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Region of Human and Mouse *Pkr* Genes

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The RNA-dependent protein kinase (PKR) is inducible by interferon (IFN) and is implicated in the antiviral and antiproliferative actions of IFN. We have now isolated human genomic clones that contain the promoter region required for transcription of the *Pkr* gene. Transient transfection analyses, using chloramphenicol acetyltransferase (CAT) as the reporter in constructs possessing various 5′-flanking fragments of the *Pkr* gene, led to the identification of a functional TATA-less promoter that directed IFN-inducible transcription of CAT. Sequence determination and deletion analysis of the promoter region revealed an element (5′GGAAAACGAAACT3′) involved in IFN inducibility that corresponds to the consensus sequence of the IFN-stimulated response element (ISRE). Comparison of the promoter sequence of the human *Pkr* gene to that of the mouse homolog identified a novel element (5′GGGAAGGCGGAGTCC3′) immediately upstream of the ISRE element which so far is unique to the human and mouse *Pkr* gene promoters. We have designated this new motif as KCS, for kinase conserved sequence. Deletion and substitution mutants of the *Pkr* promoter region showed that the ISRE element was required for transcriptional induction by type I IFN, whereas the KCS motif increased promoter activity mediated by the ISRE. Additional potential regulatory cis-elements were identified in the human *Pkr* promoter that are commonly associated with growth control regulation and differentiation. Other than the ISRE and novel KCS elements, the overall organization of potential binding sites for transcription factors was not well conserved between the IFN-inducible promoters of the human and mouse *Pkr* genes. The strict conservation of sequence, distance, and position of KCS, relative to ISRE, together with mutagenesis results, suggest an important functional role for the newly recognized KCS motif.

INTRODUCTION

Interferons (IFN)[2] are a family of regulatory cytokines that possess a wide range of biologic activities (Vilcek and Sen, 1996). These activities include the ability to mediate establishment of a potent antiviral state in uninfected animal cells, the regulation of cell proliferation and differentiation, and the modulation of the immune response. Among the IFN-inducible genes responsible for the actions of IFN is an RNA-dependent protein kinase, designated PKR (Samuel, 1993; Clemens, 1996). PKR is a central component of the IFN-induced antiviral response (Samuel, 1991; Vilcek and Sen, 1996) and is implicated in the control of cell proliferation (Lengyel, 1993). For example, the replication of encephalomyocarditis virus (Meurs *et al.,* 1992) and vaccinia virus (Lee and Esteban, 1993) are reduced in mouse 3T3 cells by expression of the cDNA encoding wild-type PKR, but not by expression of the PKR(1–551)K296R catalytic subdomain II point mutant which lacks kinase activity. Stable transformants of 3T3 cells overexpressing catalytically inactive human PKR proteins display a transformed phenotype and are highly tumorigenic when injected into nude mice (Koromilas *et al.,* 1992; Meurs *et al.,* 1993), and in yeast the wild-type but not mutant PKR cDNA mediates a growth suppression phenotype (Chong *et al.,* 1992; Romano *et al.,* 1995). However, in *Pkr* knock-out mice devoid of functional PKR, no evidence of tumor suppressor activity of PKR was observed (Yang *et al.,* 1995).

Protein phosphorylation catalyzed by PKR serves as an important mechanism for the regulation of gene expression in both interferon-treated and virus-infected animal cells (Samuel, 1991; Clemens, 1996). Biochemical studies have established that PKR is a protein serine/threonine kinase which acquires enzymatic activity following autophosphorylation, a process mediated by RNA with double-stranded character (Samuel, 1993). Protein synthesis initiation factor eIF-2 is the best characterized of the PKR substrates (Clemens, 1996). Phosphorylation of eIF-2 on serine 51 of the α subunit (Samuel, 1979; Pathak *et al.,* 1988) leads to an inhibition of translation (Samuel, 1993; Clemens, 1996). Phosphorylation of transcription factor inhibitor I-κB, which leads to activation and nuclear translocation of the transcription factor NF-κB (Thanos and Maniatis, 1995), also is catalyzed by PKR (Maran *et al.,* 1994). The availability of cDNA clones for

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[2] Abbreviations used: PKR, the RNA-dependent eIF-2α protein kinase inducible by interferon; IFN, interferon; FISH, fluorescence *in situ* hybridization; dsRNA, double-stranded RNA; bp, base pair; nt, nucleotide; UTR, untranslated region.

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human and mouse PKR proteins (Meurs *et al.,* 1990; Icely *et al.,* 1991; Thomis and Samuel, 1992; Baier *et al.,* 1993) facilitated the identification and characterization of domains of the kinase responsible for regulation and for catalysis, including RNA binding subdomains in the amino terminal region of the kinase and catalytic subdomains in the carboxyl terminal region (Samuel, 1993; Clemens, 1996). The expression and function of PKR is regulated in several ways. Regulation of PKR activity in cells has so far been demonstrated at the transcriptional level by interferon treatment (Meurs *et al.,* 1990; Thomis *et al.,* 1992; Tanaka and Samuel, 1994); at the translational level through an autoregulatory mechanism (Thomis and Samuel, 1992; Barber *et al.,* 1993a); at the posttranslational level by the RNA-mediated autophosphorylation (Samuel, 1979; Kostura and Mathews, 1989; Thomis and Samuel, 1993); and also at the posttranslational level by protein complex formation, with another PKR molecule (Ortega *et al.,* 1996), the TAR cellular RNA-binding protein TRBP (Cosentino *et al.,* 1995), or the p58 cellular protein inhibitor of PKR (Lee *et al.,* 1994).

Because of the possible role of PKR as a tumor suppressor (Lengyel, 1993) and because of the central role of PKR in the antiviral actions of IFN (Samuel, 1991), it is important to define the cis-elements and trans-acting factors responsible for *Pkr* transcriptional control. As a step toward this goal, we report herein the isolation of genomic clones that contain the functional IFN-responsive promoter region of the human *Pkr* gene. Sequence analysis revealed an ISRE as well as multiple candidate recognition sites for transcription factors implicated in the regulation of cell proliferation and differentiation. Comparison of the human *Pkr* gene promoter to that of its mouse homolog (Tanaka and Samuel, 1994) revealed the existence of a novel kinase conserved sequence designated KCS, so far unique to the human and mouse *Pkr* gene promoters, which is required for optimal IFN-inducible expression of PKR.

MATERIALS AND METHODS

Promoter cloning

Genomic clones of *Pkr* were isolated by screening two types of libraries, a λ-phage library and a P1-phage library. A human genomic library in the λ phage vector EMBL-3 SP6/T7 prepared from human placenta DNA (Clontech) was screened by filter hybridization using random primed ^{32}P-labeled cDNA fragments of the human PKR cDNA as the probes (Sambrook *et al.,* 1989; Thomis *et al.,* 1992). λ-Phage DNA was prepared from twice-rescreened plaques and genomic inserts were characterized by restriction mapping and Southern blot analysis (Sambrook *et al.,* 1989; Tanaka and Samuel, 1994). Restriction fragments of positive genomic clones were subcloned into the pBluescript plasmid (Stratagene) for detailed restriction mapping and DNA sequencing. The human P1-phage genomic library in the pAD10SacBII vector (Sheperd *et al.,* 1994) was screened by polymerase chain reaction using synthetic oligonucleotide primers based on sequences determined previously from λ genomic clones that amplified a 395-bp DNA fragment corresponding to part of intron XI and exon 12.

Southern gel-blot analysis

λ- and P1-genomic clone DNA were digested with restriction endonucleases, fractionated, and transferred to Hybond-N filter membranes (Amersham) by the method of Southern (1975). Filters were probed with the ^{32}P-labeled *Hin*dIII–*Pst*I fragment of human PKR cDNA corresponding to nt −30 to +1783 which includes the entire coding region.

Sequence analysis of genomic clones

Plasmid subclones of the genomic DNA were sequenced by the Sanger dideoxynucleotide procedure (Sanger *et al.,* 1977) using the Sequenase protocols from United States Biochemical. Universal primer sites in the pBluescript plasmid were used as well as custom PKR primers obtained commercially from BioSynthesis (Lewisville, TX) or were synthesized using a Millipore Cyclone Plus automated DNA synthesizer. Sequences were analyzed using the University of Wisconsin Genetics Computer Group programs on a Silicon Graphics IRIS 4D/340VGX computer. The FINDPATTERNS program and the transcription factor data base versions 6.5 and 7.3, and the TRANSFAC data base version 2.3 (Quandt *et al.,* 1995), were used to analyze the sequence for potential transcription factor binding motifs.

Determination of the 5′-cDNA region

The 5′-region of the PKR cDNA was obtained by the 5′-RACE procedure (Frohman *et al.,* 1988) using the Marathon-Ready cDNA system (Clontech) according to the manufacturer's recommendations. An uncloned library of adaptor-ligated cDNA prepared from human placenta was used to amplify the 5′-end of the PKR cDNA. First round PCR was performed with a PKR cDNA-specific minus primer corresponding to PKR antisense nt 1045 to 1028 (5′CAGGATCATAATCACTGC3′) and the plus anchor primer AP1 supplied by Clontech. Nested PCR then was performed with either one of two additional cDNA-specific minus primers corresponding to PKR antisense nt +177 to +157 (5′TGATCTACCTTCACCTTCTGG3′) and nt +10 to −10 (5′CACCAGCCATTTCTTCTTCC3′) along with the Clontech plus primer AP2. The products were characterized by cloning, sequencing, and comparison of the sequence results with those obtained for genomic clones.

Construction of reporter gene plasmids

The pCAT-Basic promoter-less plasmid (Promega) containing the chloramphenicol acetyltransferase (CAT)

gene was used for construction of the reporter gene plasmids for analysis of the *Pkr* gene promoter function. Reporter gene parent plasmids were prepared by inserting the indicated genomic DNA restriction fragment from the 5′-flanking region of the *Pkr* gene, either 0.5-kb *Hin*dIII–*Hin*dIII (0.5 H/H), 2.4-kb *Hin*dIII–*Hin*dIII (2.4 H/H), or 6.7-kb *Hin*dIII–*Hin*dIII (6.7 H/H) (see Fig. 1 schematic), into the *Hin*dIII site of pCAT-Basic following standard cloning procedures (Sambrook *et al.,* 1989). Deletions (Figs. 2 and 5) were subsequently made from the parent reporter plasmids using appropriate restriction enzymes; the structures of *Pkr* promoter deletion constructions were confirmed by sequencing.

Oligonucleotide-directed mutagenesis

Nucleotide substitution mutants within the ISRE element and the KCS motif of the human *Pkr* 5′-flanking region were prepared by a PCR-based method for site-directed mutagenesis. Oligonucleotides used for mutagenesis were as follows, with the mutated bases underlined: KCS (+), 5′CGGCTGCAGGGAAGG(C → A,G → C, G → T)AGTCCAAGGG3′; and ISRE(+), 5′CGGCTGCAGGGAAGGCGGAGTCCAAGGGGAAAAC(G → T)AAACTG3′. The minus primer was the pCAT-Basic (−) oligonucleotide 5′CAACGGTGGTATATCCAG3′. The symbol "+" indicates the sense primer, and the symbol "−" indicates the antisense primer. For each mutant, PCR (Saiki *et al.,* 1985) was performed using native *Taq* DNA polymerase and conditions specified by the manufacturer (Perkin–Elmer) to generate products possessing the desired site-specific mutations flanked by suitable restriction sites to facilitate subcloning; the template DNA for PCR was the *Sma–Pst* fragment of the 5′-flanking region (Fig. 2) subcloned into the pCAT-Basic plasmid. The presence of the engineered site-directed mutations within the ISRE element and the KCS motif of the *Pkr* promoter was confirmed by sequencing.

Cell maintenance and interferon treatment

Human amnion U cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (HyClone) at 5% (vol/vol), 100 units of penicillin per milliliter and 100 μg per milliliter of streptomycin. Where indicated, interferon treatment was with 1000 IU per milliliter of IFN-α for 24 hr. Parallel cultures were left untreated as controls.

Transfection and reporter assays

For the transient expression assay of *Pkr* promoter function, U cells (60-mm dishes) at a density of approximately 5×10^5 cells per plate were transfected by the DEAE-dextran-chloroquine phosphate transfection method (Luthman and Magnusson, 1983) using 10 μg of the *Pkr*-CAT reporter gene construct and 5 μg of the internal reference plasmid pRSV2-βgal (generously provided by J. Nevins, Durham, NC). For comparative purposes, the pCAT-Control (Promega) plasmid containing the simian virus 40 promoter and enhancer and the pCAT-Basic promoter-less plasmid were routinely analyzed in transfection experiments. All DNA plasmids used in transfections were purified by cesium chloride equilibrium centrifugation and were analyzed by agarose gel electrophoresis to verify plasmid integrity. Treatment with IFN was carried out approximately 24 hr posttransfection. For analysis of CAT and β-galactosidase activity, cell cultures were harvested 65 hr after transfection, extracts prepared by repeated freeze-thaw cycles, and enzymatic assays performed as described (Sambrook *et al.,* 1989). The protein concentration of extracts was determined by the Bradford method (Bio-Rad). CAT activity was quantified after thin layer chromatography by direct measurement of the ^{14}C-acetylated chloramphenicol products using a Beckman LS1801 liquid scintillation system to determine the radioactivity associated with the excised product spots localized using an autoradiogram of the TLC plate. CAT activity values, calculated as percentage conversion of [^{14}C]chloramphenicol to the acetylated derivatives, were normalized by β-galactosidase activity to control for variation in transfection efficiency.

Nucleotide sequence accession number

The sequences reported in this paper have been deposited in the GenBank database. The 5′-flanking genomic sequence of the human *Pkr* gene, including the promoter region, has been assigned Accession No. U51035. The sequences of the 17 exons including the 5′-untranslated region, and the intron junction sequences, have been assigned Accession Nos. U50632–U50648.

Materials

Unless otherwise specified, materials and reagents were as described previously (Thomis *et al.,* 1992, Tanaka and Samuel, 1994; Patterson and Samuel, 1995).

RESULTS

Cloning of the 5′-untranslated region of the human PKR cDNA

The sequence of the complete 5′-UTR of the human PKR mRNA is not known. The nucleotide sequence for the most 5′ cDNA clone previously determined for the human PKR cDNA included sequence of 186 nt flanking the ATG translation start site (Meurs *et al.,* 1990). To obtain the sequence of the 5′-UTR, 5′-RACE was performed as described under Materials and Methods using as the PKR primer antisense nt 177 to 157 and the nested PKR primer +10 to −10 that included the ATG translation initiation codon, with the A of the ATG designated as nt +1 (Thomis *et al.,* 1992). The 5′-RACE-derived cDNA clones included the previously reported 186-nt 5′-UTR sequence upstream of the PKR coding region as well as

an additional 249 nt of 5′-UTR sequence. Therefore, the 5′-UTR of the human PKR cDNA is 435 nt.

Isolation of *Pkr* genomic clones and determination of the structural organization of the 5′-flanking region

A human placenta genomic library in the λ vector EMBL3 SP6/T7 was screened using fragments from the 5′ end of the human PKR cDNA clone as probes (Thomis *et al.,* 1992). Two overlapping λ phage clones were isolated that contained the 5′-region of the PKR cDNA as established by detailed restriction mapping, Southern blot analysis, and sequence analysis. These two genomic clones, designated λ1 and λ8, contained approximately 15-kb inserts. Both λ clones possessed exons 1 through 4 of the *Pkr* gene (Fig. 1). Genomic clone λ1 extended about 3 kb more 5′ than did clone λ8. A human fibroblast foreskin genomic P1 library in the pAD10SacBII vector (Shepherd *et al.,* 1994) was screened by PCR and two clones were isolated, designated 202 and 963 (Fig. 1B). The P1 genomic clones included the entire PKR coding region as well as the 5′-flanking region contained within the λ1 and λ8 clones. The precise *Pkr* exon–intron organization of the human gene (Fig. 1A) was established by sequencing plasmid subclones and by comparison of the genomic sequences to the previously determined cDNA sequences (Meurs *et al.,* 1990; Thomis and Samuel, 1992). When analyzed by fluorescence *in situ* hybridization (FISH) with heat denatured metaphase spreads from normal human lymphocytes (Kuhen *et al.,* 1996), the λ genomic clones showed signals on the short arm of chromosome 2, in agreement with the previously determined assignment of the human *Pkr* gene to a single locus at 2p21-22 (Barber *et al.,* 1993b; Squire *et al.,* 1993).

Functional analysis of the human *Pkr* 5′ region

As an approach to isolate the human *Pkr* gene promoter, various restriction fragments from the 5′-flanking region of the human *Pkr* gene (Fig. 1C) were fused upstream of a CAT reporter gene in the pCAT-Basic plasmid vector. The constructs were transfected into human amnion U cells and CAT activity was measured in extracts prepared from untreated and from IFN-treated cells (Fig. 1D). The 5′-proximal 2.4-kb H/H construct derived from the 5′-flanking region of the *Pkr* gene included in the λ1 genomic clone (Fig. 1C) exhibited IFN-inducible promoter activity in transfected U cells (Fig. 1D). This 2.4-kb H/H fragment displaying inducible promoter activity was located about 7.5 kb upstream of the ATG translation start site, within exon 3 of the *Pkr* gene (Figs. 1A and 1C). None of the other fragments from the 5′-flanking region of the λ genomic clones supported reporter transcription in the transient transfection assay (Fig. 1D). The activities of these other CAT reporter constructs were comparable to the promoter-less pCAT-Basic plasmid vector. As a negative control, the promoter-less pCAT-Basic plasmid vector without inserted human genomic DNA exhibited low CAT activity (<2% conversion). By contrast, the positive control plasmid pCAT-Control, which contains the simian virus 40 promoter and enhancer, displayed high CAT activity levels (>90% conversion) in our transfection assay. Neither pCAT plasmid vector, the promoter-less pCAT-Basic, or the SV_{40} pCAT-Control, showed IFN inducibility of the CAT reporter (data not shown).

A series of deletions of the 5′-proximal 2.4-kb H/H construct were generated using appropriate restriction sites and analyzed for IFN-inducible CAT reporter expression (Fig. 2). The 1.72-kb H/P deletion mutant showed both increased basal CAT activity (9.8% conversion) and increased IFN-inducible CAT activity (47.2% conversion) relative to the 2.4-kb H/H construct; the induction by IFN was about fivefold (Fig. 2). By contrast, the 1.5-kb H/B deletion mutant which lacks the 3′-terminal 237 nt of the 1.72-kb H/P construct did not display IFN-inducible promoter activity, suggesting that elements required for IFN-inducible transcription were present within the 237-nt region 3′ of the *Bam*HI site. Consistent with this notion, the 1.42-kb A/P deletion mutant which lacks the 300-bp *Hin*dIII to *Acc*I 5′-region of the 2.4-kb H/H construct retained basal CAT activity (10.6% conversion) and showed significant IFN-induced CAT activity (51.8% conversion). Likewise, the 368-nt Sm/P deletion mutant which lacks the large 1.34-kb *Hin*dIII to *Sma*I 5′-region also displayed strong IFN-inducible promoter activity (41.1% conversion). Further deletion of the region between the *Sma*I and *Bam*HI sites to generate the 238-nt B/P deletion mutant led to a small reduction in both basal and IFN-inducible CAT activity (Fig. 2). Thus, all CAT reporter constructs that exhibited IFN-inducible promoter activity possessed the 237-nt *Bam*HI–*Pst*I region, suggesting that it contains an IFN-inducible element.

DNA sequence of the human *Pkr* promoter region

The entire 2.4-kb *Hin*dIII genomic fragment that possessed the necessary functional elements to support basal and IFN-inducible transcription was sequenced; 800 nt of the sequence are shown in Fig. 3. Comparison of the *Pkr* genomic DNA sequence obtained from the λ1 clone to 5′-UTR sequence of the PKR cDNA revealed an exact match of the 5′-proximal 18 nt that constitute exon 1. Four independently isolated PKR cDNA clones obtained by 5′-RACE, representing the 5′-most cDNA clones, all possessed the 18 bp of sequence corresponding to nt positions -435 to -418 of the cDNA. Sequence analysis of 10 additional independently isolated cDNA clones confirmed the position of the exon 1–intron I junction, but revealed that exon 1 possessed multiple 5′-ends. Following the first intron, the 401-bp exon 2 of the 5′-UTR corresponding to nt position -417 to -17 of the cDNA also matched the 5′-flanking genomic DNA sequence (data not shown). Intron I, which conformed to the GT — AG rule (Padgett *et al.,* 1986), was 7.4-kb pairs (Fig. 1A).

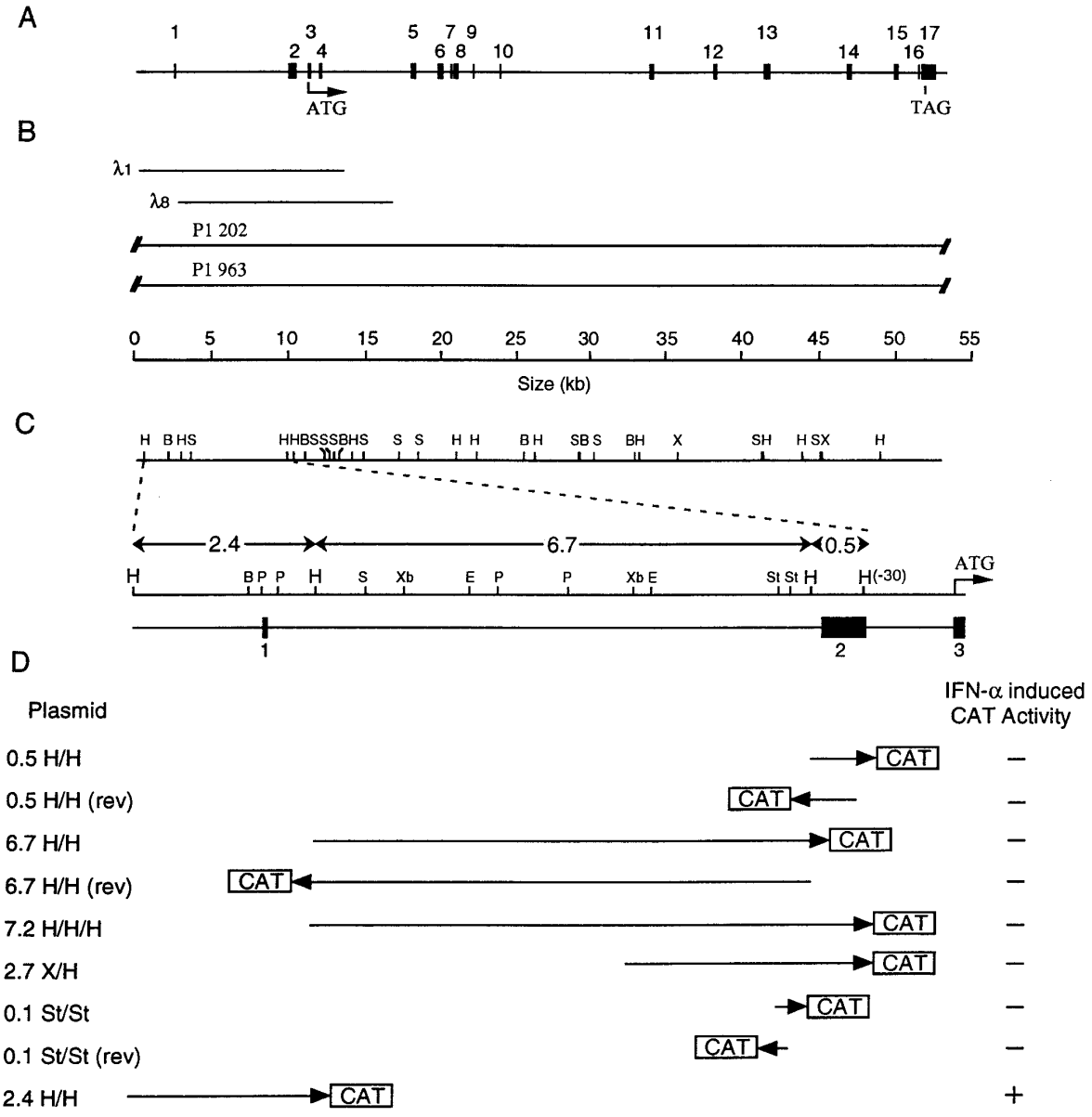

FIG. 1. Physical map of the human *Pkr* gene. (A) The structure of the gene is represented with regard to the organization of the exons and introns. Exons are indicated to scale by filled boxes, numbered 1–17; introns and the 5′ and 3′ flanking regions are indicated by the solid lines. The entire gene spans approximately 50 kb in length and contains 17 exons. The ATG for translation initiation is in exon 3, and the TAG for termination is in exon 17 as indicated. (B) Shown to scale are two P1-phage genomic clones (P1-202, P1-963) that span the entire length of the *Pkr* gene and continue into flanking sequences and two overlapping λ-phage genomic clones (λ1, λ8) that include the 5′-flanking promoter region. (C) The restriction maps show cleavage sites for endonucleases *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sac*I (S), *Sty*I (St), *Xba*I (Xb), and *Xho*I (X). The upper map represents the entire *Pkr* gene, and the lower expanded map corresponds to the 5′-terminal region of the gene defined by the three *Hin*dIII restriction fragments of 2.4, 6.7, and 0.5 kb size. The *Hin*dIII site denoted by -30 corresponds to the *Hin*dIII site in the 5′-UTR at nt position -30 in the cDNA (Meurs *et al.,* 1990; Thomis *et al.,* 1992). (D) Transient transfection analysis of the *Pkr* gene 5′-flanking region constructs. The *Hin*dIII fragments from the 5-flanking region of the *Pkr* gene were used to generate the reporter gene constructs as illustrated by the schematic. Cells were transfected with the indicated CAT reporter plasmid and to control for transfection efficiency were cotransfected with a β-galactosidase reporter gene. Transfected cells were left untreated or were treated with IFN-α, and then analyzed for expression of chloramphenicol acetyltransferase and β-galactosidase enzyme activities as described under Materials and Methods.

The 800-nt 5′-flanking promoter region sequence is (G + C)-rich, 64.5%, and contains 52 CpG pairs. Computer analysis of the sequence revealed numerous candidate binding sites for transcription factors. Among the potential transcription factor sites identified were several possibly relevant to the regulation of *Pkr* constitutive and cytokine-inducible gene expression. A candidate IFN-α/β-stimulated response element (Williams, 1991; Schindler and Darnell, 1995) is present 50 nt upstream of the 5′-end of exon 1. A candidate IFN gamma-activated sequence (GAS) (Pearse *et al.,* 1993; Schindler and Darnell, 1995) was present at nt 563 to 571 (Fig. 3). A 19-bp

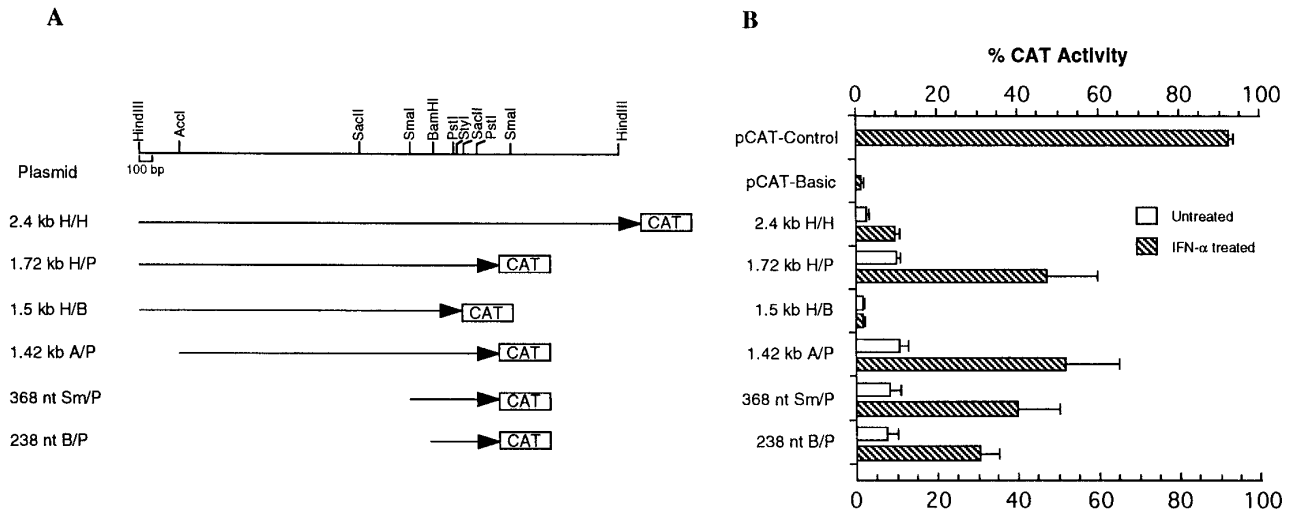

FIG. 2. Functional analysis of the human *Pkr* gene promoter by transient transfections with reporter gene constructs. (A) Schematic representation of the CAT reporter plasmids constructed by insertion of the indicated genomic DNA restriction fragments from the 5′-flanking region of the *Pkr* gene into the promoter-less pCAT-Basic plasmid. (B) Promoter activities observed in human amnion U cells transfected with the indicated CAT reporter plasmids. Promoter activities of deletion constructs are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon beginning at 24 hr after transfection. Transfections were repeated three to five times in independent experiments to allow for calculation of a mean value and standard deviation. pCAT-Control, the CAT reporter gene linked to the simian virus 40 promoter and enhancer; pCAT-Basic, the promoter-less plasmid vector without inserted human genomic DNA.

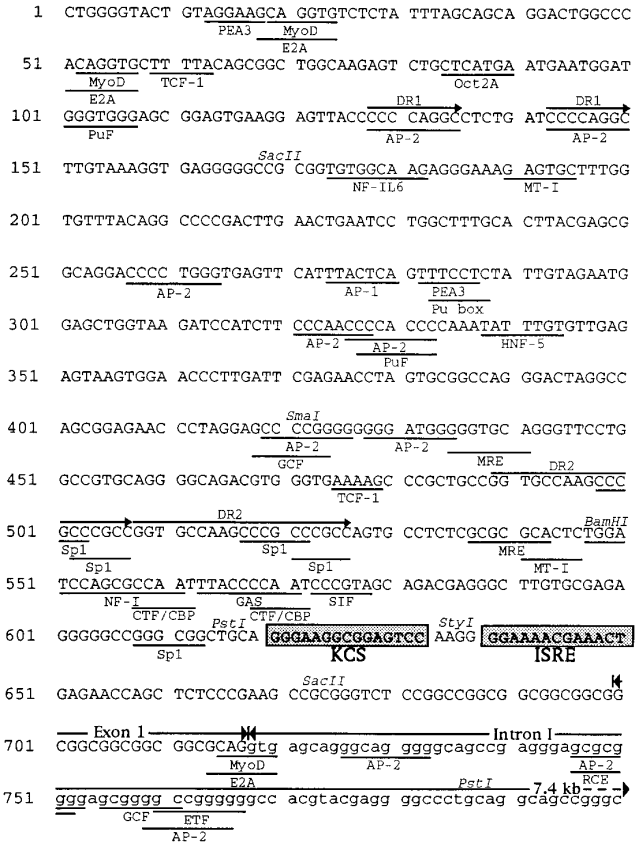

FIG. 3. Nucleotide sequence of the 5′-flanking region of the human *Pkr* gene. The sequence of the promoter region as well as the sequence of the 18-nt exon 1 of the 5′-untranslated region and a portion of the 7.4-kb intron I are shown. The nucleotide numbers are relative, and those of exon 1 correspond to nt -435 to -418 of the cDNA as defined by 5′-RACE. Several potential transcription factor binding sites as described in the text are shown, along with landmark restriction endonuclease sites and the direct repeat (DR) regions. The perfect interferon-stimulated response element (ISRE) and the newly identified kinase consensus sequence (KCS) are boxed.

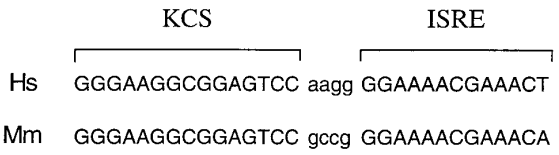

FIG. 4. Comparison of the KCS and ISRE elements from the human and mouse *Pkr* promoters. The sequence of the human (Hs) *Pkr* promoter region corresponding to the KCS and ISRE elements is taken from Fig. 3; the mouse (Mm) *Pkr* promoter sequence is from Tanaka and Samuel (1994).

perfect tandem direct repeat was found approximately 140 nt upstream of the ISRE that possesses candidate Sp1 factor binding sites. Other putative factor motifs (Faisst and Meyer, 1992; Quandt *et al.,* 1995) identified in the 5′-flanking region include PEA3 of the *Ets*-1 family, AP-1, AP-2, CTF/CBP, HNF-5, Myo-D, MRE, MT-I, NF-IL6, NF-I, Oct-2A, ETF (an activator of basal transcription), and GCF (an inhibitor of basal transcription) (Fig. 3).

Comparison of the 5′-flanking promoter region sequence determined for the human *Pkr* gene (Fig. 3) with that previously established for the mouse *Pkr* gene (Tanaka and Samuel, 1994) revealed that the overall sequence and organization of potential binding sites for transcription factors surprisingly were not well conserved between the two species, with two exceptions. First, both the human and the mouse promoters possessed the consensus ISRE element, as anticipated for a type I IFN-α/β inducible gene (Williams, 1991; Schindler and Darnell, 1995). Second, we identified a novel 15-nt sequence immediately upstream of the ISRE element that was absolutely identical between the human and mouse promoters (Fig. 4). A search of the sequence databanks revealed that this newly discovered 15-nt sequence so far is unique to the human and mouse *Pkr* gene promoters. We have designated this sequence, 5′-GGGAAGGCGGAGTCC-3′, as KCS or kinase conserved sequence. The strict conservation of sequence, position, and distance of the KCS motif relative to the ISRE, in both the human and the mouse *Pkr* promoters, suggests a functional role for KCS in transcription of the *Pkr* gene.

Functional analysis of the ISRE and KCS motifs

Deletion constructs were generated to examine the roles of the two motifs in transcription using the CAT reporter (Fig. 5). The 368-nt Sm/P construct includes both the ISRE (I) and the KCS (K) motifs, and this construct reproducibly showed IFN-inducible promoter activity (Figs. 2 and 5). The 368-nt Sm/P (ISRE *del*) construct was generated by deletion of the 40-nt *Sty*I through *Sac*II region that included the ISRE element from the 368-nt Sm/P construct (Fig. 5A). This construct lacking the ISRE displayed the expected loss of type I IFN inducibility (Fig. 5B). The *Sty*I through *Sac*II region alone, which possesses the ISRE element, was sufficient to confer IFN-inducible expression of CAT as demonstrated by the 40-nt Sty/Sa promoter deletion mutant. Although the increase in CAT activity observed following IFN treatment was 4.3-fold, the overall promoter activity observed with the 40-nt Sty/Sa construct was significantly reduced relative to the 368-nt Sm/P construct (Fig. 5B). Fusion of the 5′-flanking 23-nt fragment containing the novel 15-bp KCS motif to the 40-nt Sty/Sa promoter deletion mutant to generate the 63-nt P/Sa construct that includes both the KCS and ISRE restored overall promoter activity to a level comparable to that observed for the 368-nt Sm/P and 503-nt Sa/Sa constructs, two constructs which possess both the KCS motif and the ISRE element. Conversely, deletion of the KCS from the 503-nt Sa/Sa construct to generate the KCS *del* construct that lacks the KCS motif but retains the ISRE element reduced transcriptional activity (Fig. 5B). Results obtained with these constructs suggest that maximal basal and IFN-inducible activity was observed in the transient transfection assay when the KCS motif was present. Responsiveness to IFN was retained upon reversing the orientation of the 63-nt P/Sa sequence relative to the CAT reporter, although the overall promoter activity was weaker in the reverse orientation.

To further investigate the possible functional role for the KCS motif, two substitution mutants in the background of the 503 Sa/Sa construct were prepared: KCS *sub* which possesses a wt ISRE element but the indicated C → A, G → C, G → T triple nucleotide substitution within the KCS motif; and ISRE *sub* which possesses a wt KCS motif but the indicated G → T substitution mutation within the ISRE element. As expected (Schindler and Darnell, 1995), the ISRE *sub* mutant lacked IFN-inducible transcriptional activity relative to the wt parent construct (Fig. 6). Surprisingly, the IFN-inducible promoter activity of the KCS *sub* mutant likewise was substantially reduced relative to the wt parent construct. The activity of the KCS *sub* mutant and the ISRE *sub* mutant were comparable, and low, in the transient transfection assay (Fig. 6). These data further support the notion that the KCS motif has a positive regulatory role in constitutive and IFN-inducible transcription of the *Pkr* gene mediated by the ISRE motif.

DISCUSSION

The IFN-inducible RNA-dependent protein kinase (PKR) plays a central role in the control of translation and is implicated in both the antiviral and the antiproliferative actions of IFN (Samuel, 1991, 1993; Lengyel, 1993; Clemens, 1996). As a step toward gaining further insights into the regulation and function of PKR in human cells, we have isolated genomic clones of the human *Pkr* gene (Kuhen *et al.,* 1996). We now have identified the 5′-flanking promoter region of the human *Pkr* gene that contains all of the elements necessary to support basal and IFN-inducible transcription. Characterization of the human *Pkr* promoter as reported herein revealed two important

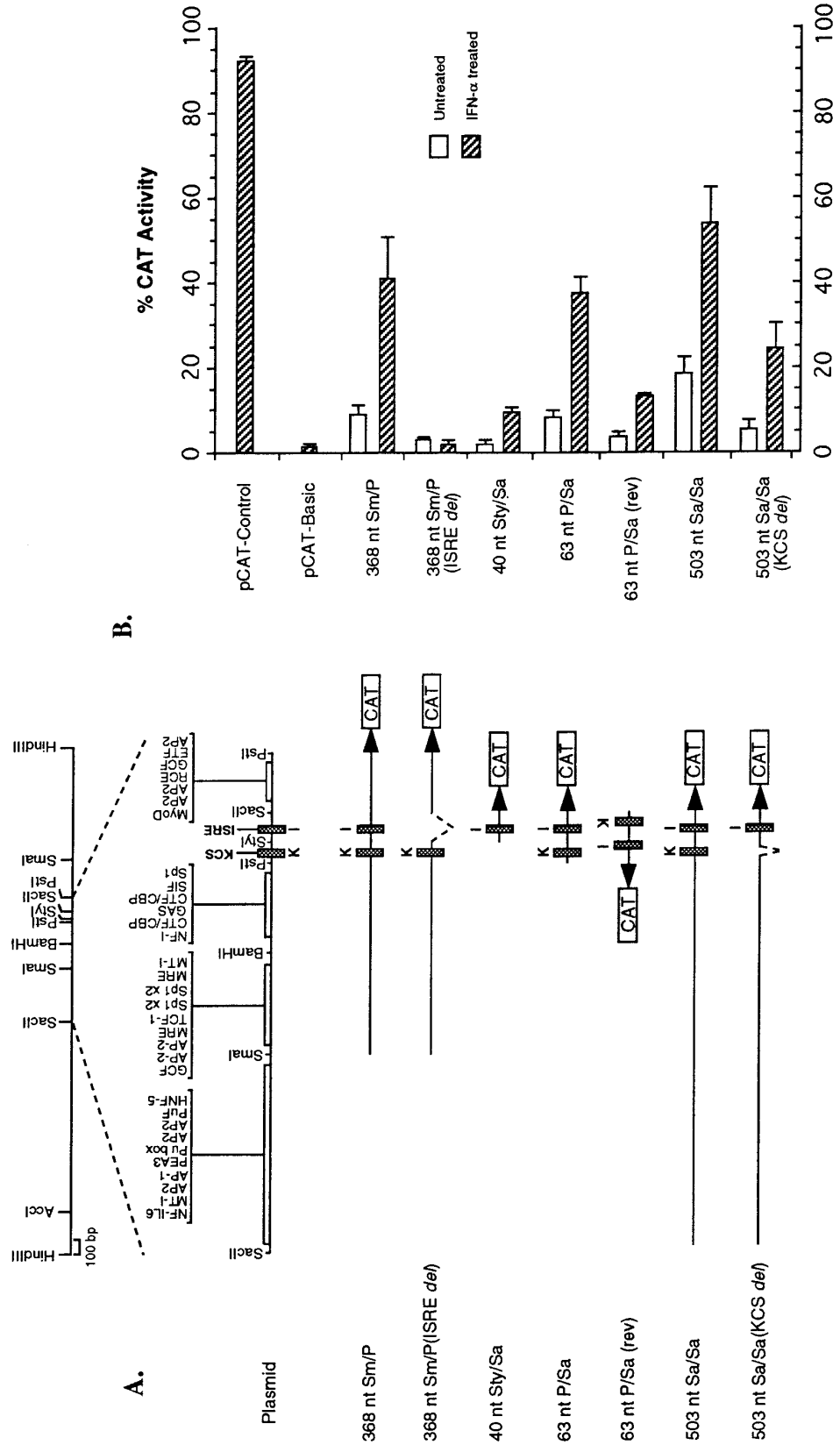

FIG. 5. Deletion of either the ISRE element or the KCS motif reduces *Pkr* promoter activity. (A) Schematic representation of the CAT reporter plasmids constructed by insertion of the indicated human *Pkr* promoter fragments into the promoter-less pCAT-Basic plasmid. The presence or absence of the ISRE and the KCS in the deletion constructs are indicated by the boxes labeled I and K, respectively. (B) Promoter activities observed in human amnion U cells transfected with the indicated CAT reporter plasmids. Promoter activities of deletion constructs are shown as percentage of the conversion of [^{14}C]-chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon beginning at 24 hr after transfection. Transfections were repeated three to five times in independent experiments to allow for calculation of a mean value and standard deviation. pCAT-Control, the CAT reporter gene linked to the simian virus 40 promoter and enhancer; pCAT-Basic, the promoter-less plasmid vector without inserted human genomic DNA.

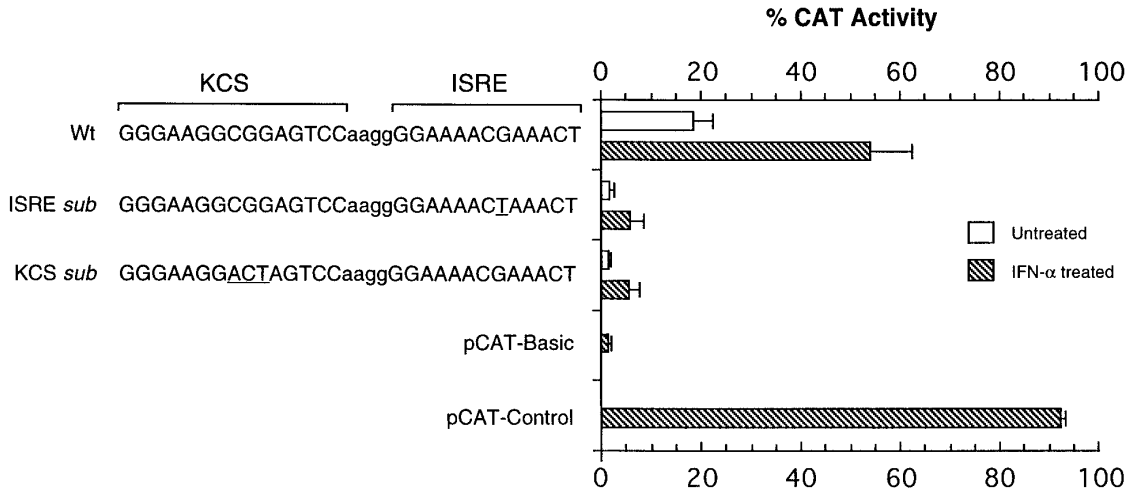

FIG. 6. Nucleotide substitution within either the KCS motif or the ISRE element reduces *Pkr* promoter activity. Mutant CAT reporter plasmids were derived from the 503 nt Sa/Sa wild-type (wt) parent construct. For ISRE *sub,* the construct possessed a wt KCS motif but the indicated G → T substitution mutation was introduced within the ISRE element. For KCS *sub,* the construct possessed a wt ISRE element but the indicated C → A, G → C, G → T triple substitution mutation was introduced within the KCS motif. Mutated nt positions are underlined. Promoter activities were determined in human amnion U cells transfected with the indicated CAT reporter plasmids; activities of the constructs are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon beginning at 24 hr after transfection. pCAT-Control, the CAT reporter gene linked to the simian virus 40 promoter and enhancer; pCAT-Basic, the promoter-less plasmid vector without inserted human genomic DNA containing the *Pkr* promoter.

and novel points. First, the human *Pkr* promoter contains, in addition to an ISRE element that is required for IFN-inducible transcription, a novel motif that we have identified and designated KCS for kinase conserved sequence. The 15-nt KCS motif is exactly conserved in sequence, distance, and position, relative to the 13-nt ISRE, in the human and mouse *Pkr* promoters (Fig. 4). The KCS was required for optimal transcriptional activity of the human *Pkr* promoter. Second, despite extensive conservation of the exon organization of the human and mouse *Pkr* genes in the coding region of the PKR proteins (Tanaka and Samuel, 1994; Kuhen *et al.,* 1996), the human and mouse *Pkr* promoters show striking differences in DNA sequence and thus in the structural organization of the predicted transcription factor binding sites, aside from the ISRE and KCS sequences that are conserved between the human and the mouse promoter homologs.

Sequence of the human *Pkr* promoter region and comparison to the mouse homolog

Several candidate transcription factor binding sites were identified by computer analysis of the DNA sequence of the human *Pkr* promoter region. Not unexpected, an interferon-stimulated response element (ISRE) involved in IFN inducibility was identified. The sequence of the ISRE element (GGAAAACGAAACT) within the human *Pkr* promoter (Fig. 3) matches exactly the consensus ISRE sequence (GGAAAN$_{(1\text{-}2)}$GAAACY) (Williams, 1991) and differs from the mouse ISRE element which possesses the purine A instead of a pyrimidine in the 3′-terminal nt position (Tanaka and Samuel, 1994). Regulation at the transcriptional level of type I IFN responsive genes in many cases is mediated through the ISRE element located in the 5′-flanking sequences (Schindler and Darnell, 1995).

GAS elements often are involved in mediating transcriptional induction of type-II (γ) IFN-stimulated gene expression (Schindler and Darnell, 1995). The human *Pkr* promoter, like the mouse *Pkr* promoter (Tanaka and Samuel, 1994), possessed a candidate GAS sequence upstream of the ISRE element that conforms to the consensus TTnCnnnAA motif (Pearse *et al.,* 1993). However, the human *Pkr* GAS-like sequence, unlike the mouse *Pkr* GAS sequence, does not show a perfect match with previously identified GAS core sequences. This may explain, in part, the poor inducibility of *Pkr* transcription by IFN-γ observed in human cells by Northern analysis (Thomis *et al.,* 1992) and also in transient transfections with reporter CAT constructs.[3] This GAS-like element potentially could serve as an IL-6 responsive element, as both cytokines have been observed to mediate their effects through TTnCnnnAA-type motifs (Harroch *et al.,* 1994). An additional candidate IL6-responsive element, NF-IL6, was identified in the human *Pkr* promoter region. This element was also present in the mouse *Pkr* promoter (Tanaka and Samuel, 1994). However, we so far have been unable to demonstrate IL6-mediated transcriptional activation of the *Pkr* promoter in either the human amnion U or hepatoma HepG2 cell lines or the mouse L cell line,[3] although it is conceivable that IL-6

[3] Borthwick, E. B., Kuhen, K. L., and Samuel, C. E., unpublished observations (1996).

still may induce PKR expression under conditions different from those which we tested.

Examination by sequence analysis of 14 independently isolated cDNA clones using the 5′-RACE procedure localized the 5′-end of the human PKR mRNA to about 50 bp downstream of the ISRE element. In most of the characterized type I IFN responsive genes, the transcription initiation site(s) are usually located within 200 bp downstream of the ISRE element (Williams, 1991; Schindler and Darnell, 1995). The human *Pkr* promoter region that contains all of the elements sufficient for basal and IFN-inducible transcription, including the ISRE, lacked the classical consensus sequences such as a TATA box (Breathnach and Chambon, 1981) or CTCANTCT initiator positioning sequence (Smale *et al.,* 1990). In this context, the human *Pkr* promoter region was similar to that of the mouse *Pkr* promoter region (Tanaka and Samuel, 1994) and also that of several other protein kinase genes from mammalian cells (Voss *et al.,* 1991). However, the human *Pkr* promoter did possess two CCAAT boxes (McKnight and Kingsbury, 1982) approximately 130 to 150 nt upstream of the 5′-end of exon 1 (Fig. 3), whereas the mouse promoter lacked candidate CCAAT boxes.

Comparison of the organization of the human *Pkr* promoter (Fig. 3) with that of its mouse homolog (Tanaka and Samuel, 1994) revealed that the two promoters are surprisingly quite distinct from one another at the level of DNA sequence with the exception of the KCS and ISRE sequences. Thus, not unexpectedly, some of the putative transcription factor binding motifs predicted from the DNA sequence differ substantially between the human and the mouse *Pkr* promoters. Two potential binding sites were detected for the PEA3 transcription factor in the human *Pkr* promoter region. It is curious that promoters of other IFN-regulated genes including the mouse *Pkr* gene (Tanaka and Samuel, 1994), both the human and mouse 2′,5′-oligoadenylate synthetase genes (Williams, 1991), and the *ISG*54, 202, 9-27, H-2Dd, H-2Ld genes (Williams, 1991; Schindler and Darnell, 1995) all contain *Ets* sites. The Ets-family of proteins, which includes PEA3, are important in the regulation of cell proliferation and differentiation during hematopoeisis (Wasylyk *et al.,* 1993, Shen-Ong, 1990). Numerous consensus binding sequences for the enhancer binding proteins Sp1 and AP-2 were also found in the human *Pkr* promoter; these sites likewise are present in the mouse promoter region (Tanaka and Samuel, 1994).

Functional identification of the IFN-responsive human *Pkr* promoter

Our results of the functional analysis of 5′-flanking sequences of the human *Pkr* gene fused to a CAT reporter gene provides strong evidence in support of the conclusion that we have correctly identified the human *Pkr* promoter. Analysis of deletion mutants (Figs. 2 and 5) of the 5′-proximal 2.4-kb H/H construct (Fig. 1D) showed that the ISRE element, along with 23 nt of upstream sequence that included the novel KCS motif, were necessary and sufficient to mediate the maximal level of type I IFN-inducibility observed among the promoter constructs we analyzed. The *Pkr* ISRE retained function as an IFN-inducible element upon reversing its orientation as established with the 63-nt P/Sa and 63-nt P/Sa(rev) constructs. It is well-established that the ISRE sequence is necessary and sufficient to mediate IFN-inducible transcription of a heterologous promoter in reporter gene constructs (Williams, 1991; Schindler and Darnell, 1995). Our results obtained with the human *Pkr* promoter constructs 368-nt Sm/P ISRE *del* and its converse, the 40-nt Sty/Sa, are in full agreement with this earlier finding.

Northern analysis shows about a fivefold increase in the steady-state level of PKR message in various human cell lines treated with type I IFN as compared to untreated cells, including the human U, HeLa, and Daudi cell lines (Meurs *et al.,* 1990; Thomis *et al.,* 1992). A comparable level of induction by IFN of reporter activity (fivefold induction) was observed for U cells transfected with promoter constructs possessing both the KCS motif and the ISRE element, for example, the 1.72-kb H/P, the 1.42-kb A/P, the 368-nt Sm/P, and the 63-nt P/Sa constructs. These results are consistent with the notion that the increased levels of *Pkr* mRNA and PKR protein observed in IFN-treated cells (Samuel, 1993; Clemens, 1996) are largely due to transcriptional activation of the *Pkr* gene, rather than posttranscriptional regulatory events.

Although the PKR kinase is inducible by IFN (Samuel, 1993; Clemens, 1996), Northern analysis and PKR autophosphorylation assays have consistently revealed a clearly detectable basal level of *Pkr* mRNA expression and *Pkr* kinase activity in human cell lines not treated with exogenously added IFN (Meurs *et al.,* 1990; Thomis *et al.,* 1992). Candidate transcription factor binding sites found in the *Pkr* promoter region that may positively effect basal *Pkr* constitutive gene expression include, for example, Sp1, AP2, NF-I, CTF/CBP, ETF, and the novel KCS described herein. The presence of an ETF site within the *Pkr* promoter, which lacks a TATA box, would be in agreement with the original observations of Kageyama *et al.* (1989) who found ETF specifically stimulated transcription from TATA-less promoters. The loss of cytokine-independent promoter activity when the 40-bp *Sty*I through *Sac*II region was deleted from the 368-nt Sm/P mutant suggested that this region, which included the ISRE element, was likely involved in the assembly of the basal transcription complex. The 40-nt Sty/Sa and the 238-nt B/P deletion mutants showed similar inducibility by IFN. However, the 238-nt B/P construct displayed much stronger promoter activity, suggesting sequences therein have a functional role in the constitutive level of *Pkr* gene expression. The 238-nt B/P construct which possessed significant basal and inducible promoter ac-

tivity nevertheless was not as active as the 368-nt Sm/P construct (Fig. 2). Conceivably the 19-bp tandem direct repeat within the 368-nt Sm/P construct, with each repeat possessing two overlapping Sp1 binding sites (Fig. 3), or the putative AP2 binding site, is responsible for this difference in promoter strength and inducibility by IFN.

Functional importance of the novel KCS motif

The strict conservation of sequence, distance, and position, relative to the ISRE element, of the KCS motif which we have identified by sequence comparison of the human and mouse *Pkr* promoters is consistent with a possible functional role for this newly recognized motif. Our results of reporter gene expression analyses of KCS deletion and substitution mutants support this notion. Addition of the KCS motif to a construct that possesses an ISRE element increased both basal and IFN-inducible CAT activity in transfected cells (Fig. 5). Induction by IFN of the reporter gene activity in the case of the 63-nt P/Sa construct was about fivefold, comparable to the IFN inducibility observed for the 368-nt Sm/P, 1.42-kb A/P, and 1.72-kb H/P constructs. The data obtained for the 40-nt Sty/Sa and 63-nt P/Sa constructs without and with the KCS motif, respectively, suggest that the presence of the KCS sequence strengthens constitutive *Pkr* gene expression as well as IFN-inducible expression mediated by the ISRE element located immediately downstream of the KCS motif. Either deletion of the entire KCS motif from the 503-nt Sa/Sa promoter construct or the introduction of nucleotide substitutions within the KCS motif resulted in a decrease in both basal and inducible CAT activity (Figs. 5B and 6). These results are consistent with an important functional role for the KCS motif in basal *Pkr* transcription in the absence of cytokine treatment as well as inducible transcription.

It is now of utmost importance to identify, by systematic mutational analysis, which of the nt positions of the newly identified 15-nt KCS motif are of primary importance for promoter activity. It is also of central importance to attempt to identify the protein factors which may interact with KCS and under what conditions of cell growth and cytokine treatment those interactions occur.

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