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Regulatory regions of rat insulin I gene necessary for expression in transgenic mice

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ABSTRACT

Ten transgenic mouse lines harboring the −346/−103 fragment of the rat insulin I enhancer linked to a heterologous promoter and a reporter gene (Eins-Ptk-CAT construct) were produced. Expression of the hybrid transgene was essentially observed in pancreas and to a lesser extent in brain. These results indicate that the rat insulin I promoter is dispensable for pancreatic expression. This insulin gene sequence is the shortest fragment described as conferring tissue-specific expression in transgenic mice. Two short homologous sequences in the rat insulin I enhancer fragment used, IEB2 and IEB1, have been described as playing a dominant role in the regulation of HIT hamster insulinoma cell-specific transcription of the insulin gene (1). We investigated whether the combination of IEB2 and IEB1 sequences is sufficient to confer specific expression in transgenic mice to a IEB2-IEB1-Ptk-CAT gene construct. No CAT activity was observed neither in pancreas nor in any other organ examined in 19 different transgenic mice. Moreover in transient expression experiments in RIN2A rat insulinoma cells, the IEB sequences had a very weak or no enhancer activity. These observations contribute to the conclusion that DNA regulatory elements other than the IEB sequences are necessary for gene expression *in vivo*.

INTRODUCTION

There are two nonallelic insulin genes in rat (as well as in mouse) which code two very homologous proteins. The rat insulin II gene has two introns, while the rat insulin I gene has only one. and might result from the retrotransposition of a partially processed ancestral insulin II gene transcript (2−4)

The 660 base pairs (bp) located upstream to the rat insulin II gene conferred pancreatic β-cell specific expression in transgenic mice to a hybrid gene (5), but the specificity of expression of rat insulin I gene was not yet examined in living animals. The regulation of the two genes was extensively analyzed in transient expression experiments over the past years in hamster insulinoma (HIT) cells (1,6−12), which led to the identification of several sequences important for their expression. These experiments have revealed differences in the 5′ regions involved in the regulation of the two genes. A distal regulatory element (IEB2) essential for insulin I gene expression appears unimportant for insulin II gene. On the other hand, an element (RIPE3b) located 5′ to a regulatory box (IEB1/RIPE3a) involved in the expression of the two genes,is crucial for insulin II, and not for insulin I gene (1,8−11) . Moreover, if the two rat genes are usually expressed *in vivo*, selective expression can be observed in rat insulinoma cell lines and might result from alterations in levels of transactivating factors (13). This observation also suggests some distinct regulation pathways for the two nonallelic genes.

The relevant question was to determine whether the DNA sequences sufficient to confer HIT-cell specific expression in transient experiments are competent in transgenic mice.

We first determined whether the DNA sequence included in the −346/−103 fragment of rat insulin I gene was sufficient to confer pancreatic specific expression in transgenic mice to a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a herpes virus thymidine kinase promoter (Ptk). This −346/−103 sequence was reported to display all the enhancer activity located upstream to the gene (7). Then, we constructed transgenic mice carrying the IEB2-IEB1-Ptk-CAT hybrid gene (1). IEB2 and IEB1, corresponding to −238/−229 and −113/−103 sequences, respectively, of insulin I gene, were the shortest sequences reported as playing a major role in the expression of a reporter gene in HIT cells. Block mutational analysis of the region upstream to the transcriptional start site have revealed that two short sequences are necessary for specific enhancer activity of this region. Replacement of either of these sequences resulted in 5- to 10-fold reduction of the activity, and mutation of both sequences almost abolished it (8). The two sequences include the same 8-bp motif (GCCATCTG), and they bound an insulinoma-cell specific protein (14). Moreover, IEB1 as well as IEB2 can stimulate a heterologous promoter specifically in HIT cells, and the association of both elements can confer about 40% of the activity of the rat insulin I enhancer (1). We found that, while the −346/−103 enhancer fragment was active, the combination of IEB2 and IEB1 elements alone failed to confer any clear pancreatic gene activity in transgenic mice.

MATERIALS AND METHODS

Gene constructs

The constructs containing IEB1-IEB1-Ptk-CAT; IEB2-IEB2-Ptk-CAT; IEB2-IEB1-Ptk-CAT; the rat insulin I enhancer (−346/−103)-Ptk-CAT (Eins-Ptk-CAT); the Moloney murine sarcoma virus enhancer-Ptk-CAT (MSV-Ptk-CAT); and Ptk-CAT (pOK2) were described by Karlsson et al. (1), and were kindly provided by T.Edlund.

A plasmid for luciferase expression (pRSV-L) (15) was used as an internal standard in some transfection experiments.

Tandem repeat polymers of the IEB1 sequence were constructed from two partially complementary synthetic oligonucleotides 5'-gatcCGCCATCTGCCTCGCCATCTGCC-TCGCCATCTGCCTa-3', and 5'-gatctAGGCAGATGGCGA-GGCAGATGGCGAGGCAGATGGCg-3', each containing three copies of IEB1. The oligonucleotides were treated with polynucleotide kinase, annealed, and ligated with T4 DNA ligase. The ligated polymers were inserted into pOK2 plasmid previously digested with *Bam* HI and *Bgl* II. The selected pFD31 clone was a direct 5'-3' repeat of 9 IEB1 copies.

Transgenic mice

The DNA fragments used for microinjection, obtained by *Kpn* I and *Xba* I double digestion of the plasmids followed by Biotrap (Schleicher and Schuell) electroelution, were diluted in Tris 10 mM, pH 7.5, EDTA 0.1 mM. Pronuclear microinjections were performed into (C57BL/6×DBA/2) F2 zygotes as described in Hogan et al. (16). About 1 pl of DNA solution containing 2400 copies were injected. The microinjected eggs were transferred into pseudopregnant females. The progeny obtained by this procedure was screened by a dot-blot assay of DNA (10 μg) prepared from tail biopsies, using as a probe the *Xba* I-*Kpn* I DNA fragments prepared for microinjection, labeled by random priming with either α (^{32}P)dATP or α (^{32}P)dCTP (the specific activity was about 2×10^9 cpm/μg DNA). The final wash of the filters was in 0.1×SSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7), 0.1% sodium dodecylsulphate, at 65°C. The filters were exposed with Hyperfilm-MP (Amersham).

Cell culture and DNA transfection

The different cell lines used, RIN2A (17), HIT M 2.2.2 (7), and LM TK$^-$Cl.1D (Cl.1D) were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum. Twenty-four hours before transfection, the cells were seeded at 1.2×10^6 cells per 100-mm diameter tissue-culture dish. DNA transfection was performed using calcium phosphate/DNA coprecipitates (18), with 8 μg of either plasmidic DNA per dish. Some transfection experiments included in addition for internal control pRSV-L DNA (3 μg). Cells were collected for transient expression analysis 48 h later.

CAT assays

CAT assays were performed (a) on homogenates prepared from various organs of transgenic mice, and (b) on cell extracts upon DNA transfection experiments.

(a) Some transgenic mouse founders were directly analyzed. Others were crossed with (C57BL/6×DBA/2) F1 mice and the transgenic mice from G1 progeny were examined for CAT expression. Various organs were homogeneized in 2 ml Tris 250 mM, pH 8, with a Polytron tissue homogenizer. The samples were clarified by centrifugation at 3600 g for 10 min, heated at 65°C for 10 min, and centrifugated again at 11000 g for 10 min. Protein concentration was determined by the Bio-Rad protein assay. For every organ extract, aliquots containing 70 μg proteins were tested for CAT activity at 37°C for 1 h essentially as described by Gorman et al. (19). (^{14}C) chloramphenicol acetylation was determined by counting the radioactivity of the different regions of the chromatograms in a scintillation counter.

Table 1. Expression of rat insulin I - Ptk - CAT transgenes

Rat insulin I fragments	Mouse number**	number of copies	CAT activity*									
			Pancreas	Spleen	Liver	Brain	Heart	Kidney	Salivary gland	Thymus	Testis or Ovary	Lung
-346/-103	3-22	25	12.8	- ***	-	4.6	-	-	-	-	-	-
	6-17	15	15.4	-	-	16.6	-	-	-	-	-	-
	17-1	2	0.7	-	-	-	-	-	-	-	-	-
	20-6 and 20-19	5	-	-	-	-	-	-	-	-	-	-
	28	20	98.2	-	-	1.3	-	-	-	-	-	-
	32	5	76.5	-	-	0.9	-	-	-	0.6	-	-
	34	3	3.2	-	-	1.7	-	-	-	-	-	2.1
	38	3	32.2	-	-	2.4	-	-	-	-	-	-
	40-2	10	3.7	-	-	5.8	-	-	-	-	-	-
	47-9	5	11.3	-	-	10.7	-	-	-	-	-	-
IEB2-IEB1	nineteen different transgenic mice	2-50	-	-	-	-	-	-	-	-	-	-
	37-6	20	1.0	-	-	-	-	-	-	-	-	-
	25	5	0.5	-	10.0	1.1	-	-	4.4	-	1.4	-

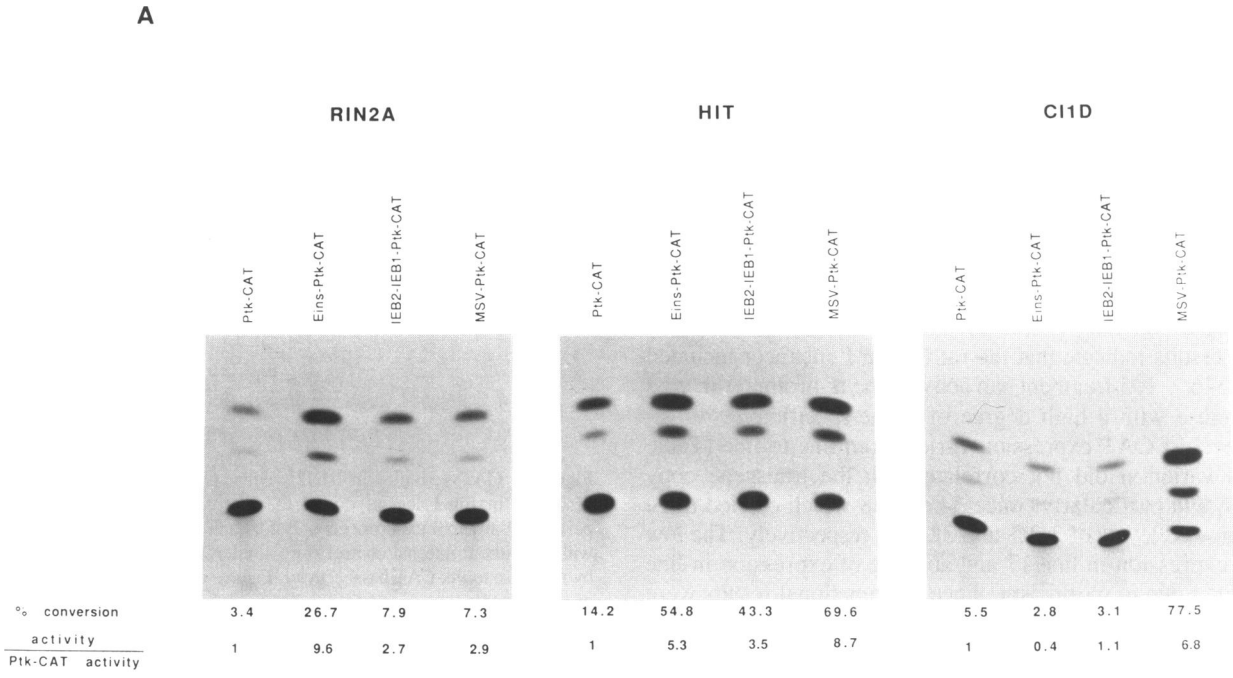
* Percentage of (^{14}C) chloramphenicol conversion to acetylated forms. All the tests were performed with 70μg of protein extracts

** Transgenic mice analyzed were either the founders (one-figure numbers) or G1 progeny (two-figure numbers)

*** ≤ 0.5% conversion

b) Cells collected 48 h after DNA transfection were resuspended in 100−160 µl Tris 250mM, pH 8, and lysed by three cycles of freezing-thawing. The extracts were prepared as above. CAT assays were generally performed with samples of 80 µg proteins for RIN2A cells, 160 µg for Cl 1D, and 8 µg for HIT. Enzymatic reaction was allowed to proceed at 37°C for 2 h, except for HIT cell extracts (30 min).

Luciferase activity was determined as described by Nguyen



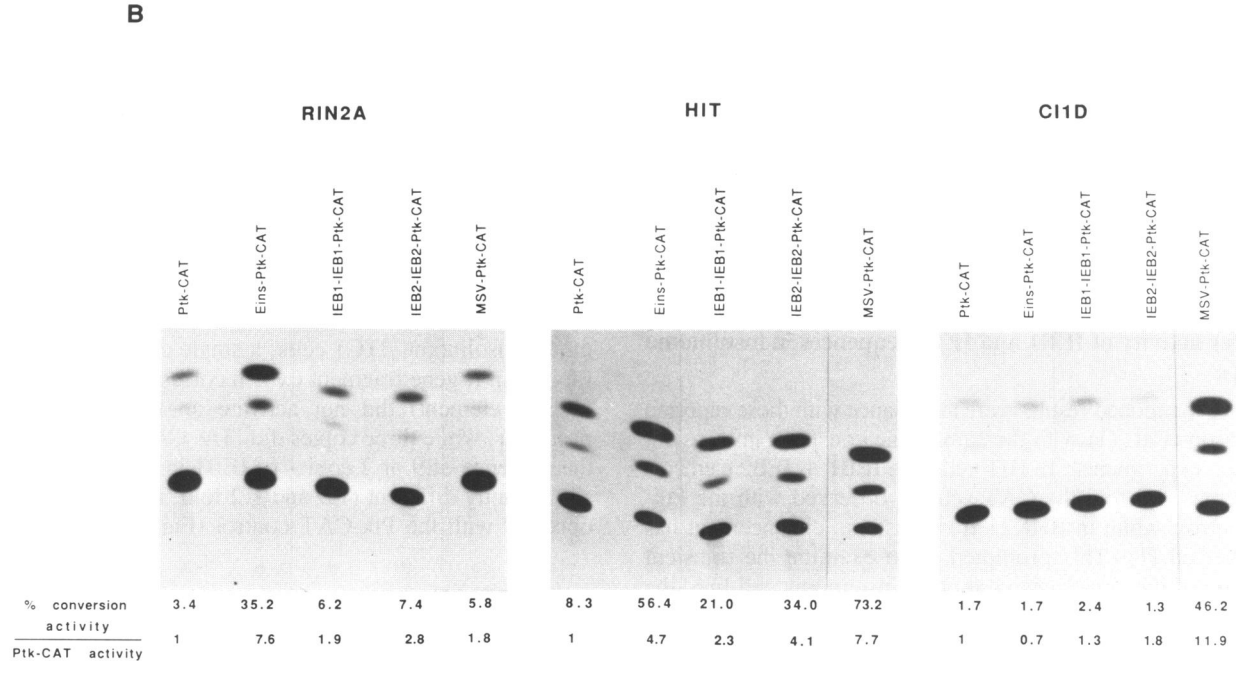


Figure 1. CAT activities obtained with various IEB combinations in RIN2A and HIT insulinoma and Cl1D fibroblast lines (A) The CAT activity elicited by the IEB2-IEB1-Ptk-CAT hybrid gene was compared with the rat insulin I and MSV enhancer activities. The percentage of (^{14}C)chloramphenicol conversion into acetylated forms calculated after counting the different spots in a scintillation counter is indicated. The activity of each construct tested was calculated as follows:

$$\frac{\text{percentage chloramphenicol conversion in the sample}}{\text{percentage conversion in the Ptk-CAT control}} \times \frac{\text{luciferase activity with the Ptk-CAT control}}{\text{luciferase activity in the considered sample}}$$

(B) The CAT activities obtained with the IEB1-IEB1-Ptk-CAT and the IEB2-IEB2-Ptk-CAT gene constructs were determined in the same cell lines.

et al. (20), using a LKB 1209 rackbeta scintillation counter. The CAT activity values were corrected following the luciferase activities detected in the same cell extracts.

RESULTS

Expression of the insulin gene constructs in transgenic mice

Ten transgenic mice carrying Eins-Ptk-CAT were obtained. They carried 2 to 25 copies of the transgene (Table 1). CAT activity was determined in pancreas, spleen, liver, brain, heart, kidney, salivary glands, thymus, testis or ovary, and lung (Table 1). CAT activity was detected in the pancreas of 9 of the 10 lines tested. In 6 lines, the conversion of unacetylated (^{14}C) chloramphenicol into acetylated forms was higher than 10%. In other tissues, CAT activity was essentially found in brain. In most instances, the level of expression in brain was lower than in pancreas. Transgenic mouse 32 had in addition a very low CAT activity in thymus, and mouse 34 in lung (Table 1).

These results indicate that the rat insulin I enhancer included in the −346/−103 fragment can activate the tk promoter in vivo in the mouse with a high degree of tissue-specificity.

The level of CAT expression varied from line to line (Table 1). This variation did not correlate with the transgene copy numbers, and particularly, mice 34 and 38 which carried three copies showed levels of 3.2% and 32.2%, respectively. The low level of expression in line 17 and absence of expression in line 20 were not due to mosaicism, since the functional results were obtained in G1 mice (Table 1).

In order to determine whether IEB2 + IEB1 sequences of the rat insulin I enhancer are sufficient to activate the tk promoter specifically in pancreas, we have constructed transgenic mice carrying the IEB2-IEB1-Ptk-CAT gene construct. Twenty-one transgenic mice were obtained, and CAT activity was checked in homogenates of different organs (Table 1). In 19 mice, no CAT activity was detected in pancreas and in any other organ neither. Mouse 25 had CAT activity in several organs, which suggests that the low activity found in pancreas of mouse 25 resulted from a transgene insertion near a strong endogenous promoter. Only mouse 37−6 had low CAT expression restricted to pancreas. These results indicate that the IEB1 + IEB2 sequences of the rat insulin I enhancer are not sufficient by themselves to activate the tk promoter *in vivo*.

Enhancer activity of IEB1 and IEB2 sequences in insulinoma cell lines

The above mentioned results were at variance with those reported by Karlsson et al. (1) with the same gene construct in transient expression experiments . In HIT cells, the IEB1 + IEB2 elements have increased 8-fold the CAT activity observed with the Ptk-CAT control, while in BHK21 fibroblasts no enhancement has been detected (1). This prompted us to examine the transient expression of the constructs in another insulinoma cell line, the RIN cells.

CAT activity elicited in RIN2A cells upon transfection of Eins-Ptk-CAT was 9.6-fold the basal activity obtained with Ptk-CAT (Fig. 1A). In contrast, the enhancement with IEB2-IEB1-Ptk-CAT was only 2.7 higher (Fig. 1A), i.e., only 28% of the enhancer activity of the −346/−103 fragment. In our hands, the IEB2-IEB1-Ptk-CAT gene elicited only 3.5-fold the basal CAT activity in HIT cells (Fig. 1A), but this represented nevertheless 66% of the −346/−103 enhancer fragment activity in the same cells. Also, in RIN2A cells, IEB1-IEB1-Ptk-CAT and IEB2-IEB2-Ptk-CAT restored only 25% and 37%, respectively, of the −346/−103 enhancer fragment activity, while in HIT cells the values observed were 49% and 87%, respectively (Fig. 1 B). No enhancement was observed with any of the insulin constructs tested neither in Cl 1D (Fig. 1 A and B), nor in BHK21 fibroblasts (data not shown), while the MSV enhancer stimulated the tk promoter in both fibroblast cell lines (Fig. 1 and data not shown).

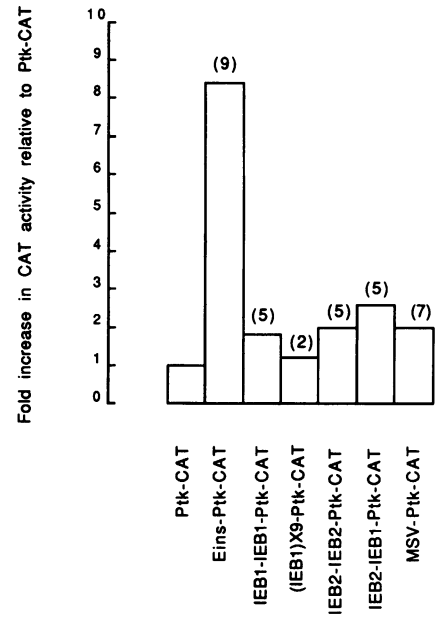


Figure 2. CAT activities in RIN2A cells. CAT activities were calculated as described in Fig.1 legend except that correction for luciferase activity was performed in 1 to 3 experiments only for each construct. The mean CAT values with or without internal control were similar. The fold increase was calculated by reference to Ptk-CAT basal activity. Figures in parentheses indicate the numbers of experiments.

Our results with RIN2A cells, reproduced in several independent experiments, are summarized in Figure 2. The IEB sequences appeared less effective in RIN2A than in HIT cells. In order to clarify the possible enhancer activity of the IEB sequences in RIN2A cells, 9 copies of IEB1 were introduced upstream to Ptk-CAT. Whelan et al. (10) have reported that in mouse insulinoma βTC1 cells, a single copy of the −100/−91 rat insulin II gene fragment (which corresponds to the rat insulin I IEB1 element) did not activate an heterologous β-globin promoter, while three copies did. The CAT activities elicited in the presence of 9 or 2 copies of IEB1 in RIN2A cells were not significantly different (1.9 and 1.2 fold, respectively) from that obtained with the Ptk-CAT control (Fig. 2).

DISCUSSION

Our results indicate that the −346/−103 DNA sequence of the rat insulin I gene can specifically activate a heterologous promoter in pancreas of transgenic mice. Of the 10 transgenic mice carrying the Eins-Ptk-CAT gene examined, 9 showed CAT activity in pancreas, of which three (mice 28, 32, and 38, see Table 1) at high levels.

This 250 bp enhancer fragment represents up to now the shortest sequence of an insulin gene conferring tissue-specific expression in transgenic mice. For the human insulin gene, cell-

specific expression was obtained with a −168/+7000 DNA fragment (21), or for a 1867 bp DNA fragment flanking immediately in 5′ of the translational start site (22) . For the rat insulin II gene, the cell specificity was obtained with a 660-bp DNA fragment of 5′ sequences (5).

Eight out of the 9 mice expressing the Eins-tk-CAT transgene in pancreas had also CAT activity in brain, generally at lower levels than in pancreas (Table 1). Novel tissue specificity was already observed with other hybrid genes (23,24), and the association of heterologous sequences might account for this phenomenon. Another explanation could be that there is a positive regulatory factor in mouse brain capable of activating a DNA regulatory element of rat insulin gene. Alpert et al. (25) have reported the transient expression in brain during development of a transgene including a 660 pb 5′-fragment of rat insulin II gene associated to the coding sequence of simian virus 40 large T antigen (ins-Tag transgene). This transient expression was found in the neural tube and neural crest, at sites where catecholamines are synthesized later. Because tyrosine hydroxylase, the enzyme of the first step in catecholamine biosynthesis, is transiently found in pancreatic endocrine cells (26), it was suggested that common elements are involved in the regulation of both insulin and tyrosine hydroxylase genes (25). We are currently attempting to localize in brain the Eins-Ptk-CAT transgene activity, in order to determine whether it is expressed in the same cells as the ins-Tag transgene.

The IEB2-IEB1-Ptk-CAT transgenic mice had no CAT activity in pancreas in twenty different lines. Only one transgenic mouse had a low CAT activity in pancreas. With one exception no activity was detected in any other organ examined (Table 1). This result strongly suggests that the IEB2 + IEB1 sequences are insufficient by themselves to activate the tk promoter, neither in pancreas, nor elsewhere.

These results contrast with those obtained with the same hybrid gene in HIT cells, where the combination of IEB2 and IEB1 sequences confer about 40% of the activity of the −346/−103 enhancer fragment (1). We have reproduced this enhancer activity in the same cells, but in another insulinoma, the rat RIN2A cell line, the IEB sequences had a very weak or no enhancer activity (Fig. 1 and 2). In contrast, the −346/−103 enhancer sequence was more efficient in RIN2A than in HIT cells (Fig. 1 and 2). Walker et al. (6) have suggested that the regulatory factors necessary for high expression of genes introduced by transfection are underrepresented in HIT cells. One can speculate that the relative amounts of the particular factors interacting with the IEB sequences in these cells are high among the other factors interacting with the complete rat insulin I enhancer, a feature that is found neither in RIN2A cells, nor in transgenic mice. The better expression of the transfected Eins-tk-CAT gene in RIN2A cells might also result from the exclusive activity of the endogenous insulin I gene in these RIN2A cells (13).

Attempts to isolate regulatory proteins interacting only with the IEB sequences resulted until now in the cloning of an ubiquitous protein (27,28). The rat protein Isl-1 recently cloned by Karlsson et al. (29) is pancreatic β-cell specific, and binds to the −222/−211 region of rat insulin I gene, but not to the IEB sequences. The −222/−211 sequence has been already described by Karlsson et al. (1) as potentiating the IEB2 activity in HIT cells.

Altogether, these results imply that the role of the IEB sequences in the positive regulation of rat insulin I gene expression might be less predominant than suggested (1,14).

Deletion analysis in transgenic mice of the human insulin gene (21) showed that the sequences corresponding to IEB also do not play a predominant role in the human gene. When the regions corresponding to IEB1 and IEB2 were maintained (deletion −258), the transgenic mice had 10-fold less human insulin in pancreatic islets than those which harbored a transgene with 4 kb upstream to the transcription unit. When the transgene started at −168, i.e., had still the region corresponding to IEB1, its expression was virtually suppressed (21).

The specific pancreatic expression was indeed obtained with a larger fragment encompassing the −346/−103 sequence of the rat gene. This demonstrates that the insulin I promoter is dispensable for pancreatic expression. However, this tissue specificity was not absolute, since most of the Eins-Ptk-CAT transgenic mice had also CAT activity in brain (Table 1). In contrast, transgenic mice carrying the human gene starting at −353 had together a high level of expression in pancreatic β cells and no obvious expression in other organs (21). In the present results, the use of a very sensitive reporter gene might result in apparent levels of expression higher than would have been detected with the insulin gene itself. The sequences 3′ to −103 either in the insulin gene promoter (−103/+1), or even downstream may also include elements capable of suppressing the low extrapancreatic expression observed. This question was not raised by the results of Karlsson et al. (1) since these authors found in transfection experiments that the −346/−103 fragment was not active in fibroblasts. However, in transgenic mice, Hammer et al. (30) have described in the rat elastase I enhancer a sequence spanning positions −205 to −150 which deletion suppressed the specific expression in pancreatic exocrine cells.

Edlund et al. (7) have shown that the −103/+1 fragment of the rat insulin I gene includes an element conferring HIT cell specific expression to a reporter gene in the presence of an heterologous enhancer. In human, two sequences CT II and CT I, at positions −217/−210, and −84/−77, respectively, interact with insulinoma specific factors (31,32). The equivalent of the CT II region in the rat insulin I gene is the target sequence for the Isl-1 protein (29) and corresponds to the sequence that potentiates IEB2 activity (1). The rat insulin I gene region homologous to CT I (−83/−76) included in the −103/+1 fragment might also be important for the pancreatic gene expression at physiological levels.

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