Regulation of Protein Kinase C (PKC) Expression by Iron: Effect of Different Iron Compounds on PKC-β and PKC-α Gene Expression and Role of the 5′-Flanking Region of the PKC-β Gene in the Response to Ferric Transferrin

By Orlando Alcantara, Lina Obeid, Yusuf Hannun, Prem Ponka, and David H. Boldt

We have studied effects of ferric transferrin (FeTF), ferric lactoferrin (FeLF), ferric complexes of pyridoxal- or salicyldihyde isonicotinyl hydrazone, (Fe-PIH, Fe-SIH), and ferric ammonium citrate (FAC) on expression of protein kinase C (PKC) mRNA transcripts in a variety of cultured cell lines. FeTF supported an increase of PKC-β mRNA transcripts in T-lymphoblastoid (CCRF-CEM; Jurkat), B-lymphoblastoid (Daudi; Raji), promyelocyte (HL-60), erythroleukemia (K562), and monocyte (U937) cell lines. By contrast, FeLF, Fe-PIH, and Fe-SIH did not support an increase of PKC-β mRNA transcripts in any of these cell lines. Furthermore, FAC supported an increase of PKC-β mRNA transcripts in HL-60, K562, and U937 cells only. Preincubation of cells with desferrioxamine (DF), a cell-permeable iron chelator, abolished the increments of PKC-β mRNA observed in response to FeTF or FAC. In contrast to results with PKC-β, neither FeTF nor FAC caused an increase of PKC-α transcripts in any cell line. To locate iron-responsive DNA regulatory elements of the PKC-β gene, we prepared genetic constructs containing various portions of the human PKC-β 5′-flanking DNA linked to the firefly luciferase gene. Constructs were cotransfected with the neomycin resistance plasmid, Pwldneo, into HRE H9 cells, and stable transfectants were selected in G418. Treatment with FeTF of transfectants bearing chimeric gene constructs with 2,200 bp of the PKC-β 5′-flanking region increased luciferase activity and mRNA transcripts 2.5-fold. This increase was blocked by DF. Neither luciferase activity nor mRNA increased with FeTF in stable transfectants bearing constructs with 342 bp or 587 bp of the PKC-β 5′-flanking region. These data provide direct confirmation that iron is involved in regulation of PKC-β but not PKC-α gene expression in many cell lines. The form in which iron is presented to these cell lines appears to affect its availability for this function, and cells vary in their capabilities to use nontransferrin iron to support PKC-β gene expression. Finally, transcriptional upregulation of PKC-β by FeTF is mediated by DNA sequences located between −2200 bp and −587 bp in the 5′-flanking region of the human PKC-β gene. This is a US government work. There are no restrictions on its use.

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PKC-β mRNA, and increased PKC enzyme activity in the lymphoblastoid T-cell line, CCRF-CEM. This effect required iron delivery to the cells and was not mediated via a classic signal transduction pathway.

We now report that iron regulation of PKC gene expression is a characteristic of many hematopoietic cell lines. Induction of PKC-β gene expression by iron is highly dependent on the form in which iron is presented to a cell. The data also indicate that the PKC-β subspecies is responsive to iron regulation, whereas the PKC-α subspecies is not.

Finally, transfection experiments with PKC-β-luciferase gene constructs have been used to show that the 5′-flanking region of the human PKC-β gene contains iron-responsive transcriptional regulatory elements.

MATERIALS AND METHODS

Cell cultures. The following cell lines were used in these experiments: T-lymphoblastoid, CCRF-CEM and Jurkat; B-lymphoblastoid, Daudi and Raji; promyelocytic, HL-60; erythroleukemia, K562; monocyte, U937; and fibroblast (adenovirus type 5-transformed human embryonal kidney), 293. Suspension cells in logarithmic growth phase were seeded in culture at 5 × 10^4 to 1 × 10^5/mL in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 mmol/L L-glutamine, and antibiotics. Fibroblast cells, 293, were grown to confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS, L-glutamine, and antibiotics. The 293 cultures were split every 3 days.

For treatment with FeTF, ferric ammonium citrate (FAC), or ferric lactoferrin (FeLF), plateau-phase cells were washed twice in one volume of ice-cold culture medium. The cells were resuspended in the same medium supplemented with 200 mmol/L L-glutamine, and 9.5% bovine serum albumin (BSA) at a concentration of 1.0 × 10^7 cells/mL. For treatment of fibroblasts, adherent cells were washed...
twice as above; then the medium was replaced with Dulbecco's modified Eagle's medium containing 200 mmol/L L-glutamine and 0.5% BSA. All cultures were equilibrated at 37°C for 30 minutes and then were treated with 100 μg/mL of human FeTF, 75 μmol/L FAC, or 15-100 μg/mL of human FeLF for the times indicated. In certain experiments, desferrioxamine (DF) or ferrioxamine (50 μmol/L) were added before addition of FeTF, FAC, or FeLF. For all cell culture experiments, control cells were treated and incubated in parallel under identical conditions without addition of FeTF, FAC, or FeLF to the culture medium.

Ferric complexes of pyridoxal isonicotinoyl hydrazone (Fe-PIH) and salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) were prepared as previously described.14,15 Chelates were freshly prepared for each culture and were used at concentrations of 5 to 50 μmol/L.

RNA hybridization analyses. Total cellular RNA was isolated by lysis in a guanidinium isothiocyanate solution and by pelleting through cesium chloride.20 Integrity of RNA was assessed by visualization of ethidium bromide-stained gels. For Northern analyses, total cellular RNA was electrophoresed through a 1% agarose gel containing formaldehyde. The gel was rinsed in water and 500 mL of 10X SSC (0.15 mol/L NaCl, 15 mmol/L sodium citrate dihydrate) to remove formaldehyde; then RNA was transferred to nitrocellulose filters presoaked in 20X SSC. The filters were hybridized with 32P-oligolabeled cDNA probes. Hybridization was performed overnight in hybridization solution containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 100 μg/mL sheared carrier DNA, 6X SSC, 2X Denhardt’s solution, and 10% dextran sulfate. After hybridization, the filters were washed for 10 minutes in each of 3 changes of 2X SSC and 0.1% SDS and in 2 changes of 0.2% SSC and 0.1% SDS (45°C, 1 hour). After washing, filters were exposed to Kodak X-OMAT film (Eastman-Kodak, Rochester, NY) at -70°C with Dupont Cronex intensifying screens (Dupont, Wilmington, DE). Relative intensities of autoradiographs were determined by scanning densitometry.

Oligonucleotide probes. Probes used were PKC-α, 2.0- and 1.25-kb Pst I fragments from the bovine phPKC-α 7 clone;22 PKC-β, 1.4- and 0.68-kb Pst I fragments from the bovine phPKC-β 1-15-Eco clone; and α-actin, a 3.8-kb EcoRI-HindIII fragment from the human α-actin genomic clone.22

Promoter constructs. Obeid et al.27 cloned the 5'-terminal region of the human PKC-β gene from a human genomic library in EMBL-3 and determined the coding sequence for 559 bp of the 5'-flanking region.21 Promoter constructs were made using the promoterless luciferase vector pSVOA that was provided by Dr Suresh Subramani (University of California, San Diego, CA).24 Constructs are depicted in Fig. 1. Construct 1 was an EcoRI/Mae III fragment spanning 371 bp of the 5' region of PKC-β. This was cloned into the HindIII site of pSVOA in front of the luciferase reporter gene by using HindIII linkers. Constructs no. 2 and 3 were produced by polymerase chain reaction (PCR) cloning using oligonucleotides homologous to the 5' region of the PKC-β gene. These constructs were subcloned into the HindIII site of pSVOA by inserting HindIII sites at the 3' ends of the PCR oligonucleotides. Orientation was verified by restriction-mapping or sequencing. Polymerase chain reaction-generated construct no. 2 was sequenced, and construct no. 3 was verified by restriction-mapping and partial sequencing.

Transfection experiments. HRE H9 cells were transfected using the CaPO4 method.25 For transfections, 15 μg of cesium chloride-gradient-purified plasmids were used. The neomycin-resistant plasmid, PWL neo (Stratagene, LaJolla, CA), was cotransfected with PKC-β-luciferase plasmids at a 1:3 ratio. Cells were exposed for 12 hours to the CaPO4/DNA precipitate, washed, incubated with fresh media supplemented with 10% FBS for 48 hours, and then treated with G418 (400 μg/mL) for 30 days before beginning the following experiments. To test the effect of iron on luciferase expression by stable transfecnts, transfected cells in 100-mm dishes were incubated for 48 hours at 37°C and 5% CO2 in α-minimum essential medium containing 10% heat-inactivated FBS. FeTF (100 μg/mL), with or without DF (50 μmol/L), was included in certain cultures. After incubation, cells were lysed using a Triton X-100–based lysis reagent for measurement of luciferase activity, or cells were harvested for extraction of mRNA.

Luciferase activity in lysates was measured with the Promega (Madison, WI) Assay System using 20 μL of lysate in a Turner TD-20e luminometer (Turner Designs, Mountain View, CA) for 20 s of integrated time. To detect luciferase mRNA, Northern blots were probed with 32P-oligolabeled linearized plasmids or luciferase cDNA excised from pSV0A by EcoRI.

FeTF binding studies and measurement of cellular ferritin content. Radioligand binding studies were performed as we have previously described.28 Iron-saturated human FeTF was radiolabeled with 125I by the chloramine-T method and then incubated with HRE H9 cells in medium containing 0.5% BSA for 90 minutes at 4°C. Parallel reactions to correct for nonspecific binding included more than 200-fold excess of unlabeled FeTF. Reactions were terminated with cold phosphate-buffered saline, and cells were collected by filtration on Whatman (Maidstone, UK) GF/C glass fiber filters. Specific binding was taken as the difference in counts per minute bound between parallel reactions lacking or including unlabeled FeTF. Data were analyzed by the method of Scatchard.23 Ferritin content of whole cell lysates was determined using the Becton Dickinson ferritin monoclonal antibody solid-phase component system (Becton Dickinson Co, Orangeburg, NY).

RESULTS

Effect of FeTF on PKC-β and PKC-α mRNA transcripts in various hematopoietic cell lines. Exposure of CCRF-CEM lymphoblastoid T cells to FeTF produced increased transcription of the PKC-β gene, increased PKC-β mRNA transcripts, and increased whole-cell PKC enzymatic activity.8,9 We wished to determine if transcriptional regulation of PKC-β expression by FeTF was shown by other cell types and if PKC-α was similarly responsive to FeTF in these
same cell lines. Therefore, we have examined the effect of FeTF on PKC-β and PKC-α mRNA transcripts in seven hematopoietic cell lines representative of T and B lymphocytes, promyelocytes, monocytes, and erythroleukemia cells. A single nonhematopoietic cell line, 293, also was studied. Results are shown in Fig 2. In each hematopoietic cell line, FeTF supported a threefold to fivefold increase in steady state level of PKC-β mRNA transcripts after 60 minutes. This increase was abolished by preincubation of cells with DF, indicating that the effect was dependent on delivery of Fe3+ (Fig 3). Ferrioxamine had no effect (data not shown).x We have previously shown that the increase of PKC-β mRNA transcripts induced by exposure to FeTF was sustained up to 72 hours.3 In contrast to the results given by each hematopoietic cell line, no increase of PKC-β mRNA transcripts was observed in the fibroblast line, 293, when treated under identical conditions or when exposed to FeTF for up to 24 hours (data not shown).

All eight cell lines used for these experiments expressed both PKC-β and PKC-α isofoms (Fig 2), thereby permitting additional experiments to test effects of FeTF on PKC-α mRNA transcripts. No increase of PKC-α transcripts was observed in any of the cell lines after exposure to FeTF for up to 48 hours. These data indicate that the PKC-α and -β genes differ in their susceptibilities to regulation by FeTF.

Effect of treatment with other iron compounds on PKC-β and PKC-α mRNA transcripts. Previously, we had observed that FAC did not produce an increase of PKC-β mRNA transcripts in CEM cells.8 Results of treating various other hematopoietic cell lines with FAC (75 μmol/L) are shown in Fig 2B. No increase of PKC-β mRNA transcripts was observed in T- or B-lymphoblastoid cell lines when treated with FAC under the standard experimental conditions or for 24 hours. By contrast, in the nonlymphoid hematopoietic cell lines (HL-60, K562, and U937), FAC induced a twofold to fourfold increase in steady state level of PKC-β transcripts after 60 minutes. DF again abolished this effect (Fig 3). FAC did not induce an increase of PKC-β transcripts in the fibroblast line, 293, after 1 or 24 hours. Also, FAC did not cause an increase of PKC-α transcripts in any of the seven hematopoietic cell lines. These data indicate that differences exist between these lymphoid and nonlymphoid hematopoietic cell lines with respect to their abilities to use nontransferrin iron for regulating PKC-β expression. In addition, they indicate that PKC-α differs from PKC-β in its absence of regulation by iron delivered either as FeTF or FAC.

The different effects on PKC-β transcripts observed in some cell lines with FeTF and FAC suggested that the route by which iron is delivered to a cell may effect the subsequent use of the metal. To examine further the influence of the route by which iron is presented, we examined effects of FeLF and of Fe-PIH and Fe-SIH on PKC-β mRNA transcripts by these cell lines. FeLF, Fe-PIH, and Fe-SIH did not support an increase of PKC-β mRNA transcripts in any of the cell lines during incubations of 1, 24, or 48 hours. Representative results for some cell lines are presented in Fig 4. We measured cellular ferritin levels in cells before and 48 hours after treatment with Fe-PIH and Fe-SIH (Table 1). With the exception of the Daudi cell line, ferritin levels increased significantly indicating that iron delivered by these complexes was taken into the cells. These data lend additional support to the observations with FeTF and FAC (Fig...
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A

Fig 4. Effect of FeLF, Fe-PIH, and Fe-SIH on PKC-β mRNA transcripts in cultured cell lines. Cells were incubated with FeLF (A), Fe-PIH, or Fe-SIH (B) for 48 hours under conditions described in Materials and Methods. RNA was extracted, and Northern blot analyses were performed as described in Materials and Methods using a PKC-β cDNA probe.

2) that the form in which iron is presented to cells influences the subsequent use of that iron for various cellular functions. In this case, cells are unable to use ferric iron presented as either FeLF, Fe-PIH, or Fe-SIH to regulate expression of PKC-β. By contrast, cells are able to use ferric iron presented as FeTF for this purpose, and some cells, notably HL60, K562, and U937 cell lines, also are able to use ferric iron presented as FAC for a similar function.

Development of stable transfectants expressing PKC-β-luciferase fusion genes. The tartrate-resistant acid phosphatase (TRAP) gene is transcriptionally regulated by FeTF in a manner similar to that for PKC-β. We previously have used the cell line HRE H9 to stably express murine TRAP-luciferase gene constructs and to study their response to FeTF. These experiments showed the presence of iron-responsive DNA sequences in the 5′-flanking region of the mouse TRAP gene. Therefore, HRE H9 cells were chosen to develop stable transfectants expressing human PKC-β-luciferase gene constructs composed of various segments of the PKC-β 5′-flanking region (Fig 1). Stable transfectants bearing the various constructs (shown in Fig 1) or the promoterless plasmid, pSVOA, were cultured, and luciferase activity in logarithmically growing cultures was determined. Results, shown in Fig 5, indicate that all 3 fragments of the human PKC-β 5′-flanking region were capable of driving the luciferase reporter gene. The two larger fragments were more efficient than the shorter, but these, in turn, were only 10% as efficient as the SV40 promoter.

Effect of FeTF on luciferase expression by stable transfectants. Radioligand binding studies with 125I-FeTF were performed to confirm that human FeTF interacted with HRE activity.

Table 1. Ferritin Content of Cells Treated With the Iron Chelates, Fe-SIH and Fe-PIH

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>Fe-SIH</th>
<th>Fe-PIH</th>
</tr>
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<tbody>
<tr>
<td>CCRF-CEM</td>
<td>236 ± 8</td>
<td>994 ± 21</td>
<td>319 ± 15</td>
</tr>
<tr>
<td>Jurkat</td>
<td>30 ± 1</td>
<td>207 ± 8</td>
<td>81 ± 10</td>
</tr>
<tr>
<td>Daudi</td>
<td>46 ± 2</td>
<td>44 ± 11</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Raji</td>
<td>67 ± 2</td>
<td>229 ± 4</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>K562</td>
<td>125 ± 11</td>
<td>454 ± 68</td>
<td>335 ± 43</td>
</tr>
<tr>
<td>HL60</td>
<td>84 ± 5</td>
<td>390 ± 56</td>
<td>318 ± 39</td>
</tr>
<tr>
<td>U937</td>
<td>102 ± 2</td>
<td>533 ± 10</td>
<td>255 ± 5</td>
</tr>
</tbody>
</table>

Cells were incubated for 48 hours in serum-free medium with no addition, with Fe-SIH (5 μmol/L), or with Fe-PIH (5 μmol/L). Cellular ferritin was measured in cell lysates with a commercial immunoradiometric assay.
Fig 6. Effect of FeTF on expression of luciferase activity by HRE H9 transfectants. Stably transfected cells bearing PKC-β-luciferase gene constructs were incubated for 40 hours with no additions or with the indicated concentrations of FeTF. Luciferase activity then was determined in cell lysates. Symbols indicate results of separate experiments.

H9 cells. Results of these studies indicated that there were approximately $6 \times 10^5$ high-affinity ($K_a = 3.2 \times 10^9$ M$^{-1}$) FeTF binding sites per cell (data not shown).

Figure 6 shows results of individual experiments in which HRE H9 transfectants bearing constructs no. 1, 2, or 3 were incubated with various concentrations of FeTF for 40 hours. Construct no. 3 containing $-2,200$ bp to $+43$ bp of the human PKC-β gene responded to FeTF with the largest increase of luciferase activity. Construct no. 2 containing $-587$ bp to $+43$ bp of the PKC-β gene responded less well, whereas construct no. 1 containing $-342$ bp to $+29$ bp responded little if at all to FeTF. Figure 7 shows results of additional experiments performed with transfectants bearing construct no. 3, construct no. 4 (SV40 promoter), or the promoterless plasmid pSVOA. In these experiments FeTF failed to induce an increase of luciferase activity in cells transfected with pSVOA or construct no. 4. The increase of luciferase activity produced by FeTF in cells transfected with the PKC-β-luciferase plasmid construct no. 3 was inhibited by DF. Representative Northern blot analyses of RNA extracted from transfectants grown with or without FeTF and probed with linearized pSVOA plasmids are shown in Fig 8, and a summary of several such experiments is given in Table 2. These data indicate that luciferase mRNA transcripts consistently were increased by FeTF in transfectants bearing construct no. 3 and that this increase was blocked by DF. Taken together, the results of the transfection experiments indicate that the effect of FeTF is mediated via the PKC-β 5'-flanking region and is dependent on the intracellular delivery of iron. Furthermore, it appears that DNA sequences located between $-2,200$ bp and $-587$ bp are most important for this iron regulation.

**DISCUSSION**

These studies expand on our previous observation that PKC-β gene transcription was supported in CCRF-CEM cells by iron delivered as FeTF. In those studies, nuclear run-on assays showed a sevenfold increase in the rate of PKC-β gene transcription within 3 hours of treatment with FeTF. Increase of gene transcription was associated with sustained (up to 72 hours) steady state levels of PKC-β
Fig 8. Northern blot analyses of total RNA from HRE H9 transfectants bearing PKC-β-luciferase gene constructs. Total RNA extracted from cells grown for 40 hours with or without FeTF, (100 μg/mL) was used in Northern blot analyses. Blots were probed with 32P-labeled linearized pSVOA plasmids. (A) The upper panel shows an autoradiograph of a blot probed with 32P-pSVOA: (1), construct no. 1; (2), construct no. 2; (3), construct no. 3; (C), untransformed cells; and (FT), cells incubated with FeTF. The lower panel shows the ethidium bromide-stained gel shown in (A). (B) The upper panel shows an autoradiograph of a blot probed with 32P-pSVOA: (1), untreated cells; (2), construct no. 3 + FeTF (100 μg/mL) + DF (50 μmol/L); and (3), construct no. 3 + FeTF (100 μg/mL). The lower panel shows the ethidium bromide-stained gel shown in (B).

mRNA transcripts and increase of both PKC enzyme activity and phorbol dibutyrate binding. The requirement for iron in this regulation was established by showing that DF but not ferrooxamine abolished the FeTF-stimulated increase of PKC-β transcripts and that neither gallium transferrin nor apo-transferrin supported a similar increase.

We now have shown that six other human hematopoietic cell lines, both lymphoblastoid and nonlymphoblastoid, show a similar capability to use iron from FeTF to support an increase of PKC-β mRNA transcripts (Figs 2 and 3). The fact that the fibroblast line, 293, did not show such a response indicates that not all cell types are capable of using transferrin iron in this capacity. It is of interest that all seven hematopoietic cell lines, including T (CCRF-CEM, Jurkat) and B (Daudi, Raji) lymphoblastoid, erythroleukemia (K562), monocyte (U937), and promyelocyte (HL-60), do possess this capacity. However, this capacity is not limited to hematopoietic cells because the HRE H9 rabbit endomtrial cell line used for transfections used transferrin iron for gene regulation (Figs 6, 7, and 8).

It is of considerable further interest that, unlike PKC-β, expression of the PKC-α gene was not regulated by iron in any cell line examined (Fig 2). This differential regulation of expression of PKC isoforms in response to iron availability provides a mechanism whereby iron may participate in the control of certain cell activities that might include differentiation and function of hematopoietic cells.

An additional observation from these studies is that not all iron delivered to cells becomes available for regulation of PKC-β expression and, further, that cells differ in their abilities to use nontransferrin iron for this function (Figs 2 and 4). Thus nonlymphoblastoid cell lines, K562, Hl-60, and U937, responded to treatment with FAC by increases of PKC-β mRNA transcripts, whereas lymphoblastoid cell lines did not (Fig 2). Because this pattern of response to FAC was observed consistently on repeated occasions, we do not believe that the different responses of different cell lines to FAC reflect an artifact caused by unreliable solubility of FAC. We have shown previously that treatment of CCRF-CEM cells with FeTF or FAC resulted in equivalent whole cell increases in ferritin, indicating that uptake of iron occurred from either compound.

None of the cell lines tested used iron from FeLF or from the ferric complexes, Fe-PiHo or Fe-SiH, to increase PKC-β mRNA transcripts (Fig 4). Data indicate that FeTF and FeLF bind to separate receptors and suggest that, unlike FeTF, FeLF may not serve as an efficient iron donor to intracellular pools. Nonetheless, FeLF effectively may support cell proliferation. It has been suggested that FeLF may mimic the effects of certain impermeable membrane oxidants that are believed to stimulate serum-free cell growth by activating plasma membrane oxidase and Na⁺/H⁺ antiport activity. Recently, a similar mechanism for FeTF in supporting cell growth has been proposed. The observation that FeLF did not stimulate an increase of PKC-β mRNA transcripts in any cell line may be taken as support that iron does not activate PKC-β gene expression by stimulating plasma membrane oxidoreductase activity.

Fe-PiHo and Fe-SiH have been shown to support transferrin-independent iron uptake and utilization in a variety of in vitro systems. Of particular relevance to this study are the observations that Fe-PiHo may support transferrin-inde-
determined coding sequences for 559 bp of the S'-flanking region. This area of the gene contains a number of potential promoter and regulatory sites that may confer a high level of promoter activity. In addition, Oria et al. have shown that iron acquired from FeTF and iron acquired by phagocytosis were handled differently.

Obeid et al. cloned the 5'-terminal region of the human PKC-β gene from a human genomic library in EMBL-3 and determined coding sequences for 559 bp of the 5'-flanking region. This area of the gene contains a number of potential cis-acting regulatory elements including an octamer-binding motif, Sp1-, Ap1-, and Ap2-binding sites, and E boxes. A 630-bp fragment extending from -587 to +43 was able to drive expression of a luciferase reporter gene in transient transfections of human hematopoietic cells. Deletion analysis showed that a fragment -111 to +43 bp was necessary and sufficient for promoter activity.

Analyses of these sequence data or of the additional PKC-β sequence data reported by Nino et al. showed no sequences with the features of previously described iron regulatory elements. Therefore, to locate iron-responsive DNA sequences within the PKC-β promoter region, we developed a series of stably transfected cell lines bearing human PKC-β-luciferase fusion genes constructed with various portions of the PKC-β 5'-flanking region (Fig 1). Treatment with FeTF of stable transfectants bearing PKC-β-luciferase constructs containing 2,200 bp of the human PKC-β 5'-flanking DNA showed a twofold to 2.5-fold increase of luciferase activity and a corresponding increase of luciferase mRNA transcripts (Figs 7 and 8 and Table 1). Increases of luciferase activity and mRNA in FeTF-treated cultures were abolished by DF. Little or no increase in response to FeTF was observed in cells stably transfected with constructs containing 342 or 587 bp of the 5'-flanking PKC-β DNA. These data provide strong evidence that intracellular iron levels are importantly involved in regulation of PKC-β expression and that the effect of iron is mediated by nucleotide sequences between -2,200 and -587 bp in the 5'-flanking region of the PKC-β gene. It is of considerable interest that a recent report indicated that PKC-mediated phosphorylation of the iron-responsive element binding protein during in vitro differentiation of HL-60 cells.

Other eukaryotic genes whose expression is regulated by iron include transferrin receptor and ferritin, but in these instances iron regulation occurs predominantly (but not exclusively) at a posttranscriptional level. However, we have recently shown that expression of the TRAP gene is regulated by iron in a manner similar to PKC-β. The mouse TRAP gene 5'-flanking region has been sequenced to -1,846 bp that encompasses the region involved in this iron-dependent regulation. No candidate iron response elements have been identified in this region, strongly suggesting that a novel iron-responsive transcription regulatory mechanism is involved. A similar mechanism may be involved in the iron responsiveness of the PKC-β gene. Therefore, PKC-β and TRAP may represent a new class of iron-regulated genes.

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