Identification and Functional Characterization of an Intragenic DNA Binding Site for the Spumaretroviral trans-Activator in the Human p57<sup>Kip2</sup> Gene*

Kenji Kido, Anja Doerks, Martin Löchelt, and Rolf M. Flügel‡

From the Division of Retroviral Gene Expression, Research Program Applied Tumor Virology, German Cancer Research Center, Im Neuenheimer Feld 242, 69009 Heidelberg, Germany

Expression of the human cyclin-dependent kinase inhibitor p57<sup>Kip2</sup> gene was previously shown to be specifically and strongly activated by the retroviral trans-activator Bel1 of human foamy virus by means of expression profiling, Northern, and Western blot analysis. Here we report that Bel1-mediated trans-activation was conferred by a Bel1 response element (BRE) located in the second exon of p57<sup>Kip2</sup>. The intragenic Kip2-BRE was capable of trans-activating the luciferase reporter gene upon cotransfection with Bel1. In electrophoretic mobility shift assays using 293T nuclear extracts or a purified glutathione S-transferase (GST)-Bel1 fusion protein, we identified the 55-nucleotide-long Kip2-BRE site that mainly consists of three direct repeats of 14mers partially homologous to a functionally active BRE in the viral internal promoter. The specificity of the transactivator-DNA binding was shown by mutated and shortened Kip2-BRE oligodeoxynucleotides in competition experiments with the authentic viral internal promoter and by Bel1-specific antibody that led to a supershift of the nuclear protein-Kip2-BRE and GST-Bel1-Kip2-BRE complex. The data indicate that Bel1 can directly bind to BRE sites. The cellular Kip2-BRE can be used to predict those human genes that are directly or indirectly activated by the Bel1 trans-activator.

The cell cycle is predominantly regulated by a group of protein kinases. These serine/threonine kinases are controlled by various mechanisms including proteins that act as kinase inhibitors. There are two known families of protein kinase inhibitors that comprise the p16<sup>ink4a</sup> and p21<sup>Cip1</sup> families (1). The three known members of the second family, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>, share structural and functional properties, and each member seems to play a distinct role during cell cycle progression and development. p57<sup>Kip2</sup> knockout mice die immediately after birth due to dyspnea resulting from cleft palate, abdominal muscle defects, and skeletal abnormalities (2–4). However, p57<sup>Kip2</sup> also known as Cdkn1c is the only member that maps to the human chromosomal locus at 11p15.5, a region implicated in both sporadic cancers and Beckwith-Wiedemann syndrome (5, 6). This locus is especially interesting because it contains several genes that correlate with cell proliferation, growth, and specific tumors. It maps close to a major region that is controlled by genomic imprinting. Paternal versus maternal imprinting via DNA methylation has opposite effects on expression of the insulin-like growth factor 2 (IGF-II) and the negative cell cycle regulator p57<sup>Kip2</sup> (7).

Complex retroviruses code for proteins that specifically recruit the cellular transcription machinery to viral promoters. By virtue of viral trans-activators, such as Tax and Bel1, cellular transcription programs are specifically affected or even reprogrammed in a way favorable for viral replication (8–10). The Bel1 trans-activator of the human foamy virus (HFV), also called spumaretrovirus, binds directly to DNA target sites with no or low sequence conservation located in both the 5’-long terminal repeat and the internal promoter (IP) (11, 12). The trans-activator Tas of simian foamy virus type 1 also binds directly to corresponding DNA target sites that are not homologous to those of Bel1 (13). DNA binding of Bel1 and full trans-activation activity depend on cellular factors that have not been identified so far. The DNA binding domain has been mapped to a central region of the 300-amino-acid-long HFV Bel1 (14). The COOH-terminal domain functions in transcriptional trans-activation and belongs to the acidic class of eukaryotic trans-activators with VP16 as one member (15, 16). Expression profiling by cDNA arrays, Northern, and immunoblot analyses were carried out to determine whether HFV infection or cotransfection with the Bel1 trans-activator alters the expression of defined human genes. Using these methods, we found that HFV infection and Bel1 strongly increased expression of distinct sets of cellular genes including IGF-II, p57<sup>Kip2</sup>, early growth response gene (EGR-1), COUP-TF1, and the tyrosine kinase receptor EPH3 (17). While the induction of some genes appeared to be mediated by Bel1-independent mechanisms, the human p57<sup>Kip2</sup> gene seemed to be directly activated by cotransfection of a Bel1 expression plasmid (17).

In the present study it was our aim to define the sequence elements that conferred Bel1 responsiveness to the p57<sup>Kip2</sup> gene. Surprisingly the Bel1 DNA target site was found to be located in the second exon of p57<sup>Kip2</sup>. Binding of nuclear proteins from human cells transfected with Bel1 or of purified GST-Bel1 fusion protein to this DNA target site was specific.

* This work was supported by Grant BEO 0311714 from the Bundesministerium für Forschung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Division of Retroviral Gene Expression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, INF 242, 69009 Heidelberg, Germany, Tel.: 49-6221-424611; Fax: 49-6221-424865; E-mail: r.m.fluegel@dkfz-heidelberg.de.

Received for publication, September 11, 2001, and in revised form, December 18, 2001
Published, JBC Papers in Press, January 28, 2002, DOI 10.1074/jbc.M108747200

1 The abbreviations used are: IGF-II, insulin-like growth factor 2; BRE, Bel1 response element; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HFV, human foamy virus; IP, HFV internal promoter; Kip2, cyclin-dependent kinase inhibitor 2; luc, luciferase; nt, nucleotide(s); CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; EGR-1, early growth response gene; CNBP, cellular nucleic acid-binding protein.
This response element showed an unexpected complex repeat structure.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Plasmids, and Transfection—*Human 293T cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin and streptomycin and 10% fetal calf serum. Plasmids pUC18, pCMVβ-gal (18), pβ-gal-plasmid, and pβ-gal-pro-kip2 derivatives (20) were transfected into 293T cells using the coexpression method of Chen and Okayama (21). In general, 3–6 μg of plasmid DNA was transfected into 293T cells grown in Petri dishes with a diameter of 6 mm.

*Construction of Eubaryotic p57kip2-based Luc Reporter Plasmids—*Nucleotide numbering of the human p57kip2 gene (accession no. D64137, EMBL data base) was used according to Tokino (20). Recombinant DNA techniques were used as described previously (21, 22). Reporter constructs containing parts of the p57kip2 promoter were constructed by PCR-mediated amplification of defined promoter fragments. Sense primers were: Ks1 (983 to 964), 5′-GATGAGCTGTCATCTGCGCTG-3′; Ks2 (760 to 742), 5′-GATGAGCTGCCCCGATCGAGGGCTTTAG-3′; Ks3 (609 to 582), 5′-GATGAGCTGCTTCG-TCAGCTGGCGCAGGAG-3′; Ks4 (456 to 438), 5′-GATGAGCTGCCTCAGAAAGGACCC-3′; Ks5 (322 to 305), 5′-GATGAGCTCCCGCCTGCCCCCCTTC-3′; and Ks6 (228 to 211), 5′-GATGAGCTGCGTGGTCGGCGCAGGA-3′. The antisense primer was: Kas (277 to 257), 5′-GATGATATCTTGTTGAGGTCGAGGAGAAG-3′. The location of the primers relative to the position of the p57kip2 cap site are schematically shown in Fig. 1A. PCRs using different sense primers and antisense primer Kas were done with Pfu polymerase (Stratagene, Heidelberg, Germany) and pKip2 plasmid DNA (20). PCRs were carried out with the buffer recommended by the supplier supplemented with 5% Me2SO at 1 min 96 °C, 1.5 min 55 °C, 5 min 72 °C for 35 cycles. The resulting blunt-ended PCR amplions were digested with BglII and inserted into the BglII- and EcoRI site of 136II-digested reporter plasmid pGL2 basic (Promega, Mannheim, Germany). The resulting reporter construct was designated pGL2-K1 to -K7. Separately pGL3-promoter plasmids were constructed. These plasmids were designated pGL3-pro-kip2 derivatives as shown in Fig. 1C.

The complete p57kip2 coding sequences from plasmid pKip2 was excised with BamHI located within the vector (20) and NheI and inserted into the NheI- and BglII-digested plasmid pGL3-promoter. The resulting luc reporter plasmid pGL3-pro-kip2ds contains the p57kip2 coding sequence in the sense orientation upstream of the luc gene. The PvuII-PvuII p57kip2 fragment (+649 to +968) was inserted into the EcoRI site of pGL3-promoter in the sense orientation. Sequences from +649 to +968 were deleted from the plasmid pGL3-pro-kip2s plasmid by PvuII digestion and religation. The oligodeoxynucleotide Kip2-BRE (Fig. 1C) was mixed with the corresponding antisense oligodeoxynucleotides, heat-treated, and inserted into the KpnI digested EcoRI-digested pGL3-promoter plasmid to generate reporter plasmid pGL3-pro-BRE promoter in both orientations. Oligodeoxynucleotides Kip2-BRE-38, those containing three and four direct repeats of 14-mers, HFV-IP-BRE, and IP-BRE37 were synthesized as oligodeoxynucleotides, cloned into the pGL3-promoter and used for luc assays. To express p57kip2 from the minimal SV40 promoter, plasmid pkip2 was digested with SmaI and BamHI. The p57kip2 sequencing was used to replace the luc gene in plasmid pGL3-promoter. To this end, pGL3-promoter was digested with HindIII, blunt-ended, and digested with BamHI. The resulting plasmid pSV40pro-kip2s contains the p57kip2 coding sequence in the sense orientation. Homologies shown in Table I were obtained by pairwise aligning of the genes in both orientations with the BESTFIT algorithm of the Wisconsin Package, Version 10.3 (Genetics Computer Group, Madison, WI).

EMSAs — EMSAs experiments were performed according to Soto et al. (23). The probes used for EMSAs included the Kip2-BRE and IP-BRE (Fig. 3, lines 5 and 1). These oligodeoxynucleotides were synthesized, annealed, and end-labeled using γ−ATP (3000 Ci/nmol, Amersham Biosciences) with T4 polynucleotide kinase (New England Biolabs). The labeled probe was purified by electrophoresis on a 15% polyacrylamide gel. Further orations were prepared as described previously (23, 24). Protein concentrations were determined with the detergent-compatible protein assay (Bio-Rad). Nuclear extracts (2 μg) were preincubated for 5 min at room temperature in a volume of 40 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 2.5 mM MgCl2, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.2 mM phenylmethylsulfonyl fluoride, 5% glycerol, 5 ng/μl poly(dA:dT)-poly(dA:dT) (Amersham Biosciences). Labeled DNA probe (20,000 cpm) was added and incubated for 30 min at 25 °C. For competition experiments, unlabeled competitor oligonucleotides were added in 200–300-fold molar excess at the preincubation period. Antibody against Bel1 or control preimmune serum used in supershift assays was added at 1:200 dilution 30 min after addition of the labeled probe and further incubated for 1 h at 4 °C. GST-Bel1-(1–228) fusion protein and GST protein was bacterially expressed and purified on glutathione-Sepharose 4B as described previously (12, 35). The DNA-protein complexes were resolved in a 5.5% nondenaturing polyacrylamide gel, dried, and exposed overnight to Kodak BioMax MR films.

*Luc Expression Assays—*Plasmids pCMVBgal directing β-galactosidase expression from the CMV-β-gal promoter were used for normalization of transfection efficiency. Luciferase reporter gene assays were performed and quantified as described previously (24) using a Luminoskan TL, Plus luminometer (Labsystems, Frankfurt, Germany). Cells were harvested 24 h after transfection.

*Immunoblotting—*Cells were harvested 2 days after transfection by lysis in 1% SDS, and the protein concentration was determined using the detergent-compatible protein assay (Bio-Rad). Identical amounts of proteins were separated by SDS-PAGE, blotted, incubated with monoclonal serum directed against p57kip2 (PharMingen, Hamburg, Germany), and detected by enhanced chemoluminescence as described previously (17).

**RESULTS**

Characterization of the 5′-Upstream p57kip2 promoter—To study whether the DNA target site of Bel1 is in the kip2 promoter region between −983 and +277, the reporter plasmids pGL2-K1 to -K7 were cotransfected with the eukaryotic expression plasmid pβ-gal and pUC18 control DNA (Fig. 1A). None of the plasmids showed any Bel1-dependent enhancement of transcriptional activity of luc reporter gene expression (data not shown). This result indicates that a potential Kip2-BRE should be located further downstream of position +277 within the p57kip2 gene.

Mapping the Bel1 response element of p57kip2 (Kip2-BRE)—To map the Kip2-BRE site, the complete p57kip2 coding sequence (nt +14 to +2076) was placed in sense orientation into the luc reporter plasmid pGL3-promoter (Fig. 1C, line 2). Luc gene expression of the resulting plasmid pGL3-pro-kip2 was 7-fold trans-activated by Bel1 (Fig. 1C, line 2). To define the location of the BRE more precisely, deletions were introduced into pGL3-pro-kip2 and analyzed for Bel1 responsiveness (Fig. 1C). Deletion of the PvuII-PvuII DNA fragment located between +649 and +968 nt relative to the p57kip2 cap site within the first coding exon of p57kip2 virtually abolished Bel1 trans-activation (Fig. 1C, line 4). Consistent with this result, the PvuII-PvuII DNA fragment conferred a 7-fold enhancement of Bel1 trans-activation to the minimal SV40 promoter (Fig. 1C, line 3). To select and define the Kip2-BRE more precisely, sequence alignments with the known viral BRE sequences were performed. A candidate Kip2-BRE sequence located about 750 nt downstream of the cap site and within the PvuII-PvuII insert identified above showed homology to the known minimal and functional IP-BRE (see below).

To confirm unambiguously that a functional Kip2-BRE maps within the PvuII-PvuII insert and specifically confers Bel1 responsiveness to a heterologous promoter, a 55-nt-long oligodeoxynucleotide, Kip2-BRE, was synthesized, cloned upstream of the minimal SV40 promoter of the pGL3-pro plasmid in both orientations, and analyzed for luc gene expression. The results shown in Fig. 1C, line 5 demonstrate that the synthetic Kip2-BRE oligodeoxynucleotide is capable of enhancing the transcriptional activity of Bel1 in sense orientation. In comparison, the corresponding antisense plasmid, pGL3-pro-Kip2BREas, had only 50% of that activity (Fig. 1C, line 6). When the Kip2-BRE length was shortened from 55 to 38 bp and used in luc assays, a 3-fold enhancement of activation was found (Fig. 1C, line 7). A direct comparison of the level of
transactivation between the HFV IP-BRE with Kip2-BRE revealed that the minimal viral IP-BRE had a lower activity (Fig. 1C, lines 11 and 5) since luc gene expression of plasmid pGL3-pro-kip2.BREEs was 14-fold trans-activated by Bel1 (Fig. 1C, line 5). Apparently the minimal Kip2-BRE (Fig. 1C, lines 5 and 6) and the HFV IP-BRE (Fig. 1C, lines 11 and 12) seem to preferentially function in an orientation-dependent manner as the levels of transactivation of Kip2-BREAs and of IP-BREAs were relatively low (Fig. 1C, lines 6 and 12). This appeared to be similar to the complete viral internal enhancer-promoter that has been reported to transactivate in cis to higher levels in mammalian cells (26). However, the level of activation of the plasmids that contained the HFV IP-BRE in the antisense orientation was critically dependent on the length of the enhancer (Fig. 1C, bottom line). We conclude that the long IP-BRE reached similar levels of transactivation in both orientations under the conditions used. In contrast, the Kip2-BRE predominantly acted in cis.

To determine the expression of the authentic human p57kip2 at the protein level, plasmid SV40-pro-Kip2 that contained the complete coding sequence of p57kip2 under the control of the heterologous SV40 promoter was transfected into 293T cells with and without the Bel1 expression plasmid pbel1s. Cell extracts were harvested 2 days after transfection and equal amounts of protein were analyzed for p57kip2 expression by immunoblot analysis. Bel1 induced p57kip2 (Fig. 2, lane 2); without Bel1, enhancement of p57kip2 expression was not detectable (Fig. 2, lane 1). This result confirms that the Kip2-BRE regulatory element required for p57kip2 expression should reside at an intragenic location. Controls were carried out by cotransfection of two unrelated expression plasmids that did not detect p57kip2 expression (Fig. 2,

---

**Fig. 1.** Diagrams of the human p57kip2 gene and results of luc reporter assays for mapping the Kip2-BRE. A and B, structure of the human p57kip2 gene. The noncoding exons 1 and 4 of p57kip2 mRNA are presented as open boxes, and coding exons are presented as filled boxes. Locations of the TATA box, cap site, and AUG initiation codon are shown. The right-angled indicates the start site and direction of p57kip2 transcription. Horizontal arrows mark the positions and orientations of oligodeoxynucleotides used for PCRs; pGL2-K1 to -K7 represent plasmids with successively shortened promoter regions. B, locations of restriction sites used; the Kip2-BRE site is shown by a filled rectangle. C, results of transient expression assays with names and structures of the luc reporter plasmids. Numbers refer to the start and end points of the different human p57kip2-derived inserts relative to the cap site of the p57kip2 mRNA (20). Gaps mark the deletions discussed in the text; the orientation of the inserts is indicated by "s" (sense) and "as" (antisense). The interrupted and filled boxes in lines 9 and 10 present the number of direct repeats as shown in Fig. 3. The open boxes and numbers in the last 4 lines refer to the promoter sequence upstream of the HFV internal cap site (12). The number of the BRE indicates the length of the oligonucleotides in bp. SV40, SV40 minimal promoter. The data represent the mean for three separate experiments with S.D. shown by error bars. Representative results for the Bel1-mediated trans-activation by the plasmids relative to pUC18 controls are graphically shown.

---

**Fig. 2.** Immunoblot analysis of p57kip2 expression in response to Bel1. The top line shows genetic organization of the plasmid vector used; filled boxes represent the p57kip2 coding sequence. The SV40pro-kip2 plasmid was cotransfected into 293T cells with plasmids pbel1s, pBet, and pSp1 (lanes 2–4). Cell extracts were harvested 2 days later, and equal amounts of protein were incubated with p57kip2-specific monoclonal antibody. An arrow marks the position of p57kip2 protein; marker proteins were separated in parallel.

lanes 3 and 4). The sequence alignments in Fig. 3 revealed that the position and spacing of those G residues (in bold face) that had been shown to be crucial for Bel1 binding to the minimal IP-BRE (12) are completely conserved. The viral IP-BRE that had been previously determined in methylation interference experiments was almost fully conserved in the 14-mer kip2
sequence 5'-GGCTCCGGTGCCGG-3' (Fig. 3, line 2, broken underlining), whereas the octamer (Fig. 3, line 4) is virtually completely conserved in both the viral and cellular BRE except for position 4 where T is replaced by a C base. Close and comparative inspection of the kip2 sequence revealed that the BRE is present as three direct repeats of partially overlapping 14-mers (Fig. 3, broken underlining). Each 14-mer repeat contains one 12-mer that does not overlap.

The transcriptional activities of both the 5'- and 3'-halves, Kip2-BRE1 and Kip2-BRE2, of this site (Fig. 3, lines 6 and 7) were determined. The full-length DNA retained full transactivation capacity in sense orientation (Fig. 1C). Both subfragments Kip2-BRE1 and -2 conferred significantly lower Bel1 responsiveness to the pGL3-pro-luc plasmid (data not shown).

We next sought to analyze whether oligomers of the 12- and 14-mer repeats of Kip2-BRE enhanced transactivation. Three different oligonucleotides were synthesized, cloned, and subjected to luc assays. The primary structures of the oligomers are shown in Fig. 3. Luc gene expression of plasmids pGL3-pro-Kip2-BRE38s and -38as that consist of three overlapping direct repeats of 14-mers (and nonoverlapping direct repeats of 12-mers) of the authentic Kip2-BRE was slightly activated above background levels in both orientations (Fig. 1C, lines 7 and 8). Moreover, the two plasmids that contain either three or four direct repeats of 14-mers did not yield any transactivation (Fig. 1C, lines 9 and 10). This result indicates that the three overlapping 14-mer direct repeats of Kip2-BRE seem to be essential for Bel1-mediated transactivation.

To summarize this point, we have mapped the human Kip2-BRE to an intragenic kip2 DNA sequence that is located within the second p57kip2 exon (Fig. 1, B and C). Kip2-BRE is partially homologous to the viral IP-BRE and displays a structure consisting of three identical and overlapping 14-mers or of three nonoverlapping 12-mer direct repeats. Thus, a first cellular BRE has been identified with a minimal length of 55 nt that contains three 14-mer repeats (Fig. 3, line 5).

**EMSA of Nuclear Proteins from Bel1-transfected Cells That Bind to DNA Target Sites of Kip2-BRE**—To determine whether the Kip2-BRE site is recognized by the viral transactivator, EMSA experiments were carried out. Nuclear extracts from 293T cells transfected and nontransfected with Bel1 were prepared and incubated with the labeled Kip2-BRE oligonucleotide. In the extracts from Bel1-transfected cells a Kip2-BRE DNA-protein complex, C1, was clearly detectable (Fig. 4A, lane 3). Binding of proteins in the Bel1 protein-containing nuclear extracts decreased in a dose-dependent manner (Fig. 4A, lanes 3–6). Additional bands observed in nuclear extracts from pUC18-transfected cells are considered to be unspecific (Fig. 4A, lane 2) as reported previously (11–15).

To confirm the specificity of the Kip2-BRE interaction with Bel1, a polyclonal Bel1-specific antibody was used in supershift assays. The EMSAs shown in Fig. 4B indicate that in the presence of nuclear extracts from 293T cells transfected with Bel1, the C1 complex was detectable as expected (Fig. 4B, lane 3). Incubation with antibody against Bel1 resulted in a supershift of the C1 complex to positions marked by two arrows, Cα1 and Cα2 (Fig. 4B, lane 4), indicating the presence of two bands. The specificity control, preimmune serum and antibody against Bel1 did not show any effects (Fig. 4B, lanes 5 and 6).

To directly compare the authentic viral IP-BRE binding with the cellular Kip2-BRE, supershift EMSAs were performed under the same conditions. The resulting gel shifts in Fig. 4C confirmed that treatment with antibody against Bel1 yielded two complexes for both labeled oligonucleotides (Cα1 to Cα4, arrows). The supershift bands migrated with similar mobilities, whereas the complexes without antibodies (C1 of Kip2-BRE, and C2 and C3 of IP-BRE) migrated to slightly different positions.
positions. An unrelated antibody did not supershift these complexes.

**EMSA of Nuclear Extracts from Bel1-transfected Cells Incubated with Various Oligonucleotides as Competitors for Binding to the Kip2-BRE Site**—To determine whether different oligonucleotides were able to act as competitors for the formation of the Kip2-BRE-Bel1 complex, unlabeled Kip2-BRE, two subfragments, Kip2-BRE1 and BRE2; three mutated Kip2-BREs; and IP-BRE were used in EMSAs (sequences shown in Fig. 3). The data shown in Fig. 5A prove that the unlabeled Kip2-BRE and the subfragment Kip2-BRE2 functioned as effective competitors (Fig. 5A, lanes 4 and 6), whereas the half-sized oligonucleotide Kip2-BRE1 did not block Bel1 binding (Fig. 5A, lane 5). Kip2-BRE mutant 2 partially inhibited formation of complex C1 (Fig. 5A, lane 8), but mutants 1 and 3 did not (Fig. 5A, lanes 7 and 9).

To further confirm that the known functionally active IP-BRE (12) is able to compete with the C1 complex formation under our assay conditions, we used an unlabeled 27-bp-long IP-BRE DNA probe as competitor for Kip2-BRE. As shown in Fig. 5A, lane 10, the minimal viral IP-BRE was capable of inhibiting the Kip2-BRE complex C1. To examine whether the authentic IP-BRE was competed with unlabeled Kip2-BRE, the labeled IP-BRE oligonucleotide was used in EMSA with and without unlabeled Kip2-BRE. As shown in Fig. 5B, the two different IP-BRE protein complexes C1 and C2 were completely blocked by authentic IP-BRE and almost fully blocked by Kip2-BRE (Fig. 5B, lanes 4 and 5). Taken together the results indicate that nuclear extracts from Bel1-transfected cells contain proteins that specifically bind to the Kip2-BRE oligonucleotide.

To ascertain that nuclear extracts of cells cotransfected with Bel1 contain a protein that binds to the Kip2-BRE oligonucleotide directly or indirectly, a GST-Bel1-(1–228) fusion protein that contains the DNA binding domain (14) was bacterially expressed and purified by affinity chromatography (12, 35). Upon incubation of labeled Kip2-BRE with the purified GST-Bel1 protein, three complexes were generated (Fig. 6A). The three bands observed were actually due to Bel1 protein binding since purified GST protein failed to bind to the labeled probe (Fig. 6B, lane 2). The fact that three distinct protein-DNA C1 to C3 complexes were detectable is fully consistent with the data obtained by Kang et al. (12). The same authors pointed out that the appearance of three complexes might be due to the ability of Bel1 to form multimers (12). Upon prior treatment with antibodies against glutathione S-transferase, the three complexes resulted in supershift bands (marked by arrows, Co1 to Co3) indicating that the formation of the shifted Kip2-BRE complexes is GST-Bel1-specific.

To prove that the GST-Bel1-Kip2-BRE complexes can be blocked by an authentic viral IP-BRE oligonucleotide, EMSA
experiments were carried out by incubating Kip2-BRE with purified GST/Bel1 protein and different unlabeled oligonucleotides as shown in Fig. 6B. The viral IP-BRE clearly acted as a competitor of the cellular Kip2-BRE site (Fig. 6B, lane 8). The resulting three Bel1-Kip2-BRE complexes were marked by the arrows C1 to C3. Supershifts of the three DNA-protein complexes with polyclonal antibody against GST at a 1:20 dilution are shown in lane 3. B, EMSA of purified GST-Bel1 fusion protein and reaction of various oligonucleotides as competitors for binding to the Kip2-BRE site (for details, see legend to Fig. 5). As control, purified GST protein was run in lane 2. An asterisk marks the position of free probe.

**DISCUSSION**

In this report, a Kip2-BRE DNA element located within the second exon of the human p57<sup>Kip2</sup> gene has been identified that mediates a specific Bel1 trans-activation. It is worth noting that the Kip2-BRE primarily consists of direct repeats of three 14-mers that partially overlap. Kip2-BRE was sufficient to bind to nuclear extracts from 293T cells that had been transfected with Bel1 as shown by EMSA and supershift experiments. A purified GST-Bel1 fusion protein was capable of binding to Kip2-BRE; formation of the Bel1-Kip2-BRE complexes was effectively blocked by the minimal viral IP-BRE oligonucleotide. The Kip2-BRE site has a characteristic spacing of G bases in each of three direct repeats that is well conserved in the viral IP-BRE previously shown to be required for Bel1 binding (12).

The 3-fold direct 14-mer repeats of Kip2-BRE are reminiscent of the three direct repeats of the 21-bp tax promoter in the
5′-long terminal repeat of human T-cell leukemia virus type I (8, 27). Several complexes of Tax with different cellular transcription factors have been reported (27–31). In one of them, Tax seems to make limited contacts with the flanking sequences of this core DNA of octamers in the context of several transcription factors so that a specific interaction results via a Tax-CREB-CBP-P/CAF-DNA complex (27–31). On the other hand, Bel1 binds to DNA target sites directly in contrast to Tax.

The combined results of the transactivation data, the EMSAs with the native Kip2-BRE, the shortened Kip2-BRE1, and two oligonucleotides, and mutant Kip2-BREs suggest that the major part of the 55-bp Kip2-BRE sequence is necessary for specific binding of Bel1 and for an enhancement of transcriptional activity by Bel1. The data are consistent with the essential G patterns and the fact that mutant 2 (Fig. 3) is the only sequence that has the characteristic 14-mer repeats with two G bases at each 3′-end conserved and the only mutant capable of partially blocking Kip2-BRE. In contrast, in both mutants 1 and 3 the two conserved G bases at the 3′-end are replaced by two T bases (mutant 1) or one T residue (mutant 3). This points to the crucial role of these two G bases for recognition and binding of the Bel1 protein in the context of the direct repeats.

It is well known that transcription is context-dependent usually requiring recruitment of several transcription factors that can cooperate by protein-protein interactions (32). This assumption is consistent with the presence of an additional but unknown partner in the nuclear protein Tax and an unknown partner in the nuclear protein complex Cα1 and Cα2 in the supershift EMSA. Other reasons that might contribute to the formation of the complexes, e.g., Bel1 dimerization, cannot be ruled out.

The identification of a first cellular BRE site, called Kip2-BRE in this report, was used for searching this element and closely related DNA target sites in those human genes that were trans-activated by Bel1 and previously defined by means of expression profiling in cDNA arrays (17). Two criteria were used to qualify for a cellular BRE: (i) at least one repeat of octamers as in Kip2-BRE and (ii) a conserved G base pattern taking as guideline a degree of homology of about 75% between the octamer direct repeat of Kip2-BRE and the cellular BRE sites. Using both criteria, those 40 human genes of a total of 588 that were strongly activated by HFV infection were searched for homology and repeats (Table I). Among them, the genes of the transcription factors COUP-TF1, EGR-1, and MYB did contain a Kip2-BRE-like sequence with conserved G base patterns accompanied by one or more direct repeats of octamers. It is noteworthy that some of the activated genes are transcription factors that contain a putative cellular BRE either at an intragenic location, e.g., in the COUP-TF1 gene or in the corresponding promoter regions, for instance in those of EGR-1 and the p65 subunit of NFκB. In virtually all activated genes, there is at least one direct octamer repeat defined by Kip2-BRE and, in addition, a conserved set of the characteristic G base patterns previously detected by methylation interference analysis of the viral BRE sites (12). BRE sites with conserved G base pattern repeats were not found in three genes repressed by HFV infection, the glia maturation factor-β, DNA-binding protein CNBP, and an ATPase that is related to the family of SNF2/SWI2 proteins (Table I). Thus, the predictions based on the two different criteria indicate that the presence of a cellular BRE in the promoter or coding regions may lead to a Bel1-mediated trans-activation of the corresponding gene.

It is, however, clearly apparent that most of the Bel1-induced cellular transcription factors belong to the class of immediate-early genes, for instance COUP-TF1 and EGR-1. This raises the intriguing question whether the induction of distinct cellular genes by Bel1 is direct or indirect or both since these immediate-early transcription factors might be responsible for the activation of at least some of the cellular genes observed. Indeed recently published expression profiling of EGR-1 that included more than 2000 genes clearly revealed that a number of distinct cellular proteins were specifically activated by EGR-1 that had also been detected by Bel1 activation (32, 17). Among others, IGF-II and p57KIP2 were reported to be activated by both Bel1 and EGR-1 (17, 33). In the case of p57KIP2, it seems likely that both pathways were used in Bel1-transfected cells since the expression level of the kinase inhibitor is particularly high (17). The identification of several different cellular BRE sites should help clarify this issue. It might also be appropriate to take the human hepatitis virus transactivator protein, HBx, into consideration as some features of foamy viruses resemble those of hepadnaviruses (34).

Acknowledgments—We thank Peter Angel and Jennifer Reed for critically reading the manuscript, Robert Tjian and Ubaldo Soto for advice and reagents, and Harald zur Hausen for continuous support.
REFERENCES

1. Nakayama, K.-I., and Nakayama, K. (1998) Bioessays 20, 1020–1029
2. Matsuoka, S., Edwards, M. C., Bai, S., Zhang, P., Baldini, A., Harper, J. W., and Elledge, S. J. (1995) Genes Dev. 9, 650–662
3. Yan, Y., Friisen, J., Lee, M.-H., Massague, J., and Barbacid, M. (1997) Genes Dev. 11, 973–983
4. Zhang, P. Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DeFinno, R. A., and Elledge, S. J. (1997) Nature 387, 151–158
5. Lee, M. P., Hu, R.-J., Johnson, L. A., and Feinberg, A. P. (1997) Nat. Genet. 15, 181–185
6. Engemann, S. Strödicke, M., Paulsen, M., Franck, O., Reinhardt, R., Lane, N., Reik, W., and Walter, J. (2000) Nucleic Acids Res. 18, 2691–2706
7. Reik, W., and Murrell, A. (2000) Nature 405, 408–409
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
10. Tokino, T., Urano, T., Furuhata, M., Matsushima, T., Miyatsu, S., Sasaki, S., and Nakamura, Y. (1996) Hum. Genet. 97, 625–631
11. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Soto, U., Das, B. C., Lengert, M., Finzer, P., zur Hausen, H., and Rosi, F. (1999) Oncogene 18, 3187–3198
14. Goodhall, R. H., and Smolik, S. (2000) Genes Dev. 14, 1553–1557
15. Fukuyama, K., and Nakayama, K. (1998) Bioessays 20, 1020–1029
16. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
17. Tokino, T., Urano, T., Furuhata, M., Matsushima, T., Miyatsu, S., Sasaki, S., and Nakamura, Y. (1996) Hum. Genet. 97, 625–631
18. Loañez, M., Muranyi, W., and Flügel, R. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7137–7142
19. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
20. Tokino, T., Urano, T., Furuhata, M., Matsushima, T., Miyatsu, S., Sasaki, S., and Nakamura, Y. (1996) Hum. Genet. 97, 625–631
21. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Goodhall, R. H., and Smolik, S. (2000) Genes Dev. 14, 1553–1557
25. Lenzen, R., Laird, A., Dervan, E. E., and Nyborg, J. K. (1999) J. Mol. Biol. 291, 731–744
26. Yoshida, M. (1998) Annu. Rev. Immunol. 16, 475–496
27. Goodhall, R. H., and Smolik, S. (2000) Genes Dev. 14, 1553–1557
28. Lenzen, R., Laird, A., Dervan, E. E., and Nyborg, J. K. (1999) J. Mol. Biol. 291, 731–744
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
31. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
32. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
33. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
34. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169
35. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) J. Virol. 67, 161–169