Transcriptional “silencer” element in rat repetitive sequences associated with the rat insulin 1 gene locus
(negative regulation/long interspersed rat repetitive family/enhancers)

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ABSTRACT The enhancer elements from either simian virus 40 or murine sarcoma virus activate the expression of a transfected rat insulin 1 (rI1) gene when placed within 2.0 kilobases or less of the rI1 gene cap site. Inclusion of 4.0 kilobases of upstream rI1 sequences, however, results in a substantial reduction in the enhancer-dependent insulin gene expression. These observations suggested that a negative transcriptional regulatory element was present between 2.0 and 4.0 kilobases of the rI1 sequence. To test this notion, we employed a heterologous enhancer-dependent transcription assay in which the simian virus 40 72-base-pair repeat is linked to a human β-globin gene. Addition of the upstream rI1 element to this system decreased the level of enhancer-dependent β-globin transcription by a factor of 5 to 15. This rI1 “silencer” element functions in a manner relatively independent of position and orientation and requires a cis-dependent relationship to the transcription unit on which it acts. Thus, the silencer sequence seems to have a number of the characteristics of enhancer elements, and we suggest that it may function by the converse of the enhancer mechanism. The rI1 silencer sequence was identified as a member of a long interspersed rat repetitive family. Thus, a potential role for certain repetitive sequences interspersed throughout the eukaryotic genome may be to regulate gene expression by retaining transcriptional activity within defined domains.

Enhancers are cis-dependent DNA sequences that activate the transcription of viral and cellular genes in a manner relatively independent of position and orientation (1, 2). This transcriptional activation can occur over distances as great as 6 kilobases (kb) (3, 4) from either the 5' or 3' end of the gene. What appears to be more important than the distance between an enhancer and the promoter being assayed for transcription are the specific sequences that intervene. Thus, for example, if an enhancer is flanked on both sides by active promoters, its effect on more distal promoter elements is likely to be significantly reduced (4–6).

In studying the sequences upstream of the rat insulin 1 (rI1) gene, we have identified an element that appears to suppress enhancer-dependent transcriptional activity by a distinct mechanism. In fact, this negative regulatory element, which we will refer to as a silencer (see ref. 7), appears to have many of the characteristics of enhancers including position- and orientation-independent function on a heterologous gene. The identification of this rI1 silencer element as a member of a family of long interspersed rat repetitive sequences (LINES; refs. 8 and 9) suggests a potential role for some of these repetitive elements in gene regulation.

MATERIALS AND METHODS

Plasmid Constructions. The recombinant plasmids used in this study were constructed according to methods that use standard recombinant DNA technology (10). The parental vector pSVHPΔ128 (kindly provided by M. Green and T. Maniatis) contains the human β-globin gene with its promoter including 128 base pairs (bp) of 5' sequence upstream of the cap site and 500 bp of 3' flanking sequence. This plasmid also contains rSVx sequences with the simian virus 40 (SV40) origin fragment including the enhancer element (Puv II to HindIII) at the 3' end of the β-globin gene (see Fig. 1). Between the SV40 ori and the 5' β-globin flanking sequences are approximately 900 bp of prokaryotic DNA sequence necessary for ampicillin and tetracycline selection in the bacterial host MC1061/P3. Two derivative plasmids were constructed that contained Bgl II restriction sites on either the 5' side of β-globin at bp −128 relative to the cap site (pSVx3′Bgl II) or the 3' side of bp 500 from the β-globin polyadenylation signal (pSVx3′Bgl II). These two plasmids were used to construct the derivative plasmids shown in Fig. 1. Briefly, pSVxAs contains the 3.2-kb upstream flanking sequences from the rat insulin 1 gene (Puv II–Pvu II) at the 5' end of the β-globin coding sequence (Bgl II site of pSVx3′Bgl II) in the sense (pSVx3′As) or antisense (pSVx3′Aa) orientations; pSVxB contains the same fragment at the 3' end of the β-globin coding sequences (Bgl II site of pSVx3′Bgl II) in the sense (pSVxB) or antisense (pSVxBa) orientation. Two derivative plasmids were constructed from pSVxAs with internal deletions of either the Sac I sites (pSVx3′ASac) or the Bgl II sites (pSVx3′ABgl II) (Fig. 1). The plasmid pSVxD contains the BamHI–Bgl II fragment from the upstream sequences of the rat insulin gene, and pSVxE contains the 700-bp Bgl II fragment from the adjacent upstream region inserted into pSVx3′Bgl II. The plasmid pSVxC contains the 1-kb HindII fragment from the region immediately adjoining the insulin enhancer (ref. 11 and L.L., unpublished data) inserted into the parental plasmid pSVx3′Bgl II. As a control plasmid (pSVxMβG), the 3.2-kb Bgl II fragment from the 3' end of the mouse β-globin gene was isolated and inserted into the Bgl II site of pSVx3′Bgl II.

The plasmids described in Table 1, pSV40.ri1-B, pSV40.ri1-R, pSV40.ri1-H contain the segments of the rI1 gene extending from an Xba I site at the 3' end to BamHI, EcoRI, and HindIII, respectively, at the 5' end, inserted into plasmid pA10. The SV40 72-bp monomer enhancer was located at the 5' end of these constructs. The plasmids pMSV.ri1-B and pMSV.ri1-H contain the fragments de-\n
Abbreviations: SV40, simian virus 40; MSV, murine sarcoma virus; kb, kilobase(s); bp, base pair(s); LINES, long interspersed rat repetitive family.

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Fig. 1. Plasmids carrying sequences from the upstream region of the rat insulin 1 gene located on either the 5′ or the 3′ side of the human β-globin gene activated by a 3′ SV40 enhancer. πSVHPA128 is the parental plasmid from which πSVxA/Bgl II and πSVx3′/Bgl II fragment were constructed. Plasmids πSVxAs and πSVxBa contain the Pvu II-Pvu II fragment at the 3′ end of the human β-globin gene πSVxA, and πSVxB contain this fragment at the 5′ end. πSVxBgl II and πSVxAΔSac are derivative plasmids of πSVxAs containing internal deletions. Ba, BamHI; B, Bgl II; E, EcoRI; H, HindII; P, Pvu II; S, Sac I; X, Xba I.

scribed above, but with the 450-bp murine sarcoma virus (MSV) enhancer (12) located at the 5′ end of each construct. The plasmid, pRI1, contains the rI1 gene alone without any viral enhancer sequences.

Transfections, Protein, and RNA Analyses. Transfections and transient chloramphenicol acetyltransferase (CAT) assays were performed as described (12). Radioimmune assays for insulin production were performed according to the instructions from Amersham.

In transient assays for β-globin mRNA expression, 7 μg of the β-globin containing plasmid, 7 μg of a control plasmid containing the adenovirus VAI gene (pBalM kindly provided by J. Rose), and 16 μg of pBR322 DNA were transfected into monkey CV-1 or mouse L cells.

The 5′ ends of the human β-globin transcripts were analyzed by S1 analysis using an M13 synthesized single-stranded probe. The M13 clones contained 240 bp of the second exon and 168 bp of the first exon of the human globin gene (Fig. 2B). Synthesis of the radiolabeled probe was performed by standard techniques using the Klenow fragment from Boehringer-Mannheim and 32P-labeled dCTP from New England Nuclear (10). Single-stranded 32P-labeled probes were isolated by electrophoresis in 8% (wt/vol) urea/polyacrylamide gels. Hybridization with 10–40 μg of cytoplasmic RNA was performed overnight at 37°C. S1 digestion at 30°C for 30 min in 250 mM NaCl, 30 mM sodium acetate (pH 4.5), 1 mM ZnCl2 was followed by electrophoretic analysis on 10% (wt/vol) urea/polyacrylamide gels. Exposure times were typically 12 hr. Analysis of adenovirus VAI gene transcription was performed as an internal control for transfection efficiency, using slot–blot analysis of nitrocellulose retained RNA and a nick-translated double-stranded VAI gene probe.

RESULTS

Two initial sets of studies suggested the presence of a negative regulatory element upstream from the rI1 gene. First, to examine the enhancer-dependent expression of the rI1 gene (13) in nonpancreatic cells, a transient transfection assay was employed to measure the production of rat insulin by radioimmune assay. Plasmids were constructed that introduced the SV40 or MSV enhancer elements at distances of 4.0 (BamHI), 2.5 (EcoRI) and 0.2 (HindIII) kb from the insulin cap site (Fig. 1). These constructs were introduced into CV-1 cells or L cells by calcium-mediated DNA transfection, and extracts were examined 40 hr later for insulin levels (Table 1). Insulin expression was easily detected when enhancers were located at 2.5 and 0.15 kb from the cap site (14 units and 18 units per 106 transfected cells, respectively); however, no activation of insulin expression was observed when the enhancer was located 4.0 kb away. This suggested that a negative regulatory element might be located between 2.5 and 4 kb upstream from the rI1 cap site, which could interfere with the effect of the enhancer element.

Table 1. Activation of rat insulin 1 gene expression by SV40 and MSV enhancers

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Distance of enhancer from cap site, kb</th>
<th>Insulin units/106 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSV40.rI1BamHI</td>
<td>4.0</td>
<td>*</td>
</tr>
<tr>
<td>pSV40.rI1EcoRI</td>
<td>2.5</td>
<td>14</td>
</tr>
<tr>
<td>pSV40.rI1HindII</td>
<td>0.2</td>
<td>18</td>
</tr>
<tr>
<td>pMSV.rI1BamHI</td>
<td>4.0</td>
<td>*</td>
</tr>
<tr>
<td>pMSV.rI1HindII</td>
<td>0.2</td>
<td>10</td>
</tr>
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*Below the level of detection of this assay.
A second set of experiments involved constructs in which the sequences upstream from the rI genes were introduced into a plasmid (pSV2cat) (14) expressing chloramphenicol acetyltransferase. When the rI sequences located from −2.5 to −4.0 kb relative to the cap site were positioned 3' to the chloramphenicol acetyltransferase gene, a significant inhibition of gene expression (79% chloramphenicol conversion reduced to 10% conversion) was observed in transient assays (data not presented).

To determine whether or not this inhibition affected gene expression at the transcriptional level, a separate set of plasmids was constructed employing the human β-globin gene as a reporter sequence. Previous results from our laboratory (unpublished data) have suggested that the β-globin transcripts are more stable in eukaryotic cells than those encoding the prokaryotic sequence, chloramphenicol acetyltransferase as determined by S1 analysis. The plasmid pSVHPA128 contains the human β-globin gene coding sequences with 128 bp 5'-flanking sequence upstream from the cap site and 500 bp of 3'-noncoding sequence (see Fig. 1). The SV40 enhancer element is located at the 3' end of this gene in the same orientation as that of the β-globin gene coding sequences (approximately 1.1 kb from the 5' end of the β-globin gene and 0.5 kb from the 3' end of β-globin). This plasmid generates abundant transcripts from the β-globin cap site when transfected into tissue culture cells. Variations in the length of nucleotides from the 5' end of the rI gene were positioned on either the 5' or the 3' side of the β-globin coding sequences (see Fig. 1), and these plasmids were examined in the transient assay for β-globin mRNA production. Initial studies were performed in both monkey kidney CV-1 cells and mouse L cells. To ensure comparable transfection efficiencies in various experiments, the adenovirus VAi gene [a well-characterized polymerase III transcription unit (15)] was included in each transfection as an internal control. Production of VAi RNA was determined by hybridization analysis and was used to normalize β-globin transcription results for comparisons. Within the experiments presented, variations in transcription of the VAi gene were less than 20%.

Transcriptional activity of the parent plasmid pSVHPA128, containing 128 bp of sequence upstream from the cap site and the SV40 enhancer element without rI sequences, is recorded in Fig. 2A, lanes a, d, and i. The two intense bands in each panel represent protection of an M13 probe homologous to the first and second exons of the transcript (see Fig. 2B). A 3.2-kb fragment of the rI 5'-flanking sequence extending from a Pvu II site (3.8 kb upstream from the rI cap site) to a Pvu II site (540 bp upstream from the rI cap site) was inserted either at the 5' end of the transcription unit (pSVx3A) or at the 3' end of the β-globin coding sequences (pSVx3B) in either the sense (s) or the antisense (a) orientations (see Fig. 1). Each of these plasmids was separately transfected into CV-1 cells and compared to pSVHPA128 for β-globin mRNA production. When the rI sequences were positioned at the 5' end of the transcription unit in the sense (Fig. 2A, lane b) or in the antisense (Fig. 2A, lane c) orientations, a significant decrease in transcriptional activity (by a factor of ~10) was observed. Similar results were seen when this fragment was inserted at the 3' end of the transcription unit in either the sense (Fig. 2A, lane e) or antisense (Fig. 2A, lane f) orientations. Thus, we confirm that a set of sequences between 0.5 and 3.8 kb upstream from the rI gene cap site is capable of significantly depressing the levels of transcription at both the 5' and 3' ends of the human β-globin transcriptional unit in either orientation.

To show that the depression of β-globin gene transcription resulted from the insertion of specific sequences within the upstream region of the rI gene, and not simply from altering the size of the plasmid with heterologous DNA, a 3.2-kb fragment from the 3' end of the mouse β-globin gene (a presumably neutral set of sequences not known to affect transcriptional activity) was placed at the 5' end of the pSVHPΔ128 transcription unit in both orientations. Transfection of this plasmid into CV-1 cells resulted in β-globin RNA expression equivalent to that seen in the parental plasmid, pSVxHPA128 (Fig. 2A, lanes i and k). Similarly, the presence of a 1.0-kb HindIII fragment from the region adjacent to the insulin enhancer did not affect β-globin expression levels (pSVxEn; data not shown). To demonstrate that the silencer effect of the rI upstream sequences required a cis-orientation to the transcriptional unit, transfections were next performed with the pSVHP-β-globin transcriptional unit and the upstream rI gene sequences on separate plasmids. No inhibitory effect on the transcription of the β-globin sequences was observed (Fig. 2A, lanes i versus 1).

To localize the cis-acting DNA sequences responsible for depressing β-globin transcriptional activity, a series of internal deletions from the plasmid, pSVxAs, were constructed using convenient restriction enzyme sites (see Fig. 1). The plasmid pSVxΔSac contains a 200-bp deletion between adjacent Sac I sites located approximately 1.8 and 2.0 kb on the 5' side of the rI cap site (Fig. 1). Plasmid pSVxΔBglII contains a deletion of 800 bp between BglII sites located 2.8 and 3.6 kb upstream from rI cap site (Fig. 1). The plasmid containing the Sac deletions still exhibits the silencer effect (Fig. 2A, lane h). In contrast, deletion in the region of the BglII sites (Fig. 2A, lane j versus i) restores full transcriptional activity, thus abolishing the effect of the silencer. This suggests that while 'silencers' exist between −1.8 and −2.0 kb lie outside the region required for the transcriptional silencer function, sequences between the BglII sites are critical to this activity. To further localize the cis-acting sequences responsible for transcriptional depression, various subfragments of the DNA segment upstream from the cap site were tested for their ability to silence SV40 enhancer-dependent β-globin gene expression.

Neither subfragment from BamHI to BglII (SVxD) or BglII to BglII (SVxE) alone could silence enhancer function when placed at the 5' end of the β-globin gene (data not shown). This suggests that while the sequences within the three BglII sites are necessary for silencer function, the larger 700-bp BglII fragment alone is not sufficient.

While the experiments described in Fig. 2 represent results obtained in monkey kidney cells (CV-1), similar data were obtained when mouse L cells were transfected with these constructs. Preliminary experiments suggest that the rat silencer element also depresses SV40 enhancer function in SV40-transformed (HIT) pancreatic β cells, which express insulin. The silencing effect of the rat sequences, therefore, seems to be present in a large spectrum of cell types and is not specifically released in pancreatic cells.

To determine the identity of the upstream rI sequences responsible for transcriptional depression, two approaches were used. Since the 1.3-kb EcoR1 fragment from this segment was reminiscent of a distinctive 1.3-kb EcoR1 fragment (16), a probe from total rat DNA was annealed with an EcoR1 digest of the rI locus. This probe would only give a strong signal when annealing with a repetitive rat sequence. The intense band of hybridization seen in Fig. 3b comigrating with the 1.3-kb EcoR1 fragment from total rat DNA (Fig. 3d) provides clear evidence that this sequence is highly repeated. Sequence analysis by Lakshmikumaran et al. (17) confirms that this transcriptional "silencer" exists within a region of long interspersed rat repetitive DNA (a LINE family) that is present in ~50,000 copies per cell in the rat genome (8).

**DISCUSSION**

In this study we have defined a transcriptional regulatory element, a silencer, in the fragment flanking the 5' end of the rI gene. This element is capable of depressing enhancer-dependent transcription of a heterologous gene such as
human β-globin. The molecular mechanism by which this silencer functions is unclear. Nevertheless, several of its properties have been established in these experiments. The silencer sequence must bear a cis relationship to the enhanced gene. In addition, the silencer sequence functions on both the 5' and the 3' side of the transcribed gene and in both orientations in either of these positions. Although its effect on gene transcription appears to be opposite to that of classical enhancer elements, the characteristics of the silencer are otherwise quite similar. In earlier studies, it was shown that the interposition of promoter elements between an enhancer and a test gene was sometimes capable of reducing gene expression (4–6). Furthermore, prokaryotic plasmid sequences functioning as pseudpromoters have also been shown to reduce enhancer function (4, 18) by a phenomenon referred to as promoter occlusion (5). It seems unlikely that sequences in the rI, silencer fragment function simply as a pseudpromoter as the inhibitory effect is greater than that observed in promoter occlusion studies. Furthermore, the inhibitory effect is similar when the rI, element is positioned to the 5' or the 3' side of the test gene and in either orientation, and the insertion of the rat repetitive sequence neither alters the 5' end of the β-globin transcript nor produces hybrid transcripts. While the rI, silencer element functions at both ends of the transcribed β-globin gene, there is an indication that depression of transcription is greater when the silencer is located at the 3' end of the globin gene next to the SV40 enhancer (Fig. 2). The reasons for the apparent increase in silencer activity in a 3' location are presently unclear.

A negative regulatory sequence, also termed a silencer, has been identified in yeast (7). This sequence (HMRE) acts to repress genes at the HMR locus. A potential difference between the action of the yeast silencers and those described in the upstream rI, sequence is that the yeast elements require a round of replication prior to function, while the rI, silencer apparently does not. These two elements may, nevertheless, eventually be shown to function by similar mechanisms.

The mechanism by which rat silencers function remains obscure. We can only suggest that these silencers may either induce an alteration in chromatin structure making it less susceptible to the entry of the transcriptionally active molecules or perhaps may serve as polymerase exit sites. If transcription of the rat silencer sequence is critical to its function, one might suggest that the mechanism involves both (i) promoter occlusion and (ii) a reduction in total transcriptional activity of all genes on a plasmid. Such a reduction in total transcriptional activity is generally not seen when only two transcriptional units are located on the same plasmid (4–6). In either case, we suggest that in their natural setting, an interplay between enhancer and silencer elements is likely to be responsible for the overall gene activity at various times during a cell cycle or developmental program.

The silencer sequences involved in restricting enhancer-dependent transcription are members of a highly reiterated LINES (8) present in >50,000 copies in the rat genome. It is interesting that the LINE sequence is either present or absent in what constitutes a polymorphic segment upstream from the rI, gene (13, 17, 19). Variation in sequences at this locus may, in fact, influence expression of the rI, gene in the particular strain of rat. While a subset of LINE sequences are transcribed as nuclear RNA, not all LINES are thought to be transcriptionally active (20). It is difficult to determine if the rat repetitive element found upstream of the rat insulin I gene is expressed due to the extensive homologies shown by all rat LINE sequences. This homology extends to other primates as well (8) making it difficult to study expression of rat LINES in monkey cells. The frequency of these LINE sequences in the mammalian genome suggests that they may have both an important and general function (8). Based on our studies, we speculate that at least some sets of LINES may play a role in determining the level of expression of nearby genes. LINES are associated with several tissue-specific genetic loci (8). Thus, they may act as "molecular brackets" shielding a particular locus from the influence of outside regulatory elements such as enhancers. The ability to express the rI, gene in a tissue-specific manner could then result from activation of the known rI, enhancer in pancreatic β cells through a mechanism that overrides or competes with potential negative regulatory effects of the repetitive sequences.

The suggestion that certain highly repetitive sequences may act as silencers of transcriptional activity is in part supported by the study of Fanning et al. (21). These investigators demonstrated that integration of an active mouse mammary tumor virus retrovirus into the repetitive region of mouse cells resulted in the inactivation of retroviral gene expression. Nevertheless, when this virus was recloned from the integration site, it was found to be transcriptionally active. While speculation centered on methylation as an explanation for these results, it is also possible that the repetitive sequence environment may play a role in shielding the retroviral genes from expression. This argument would also be relevant to the potential activity or inactivity of the many copies of endogenous retroviral genomes interspersed throughout eukaryotic DNA.

As expected, not all repetitive sequences function as silencers of enhancer-induced transcription. The 3.2-kb Bgl II fragment from the 3' end of the mouse β-globin gene contains 2 kb of a minor repetitive sequence referred to as LLrep1 (22). In our experiments, this control fragment was shown not to depress the transcriptional activity of the human β-globin gene in the presence of the SV40 enhancer.

The identification of sequences that have the ability to depress or silence enhancer-dependent activation of transcriptional units suggests that there are elaborate mechanisms for determining, in both positive and negative ways, the level of gene expression in eukaryotic cells. It seems likely that an interaction between these two types of elements, enhancers and silencers, will play a critical role in determining the level of expression of a particular gene in both tissue-specific and developmentally regulated programs.
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