences. The −211 to −241 region seems to be an important portion of the enhancer since a duplication of the −160 to −249 sequence exhibits enhancer activity selectively in insulin-producing cells (data not shown). In the present experiments, the mutation of the −302 to −309 region decreases the activity significantly, but there is little or no effect of mutation of the adjacent sequences (−310 to −321) including the enhancer core consensus; nor have we observed any effect of the −310 to −321 mutation using a different insulin-producing cell line (JHPI cells; see ref. 37; data not shown). Perhaps the sequence plays a role that is not evident in the transient assays used here.

This systematic analysis has identified several cis-acting sequences within the 5′ flanking DNA that play a significant role in transcription of the insulin gene: two short repeated sequences centered at −108 and −237 play a critical role. We speculate that these sequences bind similar or identical trans-acting factors required to generate cell-specific transcription. The present experiments do not discriminate unambiguously between elements that are uniquely associated with insulin-specific expression in B cells as contrasted with those that are common for transcription in all cells. A deficiency in either nonspecific or specific cis-acting elements could score similarly in these experiments. Further details of the regulatory mechanism may be revealed in more sophisticated *in vitro* reconstruction experiments using defined components or in more natural expression systems such as transgenic animals. The mutant sequences developed here should aid in these studies.

We would like to thank Drs. U. Nir, L. Moss, and D. Standring for valuable discussions; Dr. A. Lernmark for providing JHPI cells; Ms. Jennifer Turner for preparing plasmids; and Ms. Chava Goffer and Ms. Leslie Spector for assistance in preparation of the manuscript. This work was supported by Swedish Grants MFR B85-13X-07149-01A and NFR 514875103-4 to T.E., by a grant from the Israel Academy of Sciences and Humanities to M.D.W., and by grants from the National Institutes of Health (AM 21344 and GM 28520) and the March of Dimes to W.J.R. M.D.W. is supported by a Career Development Award from the Juvenile Diabetes Foundation.

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