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# Regulation of rat insulin 1 gene expression: Evidence for negative regulation in nonpancreatic cells

(cell specificity/competition/repression)

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**ABSTRACT** Two *cis*-acting elements, the enhancer and the promoter, independently contribute to the cell-specific expression of the rat insulin 1 gene. The activities of these elements are presumably mediated by *trans*-acting factors. We have performed intracellular competition experiments that suggest the presence of a negative factor(s) that represses the enhancer activity in cells that do not express the insulin gene. In these experiments fibroblast cells (COS-7) were transfected with two plasmids: a test plasmid containing the gene for chloramphenicol acetyltransferase under the control of the thymidine kinase promoter and the insulin enhancer; and a competitor plasmid containing insulin enhancer sequences and the simian virus 40 origin of replication to permit its replication in the recipient cells. The presence of the competitor plasmid led to a 5- to 6-fold increase in chloramphenicol acetyltransferase activity as compared with the activity detected when insulin enhancer was absent from either the competitor or the test plasmid. A 5-fold increase in chloramphenicol acetyltransferase activity was also seen when the rat amylase enhancer was present on the competitor plasmid; in contrast the simian virus 40 enhancer exerted no effect. Efficient derepression required additional sequences downstream from those essential for enhancer activity. We propose that the activity of the rat insulin 1 enhancer is modulated by a negative *trans*-acting factor(s) that is active in cells not expressing insulin but is overridden by the dominant positive *trans*-acting factor(s) present in insulin-producing cells.

During cellular differentiation, cell types acquire the ability to stably express a characteristic set of genes. The molecular mechanisms by which this occurs are poorly understood. Several genes whose expression is restricted to a specific subset of cells contain *cis*-acting sequences required for efficient transcription in the appropriate differentiated cells (1–6). We have demonstrated (7) that 5′ flanking DNA sequences of the rat insulin 1 gene contain two such cell-specific elements. The properties of these elements are consistent with the hypothesis that differentiated cells contain positive *trans*-acting proteins (differentiators) that interact with the specific *cis*-acting sequences to stimulate the expression of the associated genes. This idea is also consistent with the observations and concepts of other workers (8–12).

On the other hand, "extinction" of the differentiated phenotype after fusion of differentiated cells with fibroblasts is frequently observed (13). We have also noted that specific sequence deletions in the insulin gene 5′ flanking sequences can lead to increases in expression in nondifferentiated cells (unpublished observations). These results are consistent with a role for repressor-like molecules in nonexpressing cells. Therefore, we designed experiments to determine whether such negative regulators exist. *In vivo* competition experiments titrating putative *trans*-acting factors with their target DNA can be revealing (34, 35); however, the method is limited because the concentration of competitor DNA is frequently very close to the toxic dose in transfection experiments. To circumvent this limitation, we have developed an intracellular amplification competition assay in which a replicating plasmid containing the target DNA sequences is cointroduced with a test plasmid containing a reporter gene whose expression can be affected by the same target DNA sequences. Such a competition using insulin gene 5′ flanking DNA led to activation of insulin enhancer activity in a cell type where it is normally totally inactive.

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## MATERIALS AND METHODS

**Plasmids.** The plasmid pTE1 has been described (7). pSV was derived from plasmid pSV2neo (14) by deleting the *Hin*dIII–*Eco*RI fragment containing the neomycin phosphotransferase gene and replacing it with the *Eco*RI–*Hin*dIII polylinker of plasmid pSP64 (Promega Biotec, Madison, WI). The 5′ deletions (7) of rat insulin 1 5′ DNA flanking sequences were introduced as *Bam*HI–*Hin*dIII fragments into *Bam*HI and *Hin*dIII sites in the vector. The 3′ deletion (7) fragments of the rat insulin 1 DNA flanking sequences were introduced as *Bam*HI fragments into the *Bam*HI site in the polylinker.

**Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay.** COS-7 cells and HIT cells (15, 16) were grown as described. We have observed optimal activation as follows. COS-7 cells ($1 \times 10^6$) were seeded in a 100-mm dish 24 hr before transfection with 5 $\mu$g of the test plasmid (pTE1 and derivatives) and 5 $\mu$g of the replicating plasmid (pSV series) by the calcium phosphate method (17). The cells were harvested 44 hr later, and extracts containing 100 $\mu$g of protein were assayed for CAT activity by incubation for 1 hr at 37°C as described (18).

**Total Cell RNA Preparation and Analysis.** Total cell RNA was extracted 44 hr after transfection by the guanidinium thiocyanate procedure (19) followed by centrifugation through a CsCl gradient (20). The SP6 riboprobe (21) analysis was performed according to the procedure described by Promega Biotec (Madison, WI).

## RESULTS

**The Rat Insulin 1 Gene Enhancer Could Be Activated in Nonpancreatic Cells.** To achieve a high molar excess of competitor over test sequences, the competing rat insulin 1 gene 5′ DNA flanking sequences were introduced into a vector, pSV, that carries the simian virus 40 (SV40) early promoter region, including the transcriptional enhancer and a functional origin of replication. This plasmid was trans-

Abbreviations: SV40, simian virus 40; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase.

ferred to COS-7 cells together with a nonreplicating test plasmid pTE1rINS1. The test plasmid contains the rat insulin 1 gene enhancer linked to the thymidine kinase (TK) promoter, which drives the bacterial gene encoding CAT (Fig. 1). The CAT activity in the transfected cells was used as a measure of the strength of the test enhancer. The presence of insulin enhancer sequences in the replicating plasmids led to a 5-fold increase in CAT activity (Fig. 2, lanes 3 and 4). The TK promoter preceded by no enhancer (Fig. 2, lanes 1 and 2) or by an inactive insulin enhancer (lanes 5 and 6) was not activated under these conditions. We have verified that the rat insulin 1 DNA sequences did not affect the replication level of the pSV plasmid, using quantitative Southern blot analysis of extrachromosomal DNA preparations (22). The cellular levels of both pSV and pSVrINS1 were estimated to be about 5–10 × $10^4$ copies per cell (data not shown).

Efficient derepression was dependent on the presence of insulin flanking DNA in the competitor plasmid. To determine whether the SV40 enhancer sequences present on pSV were also required, we constructed a plasmid containing the insulin flanking DNA and the SV40 origin of replication and promoter but no functional SV40 enhancer. Surprisingly, this plasmid was unable to efficiently derepress the insulin enhancer (data not shown). We speculate that the presence of a functional SV40 enhancer is required either to permit optimal replication or to form a nucleosome-free chromosomal environment (23) to allow the rat insulin flanking sequences to bind repressor molecules.

To establish that the TK promoter was specifically activated in these experiments, the mRNA was analyzed by RNase protection assay. In the presence of the appropriate competitor, the level of CAT RNA initiated at the correct TK RNA initiation point was preferentially increased by 4-fold (Fig. 3). As an internal control, we used a similar plasmid containing the β-actin promoter linked to CAT sequences. The levels of CAT RNA transcribed from this plasmid were not significantly affected by the inclusion of rat insulin 1 DNA sequences in the replicating plasmid.

Table 1 summarizes experiments defining the specificity of the derepression phenomenon. The Moloney murine sarcoma virus enhancer, while driving the TK promoter, was not affected by the presence of the replicating rat insulin 1

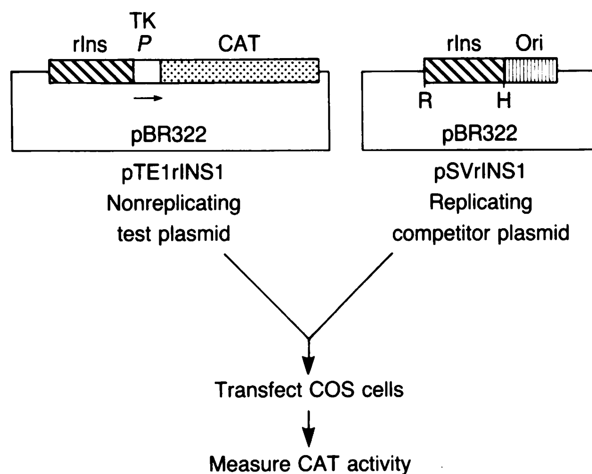


FIG. 1. Competition by *in vivo* amplification. The nonreplicating plasmids are based on the plasmid pTE1 (7) into which 5′ flanking DNA sequences of the gene for rat insulin (rIns)1 were introduced. The arrow shows the direction of CAT gene transcription under the control of the TK promoter (*P*). The replicating plasmids were derived from the vector pSV into which rInsl gene 5′ DNA flanking sequences were introduced. H, *Hin*dIII; R, *Eco*RI; Ori, origin of replication.

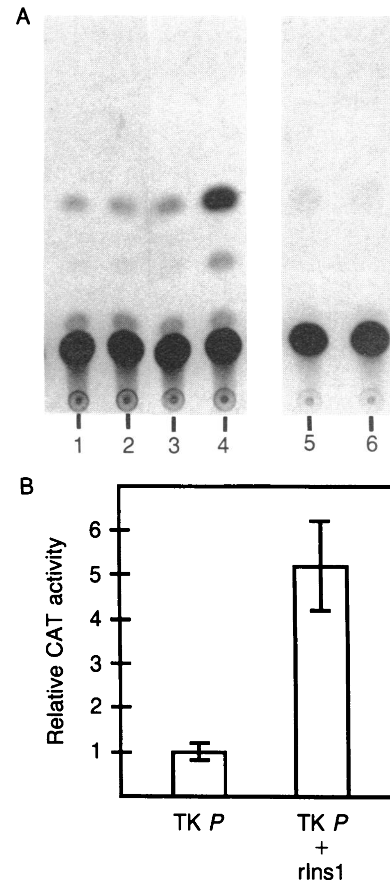


FIG. 2. Activation of rat insulin 1 (rInsl) gene enhancer in COS-7 cells. (*A*) Autoradiogram showing CAT activities in COS-7 cells transfected with the following plasmid pairs: pTE1 transferred with pSV (lane 1); pTE1 transferred with pSVrINS1, a pSV plasmid carrying rInsl gene 5′ DNA flanking sequences from nucleotide −370 to +51 (nucleotide +1 is the rInsl RNA initiation point) (lane 2); pTE1rINS1, a plasmid carrying rInsl DNA sequences from nucleotide −333 to +51 inserted in an inverted orientation to the TK promoter (12) cotransferred with pSV (lane 3); pTE1rINS1 (−333 to +51) cotransferred with pSVrINS1 (−370 to +51) (lane 4); pTE1rINS1A, a plasmid carrying rInsl DNA sequences from nucleotide −410 to −249 cotransferred with pSV (lane 5); and pTE1rINS1A cotransferred with pSVrINS1 (−370 to +51) (lane 6). Exposure time for lanes 5 and 6 was shorter than for lanes 1–4. (*B*) Ratio of CAT activity of test plasmid in the presence of replicating plasmid with or without rInsl DNA sequences. TK *P*, test plasmid containing TK promoter; TK *P* + rInsl, test plasmid containing TK promoter and rInsl gene enhancer. The experiment was repeated five times. Bars show mean values ± SEM.

sequences (Table 1, experiment 2). In contrast, insulin enhancer sequences −410 to +51 (experiment 3) or −249 to −103 (experiment 4) were derepressed and led to significant elevation in CAT activity. However, when the latter sequences (−249 to −103) were present 3′ to the CAT gene (experiment 5), no derepression was observed. This is consistent with the observed lack of enhancer activity of this fragment when placed 3′ of the CAT gene in transfected HIT cells (M.D.W., unpublished) and eliminates the possibility that the derepression resulted from homologous recombination between insulin sequences, leading to transfer of the SV40 origin of replication to the test plasmid. Significantly, the amylase 5′ flanking sequences [containing an enhancer active in pancreatic exocrine cells (27)] were also capable of derepressing the insulin enhancer sequences (experiment 8). The amylase DNA sequences contain no striking homologies with the insulin enhancer region. Thus, a strictly conserved nucleotide sequence is not required for efficient derepres-

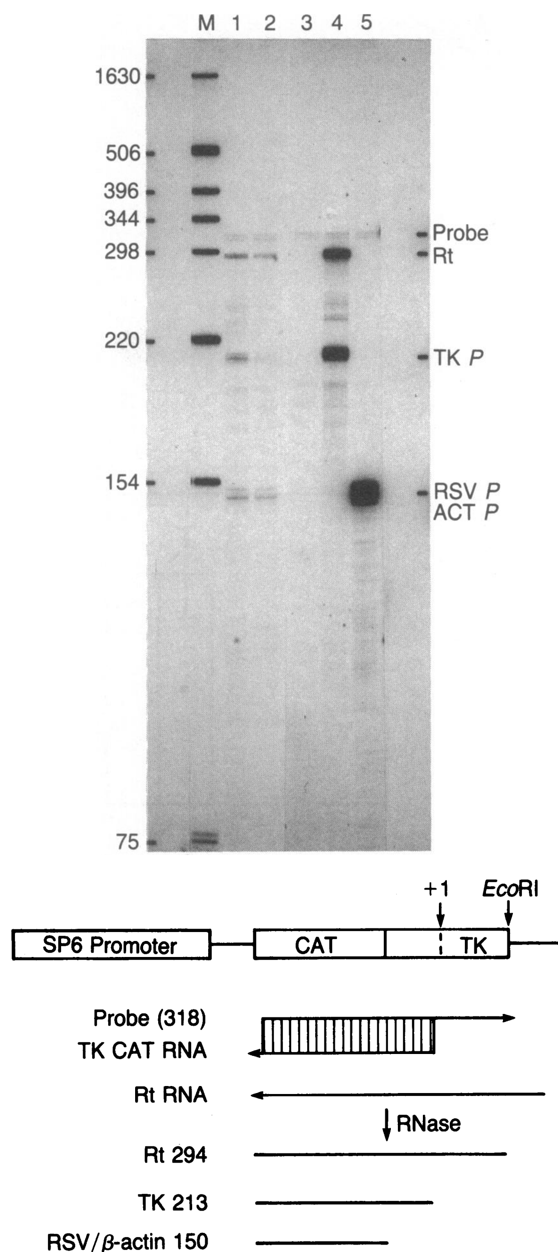


FIG. 3. Analysis of CAT RNA from transfected cells. Total cellular RNA was extracted from cells transfected with the following plasmid combinations. Lanes: 1, 5 μg of pTE1rINS1 (−333 to +51) DNA cotransferred with 5 μg of pSVrINS1 (−370 to +51) DNA [1 μg of rat β-actin CAT DNA (24) was included as an internal control; 30 μg of RNA from the transfected COS-7 cells was analyzed]; 2, pTE1rINS1 cotransferred with pSV and 1 μg of rat β-actin CAT DNA (30 μg of RNA from the COS-7 cells was analyzed); 3, analysis of 30 μg of RNA from nontransfected COS-7 cells; 4, 15 μg of RNA from HIT cells transfected with 5 μg of pTE1; 5, 5 μg of RNA from HIT cells transfected with 5 μg of RSV CAT DNA (25); M, size marker produced by *Hin*fI digestion of pBR322. The length of the fragments is given in nucleotides. Rt, readthrough transcripts; TK *P*, initiation of RNA synthesized from TK promoter; RSV *P*, initiation of RNA synthesized from Rous sarcoma virus (RSV) promoter; Act *P*, initiation of RNA synthesized from rat β-actin promoter. RNA samples were analyzed with uniformly labeled RNA probe as illustrated in the lower part of the figure. RNase digestion products were separated on 5% denaturing acrylamide urea gels. The expected lengths of the protected bands are shown.

sion. The result also supports the conclusion that homologous recombination cannot explain our observations.

**Derepression Required Sequences 3′ to Essential Enhancer Sequences.** In order to map the DNA sequences required for the enhancer derepression, progressively shorter fragments of the 5′ flanking sequences of rat insulin 1 gene were introduced into the pSV vector and cotransfected with the test plasmid pTE1rINS1 (−333 to +51). The smallest fragment tested that retained full ability to derepress mapped from −219 to +51 (Fig. 4, construct 3). Deletion of sequences from −219 to −159 (construct 4) or from +51 to −103 (construct 6) caused dramatic reductions in derepression activity. However, a replicating plasmid lacking sequences downstream from −103 was capable of derepressing an insulin enhancer fragment on the test plasmid that also lacked these sequences (Table 1, experiment 7). This raises the possibility that multiple DNA domains are involved in derepression. The fact that there was little derepression of the full-length enhancer fragment by competition with the rIns1Δ2 construct (Table 1, experiment 6; Fig. 4, construct 6) is presumably related to a higher affinity of the putative repressor to the full length as compared to the partial sequence.

## DISCUSSION

The understanding of cell-specific gene expression has advanced significantly with the identification of cell-specific transcriptional control elements (1–7). These *cis*-acting elements are responsible at least in part for specific expression of the associated gene. The results of deletion mapping and *in vivo* footprinting experiments suggest that cell specificity is mediated at least in part by *trans*-acting positive regulatory factors present in the expressing cell. The results of cell fusion experiments between muscle and nonmuscle cells are consistent with this idea (28). On the other hand, evidence for negative control elements has also been reported: when cells expressing a differentiated liver phenotype are fused to fibroblasts, one typically observes loss or extinction of the differentiated product, a result which can be most easily attributed to activity of *trans*-acting repressors (13). Negative regulation is also involved in modulating the activity of viral enhancers in embryonic cells (29) and in cells expressing the adenovirus *E1A* gene (30, 31). Further, the activity of the rat insulin 1 enhancer is reduced in pancreatic cells expressing the *E1A* gene (unpublished data).

In the present study, we have tested for repressing *trans*-acting factors interacting with the rat insulin 1 gene enhancer by competition experiments. We used intracellular amplification to achieve a high ratio of competitor to test plasmid in transfected cells without toxic effects. A sufficiently high ratio is important because standard competition protocols with nonreplicating plasmids in our system produced insignificant effects on the insulin enhancer (unpublished data). Our results suggest that the rat insulin enhancer is repressed in nonpancreatic cells: when the rat insulin 5′ flanking DNA sequences were present in high copy number, the putative repressor was titrated out. This led to derepression and activation of the enhancer (Fig. 2). The enhancer activity observed (5-fold) was substantially lower than that found in insulin-producing cells (40-fold) (7). However, a direct comparison of these numbers may be misleading because replication in COS cells may lead to titration of positive as well as negative factors.

The simplest explanation for these results is that the insulin enhancer is controlled by both positive and negative regulators. Thus, the high degree of transcriptional control of insulin gene expression seen *in vivo* may result from the activity of positive regulators in insulin-producing cells and negative regulators in nonproducing cells. Titration of the repressor(s) in nonexpressing cells led to some activation of a tissue-specific enhancer, presumably via interaction with positive factors. These positive factors might be nonspecific enhancer activator proteins or specific *trans*-activator mol-

Table 1. Rat insulin 1 (rIns1) gene enhancer activation by different replicating sequences

| | Test sequences | | Replicating sequences | | |
|---|---|---|---|---|---|
| Exp. | Enhancer | Promoter | Enhancer | Promoter | Fold activation |
| 1 | — | TK | rIns1 | rIns1 | 1 |
| 2 | Mo-MSV | TK | rIns1 | rIns1 | 1 |
| 3 | rIns1 | TK | rIns1 | rIns1 | 5 |
| 4 | rIns1Δ1-5′ | TK | rIns1 | rIns1 | 4 |
| 5 | rIns1Δ1-3′ | TK | rIns1 | rIns1 | 1 |
| 6 | rIns1 | TK | rIns1Δ2 | | 1–2 |
| 7 | rIns1Δ2 | TK | rIns1Δ2 | | 4–5 |
| 8 | rIns1 | TK | rAmy | rAmy | 5 |
| 9 | rIns1 | TK | — | human c-*myc* | 1 |

COS-7 cells were transfected with the following plasmid pairs. The fold activation represents the ratio of CAT activity observed in the presence compared to the absence of rIns1 DNA sequences on pSV. Experiments: 1, pTE1 transferred with pSVrINS1 (−370 to +51); 2, pTE1 containing Moloney murine sarcoma virus (Mo-MSV) enhancer (table 1, construct 6, in ref. 7) transferred with pSVrINS1 (−370 to +51); 3, pTE1rINS1 (−333 to +51) transferred with pSVrINS1 (−370 to +51); 4, pTE1 carrying rIns1 DNA sequences from nucleotides −249 to −103, placed 5′ to the TK promoter driving the CAT gene (rIns1Δ1-5′) transferred with pSVrINS1 (−370 to +51); 5, pTE1 plasmid carrying rIns1 DNA sequences from nucleotide −249 to −103 placed 3′ to the CAT gene (rIns1Δ1-3′) cotransferred with pSVrINS1 (−370 to +51); 6, pTE1rINS1 (−333 to +51) cotransferred with pSV plasmid carrying rIns1 DNA sequences from −410 to −103 (rIns1Δ2); 7, pTE1 plasmid carrying rIns1 DNA sequences from −410 to −103 (rIns1Δ2) cotransferred with pSVrINS1Δ2; 8, pTE1rINS1 (−333 to +51) transferred with pSV vector carrying rat amylase (rAmy) gene 5′ DNA flanking sequences from nucleotides −1000 to +31; 9, pTE1rINS1 (−333 to +51) transferred with pSV vector carrying human c-*myc* gene 5′ DNA flanking sequences from nucleotide −2300 to +1 (26).

ecules (differentiators) present at low concentrations. Full activity of the enhancer was only seen in the appropriate cell type, where the appropriate differentiator is presumably present at high concentrations (Fig. 5). The ability of mouse Ig heavy chain enhancer to compete with viral enhancers in *in vitro* transcription extracts from fibroblasts (9) is consistent with the first possibility. An analogous situation in which gene expression is controlled by the equilibrium between positive and negative factors has been described for Xenopus 5S genes (32).

The putative repressor appears to have a less-stringent binding requirement than the positive *trans*-acting factor, since the rat amylase gene 5′ flanking sequences present at high copy number could activate the rat insulin 1 gene enhancer. However, not all enhancer sequences were affected by the repressor, since neither the Moloney murine sarcoma virus enhancer nor SV40 enhancer appeared to bind the repressor. The function of the putative repressor may be to control a subset of cell-specific enhancers; therefore, it may be present also in insulin-producing cells, where its activity is overridden by positive-acting differentiators by virtue of their relative concentration or affinity (Fig. 5).

The mapping experiments suggest that the binding region of the repressor is not congruent with that of the differentiator: whereas full enhancer activity required sequences from −103 to −333 (7), efficient derepression required sequences downstream from −103 but not upstream of −219. Thus, the repressor binding domain may overlap with elements of both enhancer and promoter. Although the complete domain (−410 to +51) appeared to have the greatest derepression activity and thus perhaps the greatest affinity for repressor(s), portions of the sequences could act independently (Table 1, experiments 6 and 7). DNase I protection experiments have demonstrated that both pancreatic and nonpancreatic cells contain proteins that bind between nucleotides −110 and −210, within the binding regions of repressor and

FIG. 4. Mapping by deletion analysis of sequences capable of derepressing the insulin enhancer. DNA fragments spanning the regions indicated by black bars were introduced into the pSV replicating vector and were cotransferred with pTE1rINS1 plasmid to COS-7 cells. CAT activities were measured and compared to that seen using pSV. The values shown represent the mean of eight independent experiments. The fold activation varied by up to 30% of the mean value.

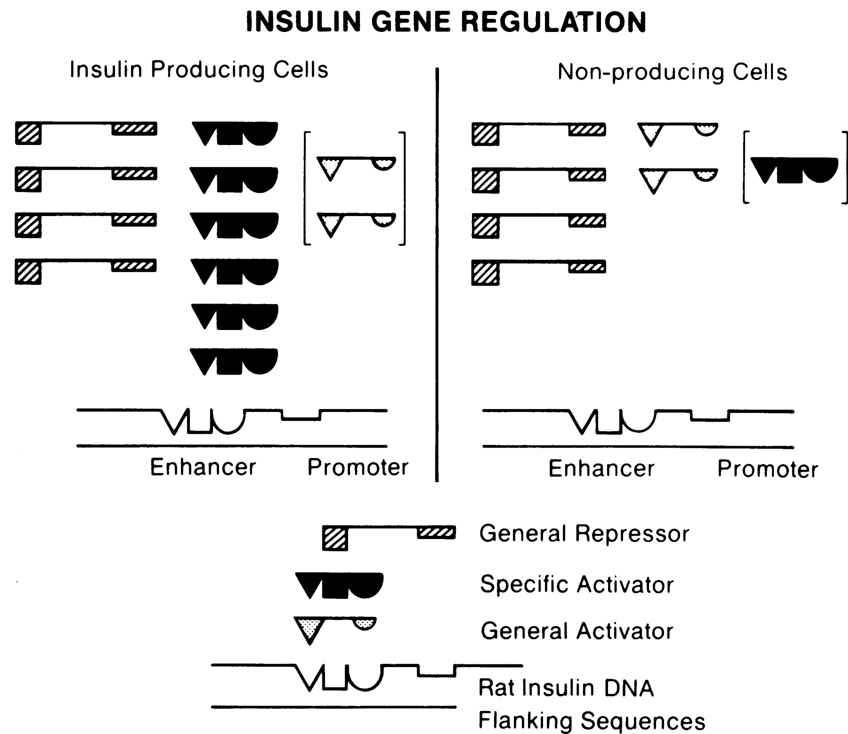


FIG. 5. Model for transcriptional regulation of the insulin gene. Insulin-producing cells contain sequence-specific positive regulators, negative regulators with less stringent sequence specificity, and possibly nonspecific enhancer activators. Nonproducing cells contain repressors, low concentrations of sequence-specific activators, and/or nonspecific activators.

differentiator (ref. 33; E. Fodor and W.J.R., unpublished results). From these experiments and those of others, it seems that the cell-specific regulation of transcription involves a number of cooperating and overlapping regulatory molecules, which can act upon a relatively small DNA domain. The study of mechanism should be greatly aided by identification of the *trans*-activators and characterization of their properties.

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