Limitation in Use of Heterologous Reporter Genes for Gene Promoter Analysis

SILENCER ACTIVITY ASSOCIATED WITH THE CHLORAMPHENICOL ACETYLTANSFERASE REPORTER GENE

Kezhong Zhang‡§, Sumiko Kurachi‡§, and Kotoku Kurachi‡||

From the ‡Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109-0618 and the §Age Dimension Research Center, the National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 308-8562, Japan

Various heterologous reporter genes have been widely used for the functional characterization of gene promoters. Many such studies often found weak to very strong silencer activities to be associated with specific parts of the basal promoter or further upstream regions. In this study, we carried out a systematic study on human blood coagulation factor IX (hFIX) and anti-coagulant protein C (hPC) genes, previously shown to have silencer activities associated with their 5′-flanking regions containing promoter sequences. With newly constructed chloramphenicol acetyltransferase (CAT) reporter vectors carrying hFIX or hPC gene promoter sequences, we confirmed the strong silencer activities associated with the regions nt −1895 through nt −416 of the hFIX gene or with the region nt −802 through nt −82 of the hPC gene. However, no such silencer activities associated with the specific regions were found when autologous hFIX cDNA, hFIX minigenes, or hPC minigenes were used as reporters in the expression vector system. Relative levels of CAT, hFIX, and hPC proteins produced in the transient assays correlated well with their mRNA levels. Human FIX minigene constructs containing a simian virus 40 (SV40) 3′-untranslated region (UTR) taken from the CAT reporter gene showed no silencer activity, indicating that SV40 3′-UTR sequence of the CAT reporter gene does not contribute to the silencer activity. Expression vectors constructed with the β-galactosidase gene under the control of hFIX gene promoter sequences also showed no silencer activity associated with the region nt −1895 through nt −416. These findings indicate that silencer activities associated with specific regions of promoter sequences as analyzed with CAT reporter genes may represent artifacts specific to the CAT reporter genes. Our findings strongly suggest a need for re-examination of promoter characterizations of many eukaryotic genes, which have been studied to date with CAT reporter genes.

Transcriptional regulation of eukaryotic genes are extremely diverse, strictly controlled and coordinated events and involve complex mechanisms for up- and down-regulation of gene expression (1, 2). Such complexity and diversity of gene expression are obviously needed for maintaining intricate balances among biological reactions and systems in response to various internal and external stimulations and stress. For characterization of promoter functions of various genes, heterologous reporter genes, such as CAT, β-galactosidase, luciferase, and green fluorescent protein genes, have often been utilized (3–5). Use of such heterologous reporter genes often gives convenience in both qualitative and quantitative analyses of promoter activities. At the same time, however, use of such heterologous reporter genes may result in significant biases in assessing subtle structure-function relationships of promoters and relevant observations, which are primarily due to the introduction of foreign elements or due to the elimination of intrinsic elements of test genes in the assay system. Any regulatory machinery including those in the promoter regions must have evolved in the context of the rest of gene structures, neighboring genomic elements as well as of higher order structures of chromatin and chromosome. Therefore, use of a heterologous reporter gene in studying regulatory mechanisms of an unrelated gene may have a risk of resulting in irrelevant observations and conclusions. To date, a large number of mammalian genes have been analyzed for their promoter functions with various heterologous reporter genes. However, literally no systematic studies have been conducted addressing the issue concerning the relevancy and limitation of commonly used heterologous reporter genes.

Many functional analyses of promoters of eukaryotic genes to date by using CAT reporter genes have shown weak to strong negative regulatory activities or silencer activities, which are associated with specific 5′-flanking regions containing promoter sequences (6, 7). Human genes for FIX and PC, important factors involved in blood coagulation and anti-coagulation pathways, respectively (8, 9), are among them (10, 11). The 5′-upstream region of the hFIX gene promoter beyond approximately nt −800 up to at least nt −1900 showed a very strong silencer activity in the CAT vector context (10). The hPC gene promoter was also shown to have a substantial silencer activity associated with the 5′-upstream region beyond nt −82 up to nt −802 (11). Literally nothing was known about the mechanisms

* This work was supported in part by Grants HL64522 and HL38644 (to K. K.) from the National Institutes of Health, Grant NIH 5-P60-AR-20557 from the Multipurpose Arthritis Center of the University of Michigan, Grant NIH 5P60 DK20572 from the Michigan Diabetes Research and Training Center, and Grant NIH MO1-RR-00042 from the University of Michigan General Clinical Research Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A recipient of an American Heart Association postdoctoral fellowship.

To whom correspondence should be addressed: The Age Dimension Research Center, the AIST Tsukuba Central 4th Site, Higashi 1–1–1, Tsukuba, Ibaraki 305-8562, Japan. Tel.: 81-298-61-2641; Fax: 81-298-61-2591; E-mail: kkurachi@umich.edu.

4826 This paper is available on line at http://www.jbc.org
by which these silencer activities are generated and what roles they play in the natural regulation of hFIX and hPC gene expression.

In this report, using transient expression assay system with HepG2 cell or HTC cell lines, we first demonstrate that CAT reporter gene constructs reproducibly show silencer activities associated with hFIX and hPC gene promoters. We then present experimental evidence that such silencer activities are irrelevant artifacts specifically associated with the CAT reporter gene, but not with other reporter genes including autologous hFIX or hPC gene as well as the β-galactosidase gene, another commonly used heterologous reporter gene.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and DNA modification enzymes were purchased from Invitrogen and New England Biolabs. Radioactive nucleotides, [α-32P]dCTP, were obtained from Amersham Biosciences. Mouse anti-hPC monoclonal antibody and rabbit polyclonal anti-hPC antibodies were purchased from Celsus Laboratory. Horse radish peroxidase-linked goat anti-rabbit IgG was purchased from Invitrogen. Anti-hFIX polyclonal and monoclonal antibodies for enzyme-linked immunosorbent assay (ELISA) were obtained from Hematologic Technologies Inc and Enzyme Research Laboratory, respectively, as previously described (12). Media, fetal calf serum, penicillin, and streptomycin for mammalian cell cultures were obtained from Invitrogen. FuGene6 transfection reagent and CAT ELISA kit were purchased from Roche Molecular Biochemicals. HepG2 cells were obtained from ATCC. Rat hepatoma cells, HTC cells, was kindly provided by Dr. Thomas Gelerter in this department. Human PC vector pUC119-hPC was kindly provided by Dr. Francis Castelino at the University of Notre Dame. o-nitrophenyl-β-D-galactoside for the hFIX gene was purchased from Sigma. All other reagents were of the highest quality commercially available.

**Construction of CAT and β-Gal Expression Vectors**—Plasmids pCH110 and pSV2CAT contain β-gal and CAT genes under the control of the SV40 early promoter, respectively (10, 13). Plasmid pUMSVOCAT, a promoter-less CAT vector with virtually no background CAT expression activity, was used as a control CAT expression vector (14). Construction of CAT expression vectors under controls of the hFIX or hPC promoters are as follows: expression vectors with hFIX 5′-flanking sequences, −1895FIX/CAT, and −416FIX/CAT, were constructed by inserting hFIX gene fragments spanning nt −1895 through nt +29 or nt −416 through nt +29, which were PCR-amplified from minigene −2331FIXm1 (15), into pUMSVOCAT at the SmaI site, respectively (Table I). Expression vectors, −802PC/CAT and −82PC/CAT, were constructed by inserting PCR-amplified fragments with SmaI linkers, which span the region nt −802 through nt +66 or nt −82 through nt +66 of the hPC gene, into pUMSVOCAT, at the SmaI site, respectively (11, 14) (Table I). A lacZ gene fragment with BamHI and HindIII sticky ends (3736 bp in size) were released from pCH110 (10, 16), followed by their insertion into pUC19 between BamHI and HindIII sites (3), thus generating pOGAL. A PCR-amplified fragment (nt −1895 through nt +29 of the hFIX gene) or 445 bp (nt −416 through nt +29 with HindIII sticky end was then inserted into pOGAL at HindIII site, thus generating −1895FIX/GAL or −416FIX/GAL. All the PCR-amplified sequences and ligation site sequences of expression vectors were subjected to automated dyeoxy sequencing to confirm their accuracy.

**Construction of hFIX and hPC Expression Vectors**—Human FIX minigene (hFIXm1) and cDNA (hFIXc) expression vectors, −1895FIXm1, −416FIXm1, −1895FIXc, and −416FIXc, were constructed with plasmid pUC19. These vectors contained 5′-end regulatory regions of the hFIX gene identical to those used in the CAT vectors. Constructs −1895FIX/CAT and −416FIX/CAT were prepared as previously described (12). Constructs −1895FIXm1 or −416FIXm1 were digested with SphI and StuI, releasing a 483-bp fragment encompassing a region nt −416 through nt +67 of the hFIX gene (the unique StuI site at nt +67 in exon I). A PCR-amplified 1467-bp fragment (nt −19 through nt +72 of the hFIX gene) with SphI and StuI sticky ends at 5′- and 3′-ends, respectively (Table I), was then inserted at the sites, thus producing −1895FIXm1 and −1895FIXc, respectively. Minigenes −1895FIXm1 and −416FIXm1 were digested by BamHI and KpnI, removing the 3′-UTR and poly(A) signal sequence of the hFIX gene, followed by treatment with Klenow enzyme. A fragment containing the SV40 early region and poly(A) signal sequence of the hFIX promoter sequence, was then inserted at the sites, thus producing −1895FIXSV40 and −416FIXSV40, respectively. Minigene −416FIXm1 was digested by SphI/NheI to remove its hFIX promoter region, followed by Klenow enzyme treatment and self-ligation, thus generating pFXm1, a promoter-less hFIX minigene control vector for hFIX expression assays. A promoter-less hFIX cDNA control vector, pFXc, was similarly digested by SphI/NheI double digestion of −416FIXc, removing the promoter region.

Human PC minigene (hPCm1) expression vectors, −802PCm1 and −82PCm1, were constructed as follows: a fragment spanning nt −82 through nt +1560 of the hPC gene (2962 bp in size) with SphI and MscI linkers at 5′- and 3′-ends, respectively, was PCR-amplified by using human genomic DNA as a template (11, 17) (Table I), and inserted into pUC119-hPC between SphI and MscI sites to replace its 5′-end portion. The 3′-end portion of the resultant construct containing the internal Sse837I site in the 3′-UTR through an EcoRI site present at the 5′-end immediately outside of the poly(A) attachment site, was released by Sse837I/EcoRI double digestion. A PCR-amplified fragment (615 bp in size, spanning nt +10494 through nt +11108 of the hPC gene) with Sse837I and EcoRI sticky ends (17), was then inserted to fill the gap, thus generating −802PCm1. Construct −82PCm1 was similarly generated by replacing the 5′-portion of −802PCm1 (nt −802 to nt +1560) with a fragment spanning nt −82 to nt +1560. The 5′-end region, non-coding exon I and partial intron 1 of the hPC gene were removed from −82PCm1 by complete and partial digestion with SacI and MscI, respectively. The remaining fragment from −82PCm1 was treated by Klenow fragment, and then self-ligated, generating promoter-less control vector, pPCm1. Expression vectors, −802PC/FIXm1 or −82PC/FIXm1, were constructed as follows: a fragment spanning nt −82 through nt +66 of the hPC gene with SphI and NheI linkers at the 5′- and 3′-ends, respectively, was PCR-amplified from −802PCm1 (Table I). After digestion with SphI/NheI, this fragment was inserted into −416FIXc, which was digested in advance with SphI/NheI, removing the hFIX promoter sequence, thus generating −802PC/FIXc. Construct −82PC/FIXc was similarly generated by replacing the 5′-portion of −82PC/FIXm1 (nt −802 to nt +66) of −82PC/FIXm1 with a 148-bp hPC fragment spanning nt −82 through nt +66 of the hPC gene. All the PCR-amplified sequences and ligation site sequences of newly constructed expression vectors were confirmed by automated dyeoxy sequencing for their accuracy.
conditions except 5% fetal bovine serum supplemented. Cell transfection was carried out with the FuGENE 6 transfection reagent as previously described (18). Plasmid pCH110 was used as a transfection internal control for CAT, hFIX and hPC expression vectors, while pSV2CAT was similarly used for β-gal expression vectors. 4–5 independent assays were carried out, and average activities were shown with S.D.

CAT and β-Gal Assays—The CAT protein assay was carried out by using a CAT ELISA kit according to the manufacturer’s instruction. β-gal activity was assayed as previously reported (19). Cell extracts obtained from cells co-transfected with pCH110 or pSV2CAT were first assayed for β-gal or CAT activities, respectively. These activities were used for normalizing transcription efficiencies among culture dishes. Amounts of cell extracts taken for activity assays were adjusted for the optimal assay range of CAT or β-gal activity.

Human FIX and hPC ELISA Assay—Human FIX produced into the conditioned culture medium was quantified by hFIX-specific ELISA as previously described (15). This ELISA system reproducibly detected hFIX antigen as low as 1 ng/ml. Human PC was produced by assaying ELISA as previously described (20). Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the detection antibody. For each expression vector, minimally three independent ELISA were carried out with duplicated assays for each diluted culture medium sample, and average values were calculated to determine the amounts of protein produced.

Northern Blot Analysis of hFIX, hPC, and CAT mRNA Levels in the Transfected Cells—Northern blot analysis of HepG2 cells transfected with hFIX, hPC, or CAT constructs was carried out as previously described (15, 20). Total RNA samples prepared from the transfected HepG2 cells were subjected to agarose gel electrophoresis (15 µg per lane). For CAT mRNA detection, a coding region fragment of CAT gene (624 bp in size) was PCR-amplified with 5’ and 3’ primers (5’-ACCAC-CGGTGTATATCC-3’ and 5’-CTGCCACTATCGCAGTA-3’, respectively) by using pUMSVOCAT as a template, and was used as a hybridization probe. A fragment (588 bp in size) prepared by SspI/BamHI digestion of −416FIXm1 was used for hFIX hybridization. Human PC hybridization probe (365 bp in size, from nt +6385 to +6749 in genomic nucleotide numbering) was prepared as previously described (20). These probes were labeled with [α-32P]dCTP by random priming (Amersham Biosciences) to a specific activity of ~1 × 106 cpm/µg. To confirm the presence of equal amount of RNAs, blotted filters were washed twice at 75 °C, each for 30 min, in 10% sodium dodecyl sulfate, and hybridized with labeled RNR 18 probe.

RESULTS

Transient Expression Activities of CAT, hFIX Minigenes, and cDNA Expression Vectors—Transient expression activities of newly generated CAT reporter gene constructs with different hFIX 5’ promoter sequences, −1895FIX/CAT and −416FIX/CAT, in HepG2 cells are shown in Fig. 1A. Construct pUMSVOCAT, a promoter-less control, gave no detectable CAT activities. Construct −1895FIX/CAT containing the region nt −1895 through nt +29 showed only 15.9% CAT activity of that of the −416FIX/CAT containing the region nt −416 through nt +29. In agreement, Northern blot analyses showed a similarly lowered mRNA level in HepG2 cells transfected with −1895FIX/CAT, −20% of that of cells transfected with −416FIX/CAT (Fig. 1D). These results indicated the existence of a strong silencer activity associated with the 5’-upstream region of the hFIX gene, confirming our previous observation (10).

Relative expression activities of hFIXm1 constructs containing the hFIX promoter sequences identical to those used in CAT constructs, −1895FIXm1, −416FIXm1, −1895FIXc, and −416FIXc, are shown in Fig. 1, B and C. Construct −416FIXm1 expressed hFIX at a level of ~40 ng/10⁶ cells per 24 h and was defined to be at 100% activity. HepG2 cells expressed non-detectable levels of hFIX as previously reported (15). As expected, the promoter-less control vector, pFIXm1, showed only minimal hFIX expression activity. Both −1895FIXm1 and −416FIXm1 showed equivalent transient expression activities, which were correlated well with similar hFIX mRNA levels in HepG2 cells transfected either with
Transient Expression Activities of CAT and hPC Minigene Expression Vectors

Silencer Activity Associated with CAT Reporter

CAT was also lowered to ~25% of ~82PC/CAT (Fig. 2C). These results indicated the presence of substantial silencer activity associated with the 5'-upstream region (nt ~82 to nt ~802) of the hPC gene, which is consistent with the previous report by Miao et al. (11).

Transient expression activities of hPC minigenes expression vectors were assayed with both HepG2 cells and HTC cells, respectively. Relative expression activities of hPC minigene constructs with that of ~82PCm1 defined as 100% are shown in Fig. 2B. HepG2 cells showed a low, but significant level of endogenous hPC expression (~12 ng/10⁶ cells per 24 h, data not shown), which correlated with the significant background expression activity of pPCm1, a promoter-less construct (average 12.8 ng/10⁶ cells per 24 h). Construct ~82hPCm1 produced 101 ng/10⁶ cells per 24 h into the culture medium. Constructs ~82PCm1 and ~802PCm1 gave equivalent hPC expression activities in HepG2 cells, correlating well with the similar levels of hPC mRNA in the transfected HepG2 cells (Fig. 2D). Relative expression activities of hPC expression vectors assayed with HTC cells, a rat hepatoma cell line with no endogenous hPC expression, agreed well with those obtained in HepG2 cells (Fig. 2B). These results obtained with native gene constructs contradict the observation with CAT reporter gene (Fig. 2A).

Possible Effects of the SV40-derived 3'-UTR on the Expression Activities of hFIX Expression Vectors—Relative expression activities of chimeric pFIXm1/SV40, ~1895FIXm1/SV40 and ~1615FIXm1/SV40 vectors, in which SV40 3'-UTR including poly(A) signal depicted by open rectangles. Expression activities relative to that of ~416FIXm1/SV40 are presented. B, transient expression assays of β-gal reporter gene expression vectors with hFIX promoter sequences; β-gal reporter gene sequences are shown by doted rectangles. The rest is identical to the descriptions in the legend to Fig. 1. Expression activities relative to that of ~416FIX/βGal are presented. C, transient expression activities of hFIX reporter gene expression vectors with hPC promoter sequences. Human PC promoter sequences are depicted by thick open bars at left with the 5'-end nt numbering. Homogenous hPC structures are shown with checkerboard rectangles with thin horizontal lines representing the first intron. Thin vertical lines in the box indicate stop codon positions. All the constructs contain homogeneous hPC 3'-UTR sequences and immediate 3'-flanking sequences shown by thick horizontal lines. Right-angle arrows indicate transcription start sites. Expression activities relative to that of ~82PCm1 are presented. C, Northern blot analysis of CAT mRNA levels in HepG2 cells. Lane 1, non-transfected HepG2 cells; lane 2, HepG2 cells transfected with pUM5/OCAT (control); lane 3, HepG2 cells transfected with ~82PC/CAT; lane 4, HepG2 cells transfected with ~82PC/CAT. The ratio of the CAT mRNA level in the cells transfected with ~802PC/CAT to that of ~82PC/CAT was ~1:4.5 after normalizing for RNA loading. CAT mRNA and 18 S rRNA are labeled with CAT and RNR 18 on the right side of the upper and lower panels, respectively. D, Northern blot analysis of hPC mRNA levels in HepG2 cells. Lane 1, non-transfected HepG2 cells; lane 2, HepG2 cells transfected with pPCm1 (control); lane 3, HepG2 cells transfected with ~802PCm1; lane 4, HepG2 cells transfected with ~82PCm1. Ratio of hPC mRNA level of HepG2 cells transfected with ~802PCm1 to that of cells transfected with ~82PCm1 was ~1:1. Positions of hPC mRNA and 18 S rRNA are shown by hPC and RNR 18, respectively.

−1895FIXm1 or with −416FIXm1 (Fig. 1E). These results indicated no appreciable silencer activities associated with the region nt ~1895 through nt ~416 of the hFIX gene. Similar to the hFIX minigene constructs, expression vectors with hFIX cDNA sequence, −416FIXe and −1895FIXe, showed equivalent hFIX expression activities in HepG2 cells, confirming no silencer activities associated with the region nt ~1895 through nt ~416 (Fig. 1C). This result also suggested that there is no specific contribution of the functional hFIX intron 1 sequence to the silencer activity shown by CAT reporter analysis.

Transient Expression Activities of CAT and hPC Minigene Expression Vectors—Transient expression activities of newly constructed CAT reporter gene vectors with hPC promoter sequences, −802PC/CAT and −82PC/CAT, are shown in Fig. 2A. The average CAT transient expression activity of −802PC/CAT was only 21.6% of that of −82PC/CAT, and in agreement, the CAT mRNA level of HepG2 cells transfected with −802PC/CAT was also lowered to ~25% of −802PC/CAT (Fig. 2C). These results indicated the presence of substantial silencer activity associated with the 5'-upstream region (nt ~82 to nt ~802) of the hPC gene, which is consistent with the previous report by Miao et al. (11).
similar hFIX expression activities, indicating no contribution by the SV40 sequences in the CAT constructs to the silencer activity observed with –1895FIX/CAT.

**Transient Expression Activities of β-Gal Expression Vectors with hFIX Promoters and hFIX cDNA Expression Vectors with hPC Promoters**—Transient expression of β-gal reporter gene constructs under the control of the hFIX gene promoter sequences, –1895FIX/βGal and –416FIX/βGal, were assayed with HepG2 cells (Fig. 3B). Construct pβGal, a promoter-less control, gave no appreciable β-gal expression in HepG2 cells. No silencer activity was found to be associated with the region nt –1895 through nt –419 of the hFIX gene in the β-gal reporter gene context. Constructs composed of hPC promoter sequences and hFIX cDNA reporter, –802PC/FIXc or –82PC/FIXc, also showed no silencer activity associated with the 5’-flanking region nt –802 through nt –82 of the hPC gene (Fig. 3C).

**DISCUSSION**

Heterologous reporter genes have been widely used for functional characterization of many eukaryotic gene promoters. Use of such reporter genes, however, may have a risk of generating irrelevant results and conclusions on gene regulation. This is the issue that we systematically addressed in the present study. Such irrelevant results may be generated due to the absence of structural elements in the heterologous reporter gene, which are present in the native test gene and required for its regulation in the context of its own promoter. Alternatively, structural elements in the heterologous genes, which are absent in the test genes, may critically affect the transcriptional regulation of test genes.

In the present studies, we first demonstrated that CAT reporter gene expression vectors reproducibly showed strong silencer activities associated with the 5’-flanking regions of the hFIX gene (Fig. 1A), confirming the previous findings (10). Such silencer activities, apparently due to transcriptional suppression as demonstrated by the similarly reduced mRNA levels (Fig. 1D), however, were not found when hFIX minigene or cDNA were used as a reporter in place of the CAT gene (Fig. 1, B and C). These results indicated that the silencer activities found to be associated with the 5’-specific upstream region of the hFIX gene may be irrelevant to the hFIX gene, and were generated through a specific combination of the hFIX promoter and the CAT reporter gene. This hypothesis was further supported by the similar findings obtained with hPC gene expression vectors (Fig. 2). Use of the CAT reporter gene in combination with the hPC promoter region gave substantial silencer activity associated with the 5’-flanking region nt –802 through nt –82 (Fig. 2A), whereas no such silencer activity was observed when the hPC minigenes were used as a reporter (Fig. 2B).

It is possible that some elements present in the hFIX minigene, such as the functional intron sequence which grossly elevates mRNA levels (12, 21), might eliminate the silencer activity observed with CAT reporter. However, this possibility was not supported, since hFIX cDNA constructs without the intron sequence did not show any silencer activities (Fig. 1C). The SV40 3’-UTR sequence used in the CAT reporter gene was also shown to be not responsible for generating the silencer activity (Fig. 3A). Furthermore, the observed correlation between levels of produced CAT, hFIX, or hPC protein and their mRNA levels eliminates the possibility of reporter gene-dependent differences in translational efficiency, intracellular protein trafficking or secretion process as a possible cause for generating CAT reporter gene-specific silencer activities (Figs. 1, D and E and 2, C and D). Examinations with another series of chimera constructs composed of promoter sequences of the hPC gene connected with the hFIX cDNA sequence also showed no silencer activity associated with the hPC promoter region nt –802 through nt –82 (Fig. 3C). It is important to emphasize that hFIX and hPC genes share a significant similarity in their coding regions (22). However, their promoter regions are grossly different from each other, suggesting that these promoter regions took different evolutionary pathways (20, 23).

Expression vectors with the β-gal gene, another common reporter gene, also do not show any silencer activity with hFIX promoter sequences, further supporting our conclusion that the silencer activities observed with the 5’-flanking upstream-specific regions of hFIX as well as the hPC gene are unique artifacts generated by use of CAT reporter genes (Fig. 3B). The detailed mechanisms responsible for generating such silencer activities associated with the CAT reporter genes in combination with the promoters of genes of interest remain to be determined. Particularly, identification of any specific parts of the CAT gene structure involved in cross-talk with the specific regions of test promoter sequences would be critical.

Other heterologous reporter genes, which are not included in the present study, may also exert unusual effects including silencer activities on various gene promoters. Furthermore, specific combinations of a tested gene promoter and a heterologous reporter gene may even generate pseudo-high activity for the promoter although to our knowledge no systematic studies are reported to date. These possibilities are yet to be tested.

Our findings indicate that observations on the promoter structure-function relationship analyzed with the commonly used CAT reporter gene may not necessarily represent the true promoter regulatory mechanisms of many genes of interest, and strongly suggest a need for their systematic re-examination. Analysis of a specific promoter may be best done, if possible, with its autologous coding and subsequent downstream structure sequences as a reporter gene.

Acknowledgments—We thank Francis Castellino for providing human protein C cDNA and Akiko Kurachi for critical reading of the manuscript.

**REFERENCES**

1. Ogboh, S. A., and Antalis, T. M. (1998) Biochem. J. 331, 1–14
2. Struhl, K. (1999) Cell 98, 1–4
3. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 16.56–16.57, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
4. Lewis, J. C., Feltus A., Easor, C. M., Ramanathan, S., and Daunert, S. (1998) J. Biol. Chem. 273, 5276–5281
5. Jain, V. K. (1996) Methods in Enzymology, Vol. 273, pp. 319–330, Academic Press, Inc., New York
6. Godbourn, S. (1960) Biochim. Biophys. Acts 1032, 53–77
7. Maaza, D. (2001) Mol. Cell 8, 489–498
8. Dahlback, B. (2000) The Lancet 355, 1627–1632
9. Esman, C. T. (2000) Biochim. Biophys. Acts 1477, 349–360
10. Salier, J-P., Hiroseawa, S., and Kurachi, K. (1990) J. Biol. Chem. 265, 7062–7068
11. Maiz, C. H., Ho, W-T., Greenberg, D. L., and Davie, E. W. (1996) J. Biol. Chem. 271, 9587–9594
12. Kurachi, S., Itomi, Y., Furukawa, M., and Kurachi, K. (1995) J. Biol. Chem. 270, 5276–5281
13. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
14. Salier, J-P., and Kurachi, K. (1989) BioTechniques 7, 30
15. Kurachi, S., Deyashiki, Y., Takeshita, J., and Kurachi, K. (1999) Science 285, 729–743
16. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) J. Mol. Appl. Genet. 2, 101–109
17. Foster, D. C., Yoshitake, S., and Davie, E. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4673–4677
18. Kurachi, S., Sze, L., and Kurachi, K. (1998) Biochemicalica 3, 43–44
19. Herbelom, P., Bourchot, B., and Yaniv, M. (1984) Cell 30, 453–462
20. Zhang, K., Kurachi, S., and Kurachi, K. (2002) J. Biol. Chem. 277, 4532–4540
21. Jallat, S., Perraud, F., Dalemans, W., Balland, A., Dieterle, A., Faure, T., Muehen, P., and Pavirani, A. (1990) EMBO J. 9, 3265–3301
22. Platek, J., Hoskins, J. A., Long, J. L., and Crabtree, G. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 546–550
23. Hsu, W., Kawamura, S., Fontaine, J-M., Kurachi, K., and Kurachi, S. (1999) Thromb. Haemost. 82, 1782–1783