**DNA Damage-binding Proteins and Heterogeneous Nuclear Ribonucleoprotein A1 Function as Constitutive KCS Element Components of the Interferon-inducible RNA-dependent Protein Kinase Promoter**

Received for publication, November 18, 2003, and in revised form, November 25, 2003
Published, JBC Papers in Press, November 28, 2003, DOI 10.1074/jbc.M312585200

Sonali Das, Simone Visosky Ward, Danielle Markle, and Charles E. Samuel†‡

From the Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, California 93106

Protein kinase regulated by RNA (PKR) plays important roles in many cellular processes including virus multiplication and cell growth, differentiation, and apoptosis. The promoter of the PKR gene possesses a novel 15-bp element designated KCS, positioned upstream of a consensus interferon (IFN)-stimulated response element, that is required for both basal and interferon-inducible transcription. Protein binding to the KCS element is not dependent upon IFN treatment and correlates with transcriptional activity of the PKR promoter. The identity of KCS-binding proteins (KBP) that selectively bind at the KCS element is largely unknown, except for the transcription factor Sp1. We now have purified KBP from HeLa cell nuclear extracts by ion-exchange and DNA-affinity chromatography steps and then identified four constituent proteins of the KBP complex by mass spectrometry and immunochemistry: KBP120 and KBP45 are the damaged DNA-binding protein subunits, p127 DDB1 and p48 DDB2, respectively; KBP100 is the transcription factor Sp1; and KBP35 is the heterogeneous nuclear ribonucleoprotein A1. The steady-state levels of these four KCS-binding proteins in human cells are not altered by IFN treatment. Components of the KBP complex bind selectively and constitutively to the KCS element in the absence of IFN treatment, both in vitro as measured by competition electrophoretic mobility shift assay (EMSA) and DNA pull-down assays and in vivo as measured by chromatin immunoprecipitation assays. Depletion of DDB2 by antisense expression reduces KBP complex formation by EMSA. These results provide new insight into the biochemical identity and activity of proteins involved in PKR promoter function.

PKR is the interferon (IFN)-inducible protein kinase whose catalytic activity is modulated by RNA-mediated autophosphorylation (1). PKR plays a central role in the control of protein synthesis in virus-infected and IFN-treated cells. Translation is down-regulated by PKR-catalyzed phosphorylation of protein synthesis initiation factor-2α on serine 51 (2–4). In addition to the role of PKR in translational control, the kinase is also involved in the modulation of cytokine signal transduction via the NF-κB and STAT transcription factors (5, 6). Because of these varied and fundamental biochemical activities, the PKR protein affects a range of physiologic processes. These include, together with the well established role of PKR in the antiviral actions of IFNs and innate immune responses (1), roles in the control of cell growth, differentiation, and apoptosis (7, 8).

Although PKR is inducible by IFN treatment and virus infection, basal expression of PKR is readily demonstrable in many cell lines and most animal tissues even in the absence of IFN treatment or virus infection (9–11). Human PKR is encoded by a single copy gene that includes 17 exons spanning 50 kb located on human chromosome 2p (11, 12). The increased expression of the PKR gene in IFN-treated cells above the basal level of expression has been firmly established by Northern blot and nuclear run-on analyses with PKR cDNA probes (13, 14), by transient transfection promoter analyses using reporter plasmid constructions (15–17), and by microarray gene profiling analyses (18–20).

Expression of the PKR gene is driven by a TATA-less promoter that directs both basal and IFN-inducible transcription (11, 15). Two DNA elements have been identified that are required for optimal promoter activity. One is a consensus 13-bp copy of the IFN-stimulated response element (ISRE) responsible for the inducibility of many different genes, including PKR, by type I IFNs (21). The trimeric transcription factor ISGF3 (STAT1, STAT2, and IRF-9) binds to the ISRE of the PKR promoter in an inducible manner (17). The second element is a novel 15-bp element, positioned upstream of the ISRE, that is required for both basal and interferon-inducible transcription. This element, designated KCS for kinase conserved sequence, is exactly conserved between the human and mouse PKR promoters in sequence and position relative to the ISRE (11). In contrast to the inducible binding of the ISGF3 factor to ISRE (17, 21), the KCS element mediates constitutive protein binding (11).

A major complex, designated KBP for KCS-binding protein complex, is formed at the KCS element with nuclear extracts from untreated as well as from IFN-treated cells. Substitution mutational studies have demonstrated that both the 5′- and the 3′-halves of the 15-bp KCS element are capable of mediating nuclear protein binding. Both basal and IFN-inducible promoter activity are impaired by single substitution mutations within either the 5′- or 3′-halves of the KCS element, muta-
tions that also reduce KBP complex formation (16, 22). Multimerization of the KCS element increases both basal and inducible transcription, whereas insertions between the two elements significantly reduce both basal and inducible promoter activity, suggesting that the KCS and ISRE elements of the PKR promoter may represent a functional unit (17). The 5′-half of the KCS element includes an Sp1-binding site, and antibody supershift studies identified the Sp1 transcriptional activator as a component of the KBP complex (22b). However, the identity of other proteins bound to the KCS elements and their possible roles in constitutive and inducible PKR expression largely remain unknown. The absence of a TATA box in the PKR promoter, together with a requirement of the KCS element for basal PKR promoter activity, raises the possibility that trans-acting KCS-binding proteins mediate, in part, basal transcription of the PKR gene.

In this report we describe the purification of the KBP complex from untreated HeLa cells and the identification of four constituent polypeptides of the heteromeric KBP transcriptional activator complex. Mass spectrometry and immunochimical analyses established the identity of three new KCS-binding protein complex components, DDB1, DDB2, and hnRNP A1, and confirmed the previously identified Sp1 protein. DDB1 and DDB2 are the large p127 and small p48 subunits, respectively, of a heterodimeric complex that has multiple roles, including UV-damaged DNA binding activity, and that also functions as a transcriptional partner (23–26). Likewise, the hnRNP A1 protein, a member of the heterogeneous ribonucleoprotein family (27), is implicated to play multiple roles in mammalian cells other than a component of spliceosomes. We provide functional evidence from experiments including DNA binding pull-down assays in vitro and chromatin immunoprecipitation assays in vivo, as well as EMSA analyses following DDB2 knock-down by antisense strategy, all of which indicate that the DDB1, DDB2, Sp1, and hnRNP A1 proteins are constitutively and selectively bound at the KCS element of the IFN-inducible PKR promoter as components of the KBP protein complex.

EXPERIMENTAL PROCEDURES

Cell Maintenance, Interferon Treatment, and Preparation of Nuclear Extracts—Human HeLa S3 cells were grown in suspension culture as described (28). DBB2-sufficient R3 and DBB2-deficient R18 HeLa cells (29), human U cells (11), and human fibroblast GM2767 cells (30) were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (Hyclone), penicillin, and streptomycin. Cultures were either left untreated or treated with interferon using 1000 IU per ml of IFN-α following priming with IFN-γ. Nuclear extracts were prepared as described (17) unless otherwise noted.

Electrophoretic Mobility Shift Assay—Protein binding activity to the KCS DNA element was measured by electrophoretic mobility shift assay (EMSA) as described previously (16, 22). Wild-type double-stranded KCS oligodeoxynucleotide (5′-CTCCAGGAGGAGCCAGGGTC-AAGG-3′) was used as the 32P-labeled probe (K-probe). Selective binding was verified by competition EMSA, using either the wild-type KCS oligo, the substitution mutant KCS(mt6A), or the mutant KCS(mtmtT) as the unlabeled double-stranded DNA competitor at 100-fold excess. The conditions for the protein-DNA binding reaction and electrophoresis using 5% native gels at 4 °C were as described (17, 22), except that fractions from the purification steps were used in place of the nuclear extract as starting material where indicated.

Preparation of KCS Sequence-specific DNA-affinity Columns—Wild-type and mutant KCS oligonucleotides biotinylated on the 5′-ends of their + strands were obtained from Sigma-Genoys. The + sequences of the wild-type and mutant KCS oligonucleotides used as double-stranded multimerized DNAs to prepare the KCS DNA-affinity columns were 5′-AGGCTCGAGAGGGAGGAGCGTCCT-GAGGAGGAGGATC-GAA-3′ for the wild-type KCS column, and 5′-AAGGCTCGAGGAGGATCCTGAGAGGGAGGAGGAGGATC-TCTAAGG-3′ for the mutant KCS column, with the three G to T substitution mutations indicated by underlines. The biotinylated + strand oligo was annealed with complementary − strand oligo, and then the biotinylated double-stranded oligonucleotides were attached to streptavidin-Sepharose beads according to the manufacturer's protocol (Amersham Biosciences). The mutated KCS sequence with T substitutions at nucleotides 6, 7, and 9 of the 15-bp KCS element used to prepare the mutant KCS DNA-Sepharose beads was verified to display mutant behavior in a competition EMSA. Unlabeled mutant DNA did not compete with nuclear extract proteins binding to a wild-type 32P-labeled K-probe, whereas the wild-type DNA did compete, consistent with prior observations (16).

Purification of DNA-Binding Proteins—Nuclei were prepared from S3 HeLa cells grown in suspension culture (28). Isolated nuclei were washed four times with 1× KHEM buffer (20 mM HEPES, pH 7.8, 1 mM EDTA, 2 mM MgCl2), and then nuclear proteins were extracted with high salt buffer (1× KHEM containing 250 mM sucrose and 400 mM KCl) by incubation on ice and vigorously vortexing for 10 s every 2 min over a period of 30 min. Following centrifugation at 30,000 × g for 3 h, the supernatant solution (40 ml, −3–5 mg of protein per ml) was used as the crude nuclear extract for purification of the KCS-binding protein complex.

Typically ~150 mg of crude nuclear extract protein was loaded at 4 °C onto a 50-ml DEAE-cellulose (Whatman) column equilibrated in 1× KHEM buffer containing 0.2 M KCl. KCS binding activity did not absorb to the DEAE matrix but rather was recovered in the DEAE flow-through fraction. Protein fractions were slowly diluted with an equal volume of 1× HEM buffer (pH 7.8, 1 mM EDTA, 2 mM MgCl2) to reduce the final KCl concentration to 0.1 M. The DEAE flow-through was then applied to a 20-ml carboxymethyl (CM)-Sepharose (Amersham Biosciences) column equilibrated with 1× KHEM buffer. The CM fractions with adsorbed KCS-binding proteins was washed with 2 column volumes of 1× KHEM buffer containing 0.2 M KCl before stepwise elution of bound proteins with KHEM buffer containing 0.3, 0.35, 0.4, or 0.45 M KCl. CM fractions with peak KCS gel shift activity (the 0.35 and 0.4 M salt eluates) were pooled and diluted to a final KCl concentration of 0.1 M KCl prior to DNA-affinity chromatography.

Sequential DNA-affinity chromatography was carried out using Sepharose beads with attached wild-type KCS DNA (1st WT), then with attached mutant KCS DNA, and finally with wild-type KCS DNA (2nd WT). Peak active fractions from the CM column were loaded onto the 1st WT DNA-affinity column, a 1-ml column of Sepharose-DNA equilibrated with 1× KHEM containing 0.1 mM KCl and 10% glycerol. The 1st WT KCS-affinity column was washed with 2 ml of 1× KHEM containing 0.2 M KCl and 10% glycerol and then with 1 ml of KHEM containing 0.3 M KCl and 10% glycerol, and finally the KCS binding activity was eluted with 3 ml of 1× KHEM containing 0.6 M KCl and 10% glycerol. 0.5-ml fractions were collected, and the peak KCS binding activity usually was found in fractions 2 and 3. The pooled peak fractions from the 1st WT KCS-affinity column were diluted to 0.45 M KCl concentration and then loaded onto a 0.4-ml mutant KCS-affinity column equilibrated with 1× KHEM containing 0.45 M KCl and 10% glycerol. The column then was washed with 1× KHEM buffer containing 0.45 M KCl. The KCS binding activity was found in the flow-through and first 0.45 M wash. The mutant column flow-through and 0.45 M wash fraction were pooled, diluted to 0.1 M KCl concentration, and then loaded onto the 2nd WT KCS-affinity column equilibrated with 1× KHEM buffer containing 10% glycerol. The adsorbed proteins were eluted following the same procedure as for the 1st WT KCS column, except that the 0.6 M KCl eluates were collected in fractions of 0.2 ml. Ten fractions were collected.

Fractions from ion-exchange chromatography and DNA-Sepharose affinity chromatography steps of the purification scheme were monitored for selective KCS DNA binding activity by competition EMSAs. SDS-PAGE using 5–15% gradient acrylamide gels was used to analyze the fractions for their protein composition, with protein detection by silver staining of the gel (BioRad).

Mass Spectrometry and Sequence Analysis—The most purified protein fractions containing KCS binding activity obtained after the 2nd WT KCS DNA-affinity column step were fractionated by SDS-PAGE. Five of the protein bands that were consistently enriched during purification in a manner that correlated with KCS binding activity were excised from unified gels for analysis by mass spectrometry. Trypsin digestion and peptide sequence analysis of three proteolytic fragments with a Finnigan LCQ DECA ion trap mass spectrometer.
KCS-binding Proteins of PKR Promoter

RESULTS

Purification of KCS-binding Proteins—The multistep scheme used to enrich for proteins binding the KCS DNA element is shown in Fig. 1A. KCS DNA binding activity was monitored through protein fractionation steps by EMSA using a 32P-labeled wild-type KCS oligonucleotide probe. Nuclear proteins bound the 15-bp KCS element selectively as established by competition EMSA. KCS binding activity measured by EMSA correlated with PKR promoter activity measured by transient transfection reporter assays (see Refs. 16 and 22 and data not shown). Because KCS binding activity is not inducible by IFN treatment (17, 22), purification of KCS-binding proteins was carried out by using nuclear extracts prepared from HeLa cells that had not been treated with IFN. Approximately 150 mg of nuclear protein obtained from ~70 liters of suspension cultured HeLa cells was used as the starting material.

KCS DNA binding activity present in nuclear extracts was enriched by sequential ion-exchange column chromatography, first utilizing DEAE-cellulose under conditions in which the KCS binding activity was not retained on the DEAE matrix (0.2 mM KCl). The DEAE flow-through was then supplemented to a column of CM-Sepharose that bound and concentrated KCS binding activity. KCS binding activity was eluted from the CM matrix using a stepwise salt gradient, with maximum activity found in eluent fractions between ~0.35 and 0.40 mM KCl (Fig. 1B). KCS binding activity recovered from the CM-Sepharose step was further fractionated by sequence-specific DNA-Sepharose affinity chromatography. Significant enrichment of KCS binding activity was achieved utilizing sequential DNA-Sepharose affinity steps beginning with wild-type, then mutant, and again wild-type KCS DNA-affinity columns. KCS DNA binding activity bound by the 1st wild-type KCS oligonucleotide affinity column was eluted with 0.6 M KCl following extensive washing with buffer containing 0.3 M KCl. The KCS binding activity recovered from the 1st wild-type affinity column was not bound by a subsequent mutant KCS oligonucleotide affinity column, but the flow-through eluate from the mutant column was bound by a 2nd wild-type KCS oligonucleotide column (Fig. 1, A and C). The enrichment in KCS DNA binding activity obtained by the purification scheme was ~200-fold from the nuclear extract fraction used as starting material.

Identification of KCS DNA-binding Proteins—The relative complexity of the active fractions at each stage of the purification scheme was assessed by SDS-PAGE using 5–15% polyacrylamide gradient gels (Fig. 2). Protein detection was done by staining with silver. The multiplicity of proteins detected in the active fractions after the 1st wild-type DNA-affinity column (Fig. 2, lane 5) was greatly reduced compared with the starting material (Fig. 2, lane 2). Five proteins most significantly enriched after the DNA-affinity chromatography steps and found consistently in the fractions with the greatest KCS binding activity possessed apparent molecular masses of ~120, ~100, ~65, ~45, and ~35 kDa (Fig. 2, lane 7). The abundance of these KBP1s increased during the purification in a manner that correlated with the increase in DNA binding activity selective for the KCS element. These proteins were designated KBP120, KBP100, KBP65, KBP45, and KBP35, respectively (Fig. 2). One of these, the KBP100 protein, was identified by Western immunoblot analysis as transcription factor Sp1 (see Fig. 4), a protein previously established as a KCS DNA-binding protein by EMSA antibody supershift analyses (22).

By using a proteomic strategy to identify additional KCS DNA-binding proteins, the most purified fractions following the DNA-affinity step were further fractionated by SDS-PAGE, and then the enriched protein bands in the most purified fractions, detected with Coomassie Blue stain, were excised from the gels...
Microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry of tryptic digests was then performed. The sequence information obtained permitted identification of three more KBP proteins. KBP120 was identified as damaged DNA-binding protein 1 (DDB1) p127 subunit (Fig. 3A), KBP45 as damaged DNA-binding protein 2 (DDB2) p48 subunit (Fig. 3B), and KBP35 as the hnRNP A1 (Fig. 3C). For these three identity assignments, the alignment of the experimentally determined tryptic peptide sequences (underlined) exactly matched those of the data base deduced sequences for the DDB1, DDB2, and hnRNP A1 proteins (Fig. 3, A–C). All tryptic peptide sequences obtained for the 120-kDa protein designated as KBP120 (Fig. 2) showed 100% sequence identity with those predicted from the cDNA deduced sequence of the p127 DDB1 protein (SwissProt accession number P33194) as illustrated by the alignment shown in Fig. 3A. Likewise, all tryptic peptide sequences obtained for the ~45-kDa KBP45 and ~35-kDa KBP35 components showed complete identity with the peptide sequences deduced for the p48 DDB2 protein (PIR accession number I38909) and the hnRNP A1 protein (SwissProt accession number P09651) as shown by the Fig. 3, B and C alignments, respectively. In addition to Sp1 identified by immunochemistry, these results suggest that DDB1, DDB2, and hnRNP A1 identified by tandem mass spectrometry are also KCS-DNA-binding proteins.

To further assess that DDB1, DDB2, A1, and Sp1 indeed display increased abundance through the protein fractionation scheme employed to purify KCS DNA binding activity, Western immunoblot analyses were carried out by using nuclear extract starting material and the active fractions obtained following DEAE-cellulose, CM-Sepharose, and wild-type DNA-Sepharose affinity chromatography (Fig. 4). All four proteins were significantly increased in their relative abundance during purification compared with that seen in nuclear extract starting material; they were especially enriched following DNA-affinity chromatography (Fig. 4, lane 4).

**Interferon Treatment Is Not Required for DDB1, DDB2, A1, and Sp1 Binding to the KCS Element in Vitro**—A pull-down DNA binding assay employing a KCS element oligomer containing three copies of KCS as the platform was used to test for the recruitment of DDB1, DDB2, A1, and Sp1 to the KCS DNA element in crude nuclear extracts. This assay strategy also was used to examine the effect of IFN treatment prior to extract preparation on the binding of these proteins to KCS DNA (Fig. 5).
man GM2767 cells were incubated with KCS element DNA linked to Sepharose beads, followed by immunoblot analysis of bound material to detect the presence of bound protein factors. DDB1, DDB2, A1, and Sp1 all bound to Sepharose beads that possessed the coupled KCS element DNA (Fig. 5, lanes 3 and 6). The levels of the DDB1, DDB2, A1, and Sp1 proteins present in nuclear extracts prepared from untreated and IFN-treated cells were comparable (Fig. 5, lanes 1 and 4). Most importantly, the amounts of the four proteins bound to the KCS DNA element as seen by the pull-down assays were relatively comparable with extracts prepared from untreated cells (Fig. 5, lane 3) and IFN-treated cells (Fig. 5, lane 6). No consistent increase or decrease in binding of DDB1, DDB2, A1, or Sp1 to Sepharose bead-linked KCS element DNA was seen between multiple independent experiments. By contrast, no significant protein binding to Sepharose beads was seen without linked KCS element DNA, irrespective of IFN treatment (Fig. 5, lanes 2 and 5). Finally, as a control, IRF-9 protein levels as well as IRF-9 binding to KCS elements were examined. IRF-9 was significantly elevated by IFN treatment as anticipated, but the IRF-9 protein was not detectably bound to KCS DNA in the pull-down assay with either extracts from untreated or from IFN-treated cells (Fig. 5).

**Protein Binding to the PKR Promoter in Vivo**—Our results obtained in vitro both from protein purification and mass spectrometry identification analyses (Figs. 3 and 4) and from DNA binding pull-down assays (Fig. 5) revealed that the DDB1, DDB2, A1, and Sp1 proteins all bound the KCS element in vitro. This finding prompted us to examine the binding of these proteins to the PKR promoter in vivo under physiologically relevant conditions. For this purpose, chromatin immunoprecipitation (ChIP) analysis was carried out (Fig. 6). The results obtained for DDB2, A1, and Sp1 were compared with those obtained for STAT1, a protein well established to bind at promoters of IFN-regulated genes. The location of primer pairs used to amplify the PKR promoter region in the ChIP assay is shown by the schematic diagram (Fig. 6A). STAT1 was observed by ChIP analysis to be associated with the PKR promoter in vivo in an IFN-dependent fashion (Fig. 6B, lanes 4 and 5). By contrast, binding of the Sp1 transcription factor was not modulated by IFN treatment (Fig. 6B). The association of the DDB2 and A1 proteins with the PKR promoter region chromatin in vivo was seen in an IFN-independent manner similar to that observed for the Sp1 transcription factor (Fig. 6B, lanes 4 and 5). These results strongly suggest that stable association of the DDB2, A1, and Sp1 proteins with the PKR promoter in vivo, like that seen for their binding to KCS element DNA in vitro (Fig. 5), occurs in the absence of IFN treatment. DDB1 binding in vivo was not analyzed by ChIP, because although the available antibody against DDB1 performed well in Western immunoblot assays (Fig. 5), it was not suitable for immunoprecipitation studies.

**DDB2 Is Required for Stable KBP Complex Formation**—Results from purification studies (Fig. 4), DNA binding assays in vitro (Fig. 5), and ChIP analysis of proteins bound in vivo to the PKR promoter (Fig. 6) all indicate that the DDB2 protein is a component of the KBP protein complex. To assess further the contribution of the DDB2 protein in the formation of a stable KBP complex detected by gel shift analysis, nuclear extracts prepared from two HeLa cell lines R3 and R18 that express greatly different DDB2 protein levels were compared both by Western immunoblot assay (Fig. 7A) and EMSA (Fig. 7B). R3 is a UV-resistant HeLa cell line characterized by a high steady-state level of DDB2 protein; R18 is a stable transformant cell line derived from R3 that has a very low steady-state level of DDB2 protein achieved by a DDB2 antisense cDNA expression strategy (29). This difference in the DDB2 protein level between R3 and R18 cells is illustrated by the Western blot shown in Fig. 7A. The level of DDB2 protein was drastically reduced in extracts prepared from R18 cells compared with R3 cells (Fig. 7A, *top panel*), as reported by Sun et al. (29). By contrast, R3 and R18 cells possessed comparable levels of A1 (Fig. 7A, *middle panel*), and Sp1 (Fig. 7A, *bottom panel*) proteins as well as DDB1 (data not shown; see Ref. 29).

Nuclear extracts prepared from the DDB2-sufficient R3 cells showed substantial KBP binding activity as measured by EMSA analysis using a 32P-labeled KCS probe (Fig. 7B, *lane 1*). By contrast, KBP complex formation was reduced with nuclear extracts prepared from the DDB2-deficient R18 HeLa cells (Fig. 7B, *lane 3*). The observed binding to the K-probe was selective as measured by competition EMSA; unlabelled KCS DNA did not (data not shown). The residual KBP complex formation seen with DDB2-deficient R18 extracts was abolished by addition of anti-Sp1 antibody. Furthermore, anti-Sp1 antibody significantly impaired KBP complex formation with nuclear extract prepared from either R3 or R18 cells. These results are consistent with those of purification, pull-down, and ChIP experiments (Figs. 3B and 4–6) that both DDB2 and Sp1 are components of the KBP protein complex, and that DDB2 contributes to stable KBP complex formation detectable by EMSA analysis (Fig. 7).

**DISCUSSION**

Type I interferon-mediated transcriptional activation of cellular gene expression is best understood in the context of the ISRE DNA element and the heteromeric transcription factor ISGF3 assembled from STAT1, STAT2, and IRF-9 (1, 21). The
promoter that drives expression of PKR not unexpectedly possesses a consensus ISRE that confers IFN inducibility (22) and binds ISGF3 (17). However, in addition to the 13-bp ISRE, a second element designated as KCS is required for both basal and IFN-inducible transcription of the PKR gene (16, 17). The KCS element DNA binds proteins that are constitutively present and have been found to be members of the DDB1 (p127 subunit), DDB2 (p48 subunit), and hnRNP A1 protein families. These observations suggest a complex mechanism for activation of the PKR promoter upon IFN treatment.

The KCS element DNA-binding proteins were identified using mass spectrometry and tryptic peptide sequence analysis. The sequence alignment of multiple individual tryptic peptides (underlined) for KBP120 correspond exactly with the deduced sequence for human damaged DNA-binding protein 1 (DDB1) p127 subunit (A); KBP45 alignment with that of damaged DNA-binding protein 2 (DDB2) p48 subunit (B); KBP35 alignment with that of human hnRNP A1 protein (C).

Figure 3 illustrates the identification of KBP protein bands by mass spectrometry and tryptic peptide sequence analysis. Samples from the wild-type DNA-affinity column were fractionated by SDS-10% PAGE, stained with Coomassie Blue without prior fixation, and proteins excised for sequence analysis by microcapillary reverse-phase high pressure liquid chromatography mass spectrometry. The sequence alignment of multiple individual tryptic peptides (underlined) for KBP120 corresponds exactly with the deduced sequence for human damaged DNA-binding protein 1 (DDB1) p127 subunit (A); KBP45 alignment with that of damaged DNA-binding protein 2 (DDB2) p48 subunit (B); KBP35 alignment with that of human hnRNP A1 protein (C).

Figure 4 shows DDB1, DDB2, A1, and Sp1 proteins copurify with KCS DNA-binding protein activity. Samples from steps of the purification scheme used for enrichment of KCS binding activity were analyzed on a 10% polyacrylamide gel containing SDS, transferred to nitrocellulose, and probed separately with antibodies specific for DDB1 (1st panel), DDB2 (2nd panel), hnRNP A1 (3rd panel), and Sp1 (4th panel) proteins as indicated. Fractions analyzed by SDS-PAGE contained ~2 μg of total protein and included the following: lane 1, nuclear extract starting material; lane 2, flow-through (FT) from the DEAE-cellulose chromatography step; lane 3, 0.35 M KCl eluate from the CM-Sepharose chromatography step; lane 4, 0.6 M KCl eluate from the wild-type (WT) KCS DNA-Sepharose affinity chromatography step.

Figure 5 demonstrates DDB1, DDB2, A1, and Sp1 proteins but not IRF-9 protein are bound at the KCS element. Sepharose beads conjugated with KCS DNA element DNA (lanes 3 and 6) or beads without linked DNA (lanes 2 and 5) were incubated with nuclear extracts prepared from either untreated (lanes 1–3) or IFN-treated (lanes 4–6) human GM2767 cells. After recovery by centrifugation and washing of the Sepharose beads as described under “Experimental Procedures,” the bound proteins were analyzed by Western immunoblot assay using antibody against DDB1 (1st panel), DDB2 (2nd panel), hnRNP A1 (3rd panel), Sp1 (4th panel), and IRF-9 (5th panel) as indicated. Input nuclear extract (7.5 μg of protein) prepared from untreated (lane 1) and IFN-treated (lane 4) cells are included as marker controls.
KCS-binding Proteins of PKR Promoter

DDB1, DDB2, and A1 proteins were identified by mass spectrometry and immuno-selectively enriched for five KCS-binding proteins. Four of the proteins were shown to bind KCS element DNA in vitro by using crude nuclear extracts. The binding was similar between extracts prepared from untreated and IFN-treated human cells. Chromatin immunoprecipitation experiments showed that DDB2, A1, and Sp1 all bound selectively at the PKR promoter in vivo and that the binding seen for these proteins was comparable with chromatin prepared from untreated and from IFN-treated cells. By contrast, the level of the ISGF3 component protein STAT1 seen bound to the PKR promoter region chromatin in ChIP experiments was significantly increased by IFN treatment. What is known about the newly identified proteins, DDB1, DDB2, and A1, that bind KCS element DNA in vitro and the PKR promoter region in vivo? Also, what is the possible novel significance of their identification in the context of our understanding of the regulation of PKR gene expression by IFN?

DDB1 and DDB2 are subunits of the heteromeric UV-induced DNA damage-binding (DDB) protein complex (36). However, currently available evidence suggests that DDB1 and DDB2 have multiple and possibly independent roles in mammalian cells in addition to their affinity for damaged DNA (37). Our findings that DDB1 and DDB2 bind at the KCS element of the PKR promoter suggest that DDB subunits may act as transcriptional cofactors. Independent observations are consistent with this possibility. For example, DDB2 interacts with the activation domain of E2F1 and in association with E2F1 stimulates transcription from E2F1-regulated promoters (25). DDB2 also associates with proteins having histone acetyltransferase activity (38), and a mammalian histone acetyltransferase complex, STAGA, has been reported to associate with both the components of UV-DDB, DDB1, and DDB2 (39). Histone acetylation plays an important role in altering chromatin structure as part of the transcriptional activation process (40).

DDB1 interacts with multiple viral transcriptional activators including the X protein of hepatitis B virus HBx (41) and the V protein of paramyxoviruses (42). Mutational studies of HBx protein reveal a correlation between DDB1 binding and its ability to stimulate viral replication (43). Because DDB1 is implicated as an important factor for basal and inducible transcription of the PKR gene, it is tempting to speculate that interaction of HBx protein with DDB1 might provide a mechanism to impair PKR gene expression, and thus subsequently antagonize PKR-
KCS-binding Proteins of PKR Promoter

dependent cellular responses. Finally, DDB1 interaction with paramyxovirus V protein correlates with a delay in cell cycle progression (44), and more recently it has been reported that V protein plays a role in antagonizing the actions of IFN by preventing the activation of STAT1 and STAT2 in a process that involves interaction with DDB1 (26, 45).

DBD subunits that we have identified as components of the KBP complex at the PKR promoter in cultured human cells also have been implicated in global genomic repair (46). Interestingly, in prokaryotes, DNA damage repair proteins are known to play a role in transcription or transcription-coupled repair. For example, the ~130-kDa mutation frequency decline gene product, also known as transcription-repair coupling factor (TRCF), is necessary for transcription-coupled repair in both Escherichia coli (47) and Bacillus subtilis (48).

The transcription factor Sp1 is known to play an important role in the transcriptional activation of both cellular and viral genes and is implicated in targeting the basal transcription machinery to TATA-less promoters (49). Because the 5’ region of the KCS element has a putative Sp1-binding site and the Sp1 factor has been shown to be a part of the KCS complex by antibody supershift EMSAs (22, 35), it was not surprising that we found Sp1 as a component of the KCS complex purified from HeLa cells by ion-exchange and KCS DNA-affinity chromatography. Interaction of Sp1 with STAT1 has been reported (50), and it is conceivable that Sp1 binding at KCS participates in the recruitment of ISGF3 to the ISRE of the PKR promoter during transcriptional activation by IFN. Consistent with this notion, it is known that increasing the 4-bp natural separation that exists between the KCS and ISRE elements by insertion of heterologous DNA decreases transcriptional activity of the PKR promoter (17).

The identification of the RNA-binding protein, hnRNP A1, as a component of the KCS complex bound at the KCS element of PKR was surprising. Heteronuclear ribonucleoproteins are known pre-mRNA-binding spliceosome components that participate in the splicing and transport of the RNA from the nucleus to the cytoplasm (27). Interestingly, the spliceosome-associated protein SAP130 also has been shown to be a component of a different transcriptional complex, the STAGA complex (39). Several lines of evidence suggest that hnRNP proteins may also act as transcriptional activators. hnRNP K, one of the major 24 hnRNPs, interacts with the basal transcriptional apparatus (51), and both K and A1 hnRNPs are implicated as potent suppressors of human thymidine kinase promoter-driven gene expression (52).

Purification of the KCS complex has been done by using highly selective oligo sequences for affinity column chromatography. However, we cannot eliminate the possibility that we have isolated more than one complex. For example, the presence of Sp1 and hnRNP A1 in the same preparation raises the possibility that we may have isolated a transcriptional initiation complex along with a part of the spliceosome machinery. Although hnRNA processing, signal transduction, and transcription are separate processes, their regulation likely is coordinated. Collectively, our findings support the notion that UV-DDB and hnRNP A1, in addition to their traditional roles in UV-damaged DNA repair and RNA biogenesis, could act as regulators of gene expression through direct DNA binding or by interacting with other proteins. Further studies will be necessary to determine the interaction of these proteins with the KCS element DNA as well as with the other co-players.

Although it is clear that the ISRE and ISGF3 factor play an important role in IFN-inducible transcription of many type I IFN-regulated genes including PKR (17, 33), it is also clear that ISRE and ISGF3 are not sufficient for inducible PKR expression based on mutational analyses (16, 17). How then are subsets of IFN-inducible genes such as PKR regulated, given that their promoters do contain ISRE-binding sites for ISGF3? The KCS element of the TATA-less PKR promoter is implicated as one important component of both the basal and IFN-inducible transcriptional responses. PKR serves to recruit proteins to the PKR promoter region that function together and possibly cooperatively with ISRE to selectively activate PKR transcription. Sp3 (33), in addition to Sp1, DDB1, DDB2, and A1 shown here, is known to bind at the KCS element. But Sp3, unlike the other four KCS-binding proteins, binds to the promoter region in an IFN-inducible manner and appears to function as part of a novel IFN-inducible complex with ISGF3 proteins as shown by EMSA and ChIP experiments (33).

PKR transcripts, like those for ADAR1 and CIITA, display a significant basal expression level in the absence of treatment with exogenous type I IFNs. This is unlike certain other IFN-regulated gene transcripts, such as Mx, that show very low if any detectable expression levels in the absence of IFN treatment (1, 53). ADAR1 (54) and CIITA (55) represent two examples of genes for which we have mechanistic understanding of the basis of the significant basal transcription seen under physiologic conditions in the absence of IFN. For both ADAR1 and CIITA, it is known that multiple promoters and alternative exons 1 splicing are utilized to generate transcripts that encode the constitutively expressed forms of these two proteins (1). A fundamentally different mechanism must be responsible for the basal transcription of the single copy PKR gene, as there is only one known PKR promoter in the mouse and the human (11, 15). Conceivably the KCS element and cognate binding proteins provide the platform that allows for the function, both basal and inducible, of the single PKR promoter, as KCS is known to play a contributing role in modulating basal transcriptional activity of the PKR gene (16, 17).

Most likely multiple transcriptional mechanisms also are involved in IFN-inducible gene expression as well as basal expression. Although type I IFN signaling events following binding to the IFN receptor leading to activation of ISGF3 have been well characterized (1, 21), the mechanism by which subsets of IFN-α/β-regulated genes are differentially regulated and the factors involved are just beginning to be elucidated. The notion that a second cis-element such as KCS and trans-acting factors such as DDB1, DDB2, A1, and Sp1 may be required in addition to the ISRE and ISGF3 factor is consistent with the finding that the BRG1 component of the SWI2-SNF2 complex selectively activates some but not all type I IFN-inducible genes (56). Surprisingly some genes that possess a single TATA-less IFN-inducible promoter as does PKR may not even utilize the TBP subunit of TFII-D in their IFN-stimulated transcription. Whereas TBP and associated factors (TAF5, S) are important for basal transcription of some TATA-less promoters (57, 58), transcription of some IFN-regulated genes has been shown to occur through a non-classical TBP-free transcriptional mechanism (59). Such diversity of molecular mechanisms involved in the transcriptional responses mediated by IFNs is of a clear evolutionary advantage to the host, given the multitude of strategies that have been identified by which viruses antagonize the IFN system either at the level of signaling or action of IFN-induced proteins including PKR (1).

Acknowledgments—We thank C. C.-K. Chao, G. Dreyfuss, S. Linn, P. Masters, N. Reich, and L. Wilson for generously providing reagents and materials.

REFERENCES

1. Samuel, C. E. (2001) Clin. Microbiol. Rev. 14, 778–809
2. Samuel, C. E. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 600–604
3. Gale, M., Jr., Tan, S. L., and Katze, M. G. (2000) Microbiol. Mol. Biol. Rev. 64, 239–280
DNA Damage-binding Proteins and Heterogeneous Nuclear Ribonucleoprotein A1 Function as Constitutive KCS Element Components of the Interferon-inducible RNA-dependent Protein Kinase Promoter
Sonali Das, Simone Visosky Ward, Danielle Markle and Charles E. Samuel

J. Biol. Chem. 2004, 279:7313-7321.
doi: 10.1074/jbc.M312585200 originally published online November 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M312585200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 25 of which can be accessed free at http://www.jbc.org/content/279/8/7313.full.html#ref-list-1