RESEARCH ARTICLE

Cell-Specific Expression of the Rat Insulin Gene: Evidence for Role of Two Distinct 5′ Flanking Elements

Thomas Edlund, Michael D. Walker
Philip J. Barr, William J. Rutter

Abstract. *The 5′ flanking DNA of the rat insulin I gene contains sequences controlling cell-specific expression. Analysis of this region by replacement of specific portions with nondiscriminatory control elements from viral systems shows that a transcriptional enhancer is located in the distal portion of the 5′ flanking DNA; its position has been mapped by deletion analysis. Additional experiments suggest that another distinct regulatory element is located more proximal to the transcription start site. The activity of both elements is restricted to pancreatic B cells. The combinatorial effect of multiple control elements could explain the cell-specific expression of insulin genes.*

The appearance and maintenance of differentiated cell types in eukaryotic organisms is largely attributable to characteristic temporal and spatial patterns of cell-specific gene expression. The control of gene expression appears to be exerted chiefly at the level of initiation of transcription (*1*). Previous studies in numerous eukaryotic systems have revealed the existence of two major classes of *cis*-acting transcriptional control elements, promoters (*2*) and enhancers (*3*). The promoter is an element located proximal to the transcription start site that is capable of initiating transcription; its activity is relatively position and orientation dependent. Enhancers are elements that stimulate initiation of transcription by increasing the activity of promoters within their domain; enhancer activity is relatively position and orientation-independent. The distinct properties of enhancers and promoters suggest a fundamental difference in their mechanism of action.

To study the mechanisms involved in cell-specific gene expression we have used the mammalian pancreas as a model system. We showed earlier that differentiated pancreatic cell lines selectively express exogenous genes containing putative pancreas-specific control regions fused to the coding region of a bacterial reporter gene, chloramphenicol acetyltransferase (CAT) (*4*). The 5′ flanking regions of the human or rat insulin genes were fused to CAT coding sequences; such recombinants elicited high activity when transfected into a differentiated insulin-producing cell line, but showed 50 to 200 times less activity in other cell lines. Likewise, recombinants containing the 5′ flanking regions of the rat chymotrypsin gene displayed much higher activity in chymotrypsin-producing tumor cells than in other cells. The results suggest that sequences within the 5′ flanking DNA function in selective gene regulation in differentiated cells. Consistent with this hypothesis, 5′ flanking sequences of elastase (*5*) and insulin (*6*) genes were recently shown by gene transfer to mouse embryos to drive selective expression of linked coding sequences in pancreatic exocrine and endocrine B cells, respectively, in adult animals.

We have now further dissected the control region of the rat insulin I gene by selective deletions, and by promoter and enhancer replacement experiments with promiscuous control elements from viral systems. For example, the insulin promoter region was replaced with the herpes simplex virus thymidine kinase (TK) promoter to further characterize the properties of the upstream regions; analogously, the insulin upstream regions were replaced by a viral enhancer derived from Moloney murine sarcoma virus (MSV) to study the characteristics of the insulin promoter region. Our result suggest that the cell specificity determinants consist of at least two elements: an upstream element that exhibits the properties of an enhancer, and another, previously unrecognized, control element more proximal to the transcription start site.

A convenient plasmid for enhancer characterization. In the plasmid pTE1 (Fig. 1) transcription of the CAT gene is controlled by the thymidine kinase promoter (TK_p). About 600 bases upstream from this promoter lies a polylinker; interposed between the polylinker and the promoter is a segment of pBR322 that can be removed by appropriate restriction enzyme treatment. The plasmid pTE1 permits convenient testing of the relative position- and orientation-dependence of putative enhancers, as well as simplifying deletion analysis.

As an internal control for variations in the efficiency of uptake of DNA among the different plates in a single experiment, we constructed a plasmid (pRSV-βGal) containing the gene coding for *Escherichia coli* β-galactosidase controlled by a powerful promiscuous promoter, derived from the Rous sarcoma virus (RSV) long terminal repeat (*7*). Inclusion of an internal control plasmid when using the CAT system permits much more reliable comparison of different CAT constructs (*8*); the standard deviation of 22 percent we observed in CAT activity measurements among duplicate plates was reduced to 8 percent when activities were normalized using β-galactosidase measurements. Under the experimental conditions used, we noted no effects of the presence of the internal control plasmid on CAT activity.

Continuous passage of some cell lines, including the insulin-producing hamster line (HIT-T15) (*9*) that we used previously, leads to significant loss in transfection competence, necessitating use of relatively freshly thawed cells (*10*). In the course of generating transformants of HIT cells with the plasmid pSV2neo (*11*), we isolated transformants whose transfection competence was considerably more stable (up to 6 months in culture, rather than 4 to 6 weeks). We therefore used one of these clonal lines

Thomas Edlund was a postdoctoral fellow in the Hormone Research Institute, University of California, San Francisco 94143, and is now an assistant professor in the Department of Applied Cell and Molecular Biology, University of Umea, S901 87 Umea, Sweden. Michael D. Walker is an adjunct assistant professor and William J. Rutter is professor and director of the Hormone Research Institute, University of California, San Francisco 94143. Philip J. Barr is a senior scientist at Chiron Corporation, Emeryville, California 94608.

(designated HIT-T15 M2.2.2) for the experiments described below.

The insulin enhancer is cell-specific and complex. A fragment of DNA containing 5′ flanking sequences (−410 to +51) of the rat insulin I gene was inserted at three different positions relative to the TK promoter of pTE1: 5′ proximal, 5′ distal and 3′ (Table 1). In all cases CAT activity was elevated in HIT cells but not in fibroblasts. In contrast, the MSV enhancer was active in both cell types, albeit at different levels. As with other enhancers (*12*), some effect of position was observed with the insulin enhancer; we found optimal enhancement of activity (42-fold) when this element was located at the 5′ proximal position.

Two series of deletion mutants were constructed and tested (Fig. 2). Since the two series of deletion mutants are oriented oppositely, the insulin enhancer activity operates in either orientation relative to the TK promoter. In the 5′ series, removal of sequences from −410 to −249 resulted in relatively minor effects on enhancer activity. However, further deletion of sequences beyond −249 caused more substantial reduction. In the 3′ series, a dramatic reduction in activity was seen when sequences between −103 and −150 were removed. When a plasmid containing −103 to −249 was constructed (*13*) and assayed, it showed 70 to 75 percent of full enhancer activity in either orientation adjacent to the TK promoter (Fig. 2, constructs 13 and 14). The mapping data show that the enhancer activity is spread over a region (−103 to −333) and that its borders are not precisely defined. Indeed, internal portions of the enhancer element (−159 to −249), which showed only low activity, stimulated full activity when duplicated (*14*), consistent with the notion that the enhancer is a composite element containing reiterated regions. However, this composite structure is not obvious from the DNA sequence.

We have tested the activity of the insulin enhancer in a number of cell types in addition to HIT and BHK cells. In the insulin-secreting RI-9 cell line [(derived from the rat insulinoma RIN cell (*17*)] 20- to 25-fold stimulation of TK promoter activity was observed. In contrast, no significant enhancement of TK promoter activity was observed in fibroblasts derived from hamster (CHO), mouse (L), rat (XC) or monkey [COS (*15*)], in rat epithelial cells [pancreatic exocrine cells (AR4-2J) (*16*), or in pituitary somatotrophs (GH_3)]. Therefore, the activity of the insulin enhancer appears to be confined to insulin-producing cells.

An additional cell-specific control element. The detection of insulin enhancer activity upstream from −103 does not eliminate the possibility that sequences downstream from this position also play an important role in cell-specific expression. Indeed, we previously observed a striking sequence conservation not only within the region of the enhancer but also in the promoters of different mammalian insulin genes (*18*). Since removal of the insulin enhancer results in undetectable promotion of CAT activity, we tested the properties of the downstream region by replacement of the insulin enhancer with the MSV enhancer. The activity of such recombinants (Table 2, constructs 6 and 7) was measured in HIT cells and BHK cells and compared with similar recombinants containing the

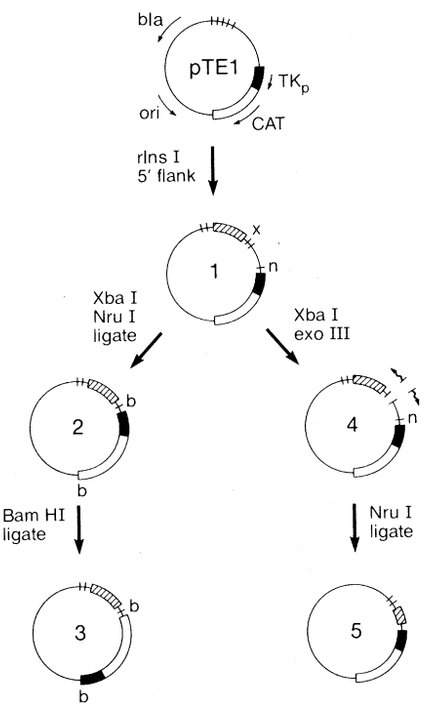

Fig. 1. Structure of plasmid pTE1 and its use for the identification and characterization by deletion analysis of the enhancer element within insulin 5′ flanking DNA. Plasmid pTE1 was constructed from pBR N/S (*43*) by incorporating the following elements, listed clockwise from the 12 o'clock position. (i) A 56-bp polylinker sequence containing the following sites: Eco RI, Cla I, Hind III, Xba I, Bgl II, Pst I, Sal I, and Bam HI. (ii) A 598-bp fragment from the pBR322 Bam HI site (375) to the Nru I site (972). (iii) The TK promoter (TKp) region (−109 to +51) containing a Bam HI site at the 5′ end and flanked at both ends by Sac I sites. (iv) A 1634-bp fragment containing CAT coding sequences linked to SV40 splice junctions and polyadenylation signals (*44*). (v) A 3390-bp pBR322 fragment from the Nru I site (972) to the Eco RI site (4361) containing the plasmid replication origin (ori) and β-lactamase gene (bla). The rat insulin I (rInsI) −410 to +51 fragment (hatched bar) was inserted in inverted orientation at the Hind III site within the polylinker portion of the plasmid, generating plasmid 1. The effect of distance and orientation on enhancement was tested by deletion of the Xba I (x) to Nru I (n) fragment (plasmid 2) and inversion of the TK.CAT Bam HI (b) fragment (plasmid 3). Plasmids containing deletions at the 5′ terminus of the insulin −410 to +51 fragments were produced by successive treatment of plasmid 1 with Xba I, DNA polymerase I (Klenow fragment), exonuclease III (exo III), nuclease S1, Nru I, and T4 DNA ligase (plasmids 4 and 5). For 3′ deletions a similar procedure was followed with a starting plasmid containing the rat insulin I gene fragment in orientation opposite to that of plasmid 1 (*50*).

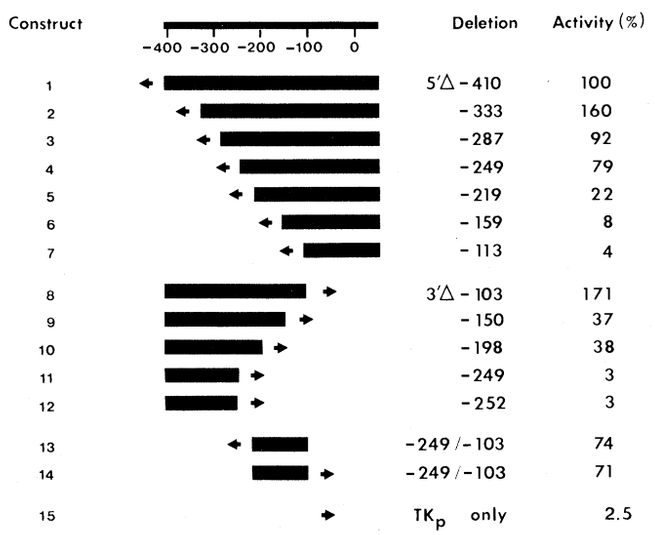

Fig. 2. CAT activities directed by the TK promoter in the presence of insulin 5′ flanking fragments containing 5′ and 3′ deletions. The insulin −410 to +51 fragment was inserted in either orientation at the Hind III site within the polylinker portion of plasmid pTE1. Successive 5′ and 3′ deletions of this fragment were produced as described in the legend to Fig. 1. The precise end points of these deletions, determined by DNA sequencing, are indicated in base pairs relative to the insulin cap site. Activities were determined after transfection of HIT cells (*46*) and are expressed relative to that of construct 1. Arrows indicate the relative location and transcriptional orientation of the TK promoter in the 5′ and 3′ series of deletions. TK_p alone indicates the activity obtained after transfection of HIT cells with plasmid pTE1.

Table 1. Effects of insulin enhancer and MSV enhancer on TK promoter activity. Relative CAT activities directed by the TK promoter in the presence of −410- to +51-bp rat insulin I gene fragment or the MSV enhancer (−490 to −150) (*19*). These two fragments were inserted at the polylinker portion of plasmid pTE1 in opposite orientation to the TK promoter. The 600 bp of pBR322 DNA which separated these fragments from the promoter in the 5′ distal configuration was removed by deleting the Xba I–Nru I fragment to yield the 5′ proximal configuration. The MSV and insulin fragments were positioned at the 3′ end of the CAT gene by either inverting the TK.CAT Bam HI fragment or by the addition of these fragments at the Bam HI site 3′ to the CAT gene. All CAT plasmids were cotransfected with pRSV-βGal and the CAT and β-galactosidase activities were assayed in the cell extracts as described (*4*, *46*). The CAT activities are expressed relative to that of plasmid pTE1.

Construct	Enhancer	Configuration	Relative CAT activity	
			HIT	BHK
1	None		1.0	1.0
2	Insulin	5′ distal	3.2	0.8
3	Insulin	5′ proximal	42.2	1.2
4	Insulin	3′ distal	11.2	1.3
5	MSV	5′ distal	3.8	3.7
6	MSV	5′ proximal	38.7	8.7
7	MSV	3′ distal	10.4	6.0

MSV enhancer and TK promoter (constructs 1 and 2), the MSV enhancer-promoter (construct 3), the RSV enhancer-promoter (construct 4), and rat β-actin promoter (construct 5). The constructs containing the insulin promoter displayed a substantial (approximately tenfold) preferential expression in HIT cells. These results are consistent with the presence in the promoter region of an additional element controlling cell-specific expression of the gene. This portion of DNA contained little or no intrinsic enhancer activity (Fig. 2, construct 7) nor was it able to augment the activity of the MSV enhancer on TK promoter (Table 2, constructs 8 and 9).

In these experiments, the MSV enhancer fragment, although devoid of promoter activity, exhibited an unexpected degree of orientation dependence. This may be due to the presence of a small segment of mink genomic DNA at the 5′ end of the fragment used (*19*) that could interfere with the transmission of the enhancer effect. Alternatively, some of the sequences at the 3′ end of the enhancer fragment might complement an intrinsic deficiency in the promoter function of the insulin fragment.

Primer extension analysis of CAT RNA. Many of the constructions used to measure enhancement of the TK promoter also contain insulin promoter sequences. In such cases, we analyzed only constructions in which the orientation of the insulin promoter was inappropriate for CAT expression (Tables 1 and 2 and Fig. 2). Nevertheless, to confirm that expected transcription start sites were used and that CAT activity determination in our experiments reflected steady-state levels of RNA, we analyzed cellular RNA after transfection of HIT cells with CAT con-

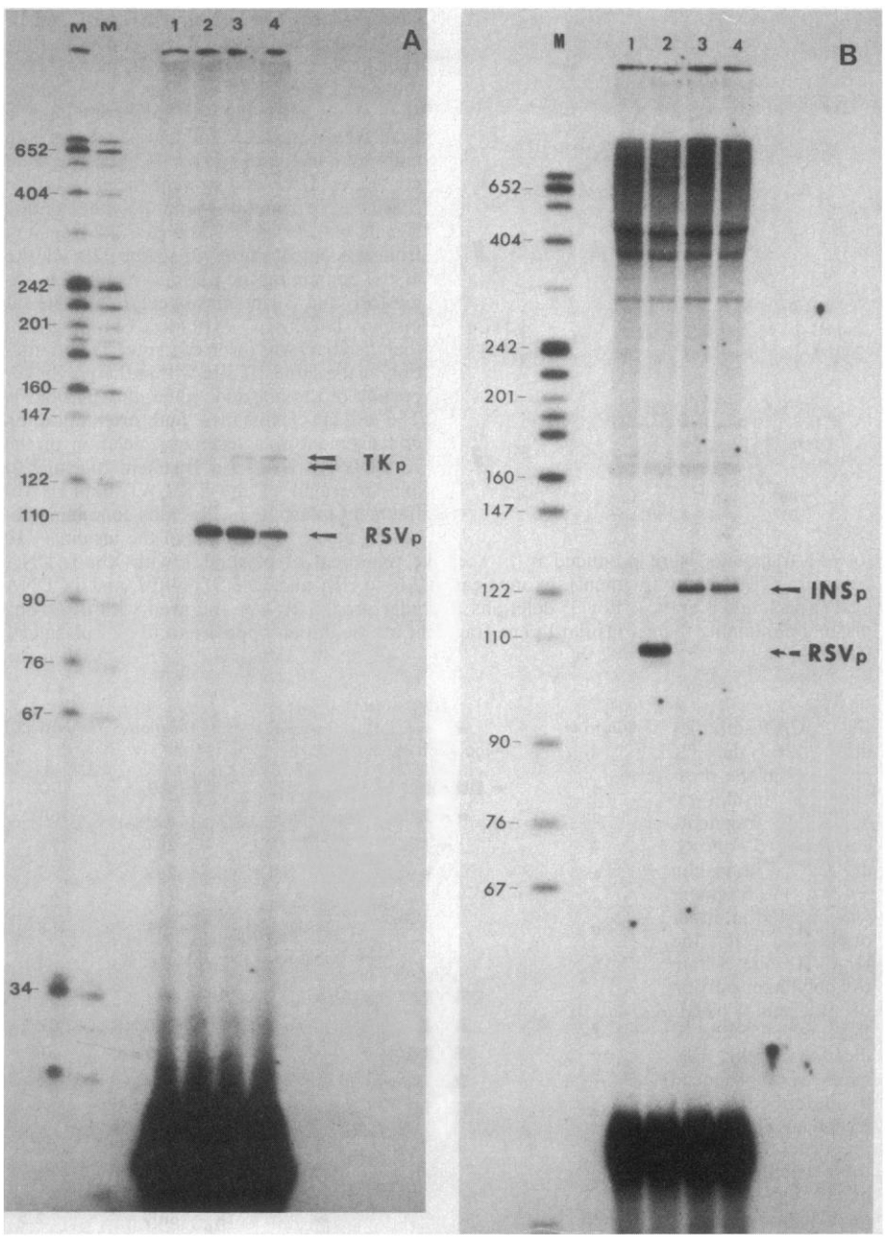

Fig. 3. Analysis by primer extension of CAT transcripts in transfected HIT cells (*4*). The autoradiograms represent data from primer extension with RNA prepared from HIT cells transfected with the following: (A) no DNA, lane 1; pTE1, lane 2; pMSV.TK.CAT, lane 3; and prIns.TK.CAT, lane 4. The construct pMSV.TK.CAT corresponds to construct 6 in Table 1, and prIns.TK.CAT corresponds to construct 3 in Table 1 and construct 1 in Fig. 2. In addition, all constructs were cotransfected with pRSV.CAT as an internal control. Arrows indicate the positions of the primer extension products of RNA initiating at the RSV (RSV_p) cap site and at the TK cap sites (TK_p), respectively. The lanes marked "M" display radioactive size markers. (B) No DNA, lane 1; pRSV.CAT, lane 2; prInsCAT, lane 3; and pMSV.Ins.CAT, lane 4. In prIns-.CAT (*4*) the CAT gene is controlled by the rat insulin I gene 5′ flanking DNA (−410 to +51). Lane M as in (A). pMSV.Ins.CAT corresponds to construct 6 (in Table 2). The arrows indicate the predicted bands corresponding to use of RSV, TK, and insulin cap sites. The predicted hybrid transcripts contain 71 bases of CAT gene sequence and either 36 nucleotides of RSV sequence (combined length 107), 49 nucleotides of insulin gene sequence (combined length 120), or 63 nucleotides of TK gene sequence (combined length 134). In the case of the TK promoter, a weaker additional band (*129 nucleotides*) is observed, which results from a reverse transcriptase pause site (*2*). The primer has been described (*4*).

structs. The sensitivity of the primer extension method did not permit us to detect RNA produced under the control of the unenhanced TK promoter (Fig. 3A, lane 2). However, in the presence of either the MSV enhancer or the insulin enhancer, transcripts were detected initiating at the expected TK cap site, showing that the insulin enhancer was indeed acting by activating the TK promoter (Fig. 3A, lanes 3 and 4). In the case of the enhancer replacement experiment, we observed that enhancement of the insulin promoter region by the MSV enhancer region occurred by activation of the natural insulin promoter (Fig. 3B, lanes 3 and 4).

Enhancers and promoters in differentiation. Upstream 5′ flanking sequences (~250 to 300 bp) of the insulin and chymotrypsin genes can drive the selective expression of a fused CAT gene in appropriate differentiated cells in a transient system (*4*). Further dissection of this region of the insulin gene by fusing selected segments with heterologous regulatory elements demonstrates that the structure determining cell specificity contains at least two distinguishable functions. The distal upstream element has the properties of an enhancer: it increases the activity of a heterologous viral promoter (TK) specifically in insulin-producing cell lines. In addition, a fragment of the insulin gene proximal to the transcription start site that is almost entirely devoid of enhancer activity, and is unable by itself to express the coupled CAT gene coding sequence, can promote cell-specific expression when coupled with a nonspecific enhancer.

Enhancer elements were discovered and have been most thoroughly studied in mammalian viruses (*3*). Comparison of viral enhancers reveal quite different overall sequences but some short stretches of homology (*20*). More recently, DNA transfection experiments have shown that sequences in the introns of immunoglobulin heavy (*21, 22*) and light (*23, 24*) chain genes and sequences in the 5′ flanking region of an Eβ MHC gene (*25*) have characteristics of enhancers. In addition to the insulin enhancer characterized here, companion experiments (*26*) identified enhancer-like elements in a similar region of the 5′ flanking DNA of the rat exocrine pancreatic chymotrypsin and amylase genes. In all the above cases of nonviral enhancers, the activities show a striking cell specificity consistent with the normal selective expression of the related gene. It is likely, therefore, that the enhancer plays an important role in controlling cell-specific expression of these genes.

Table 2. Cell-specific expression directed by the insulin promoter region. Relative CAT activities directed by the insulin (−113 to +51) and TK promoter fragments in the presence of the MSV enhancer (*47*). Arrows indicate the native and inverted configuration of the MSV enhancer and the insulin promoter. Activities were determined (*46*) and expressed relative to the activity of construct 1 (pMSV.TK.CAT). Constructs 3 and 4 are pMS.LTR2 (*48*) and pRSV.CAT (*7*), respectively. Construct 5 is pβ-CAT (*49*), which contains 1.5 kilobases of flanking DNA. The presence of an enhancer in this region has not been defined. The data shown represent the mean of two to three independent determinations; in these experiments the activity ratio varied by up to 40 percent of the mean value. For the case with lowest activity (construct 7) the signal (225 counts per minute) to background (50 counts per minute) ratio was 4.5.

Construct	Enhancer	Promoter	Relative CAT activity		Ratio
			HIT	BHK	
1	MSV→	TK	1.00	1.00	1.0
2	MSV←	TK	0.32	0.42	0.8
3	MSV	MSV	3.80	5.21	0.7
4	RSV	RSV	1.65	2.33	0.7
5		β-Actin	1.08	2.73	0.4
6	MSV→	Ins	0.41	0.05	8.2
7	MSV←	Ins	0.04	0.003	13.3
8	←Ins_p.MSV→	TK	0.56	0.61	0.9
9	←Ins_p.MSV→	TK	0.19	0.34	0.6

We also provide evidence for the existence of another cell-specific regulatory element distinct from the enhancer defined above, but functionally complementary to it. The evidence is in part derived from an "enhancer replacement" experiment. Recombinants containing the MSV enhancer linked to the insulin promoter region showed a clear (about tenfold) preference for insulin-producing cells as compared with fibroblasts. More recently we have deleted all transcribed insulin gene sequences (+1 to +51) from such constructs (*27*), and find no significant loss of cell-specific preference, suggesting that the activity is mediated at the level of initiation of transcription. Thus, these sequences contain a novel determinant that makes a contribution to the cell specificity of insulin gene expression. The importance of this region in generation of cell specificity is supported by the strong conservation of nucleotide sequence observed for several mammalian insulin gene promoters (*18, 28*).

The evidence suggests, therefore, that the insulin gene–flanking DNA contains at least two specificity determinants. Cooperation between distinct regulatory elements could produce multiplicative effects which would help to explain the degree of control and high levels of expression typically seen for the selectively expressed genes of differentiated cells. Extensive sequence conservation (>200 bp) in the proximal 5′ flanking DNA of genes from different species supports the contention that multiple regulatory elements function in other selectively expressed genes (*29–31*). In immunoglobulin genes the promoter region contains evolutionarily conserved sequences (*32*) which have been shown to be required for correct transcription of kappa chain genes (*33, 34*). Recent results (*35*) suggest that the promoter of immunoglobulin genes also displays cell-type specificity that is independent of the enhancer.

We have previously proposed that the *cis*-acting activity of 5′ flanking DNA sequences is mediated by a *trans*-acting molecule that we called a differentiator (*4*). The present results suggest that multiple such factors exist. Preliminary data (*36*) obtained with deoxyribonuclease I protection (footprinting) techniques are consistent with this idea. On the basis of the effects of deletions on enhancer activity, it is likely that *trans*-acting factors interacting with the enhancer are activators. However, we cannot exclude a role for *trans*-acting negative regulators in nonhomologous cells (*37*). Evidence accumulating from a variety of systems indicates that differentiated mammalian cells contain factors that can bind in vivo (*38, 39*) and in vitro (*40*) to DNA sequences implicated in cell-specific transcriptional control. Although such factors remain to be isolated and their activities characterized, well-established precedents do exist for activation in *trans* of both enhancers [by steroid receptor (*41*)] and promoters [by transcription factor Spl (*42*)]. The further characterization of *cis*-acting sequences determining cell specificity and the identification of their putative *trans*-acting differentiators provides a powerful approach to the study of the molecular mechanisms involved in cellular differentiation.

References and Notes

1. J. E. Darnell, *Nature (London)* **297**, 365 (1982).
2. S. L. McKnight and R. Kingsbury, *Science* **217**, 316 (1982).
3. G. Khoury and P. Gruss, *Cell* **33**, 313 (1983).
4. M. D. Walker, T. Edlund, A. M. Boulet, W. J. Rutter, *Nature (London)* **306**, 557 (1983).
5. D. M. Ornitz *et al.*, *ibid.* **313**, 600 (1985).
6. D. Hanahan, *ibid.* **315**, 115 (1985).
7. C. M. Gorman, G. T. Merlino, M. C. Willingham, I. R. Pastan, B. H. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982).
8. P. Herbomel, B. Bourachot, M. Yaniv, *Cell* **39**, 653 (1984); G. An, K. Hidaka, L. Siminovitch, *Mol. Cell. Biol.* **2**, 1628 (1982).
9. R. F. Santerre *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4339 (1981).
10. C. Gorman, R. Padmanabhan, B. H. Howard, *Science* **221**, 551 (1983).
11. P. J. Southern and P. Berg, *J. Mol. Appl. Gen.* **1**, 327 (1982).
12. B. Wasylyk, C. Wasylyk, P. Augereau, P. Chambon, *Cell* **32**, 503 (1983).
13. M. J. Zoller and M. Smith, *Methods Enzymol.* **100**, 468 (1983); G. P. Larson, K. Itakura, H. Itoh, J. J. Rossi, *Gene* **22**, 31 (1983).
14. T. Edlund, unpublished data; W. Herr and Y. Gluzman, *Nature (London)* **313**, 711 (1985).
15. Y. Gluzman, *Cell* **23**, 175 (1981).
16. N. W. Jessop and R. J. Hay, *In Vitro* **16**, 212 (1980).
17. A. Gazdar *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 3519 (1980).
18. G. I. Bell, M. J. Selby, W. J. Rutter, *Nature (London)* **295**, 31 (1982).
19. R. Dhar, W. L. McClements, L. W. Enquist, G. F. Van de Woude, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3937 (1980).
20. H. Weiher, M. Konig, P. Gruss, *Science* **219**, 626 (1983).
21. S. D. Gillies, S. L. Morrison, V. T. Oi, S. Tonegawa, *Cell* **33**, 717 (1983).
22. J. Banerji, L. Olsen, W. Schaffner, *ibid.*, p. 729.
23. C. Queen and D. Baltimore, *ibid.*, p. 741.
24. D. Picard and W. Schaffner, *Nature (London)* **307**, 80 (1984).
25. S. D. Gillies, V. Folsom, S. Tonegawa, *ibid.* **310**, 594 (1984).
26. A. M. Boulet, C. Erwin, W. J. Rutter, unpublished data.
27. Site-specific deletion mutagenesis (*13*) was performed with phage M13mp8. Deleted M13 recombinants were characterized by plaque hybridization and DNA sequencing. The double-stranded M13 replicative forms were purified by CsCl gradient centrifugation, and the appropriate fragments were subcloned to plasmid pTE1.
28. S. J. Chan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5046 (1984).
29. D. D. Moore *et al.*, *Rec. Prog. Horm. Res.* **38**, 197 (1982).
30. L. P. Shen and W. J. Rutter, *Science* **224**, 168 (1984).
31. M. A. Tavianini, T. E. Hayes, M. D. Magazin, C. D. Minth, J. E. Dixon, *J. Biol. Chem.* **259**, 11798 (1984).
32. T. G. Parslow, D. L. Blair, W. J. Murphy, D. K. Granner, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2650 (1984).
33. F. G. Falkner and H. G. Zachau, *Nature (London)* **310**, 71 (1984).
34. Y. Bergman, D. Rice, R. Grosschedl, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7041 (1984).
35. J. Foster, J. Stafford, C. Queen, *Nature (London)* **315**, 423 (1985); J. D. Mason, G. T. Williams, M. S. Neuberger, *Cell* **41**, 479 (1985); R. Grosschedl and D. Baltimore, *ibid.*, p. 885; T. V. Gopal, T. Shimada, A. W. Baur, A. W. Nienhaus, *Science* **229**, 1102 (1985).
36. E. Fodor and W. J. Rutter, unpublished results.
37. A. M. Killary, and R. E. K. Fournier, *Cell* **38**, 523 (1984).
38. A. Ephrussi, G. M. Church, S. Tonegawa, W. Gilbert, *Science* **227**, 134 (1985).
39. M. Mercola, J. Goverman, C. Mirell, K. Calame, *ibid.*, p. 266.
40. B. M. Emerson and G. Felsenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 95 (1984).
41. F. Payvar *et al.*, *Cell* **35**, 381 (1983); M. Karin *et al.*, *ibid.* **36**, 371 (1984).
42. D. Gidoni, W. S. Dynan, R. Tjian, *Nature (London)* **312**, 409 (1984); W. S. Dynan and R. Tjian, *ibid.* **316**, 774 (1985).
43. M. Haltiner, T. Kempe, R. Tjian, *Nucleic Acids Res.* **13**, 1015 (1985).
44. C. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
46. Plasmid pRSV-βGal was made by introducing a 400-bp Hind III-Nru I fragment from pRSV.CAT (*7*) containing an intact RSV enhancer-promoter unit into the Bam HI site of the plasmid pGA307 (*8*). Plasmid pRSV-βGal directed efficient production of β-galactosidase in various rodent cell lines (M. D. Walker, unpublished). In our standard transfection procedure we mixed 5 μg of pRSV-βGal with 5 μg of test CAT plasmid. Transfections and extract preparations were as before (*4*), and the β-galactosidase assay was performed as described (*8*), but with phosphate buffer at *p*H 8.0. The CAT activities were determined as described (*44*). Typical conversion activities for HIT cells (10 μg of protein, 30-minute assay) were <0.02 percent (promoterless CAT plasmid), 0.25 percent (TK promoter), and 9.5 percent (MSV-enhanced TK promoter) and for BHK cells (50 μg of protein, 2-hour assay), <0.02 percent (promoterless CAT plasmid), 0.96 percent (TK promoter), and 8.8 percent (MSV-enhanced TK promoter). The values obtained were normalized by the activities of β-galactosidase observed in the same extract. The HIT-T15 M2.2.2 cell line was isolated by its G418-resistant phenotype after cotransfection of parental HIT T15 (*9*) cells with pSV2neo (*11*) and a plasmid containing the human insulin gene.
47. Proximal insulin 5′ flanking sequences (−113 to +51) (Fig. 2, construct 7) were inserted upstream from the CAT gene in an orientation appropriate for expression. The MSV enhancer sequences (−490 to −150) were inserted immediately upstream of the insulin sequences in either normal or inverted orientations (construct 6 and 7). To test for possible cooperation between a cryptic enhancer activity in the promoter fragment of the insulin gene and the MSV enhancer, the MSV enhancer–insulin promoter sequences from constructs 6 and 7 (Table 2) were excised and inserted immediately upstream of the TK promoter of pTE1. The resultant plasmids contain the insulin promoter fragment (−113 to +51) upstream from the MSV enhancer in normal (construct 7) or inverted (construct 8) orientation relative to the adjacent TK promoter. In both cases the insulin promoter fragment has opposite orientation relative to the TK promoter. The reference plasmid in this experiment (construct 1) was made by inserting the MSV enhancer fragment to the Bg1 II site of pTE1 after removal of intervening plasmid sequences (Sal I to Nru I sites).
48. L. A. Laimins, P. Gruss, R. Pozzatti, G. Khoury, *J. Virol.* **49**, 183 (1984).
49. D. Melloul, B. Aloni, J. Calvo, D. Yaffe, U. Nudel, *EMBO J.* **3**, 983 (1984).
50. A full description of plasmids used is available from the authors.

51. We thank Drs. G. An, C. Gorman, O. Karlsson, P. Luciw, U. Nudel, R. Tjian, and K. Zaret for plasmids; Dr. H. Fong for providing the RI-9 cells; Dr. C. Craik and J. Barnett for oligonucleotides; J. Turner, C. Griffin, and A. Backman for technical assistance; and L. Spector for preparation of the manuscript. We also thank our colleagues in the Rutter laboratory for their constructive suggestions. Supported by NIH grants AM 21344 and GM 28520, a grant from the March of Dimes, and by Swedish grants MFR B85-13X-07149-01A and NFR NNFR 514875103-4.

10 July 1985; accepted 16 September 1985