Activation of the RNA-dependent Protein Kinase PKR Promoter in the Absence of Interferon Is Dependent Upon Sp Proteins*

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The protein kinase regulated by RNA (PKR) is interferon (IFN)-inducible and plays important roles in many cellular processes, including virus multiplication, cell growth, and apoptosis. The TATA-less PKR promoter possesses a novel 15-bp DNA element (kinase conserved sequence (KCS)) unique to the human and mouse PKR genes that is conserved in sequence and position. We found that Sp1 and Sp3 of the Sp family of transcription factors bind at the KCS element. Their involvement was analyzed in the activation of basal and IFN-inducible PKR promoter activity. Both the small and large isoforms of Sp3 co-purified with KCS protein binding activity (KBP) by using nuclear extracts from HeLa cells not treated with IFN. Two forms of the KCS-binding protein complex were demonstrated by electrophoretic mobility shift assay analysis; one contained Sp1 and the other Sp3. In mouse cells null for all Sp3 isoforms, PKR expression was reduced to ~50% that of wild-type cells in the absence of IFN. The IFN-inducible expression of PKR, however, was Sp3-independent but STAT1- and JAK1-dependent. Overexpression of Sp1 in human U cells resulted in increased PKR promoter activity. In Drosophila SL2 cells lacking Sp proteins, both Sp1 and Sp3 large but not small isoforms activated PKR promoter expression, with the Sp1-mediated activation dominant. Mutaional analysis of the PKR promoter region indicated a cooperative interaction between two different Sp sites, one of which is within the KCS element. These results establish that, in the absence of IFN treatment, activation of PKR basal expression is mediated by Sp1 and Sp3 proteins in a cooperative manner.

Among the interferon (IFN)-inducible proteins that play an important role in the innate defense against viral infection and pathogenesis is PKR, the protein kinase regulated by RNA (1). PKR catalyzes the phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor eIF-2 on serine residue 51 (2, 3). This modification alters the translation pattern within cells and leads to an inhibition of protein synthesis in virus-infected, IFN-treated cells (3, 4). PKR also plays a role in signaling pathways (5–7). A range of biological processes is affected by the PKR protein, including cell growth, differentiation, and apoptosis, in addition to virus multiplication. Because of these broad and basic functions affected by PKR, an understanding of the molecular mechanisms that control the amount and activity of the PKR protein, including the transcriptional activation of the PKR gene, is of fundamental importance.

Unlike many genes that are IFN-inducible, a significant basal expression level of PKR is seen in many cell lines and most animal tissues (8–10). The human PKR gene, located on human chromosome 2p, spans ~50 kb and possesses 17 exons (10, 11). Study of deletion reporter constructs derived from the 5′-flanking region of the PKR gene identified a 503-bp DNA sequence that possesses the DNA elements necessary and sufficient for both basal and IFN-inducible transcription in transfected cells. These elements include a consensus 13-bp interferon-stimulated response element (ISRE) responsible for IFN-inducible transcription (10, 12) and a novel 15-bp element designated kinase conserved sequence (KCS) that is exactly conserved between the mouse and human PKR promoters in both sequence and position relative to the ISRE (10, 13). The KCS element is required for optimal basal as well as IFN-inducible PKR expression (10). The 503-bp PKR promoter region lacks consensus sequences often associated with initiation of transcription such as the TATA box (10, 14) and CTCANTCT initiator positioning sequences (10, 15).

Activation of transcription of genes inducible by type I IFNs such as PKR is best understood in the context of the heteromeric transcription factor ISGF3 (STAT1, STAT2, and IRF9) that binds to the ISRE DNA element (1, 10, 12, 16). But in the case of the PKR gene, the 15-bp KCS element is necessary for both basal and IFN-inducible transcriptional activity (10, 16, 17). The KCS DNA element selectively binds proteins that are constitutively present in cells in the absence of IFN treatment, and in a manner that correlates with transcriptional activity in transfection reporter assays (16–18). Purification studies, KCS DNA element pull-down assays, and EMSA antibody supershift analyses have identified Sp1, DDB1, and DDB2 as trans-acting protein factors that bind the KCS element (18–20). Chromatin immunoprecipitation assays have importantly established in vivo binding of Sp1, Sp3, DDB2, STAT1, and STAT2 to the PKR promoter region (19, 20).

The 5′-flanking region of the human PKR promoter includes five Sp-binding sites (four overlapping) upstream of the KCS and ISRE elements (10). In addition, the 5′-portion of the 15-bp KCS DNA element corresponds to an Sp1-binding site (18, 19). The specificity protein (Sp) family of transcription factors that recognize the GC-rich Sp-binding sites present in the promoter 5′-flanking region includes nine known members that share the zinc finger structural motif found in the C-terminal DNA binding region (21–24). Among the Sp factors, Sp1 and Sp3...
are expressed ubiquitously and bind common DNA target sequences. The activating or repressing activity of Sp factors appears dependent upon multiple microenvironment parameters, including the relative abundance of the factors, the type of post-translational modification, if any, the nature of the cell, and the specific promoter involved (21–23).

For example, a higher level of Sp1 compared with Sp3 is thought to be important in the activation of the promoter for the vascular endothelial growth factor receptor gene (25); both Sp1 and Sp3 activate transcription of the human dopamine transporter gene (26), and the uteroglobin gene promoter is up-regulated by Sp1 but repressed by Sp3 (27). Sp1 and Sp3 are also reported to act synergistically to enhance transcription in the case of the c-met gene (28). However, little is known about the role of Sp factors in the constitutive expression of genes such as PKR that are also inducible by IFN.

Here we establish that activation of the protein kinase PKR promoter in the absence of interferon is mediated by Sp factor proteins that are constituent components of the heteromeric transcriptional activator (KBP) complex that binds at the KCS DNA element. Protein purification and immunochemical and mutational analyses established that both the large (II) and small isoforms (si) of Sp3 are KBP complex components. Sp3II and the previously identified Sp1 protein both activate the PKR promoter in mammalian and insect cells. In Drosophila cells devoid of Sp factors, the PKR promoter is silent but is activated by both Sp1 and Sp3II, whereas Sp3III neither activates nor antagonizes Sp1-mediated transactivation of the PKR promoter. These results provide new mechanistic insight into the activation of the PKR gene and unequivocally demonstrate that members of the Sp protein family of transcription factors are fundamentally important determinants of basal promoter activity of the PKR gene in the absence of IFN treatment.

**EXPERIMENTAL PROCEDURES**

Cell Maintenance and Interferon Treatment—Human amnion U cells and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco’s modified Eagle’s medium with 5 or 10% fetal bovine serum (Hyclone), respectively, and 1% sodium pyruvate (Cambrex Bio Science), 100 μg/ml penicillin, and 100 units/ml streptomycin (Invitrogen) as described previously (16, 18, 19, 29). Wild-type (WT), Stat1−/− (30), Jak1−/− (31), and Pkr−/− (8) MEF cells were generously provided by Dr. Robert Schreiber (Washington University, St. Louis) and A. Koromilas (Lady Davis Institute, Montreal, Canada). Sp3(III) and Sp3−/− MEFs were as described (32). Interferon treatment was with 1000 units/ml of IFN-α for 24 h, using either recombinant IFN-α2A/D (33) or natural leukocyte IFN. Drosophila Schneider line SL-2 cells (Drosophila melanogaster embryo, SL-2) were kindly provided by Dr. Stephen Poole of this institution and were grown in Schneider’s Drosophila medium (Invitrogen) at ambient room temperature (22–25°C).

**Plasmid Constructions**—The pCAT-Basic promoter-less plasmid (Promega) containing the chloramphenicol acetyltransferase (CAT) gene was used for construction of reporter plasmids containing WT and mutated KCS and ISRE as well as constructs with 5′-deletion in the minimal promoter region. The construction of human PKR promoter plasmids p503(WT), p63(WT), p130(WT), p503(KCSmut6A), p503(KCSmut9T), and p503(ISREmut8T) were as described previously (18). The p503(KCSdelISRE) m8T) double mutant construction was generated by insertion of a 130-bp BamHI/XbaI fragment derived from p503 (ISREmut8T) into pBluescript(Stratagene) with the PstI site in the MCS deleted (pBluescriptΔPstI). The KCS element was deleted by using PstI and Styl, followed by filling in with T4 DNA polymerase (New England Biolabs) and plasmid closure with T4 DNA ligase. The resulting BamHI/XbaI fragment containing KCSdelISREmut8T was exchanged for the corresponding BamHI/XbaI of p503 (ISREmut8T). pGL2-basic promoter-less plasmid (Promega) containing the firefly luciferase gene as the reporter was used for construction of the p503(WT)/Luc promoter plasmid that was generously provided by Dr. Ping Zhang of this laboratory. pRSV-βgal was generously provided by Dr. Joe Nevin (Duke University, Durham).

The mammalian expression plasmids pCMVSp1 and pCMVSp3 (34) were generously provided by Dr. Jonathan Horowitz (North Carolina State, Raleigh). The pCMV vector without insert was generated by deleting the Sp1 insert from the pCMVSp1 construct. The Drosophila actin promoter-driven expression plasmids pPacSp1 and pPacSp3 for WT Sp1 and Sp3, respectively, were gifts from Dr. Robert Tjian (University of California, Berkeley) and Dr. Grace Gill (Harvard Medical School, Boston). The pPac vector without insert was generated by deleting the Sp3 insert from the pPacSp3 construct. pPacSp3(D1+2AUG) was as described recently (35).

**Transient Transfection Assays**—Human U cells were transfected with the PKR promoter plasmid constructs with either the CAT or luciferase reporter by the DEAE-dextran-chloroquine phosphate transfection method (36) as described previously (10, 16, 37), using either pRSV-βgal or pGL2 as the internal reference. All DNA plasmids used in transfections were purified either by cesium chloride equilibrium centrifugation or by using a QIAfilter plasmid maxi kit (Qiagen); plasmid integrity was analyzed by agarose gel electrophoresis. Varying amounts of pCMVSp1 and pCMVSp3 expression plasmids together with added empty pCMV-4 vector to give a total of 5 μg of pCMV DNA were used for co-transfections. IFN treatment was initiated 24 h after transfection. Cells were harvested 48 h post-transfection, and extracts were prepared and luciferase, CAT, and β-galactosidase activities determined as described previously (10, 16, 37, 38). Luciferase assays were quantified using an OPTOCOMP II luminometer, and CAT assays were quantified after TLC by use of a Bio-Rad G525 molecular imager system. Protein concentrations of extracts were determined by the modified Bradford method (Bio-Rad). The data presented are the average values derived from three to five independent experiments. Drosophila SL-2 cells were transfected using Cellfectamine (Invitrogen) according to the manufacturer’s recommendations. SF-900 II SMF medium (Invitrogen) was used to dilute the DNA as well as Cellfection reagent. Briefly, SL-2 cells were plated in 60-mm dishes at ~4 × 10⁶ cells/dish 1 day prior to transfection. Cells were then transfected with 3 μg of CAT reporter plasmid and the indicated amounts of pPacSp1 or pPacSp3 expression constructs and pPac empty vector as necessary to maintain constant DNA amounts. Fresh media was added to the cells 24 h post-transfection, and cells were harvested 48 h post-transfection, and extracts were prepared by repeated freeze-thaw cycles followed by determination of CAT activity as described previously (10, 37).

**Nuclear Extract Preparation**—Nuclear extracts were prepared as described previously (19, 39). Briefly, cells were scraped into ice-cold phosphate-buffered saline and collected by centrifugation, and cell pellets were suspended in 3 volumes of lysis buffer (20 mM Hepes, pH 7.9; 10 mM KCl; 1 mM EDTA, pH 8.0; 0.2% Nonidet P-40 (v/v); 0.1 mM Na3VO4; 1 mM phenylmethylsulfonfonyl fluoride; 1 mM DTT; 10% glycerol (v/v); and 1% protease inhibitor mixture (v/v; Sigma)) followed by incubation on ice for 10 min. Cell suspensions were gently pipetted up and down, and the lysates were then centrifuged at 14,000 × g for 5 min at 4°C to obtain nuclear pellets. Nuclear pellets were washed twice with cell lysis buffer (lacking Nonidet P-40 and protease inhibitor mixture) followed by resuspension in 2 volumes of nuclear extract buffer (20 mM Hepes, pH 7.9; 10 mM KCl; 1 mM EDTA, pH 8.0; 1 mM phenylmethylsulfonyl fluoride; 1 mM DTT; 1 mM Na3VO4; 420 mM NaCl; 20% glyc-
erol (v/v) and 10% protease inhibitor mixture (v/v) (Sigma)). Nuclei were extracted by incubation at 4 °C for 30 min with gentle agitation followed by centrifugation at 14,000 × g at 4 °C for 5 min; the resultant supernatant fraction was used as nuclear extracts.

Whole Cell Extract Preparation—Whole cell extracts were prepared as described previously (19). Cells were washed, scraped, and pelleted as described above. Cell pellets were suspended in 2–3 volumes of whole cell extract buffer (20 mM Hepes, pH 7.9; 10 mM KCl; 1 mM EDTA, pH 8.0; 1 mM phenylmethylsulfonyl fluoride; 1 mM DTT; 1 mM Na3VO4; 5 mM NaF; 400 mM NaCl; 0.5% Nonidet P-40; 20% glycerol (v/v); and 1% protease inhibitor mixture (v/v) (Sigma)), incubated on ice for 10 min, and gently pipetted up and down. Cell suspensions were transferred to 4 °C for 30 min with gentle agitation, followed by centrifugation at 14,000 × g at 4 °C for 5 min to obtain whole cell extracts.

Electrophoretic Mobility Shift Assay—The sequence of the double-stranded PKR promoter KCS oligonucleotide 32P-end-labeled probe was 5′-CTGCAGGGAAGGCAGTCAAGG-3′ (+ strand). For protein-DNA binding reactions, 10 μg of nuclear extract protein was incubated with 32P-labeled probe for 25 min at room temperature in a 25-μl reaction containing 1 μg of poly(dI-dC); 20 mM Hepes, pH 7.9; 1 mM MgCl2; 40 mM KCl; 0.1 mM EGTA, pH 8.0; 1 mM DTT; and 10% glycerol (v/v). Electrophoresis using 5% native polyacrylamide gels with 0.5× Tris borate/EDTA buffer was as described previously (16, 19, 20). For supershift analyses, rabbit polyclonal antiserum against Sp1 (07-645) and Sp3 (07-107) were purchased from Upstate Biotechnology, Inc. Incubation with antibodies was carried out on ice for 45 min prior to addition of 32P-labeled probe.

Western Immunoblot Assays—Proteins were fractionated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and then probed with an appropriate dilution of primary antibody in phosphate-buffered saline containing 3% skim milk. Western blot detection was done with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody using an ECL detection reagent kit (Amersham Biosciences) or SuperSignal West Pico Chemiluminescent substrate (Pierce), according to manufacturer’s protocol. Immunoreactive bands were visualized using a VersaDoc (Bio-Rad) imaging system, and quantitation was done using the Quantity One software program. Rabbit polyclonal antibodies were used to detect Sp1 protein (07-645; Upstate Biotechnology, Inc.), mouse Sp3 protein (sc-644; Santa Cruz Biotechnology), and human Sp3 protein (07-107; Upstate Biotechnology, Inc.). Monoclonal antibody anti-hemagglutinin from Roche Applied Science (clone 12CA5) was used to detect hemagglutinin-tagged Sp1 and Sp3 proteins in extracts of transfected U-cells, and rabbit polyclonal antibody against recombinant mouse PKR expressed as a glutathione S-transferase fusion was prepared by our laboratory. When Nonidet P-40 lysates were used for Western analysis, they were prepared as described previously (40). Mouse monoclonal antibody against β-actin was from Sigma (A-5441).

Materials—Unless otherwise specified, all materials and reagents were as described previously (10, 16–19).

RESULTS

Sp3 Co-purifies with KCS DNA Binding Activity—We established earlier that Sp1, Sp3, DDB1, and DDB2 are components of a complex formed between the 15-bp DNA element, KCS, of the PKR promoter and proteins present in crude nuclear extracts (19, 20). Using as starting material nuclear extracts prepared from human HeLa cells not treated with IFN, proteins binding the KCS element were purified by a multistep scheme that included DEAE-cellulose, CM-Sepharose, and sequential DNA-Sepharose affinity chromatography steps with wild-type, then mutant, and again wild-type KCS DNA affinity columns (20). When the fractions possessing KCS binding activity (KBP) were now monitored by Western immunoblot assay using antibody against the Sp3 transcription factor, two size forms of Sp3 protein were detected (Fig. 1A). These correspond to the long (Sp3LI) and short (Sp3SI) isoforms of Sp3 that derive from alternative translation initiation sites (35, 41, 42), both of which are co-purified with KSBP activity. The Sp1 protein also co-purified with KSBP activity (Fig. 1B) as reported earlier (20).

Because the purification of KCS-binding proteins was from cells not treated with IFN, the Sp1 and Sp3 proteins interacted with the KCS DNA element in the absence of an IFN-mediated activation signal. This finding is consistent with our earlier observations that both Sp1 and Sp3 bound to KCS DNA linked to Sepharose beads comparably using nuclear extracts prepared from untreated or IFN-treated cells and that KSBP activity was IFN-independent (17, 19).

The Factors Sp1 and Sp3 Independently Bind to the KCS Element of the PKR Promoter—To assess the importance of the Sp3 transcription factor in the context of protein complex formation with the KCS DNA element of the PKR promoter, we carried out EMSAs by using extracts prepared from either wild-type MEF cells or MEF cells genetically deficient in Sp3 protein expression (32). When the KCS element was used as the 32P-labeled DNA probe in EMSA analyses with extracts prepared from wild-type cells, a KSBP complex was detected as a poorly resolved doublet band (Fig. 2A, lane 1). Treatment of extracts with antibody against Sp1 eliminated the upper KSBP complex band shift, leaving only the lower band of the KSBP complex (Fig. 2A, lane 2). By contrast, when extracts were treated with antibody against Sp3, the lower region of the KSBP band shift complex was eliminated (Fig. 2A, lane 3). Finally, in the presence of antibodies against both Sp1 and Sp3, both the upper and lower complexes were eliminated (Fig. 2A, lane 4). Taken together, these results are in agreement with the notion that two resolvable complexes constitute KSBP, one containing Sp1 and the other containing Sp3.

When extracts prepared from Sp3 null MEF cells were analyzed (Fig. 2A, right, lanes 5–8), a prominent band shift complex formation was still seen with 32P-labeled KCS probe and that corresponded to the upper region of the KSBP complex mobility (Fig. 2A, lane 5). Antibody against Sp1 eliminated entirely the formation of this band shift complex observed with extracts prepared from Sp3−/− cells (Fig. 2A, lane 6). By contrast, as a control, antibody against Sp3 had no effect on that com-

![Figure 1](image-url)
Stat1 wild-type MEF lines also were included in the immunoblot assays. Cells inducible Expression Is Sp3-independent in Mouse Embryo Fibroblast in IFN signal transduction and transcriptional activation (with defined genetic disruptions of genes that encode proteins involved purification studies that established the presence of Sp3 as a component observation, and because untreated nuclear extracts were used in the is readily detected in mouse cell lines and tissues (1, 9). In light of this small or large isoform of Sp3, whereas both Sp3li and Sp3si isoforms amounts of the heterogeneous nuclear ribonucleoprotein were found contrast, when probed with anti-hnRNPA1 as a loading control, similar MEFs (Fig. 2 lanes 5–8) were incubated with 32P-labeled KCS probe in the absence (lanes 1 and 5) or presence of antibody against Sp1 (lanes 2 and 6) or Sp3 (lanes 3 and 7) or a combination of both Sp1 and Sp3 (lanes 4 and 8). The KBP complexes are indicated by brackets. A, Western immunoblot analysis of Sp1 (upper panel) and Sp3 (lower panel) protein expression in WT (lanes 1 and 2) and Sp3−/− cells (lanes 3 and 4) using 10 μg of nuclear extract and polyclonal antibody against Sp1 or Sp3 as indicated.

Sp3 Is Necessary for Optimal Basal PKR Expression, but Interferon-inducible expression, which is dependent upon JAK1 and STAT1. A, Western immunoblot analysis of PKR protein expression in WT and mutant MEF cell lines genetically deficient in Sp3, Stat1, Jak1, and Pkr as indicated. Cells were treated with 1000 units/ml recombinant IFN (+) or left untreated (−). Nonidet P-40 cell-free extracts were prepared and then analyzed (5 μg of extract protein) as described under “Experimental Procedures” using rabbit polyclonal antibody against mouse PKR (upper panel) or monoclonal antibody against β-actin (lower panel). B, Western immunoblot analysis of Sp3 protein expression in WT and mutant MEF cell lines. Nuclear extracts (10 μg of protein) were analyzed by using polyclonal antibody against Sp3; both the long (l) and the short (s) isoforms of Sp3 were detected. C, quantitation of PKR protein expression in untreated (−) and IFN-treated (+) cells, either WT or Sp3 null MEFs as indicated. PKR quantitation is relative to β-actin for five independent experiments, presented as means ± S.D. and normalized to untreated wild-type cells. Student’s t test, *p = 0.023, untreated Sp3−/− compared with untreated wild-type.

As shown in Fig. 3A, the IFN-inducible expression of the PKR protein was Sp3-independent (lanes 2 and 4) but dependent upon the STAT1 factor (lane 8) and JAK1 kinase (lane 12), known type I IFN signal transduction pathway components. However, the basal expression of the PKR protein observed in the Sp3−/− MEF cells (lane 3) was less than that seen in all other MEF cell lines (Fig. 3A) except the Pkr−/− mutant that lacked detectable PKR (lanes 13 and 14). Most interestingly, basal expression of PKR in mutant MEFs lacking either JAK1 or STAT1 was comparable with that of wild-type MEFs (Fig. 3A). Control Western immunoblots were performed with anti-β-actin antibody (Fig. 3A, lower) and anti-Sp3 antibody (Fig. 3B) by using the same set of extracts shown for the PKR immunoblot (Fig. 3A, upper). For Sp3 Western blot, nuclear extracts were used. Sp3 protein levels were found to be similar in all of the MEF lines examined, other than the Sp3−/− MEFs that did not express either size isoform of Sp3 (Fig. 3B, lanes 3 and 4).

Quantiﬁcation of PKR protein expression in several independent experiments showed a signiﬁcant reduction (p = 0.023) in basal expression of about 50% in Sp3 null mutant MEFs compared with wild-type MEFs (Fig. 3C). Somewhat surprisingly, the IFN-inducible expression of Pkr did not differ signiﬁcantly (p = 0.127) between the mutant cells lacking Sp3 and wild-type cells. This observation is consistent with a role for Sp3 in the regulation of basal PKR expression.

Effect of Sp Factor Overexpression on PKR Promoter Activity in Human Cells—As one approach to assessing the role of Sp1 and Sp3 proteins in the regulation of PKR expression, we examined the effect of
Regulation of the PKR Promoter

FIGURE 4. Effect of Sp factors on PKR promoter activity in human U cells. A, transactivation of PKR promoter in human U cells. Varying amounts (μg) of pCMVSp1 or pCMVSp3 expression plasmid construct, as indicated, were co-transfected individually with the reporter construct containing the p503(WT) minimal PKR promoter driving expression of the luciferase reporter gene. The pRSV-gal plasmid was included as an internal control for transfection efficiency, and all data were normalized to β-galactosidase. U cells were treated with IFN-α (•) or left untreated ( – ) as indicated. Results shown are the means ± S.D. determined from five experiments. B, expression of Sp factors in transfected cells measured by immunoblotting with anti-hemagglutinin antibody. Whole cell extracts were prepared from U cells transfected with pCMVSp expression constructs for Sp1 (lane 2) and Sp3 (lane 3) proteins or empty pCMV-4 vector (lane 1) as a negative control.

FIGURE 5. PKR promoter transactivation by Sp factors in Drosophila SL2 cells. Varying amounts (μg) of pPacSp1 or pPacSp3 expression plasmid construct, as indicated, were co-transfected individually with the reporter construct containing the p503(WT) minimal PKR promoter driving expression of the CAT reporter in Drosophila SL2 cells. CAT activity measured 48 h after transfection was normalized to protein content, and the percentage conversion of [14C]chloramphenicol to acetylated derivatives was determined. Results shown are the means ± S.D. determined from five independent experiments.

FIGURE 6. Sp1 and Sp3 act synergistically to transactivate the PKR promoter in Drosophila SL2 cells. pPacSp1 and pPacSp3 were co-transfected along with the reporter construct containing the p503(WT) minimal PKR promoter driving expression of the CAT reporter in Drosophila SL2 cells. Varying amounts (μg) of pPacSp1 expression plasmid were tested either alone or with a constant amount of pPacSp3 as indicated. CAT activity was determined as for Fig. 5; results shown are the means ± S.D. determined from three independent experiments.

their overexpression on the activity of the PKR promoter in transfected animal cells. Human U cells were co-transfected with Sp1 or Sp3 cDNA expression plasmids, either pCMVSp1 or pCMVSp3, respectively, along with the PKR promoter construct p503(WT) that contains the minimal PKR promoter region driving expression of a luciferase reporter. Increasing concentrations of Sp cDNA expression plasmids were used. Overexpression of Sp1 protein led to an increase in basal as well as IFN-inducible promoter activity (Fig. 4A, center) relative to that seen in the absence of Sp1 co-transfection. Overexpression of Sp3, however, did not significantly affect promoter activity (Fig. 4A) compared with that seen with an empty vector control lacking an Sp cDNA insert (Fig. 4A, left). Sp1 and Sp3 expression in the transfected cells was confirmed by Western immunoblot analysis (Fig. 4B). For Sp3, only the large isoform (35 kDa) was detectable (Fig. 4B, lane 3; data not shown).

PKR Promoter Activity Is Sp Factor-dependent and Differentially Regulated by Sp1 and Sp3 in Drosophila Cells—Drosophila SL2 cells lack mammalian Sp-like homologues capable of activating Sp factor-responsive promoters (43, 44). These insect cells, completely null for both Sp1 and Sp3, provide a useful system examining the ability of the Sp1 and Sp3 factors to activate PKR promoter activity when expressed alone or together but more importantly in the absence of endogenous Sp factors.

The minimal PKR promoter p503(WT) construct, which includes the KCS and ISRE DNA elements and upstream 5′-flanking sequence (17), displayed very low basal activity in transfected SL2 Drosophila cells (Fig. 5, left) as compared with transfected human cells in the absence of IFN treatment (Fig. 4) (18, 19). However, co-expression of the Sp1 factor greatly activated the p503(WT) PKR promoter in SL2 cells. When increasing concentrations of pPacSp1 cDNA expression plasmid containing the mammalian Sp1 cDNA insert under the control of an actin promoter were co-transfected along with the PKR p503(WT) reporter construct, PKR promoter activity was increased greatly in an Sp1 dose-dependent manner that saturated around 1 μg of Sp1 expression plasmid (Fig. 5, center). Co-expression of the pPacSp3 cDNA expression plasmid encoding mammalian Sp3 also activated the p503(WT) PKR promoter in Drosophila cells in a dose-dependent manner (Fig. 5). However, Sp3 activated the PKR promoter much less efficiently than Sp1 in SL2 cells. Co-expression with 3 μg of pPacSp3 plasmid resulted in less activation of the PKR promoter (Fig. 5, right) than that seen with 0.5 μg of pPacSp1 plasmid (Fig. 5, center), even though the expression levels of the Sp1 and Sp3 proteins from the pPac actin promoter–driven plasmids were comparable (Ref. 45; data not shown).

Sp1 and Sp3 proteins can act synergistically or can antagonize one another, depending upon the nature of the cell and the context of the promoter–binding site (42, 46, 47). Because co-transfection of Sp3 did not increase PKR promoter activity in animal cells (Fig. 4A) and only modestly increased activity in insect cells (Fig. 5), we examined the effect of expression of Sp3 together with Sp1 in the SL2 insect cells that lack both proteins endogenously. As shown in Fig. 6, co-expression of Sp3 with Sp1 did not suppress the level of PKR promoter activation obtained with Sp1 alone. Rather, we observed that the Sp1-mediated
transactivation of the PKR promoter was increased synergistically by co-expression with Sp3 (Fig. 6).

Short Isoforms of Sp3 Do Not Affect PKR Promoter Activity—Both short and long isoforms of Sp3 protein co-purified with KCS DNA binding activity (Fig. 1A). Because Sp3i proteins lack one of the activation domains present in Sp3li (35, 48), we tested the ability of the short isoforms to transactivate or antagonize activation of PKR promoter-driven reporter gene transcription. Varying amounts of the isoforms were co-transfected into Drosophila SL2 cells with the p503(WT) reporter gene. As shown in Fig. 7, Sp3si did not transactivate the PKR promoter, and Sp3si did not repress Sp1-mediated transactivation. Similarly, Sp3si did not repress Sp3-mediated activation of the PKR promoter (data not shown). Finally, the SV40 promoter was not activated by Sp3si in Drosophila cells (data not shown), consistent with recent observations (35).

Sp1- and Sp3-mediated Activation of the PKR Promoter Is Independent of the ISRE Element but Dependent Upon the KCS Element—Two DNA elements were identified that play a role in determining basal and IFN-inducible activity of the PKR promoter, the 15-bp KCS element and the 13-bp ISRE (10, 16). The ISRE is important in conferring IFN-α/β responsiveness of transcription and is found in the promoter regions of most genes transcriptionally induced by type 1 IFN (1, 12), including PKR (10, 13). The KCS element is conserved exactly in sequence and position between the mouse and human PKR promoters, and so far is unique to them, and plays an important role in both basal and IFN-inducible transcription of PKR (10, 16). To determine the contribution of the KCS and ISREs to the Sp-mediated increase in basal PKR promoter activity, four mutants in the p503 minimal PKR promoter background that possess changes affecting the ISRE or KCS element, or both elements (Fig. 8A), were examined in co-transfection experiments using human U cells (Fig. 8B) and Drosophila SL2 cells (Fig. 9).

The ISREmt8T mutation greatly reduced IFN-inducible and basal transcription in transfected U cells (Fig. 8B), as reported previously because of impaired binding of the IFN-activated ISGF3 factor to the ISRE (18). The 5'-region of the KCS element includes a GC box, a binding site for Sp1 and Sp3. Two KCS point mutants, KCSmt6A and KCSmt9T, that possess changes in the GC box of KCS, displayed greatly reduced basal and IFN-inducible activities in transfected U cells (Fig. 8B) that correlated with impaired formation of the KBP protein complex as described previously (16, 17). Finally, both basal and IFN-inducible promoter activities were abolished in case of the p503 double mutant (KdelISREmt8T) in which the KCS element was deleted in the background of the ISREmt8T mutation (Fig. 8B).

The four p503 mutant constructs also were compared with p503(WT) in Drosophila SL2 cells, either alone or with increasing concentrations of pPacSp1 or pPacSp3 expression plasmid (Fig. 9). In the absence of either Sp1 (Fig. 9A) or Sp3 (Fig. 9B), all four mutants and the WT p503 construct showed very low promoter activity in SL2 cells. When Sp1 was co-expressed, the promoter activity of the KCSmt9T and ISREmt8T mutants increased in a comparable dose-dependent manner (Fig. 9B), as reported previously (16, 17). Finally, both basal and IFN-inducible promoter activities were abolished in case of the p503 double mutant (KdelISREmt8T) in which the KCS element was deleted in the background of the ISREmt8T mutation (Fig. 8B).

The KCS DNA element is necessary for optimal basal and interferon-inducible PKR promoter activity in human U cells. A, schematic representation of four mutant p503 PKR promoter constructs. For KCSmt6A and KCSmt9T, the indicated G6-to-A6 and G9-to-T9 substitutions are within the KCS motif (17). For ISREmt8T, the G8-to-T8 substitution is within the ISRE (10). For KdelISREmt8T, the KCS motif was completely deleted in the ISREmt8T background. B, promoter activities were determined in human U cells transfected with the indicated wild-type (WT) or mutant p503 reporter construct. Cells were either left untreated (−) or treated with 1000 units/ml IFN-α (+). Relative promoter activity is shown normalized to basal activity of the p503(WT) construct. Cells were co-transfected with the pGlu2-LUC control plasmid as an internal reference to control for transfection efficiency.
Regulation of the PKR Promoter

The transcription of the type I IFN-inducible genes such as PKR is best understood in the framework of the ISRE DNA element and the heteromeric transcription factor complex ISGF3 composed of STAT1, STAT2, and IRF9 (1, 12). Indeed, the PKR promoter possesses a consensus ISRE that binds ISGF3 (16) and that confers IFN inducibility (18). Although PKR is IFN-inducible, PKR gene expression is not restricted to IFN-treated cells or tissues. Unlike many IFN-inducible genes, significant basal expression of PKR is observed in cultured cells and mouse tissues in the absence of either IFN treatment or pathogen infection (1, 9, 49, 50). The pronounced basal expression seen for PKR no doubt reflects the broad physiologic roles played by PKR in a variety of fundamental cellular processes, including cell growth and differentiation and apoptosis, in addition to the well established role of PKR in the IFN-induced inhibition of virus growth (1). Little is known about the factors that mediate basal PKR expression. Our study was undertaken to gain

FIGURE 9. Sp1- and Sp3-mediated transactivation of the PKR promoter in Drosophila SL2 cells is dependent upon the KCS element but independent of the ISRE. Varying amounts of pPacSp1 (A) or pPacSp3 (B) were cotransfected with the indicated p503(WT) or mutant promoter construct depicted in the Fig. 7 schematic. CAT activity was measured as for Fig. 5, and the results shown are the means ± S.D. determined from three independent experiments.

(A) 

FIGURE 10. Sequence immediately 5’ of the KCS element affects Sp1- and Sp3-mediated transactivation of the PKR promoter in Drosophila SL2. A, schematic representation of the three CAT reporter plasmid constructs, p503(WT) and two derived 5’-deletions, p130(WT) and p63(WT) (18). The filled boxes 1–6 indicate the positions of the six Sp sites within the p503 minimal promoter. Varying amounts of pPacSp1 (B) or pPacSp3 (C) were co-transfected with the indicated promoter construct, p503, p130, or p63, depicted in the schematic shown in A. CAT activity was measured as for Fig. 5, and the results shown are the means ± S.D. determined from three independent experiments.

Responsiveness of the four mutant p503 constructs to co-expressed Sp3 (Fig. 9B) was similar to that seen for co-expressed Sp1 (Fig. 9A), with one exception; the activity of the KCSmt9T was reduced relative to p503(WT). Taken together, these results indicate that both KCSmt9T and ISREmt8T retain an intact Sp1-binding site and that the Sp3-binding site within KCS is positioned more 3’ toward the ISRE. In addition, because none of the mutations in the KCS or ISRE completely destroyed responsiveness to Sp factor co-expression, there may be additional GC boxes involved in Sp activation of the PKR promoter.

PKR Promoter Sequence Immediately Flanking the KCS Element Affects Sp1- and Sp3-mediated Activation—The p503 minimal PKR promoter sequence includes additional candidate Sp factor binding GC box sites within the 5’-flanking region of p503, upstream of the Sp box in the KCS element. To examine the potential involvement of upstream GC box sites in the Sp factor-mediated activation of the PKR promoter seen in Drosophila cells, and to determine whether or not the KCS and ISREs were sufficient to support PKR promoter activity mediated by Sp factors, the activities of two deletion constructs (p130, p63) were compared with that of the p503 construct (Fig. 10). The p503, p130, and p63 constructs all possess the wild-type 15-bp KCS and 13-bp ISREs (Fig. 10A). The three constructs displayed comparable activity to each other in transfected mammalian cells, both basal activity and IFN-induced activity (Ref. 18; data not shown). In Drosophila SL2 cells, none of the constructs showed significant activity in the absence of Sp1 (Fig. 10B). When Sp1 was co-expressed, comparable strong promoter activity was seen with the p503 and p130 constructs in SL2 cells. However, with the p63 deletion construct, activity was significantly reduced at all concentrations of the co-transfected Sp1 expression plasmid (Fig. 10B). These results indicate that the one additional Sp-binding site present in p130 immediately upstream from the site in KCS is important for Sp1-mediated PKR promoter activation but that the four Sp-binding sites present at the 5’ region of p503 (and absent in p130 and p63) are dispensable. Again, although Sp3-mediated promoter activation (Fig. 10B) was much less than Sp1-mediated activation, unlike Sp1 activation, the p130 construct possessed lower responsiveness to Sp3 than did the p503 construct (Fig. 10C).
understanding of the transcriptional activation of the PKR promoter in the absence of IFN treatment. Several important points emerge from our findings.

There is only one known PKR promoter, and in mouse and in human cells it is a TATA-less promoter (10, 13). Although the 13-bp ISRE DNA element is required for IFN-inducible but not basal transcription (18), the 15-bp KCS DNA element conserved exactly in sequence and position relative to the ISRE is required for both basal and IFN-inducible PKR promoter activity (10, 16–18). The basal expression of PKR observed in MEF cells as shown herein was not dependent upon either the STAT1 factor or the Jak1 kinase, two proteins normally involved in signal transduction in response to type I and type II IFNs (1, 51). These observations argue that the expression of PKR seen in the absence of exogenous treatment with IFN is not simply the result of endogenous autocrine IFN signaling. What then is the mechanistic explanation for the basal PKR expression observed in cells not treated exogenously with IFN?

One critically important element that profoundly affects both basal and IFN-inducible PKR promoter activity is the 15-bp KCS element (10, 16, 17). Transcriptional activation in reporter assays with the PKR promoter correlates with protein binding at the KCS element (17). The protein-DNA complex formed at the KCS element, the KBP (KCS-binding protein), is independent of IFN treatment (16, 18). Furthermore, electrophoretic mobility shift and antibody supershift analyses with the KCS DNA probe, and pull-down assays utilizing oligomerized KCS DNA linked to Sepharose beads, indicated that two Sp factors (Sp1 and Sp3) were components of the KBP complex (18, 19). Although GC-rich elements that are conserved within the KCS element bind predominantly if not exclusively Sp1 and Sp3. In extracts from wild-type MEF cells, KBP complex formation was essentially abolished by a mixture of antibodies against Sp1 and Sp3. Antibody cross-reactivity was not responsible for the observed inhibition of complex formation. The Sp1- and the Sp3-containing complexes were partially resolved from each other on the EMSA gels, and when the antibodies were tested individually, only the Sp1- or the Sp3-containing complex was eliminated by the cognate antibody. Furthermore, with extracts prepared from Sp3 null cells, antibody against Sp1 was sufficient to eliminate all KBP complex formation.

Purification of the KBP complex utilizing anionic and cationic ion exchange and wild-type and mutant DNA affinity chromatographic steps earlier identified Sp1 as a component of the KBP complex (20). Further analysis of the fractions obtained from the KBP complex purification scheme described for human HeLa cells (20) revealed that in addition to Sp1 the four known isoforms of the Sp3 transcription factor also co-purified with KBP binding activity. The results from the biochemical purification identifying Sp3 as a component of the KBP complex are consistent with our earlier EMSA and pull-down experimental findings (19, 20). Although the ubiquitous expression patterns, the domain structures, and the DNA binding properties of the Sp1 and Sp3 factors are similar, their transcriptional properties and physiologic roles can differ significantly (42, 46). How then is basal transcription from PKR promoter affected by these constitutively expressed components of KBP complex?

One approach that we undertook was to assess the basal activity of the PKR promoter in reporter assays following co-transfection with Sp1 and Sp3 transcription factor expression vectors, individually and in combination with each other. Human U cells, like most cultured cell lines, possess significant endogenous levels of Sp1 and Sp3. Nevertheless, cotransfection with Sp1 further increased both basal and IFN-inducible minimal promoter activity, whereas co-transfection with Sp3 did not. But when Drosophila SL2 cells that are naturally deficient in Sp factors and do not express either Sp1 or Sp3 (43) were examined, both Sp1 and the large form of Sp3 activated PKR promoter activity in a dose-dependent manner. Sp1 was significantly more potent than Sp3i, which mediated much less activation of the PKR promoter. However, the short form of Sp3 alone was completely inactive; Sp3si neither activated the PKR promoter when tested alone nor suppressed the Sp1-mediated activation when tested in combination with Sp1. Prior studies with other promoters established that Sp1 acts as a transcriptional activator, whereas Sp3 can act either as a transcriptional activator or as a repressor (42). Our findings from transfection studies with SL2 cells and Western immunoblot analyses with Sp3 null MEFs establish that Sp1 clearly is capable of activating PKR promoter activity, and that IFN-inducible PKR expression can occur in the absence of Sp3. Furthermore, our data suggest that the relative abundance of different Sp family factors is an important determinant in modulating the level of PKR transcription in the absence of IFN, with the activity of Sp1 > Sp3i >> Sp3si, where Sp3si is incapable of either activation or repression of PKR at least in SL2 cells. Conceivably, the modest activity or no activity observed for the Sp3 isoforms on the PKR promoter reflects the need for an additional Sp-interacting protein that is absent in SL2 cells or a post-translational modification such as acetylation or sumoylation that does not happen in SL2 cells (35).

The two Sp3si factors are derived from two adjacent internal translation initiation sites (35). They are N-terminally truncated and lack the N-terminal transactivation A domain present in the two larger forms of Sp3li (22, 35). Sp3si factors can act as competitive inhibitors of Sp1-mediated transcription (48). However, we did not observe that Sp3si overexpression in SL2 cells inhibited Sp1-mediated activation of the PKR promoter. Similarly, the SV40 promoter was not inhibited by Sp3si in SL2 cells in our hands.4 By contrast, long isoforms of Sp3 having two glutamine-rich activation domains do activate the SV40 promoter (which has six GC boxes), whereas the short isoforms do not (35), similar to what we observed for the PKR promoter. For some promoters that possess multiple adjacent GC sites, Sp3li also has been reported to compete with Sp1 activation and function as a negative regulator (41, 48, 52), but this was not found for the PKR promoter.

Comparison of different wild-type and mutant MEF cell lines revealed that in the absence of IFN treatment, the steady-state level of PKR in the Sp3 null cells was reduced relative to that seen in different WT MEF lines and also in Stat1- and Jak1-null MEF lines. Yet the level of PKR in Sp3 null cells following IFN treatment was comparable with that of IFN-treated wild-type cells. One possible interpretation from these observations is that Sp3 plays a role in basal transcription. But chromatin immunoprecipitation assays revealed that the binding of Sp3 at the PKR promoter region was IFN-dependent, whereas the binding of Sp1 was independent of IFN treatment (19, 20). How may the apparent discordance be explained? One possibility is that the increased amount of Sp1 can compensate for the absence of Sp3 in the null cells under conditions of IFN treatment. Additional possibilities are that the chromatin structure of the PKR promoter region is altered following IFN treatment to become more accessible to Sp3 binding directly or that the presence of the ISGF-3 complex in IFN-treated cells facilitates recruitment of Sp3 to the PKR promoter.

We found that PKR promoter activity increased about 2-fold in the presence of Sp1 expression in transfected human cells. Up-regulation of IFN-inducible promoter activity under similar conditions may be due in

4 S. Das, S. V. Ward, R. S. Tacke, G. Suske, and C. E. Samuel, unpublished data.

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part to increased recruitment of the ISGF3 complex components to the PKR promoter region by the Sp1 protein. Consistent with this possibility are the observations that STAT1 has been reported to interact with Sp1 (53), that both STAT1 and STAT2 are capable of interacting with protein components of the KBS complex (19), and that the KBS element functions in concert with the ISRE as revealed by protein binding that requires both the KBS and ISRE sequences (16). Although STAT1/2 interactions involving the ISRE could be directly with Sp1 (or other known KBS complex protein constituents at the KCS element), we cannot exclude the possibility of indirect interactions through yet as unidentified components (Fig. 11).

The minimal promoter region of the PKR gene contains six potential Sp-binding sites, with four of them positioned as overlapping Sp sites. The importance of the multiple Sp-binding sites found in the minimal PKR promoter was assessed using deletion and substitution mutant constructs. It is evident from reporter assays that the two most downstream Sp sites (Sp sites 1 and 2 shown in Fig. 10 schematic), one within the KCS element and the other positioned just upstream of KCS, play critical roles in Sp1-mediated PKR promoter activation. By using the p130(WT) promoter construct, which lacked the upstream four overlapping Sp sites, we observed similar Sp1-mediated promoter activity in SL2 cells as that seen for p503(WT). Sp3-mediated promoter activity displayed by p130(WT) was about half that of p503(WT), indicating that unlike Sp1, the Sp site immediately upstream of KCS (Sp site 2 in Fig. 10) was insufficient for complete Sp3 activation. The mutant KBSmut6A promoter construct showed that disruption of the Sp site within the KCS region caused a significant reduction in Sp factor-mediated promoter activity. A mutant with a nonfunctional ISRE, however, showed similar activity as the p503(WT) for both Sp1 and Sp3, further establishing that the binding of Sp1 and Sp3 to the PKR promoter and basal transcriptional activation by these factors are independent of the ISRE. Sp1 has been shown to function as a gene activator by recruiting TFII D (54), and the TBP-associated factor 1 (TAF1) activates the TATA-less cyclin D1 promoter by interacting with the Sp1 protein (55). The PKR promoter likewise is TATA-less (10, 13).

A major limitation in understanding how cells produce PKR in the absence of IFN has been the inability to identify the factors involved and the inability to demonstrate activation of transcription in the absence of IFN signaling. This has now been accomplished for the PKR promoter (Fig. 11). We have demonstrated that both Sp1 and Sp3 function as activators of the PKR promoter through utilization of a combination of Sp1/Sp3-binding sites. Sp1 was a much more potent activator than Sp3. Our study establishes a new role for the Sp family of transcription factors in the basal expression of PKR, which is otherwise an IFN-inducible gene. Regulation of the PKR gene, a gene that plays a major role in regulating cell growth and apoptosis, by Sp1 and Sp3 emphasizes the importance of these Sp transcription factors broadly in biology.

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