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Bel1-mediated Transactivation of the Spumaretroviral Internal Promoter Is Repressed by Nuclear Factor I*

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Gene expression of the internal and long terminal repeat promoters of the spuma retrovirus is specifically activated by the transactivator Bel1, the key regulator of viral gene expression. Bel1 directly binds to and activates DNA target sites of viral promoters and those of distinct cellular genes. To determine the contribution of cellular transcription factors to viral transactivation, the viral internal promoter (IP) was analyzed by transient expression, electrophoretic mobility shift assays), and supershifts. Here we report that Bel1-mediated transactivation of the full-length and shortened versions of the Bel1 response element (BRE) were repressed by nuclear factor I (NFI). Electrophoretic mobility shift assays using nuclear extracts from transfected 293T cells revealed that different DNA-protein complexes consisting of DNA target sites of NFI and Bel1 proteins were formed. The specificity of the repressor and transactivator DNA binding was shown by NFI- and Bel1-specific antibodies that led to supershifts of the different nuclear protein-oligodeoxynucleotide complexes. The specificity of the complexes was confirmed by using unlabeled, shortened, and mutated IP.BRE oligodeoxynucleotides in competition experiments with the authentic IP.BRE. Cotransfection of the infectious spumavirus DNA genome with a human NFI-X1 expression plasmid into cell cultures greatly reduced the expression of viral structural and Bel1 proteins. These data demonstrate the relevance of NFI-mediated repression of Bel1-driven transactivation *in vivo*.

Foamy viruses (FV)[1] also called spumaviruses belong to the orthoretroviruses and have exceptional properties with respect to replication, pol expression, and assembly (1, 2); for a review of the historical perspectives and the apparent apathogenicity of FV, see Refs. 3 and 4. FV are unconventional and complex retroviruses that code for transcriptional transactivators (5, 6). The viral transactivators specifically recruit components of the cellular transcription machinery to viral promoters so that cellular transcription programs are specifically affected and even reprogrammed in a way favorable for viral replication (7). A key regulator of viral gene expression, the Bel1 transactivator of the human foamy virus (HFV), recently renamed primate foamy virus) binds directly to DNA target sites with no or low sequence conservation that are located in both the 5′-long terminal repeat (LTR) and the internal promoter (IP) (8–11). The IP is a strong early promoter that is crucial for viral replication and critically dependent on Bel1 (11–13). Expression of Bel1 is initiated at the IP until threshold levels of the Bel1 transactivator are reached which subsequently turn on the 5′-LTR promoter to direct synthesis of virus structural proteins (13–15). The transactivator Tas of simian foamy virus type 1 binds to corresponding proximal and distal DNA target sites directly that are not homologous to the Bel1 response element (BRE) of the IP (16). Bell 1 was shown to be a nuclear protein (17, 18) that contains at least two domains, a centrally located DNA binding region and a C-terminal activation domain (19). Bel1 has a molecular size of 36 kDa (20) and can dimerize and multimerize (21). Alignments of Bel1 and Tas proteins from different species result in a remarkably low degree of homology of about 17–23% (22). The minimal IP.BRE of 27 bp is capable of directly binding Bel1 and partial transactivation *in vitro* compared with the full-length IP of 192 bp. High affinity DNA binding of Bel1 and full transactivation of the IP depend upon as yet unidentified cellular factors.

Previously, a Kip2-BRE DNA element located within the second exon of the human $p57^{Kip2}$ gene was identified that mediates a specific Bel1 transactivation (23). It is worth noting that the Kip2-BRE primarily consists of direct repeats of three 14 mers that partially overlap with the G base pattern conserved in both the IP.BRE and Kip2-BRE (10, 23). The Kip2-BRE was bound by proteins in nuclear extracts from 293T cells that had been transfected with Bel1 as shown by EMSA and supershift experiments. A purified glutathione *S*-transferase-Bel1 fusion protein was bound specifically to Kip2-BRE, and the formation of the Bel1-Kip2-BRE complexes was effectively blocked by the minimal viral IP.BRE oligonucleotide (10, 23). Cotransfection with Sp1 expression plasmids enhanced the Bel1-specific transactivation of Kip2-BRE to a limited extent.[2]

In the present study we aimed to identify cellular transcription factors and their DNA targets that either repress or activate Bel1-mediated transactivation of the viral internal promoter. We analyzed the ability of cellular transcription factors to affect the level of Bel1-driven transactivation with the clear focus on the core IP element from −166 to −116. Our investi-

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We dedicate this article to Harald zur Hausen on the occasion of his retirement as head of the German Cancer Research Center (DKFZ) with gratitude and appreciation.

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[1] The abbreviations used are: FV, foamy virus; BRE, Bel1 response element; EMSA, electrophoretic mobility shift assay; HFV, human foamy virus; IP, internal promoter; luc, luciferase; NFI, nuclear factor I; HA, hemagglutinin; m, mouse; h, human; LTR, long terminal repeat; Kip2, cyclin-dependent kinase inhibitor 2.

[2] K. Kido and R. M. Flügel, unpublished observation.

gations revealed that nuclear factor I (NFI) proteins negatively regulate spumaviral transactivation by binding to distinct core IP DNA target sites. Cotransfection of the infectious HFV DNA genome with a human NFI-X1 expression vector into two different human cell lines almost completely abolished viral gene expression.

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, pbel1s (24), pGL3-basic-HFV-IP (−192 to −1), pGL3-pro-IP.BRE −166 to −116 and −166 to −140 derivatives (23) were transfected into 293T cells using the coprecipitation method of Chen and Okayama (25). In general, 6 μg of plasmid DNA were transfected into 293T cells grown in 6-cm Petri dishes. The human fibrosarcoma cell line HT-1080 (ATCC number CCL-121) was propagated as described by the supplier and transfected using LipofectAMINE plus (Invitrogen).

Construction of Eukaryotic IP-based luc Reporter Plasmids—Reporter constructs containing the HFV internal promoter were constructed by PCR-mediated amplification of the defined promoter fragments. Sense primer was: IPs (−192 to −173), 5′-CGTCATGCTTTGGACTGGAC-3′; the antisense primer was: IPas (−1 to −22), 5′-GATAGATCTCAGCTTTTGCTCTTTCAATCTG-3′. PCR reactions using sense primer IPs and antisense primer IPas were done with *Pfu* polymerase (Stratagene) and pHSRV13 (12). PCR reactions were carried out with the buffer recommended by supplier supplemented for 1 min at 94 °C, 1 min at 56 °C, 2 min at 72 °C for 35 cycles. The resulting blunt-ended PCR amplicon was digested with *Bgl*II and inserted into the *Bgl*II- and *Ecl*136II-digested reporter plasmid pGL3 basic (Promega). The resulting reporter construct was designated pGL3-basic-HFV-IP. Separately, pGL3-promoter plasmids were constructed. The core IP.BRE (−166 to −116), 5′-AGGCCACTGGTTGCGGAAGAAAGATTGAGCTTTGAGCCACGACTGCCAA-3′ (or the IP.BRE from −166 to −140) was mixed with the corresponding antisense oligodeoxynucleotides, heat-denatured, annealed, and inserted into *Ecl*136II-digested luc reporter plasmid pGL3-promoter in the sense orientation. These plasmids were designated pGL3-pro-IP.BRE-166 to −116 or −160 to −140, respectively.

EMSAs, Supershifts, and Competition EMSAs—EMSA experiments were performed according to Soto *et al.* (26, 27) and modified as described below. The probes used for EMSAs included the IP.BRE (Fig. 1*B*, line 3). The oligodeoxynucleotides were synthesized, annealed, and end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Biosciences) with T4 polynucleotide kinase (New England Biolabs). The labeled probe was purified by electrophoresis on a 15% polyacrylamide gel. Nuclear extracts were prepared as described (28, 29). Protein concentrations were determined with the DC protein assay (BioRad). The binding reaction was carried out with 2 μg of nuclear extracts that 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, 2.5 mM $MgCl_2$, 1 mM EDTA, 1.0 mM dithiothreitol, 1.0 mg/ml bovine serum albumin, 0.2 mM phenylmethylsulfonyl fluoride, 25 ng/μl poly(dA-dT)·poly(dA-dT) (Amersham Biosciences) as indicated. Labeled DNA probe, 20,000 cpm, were added and incubated for 30 min at 25 °C. For competition experiments, unlabeled competitor oligodeoxynucleotides were added in 1000-fold molar excess at the preincubation period (29). Polyclonal antibody against Bel1 (23) and monoclonal anti-hemagglutinin (HA) antibody (Roche Molecular Biochemicals) used in supershift assays were added at a 1:80 dilution or 5 ng/μl respectively 30 min after addition of the labeled probe and further incubated for 1 h at 4 °C. Oligodeoxynucleotides FIB2.6, (Fig. 1*B*) and AP-1, 5′-CGCTTGATGAGTCAGCCGGAA-3′ were used in competition EMSAs (30). The DNA protein complexes were resolved in a 5.5 or 6.5% non-denaturing polyacrylamide gel, dried, and exposed for 4 h or overnight to Kodak BioMax MR1 films.

luc Expression Assays—Plasmid pCMVβgal directing β-galactosidase expression from the CMV-IE promoter was used for normalization of transfection efficiency. luc reporter gene assays were performed and quantified as described (23) using a Luminoskan TL Plus luminometer (Labsystems, Frankfurt, Germany). Cells were harvested 24 h after transfection.

Immunoblotting—Cells were harvested 2 or 3 days after transfection by lysis in 1% SDS, and the protein concentration was determined using the DC protein assay (BioRad). Identical amounts of proteins were separated by SDS-PAGE, blotted, reacted with monoclonal serum directed against the HA epitope of the four different HA-tagged NFI proteins (Roche) or against hNFI-X1 protein (sc870X, Santa Crux), or polyclonal sera directed against Bel1/Bet, Gag, or RNase H proteins (23), and detected by enhanced chemiluminescence as described previously (23).

FIG. 1. **Diagram of the foamy viral internal promoter.** *A*, the internal promoter is shown from position −192 to the −1 cap site determined previously (12). The core IP.BRE from position −166 to −116 is in BOLD FACE and *underlined*; the minimal IP.BRE (from −166 to −140) of 27 bp is marked by *broken* underlining, and the IP.NFI from −142 to −116 by *solid underlining*; six predicted NFI half-sites are marked by *overlining* and *numbered*; NFI half-sites s1, s2, and s7 (identified here) are *boxed* and were analyzed in more detail (see "Results"). Locations of the TATA box and cap site are shown; the *rectangular arrow* indicates the direction of Bel1 transcription. *B*, nucleotide sequences of oligodeoxynucleotides representing NFI half-sites (*underlined*) and IP.BRE mutants used; mutated bases in NFI sites are *boxed* and in *italics*. Double-stranded oligodeoxynucleotide FIB2.6 was taken from (31).

RESULTS

Characterization of the Repressive Effects of Different NFI Family Members on the Full-length Viral Internal Promoter—The minimal HFV IP has been reported to comprise 27 bp but longer promoter regions showed higher activities when assayed by transient expression assays. In the first step of our analysis, we decided to use a longer IP that extends from −192 to the internal transcriptional start site of HFV (Fig. 1*A*). To analyze Bel1-mediated transactivation of the pGL3-HFV-IP, cotransfections with and without the eukaryotic expression plasmid pbel1s DNA were carried out. Since we had identified putative NFI binding sites within the IP, we tested the ability of NFI transcription factors to affect expression from the IP. The effect of human NFI-X1 (hNFI-X1) was determined by comparing the results of the normalized luc reporter assays in the presence and absence of hNFI-X1 expression (Fig. 2*A*). These data show that hNFI-X1 repressed Bel1-driven transactivation by about 10-fold. Since vectors expressing the products of the four known human NFI genes were not available, this analysis was

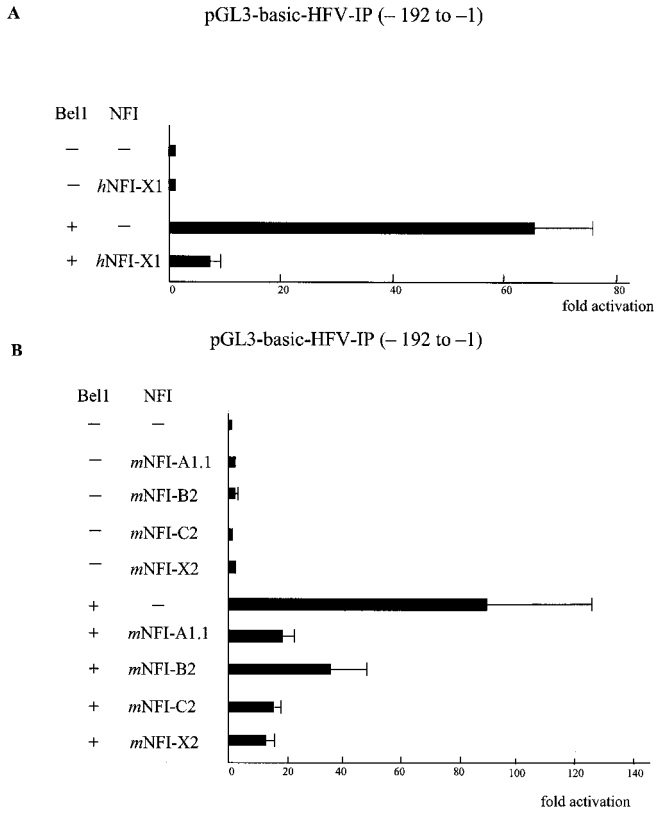


FIG. 2. **Repression of Bel1-mediated transactivation by different NFI plasmids as determined by luc reporter assays.** *A*, the pGL3-basic HFV-full-length internal promoter (HFV-IP from −192 to −1, see Fig. 1) was cotransfected with plasmids pbel1s into 293 T cells with and without human NFI-X1 (32). *B*, four different murine NFI plasmids were separately cotransfected with pbel1s; luciferase activity was determined as described previously (23). The data represent the mean ± S.D. for three experiments shown by *error bars*.

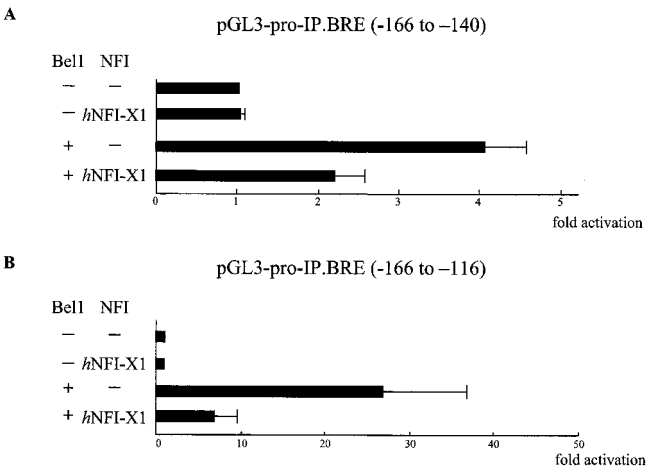


FIG. 3. **Repressive effects of Bel1-mediated transactivation by human NFI-X1 after cotransfection with pbel1s and two pGL3-pro-IP.BRE plasmids of different promoter/enhancer lengths as determined by luc assays.** *A*, the pGL3-pro-IP.BRE plasmid from −166 to −140 was cotransfected with the Bel1 expression plasmid pbel1s into 293T cells with and without hNFI-X1 (32). In *panel B*, the pGL3-pro-IP.BRE from −166 to −116 was used; after cotransfection with pbel1s and hNFI-X1, luciferase activity was measured.

extended using well characterized vectors that express products of the four murine NFI genes. The results shown in Fig. 2*B* indicated that the four different NFI proteins, NFI-A1, -B2, -C2, and -X2, each inhibited the Bel1-mediated transactivation of the full-length promoter 4- to 6-fold (*lower panel*, Fig. 2*B*). In contrast, transfection of the four different NFI family members separately or alone did not affect the basal activity of the full-length IP.BRE (*upper panel*, Fig. 2*B*).

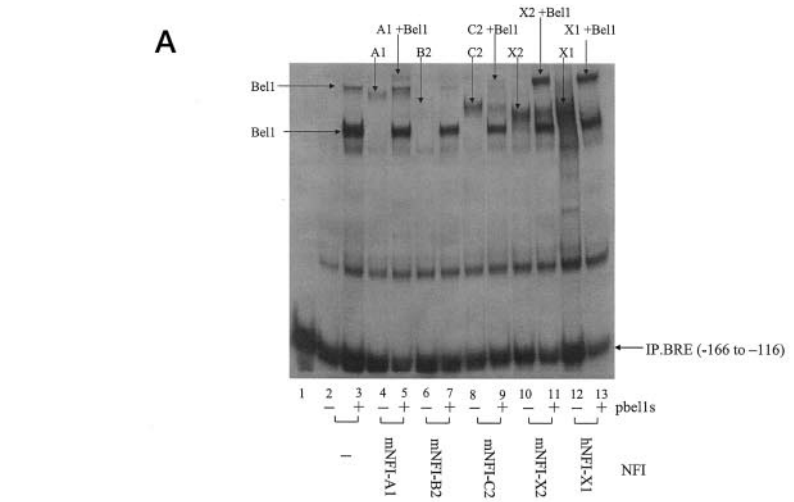
To assess expression levels of the Bel1 and NFI proteins, aliquots of nuclear extracts from transfected 293T cells were analyzed on Western blots. The results of immunoblotting showed that the expression levels of the four mouse (m) NFI, hNF-X1, and Bel1 proteins were similar under the conditions used (data not shown). This allowed us to compare the level of repression of hNFI-X1 to that of the mNFI-X2 (*bottom lines* in Fig. 2, *A* and *B*). Since this comparison showed that minor differences in repression were not due to differences in expression levels of the four mNFI and Bel1 proteins or to differences of the NFI-X protein sequences (30), we conclude that NFI-X1 and -X2 proteins from both species repressed Bel1-mediated transactivation to similar degrees.

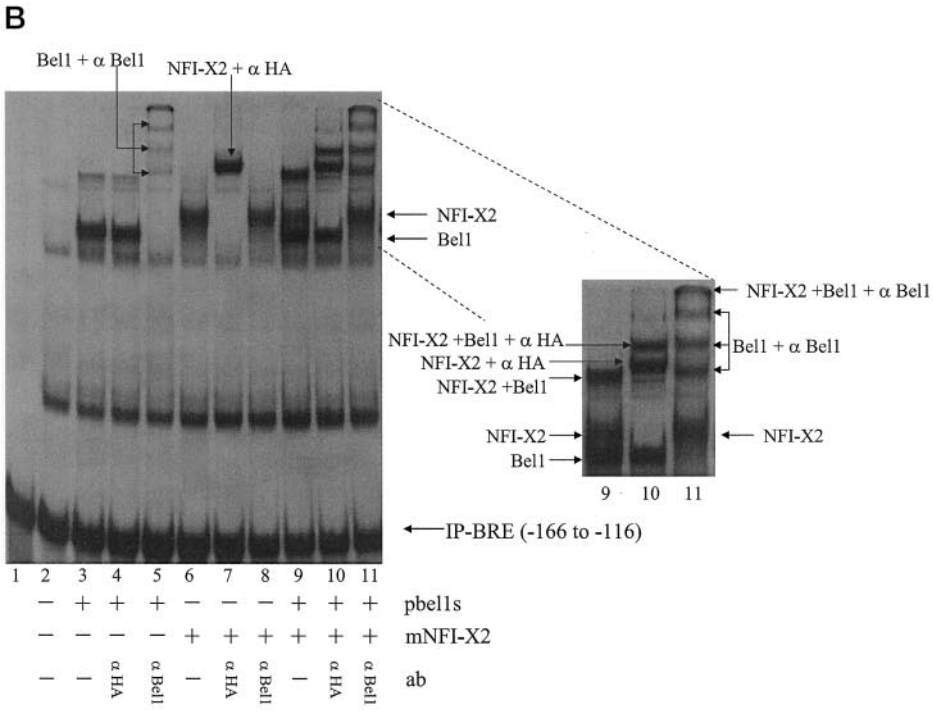
To determine more precisely the region of the IP that is responsible for the NFI-repressive effects, shorter regions of the internal promoter, two IP.BRE DNA fragments from positions −166 to −140 and −166 to −116 were cloned into the corresponding luc reporter plasmid pGL3-pro (Fig. 1*A*, *broken* and *solid underlining*, respectively). When the IP.BRE was shortened to the minimal IP.BRE of 27 bp encompassing the region from −166 to −140, the level of NFI-mediated repression of Bel1-driven transactivation decreased to a relatively very low level compared with the full-length internal promoter (Figs. 2 and 3*A*).

To further identify the region of the IP subject to repression by NFI-X, the longer core IP.BRE DNA from −166 to −116 was cloned into the pGL3-pro plasmid and cotransfected with pbel1s and phNFI-X1, and luciferase activity was subsequently determined. Bel1-dependent transactivation of the core IP was about 7-fold higher on the longer core IP.BRE compared with the minimal IP.BRE and was repressed to a much greater extent (Fig. 3*A* and *B*). When luc reporter assays were done with the core IP as template and the four mNFI plasmids, similar levels of transactivation and repression were obtained after cotransfection with Bel1 (data not shown). We reasoned that the difference in the level of repression between the two different length promoters might be due to a putative additional NFI site 2 (s2) located close to the 3′-end of the IP.BRE at position −122 to −118 (Fig. 1). As the NFI-s2 GCCAA is a perfect consensus NFI half-site, we decided to subject the core IP.BRE from −166 to −116 to a more refined analysis of the repressive effect of NFI to gain more insight into the functional role of this region.

EMSA of Nuclear Proteins from NFI-transfected Cells That Bind to NFI DNA Target Sites of the Core IP.BRE (−166 to −116)—To determine whether the longer core IP.BRE region is recognized by NFI proteins, EMSAs were carried out. Nuclear extracts from 293T cells transfected with vectors expressing NFI proteins, with or without Bel1, were prepared and incubated with the ^{32}P-labeled IP.BRE (−166 to −116) oligodeoxynucleotide. In pilot experiments, we had observed that synthetic polymers poly(dA-dT)·poly(dA-dT) instead of poly(dI-dC)·poly(dI-dC) markedly improved the detection of the different DNA-nuclear protein complexes. In nuclear extracts from Bel1-transfected cells, one intense and broad and another minor and slower migrating, Bel1-IP.BRE bands were clearly detectable (Fig. 4*A*, *lanes 3*, *5*, *7*, *9*, *11*, and *13*, *long arrow* marked Bel1). The minor Bel1-IP.BRE band of low intensity likely presents a complex consisting of Bel1, DNA, and an unknown endogenous nuclear protein migrating close to the NFI-C2-Bel1-DNA band (see below).

Distinct bands were detected in nuclear extracts of cells transfected with vectors expressing mNFI-A1, -B2, -C2, -X2, and hNFI-X1 proteins in the absence of cotransfected Bel1





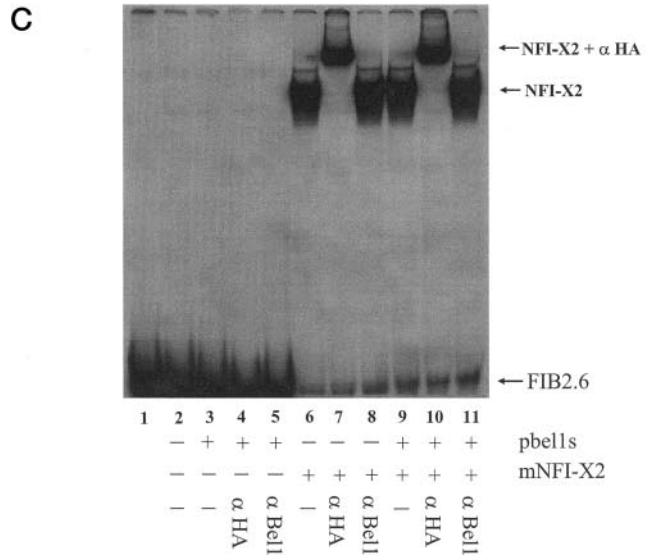


FIG. 4. **EMSA of nuclear proteins from NFI- and Bel1-transfected 293T cells that bind to DNA target sites of the core IP.BRE from −166 to −116.** *A*, the core IP.BRE oligonucleotide from −166 to −116 (see Fig. 1) was used. EMSAs were carried out with nuclear extracts from 293T cells transfected with the Bel1 expression plasmid pbel1s or cotransfected with one of five different NFI plasmids as indicated. The oligonucleotide IP.BRE probe was labeled with [γ-^{32}P]ATP and incubated with 2 μg of nuclear extract and 25 ng/μl poly(dA-dT)·poly(dA-dT) and processed as described under "Experimental Procedures." *Arrows* in *lanes 3*, *5*, *7*, *9*, *11*, and *13* mark the Bel1 protein-DNA complexes at distinct positions. *B*, supershift EMSA of the DNA protein complexes reacted with either the monoclonal antibody (anti-HA 5 ng/μl) against the HA-tagged NFI or polyclonal antiserum against Bel1 at 1:80 dilution. The core IP.BRE oligodeoxynucleotide from −166 to −116 (see Fig. 1) was used. Conditions were as described in *panel A*. Minus signs indicate absence of pbel1s or NFI-plasmids. *Inset* presents enlargement of upper part of *lanes 9–11*. *C*, the ^{32}P-labeled FIB2.6 oligonucleotide was used as probe. Nuclear extracts from cotransfected cells were used as described in *A* and supershifts were done as described in *B*.

expression plasmid (Fig. 4*A*, *lanes 4*, *6*, *8*, *10*, and *12* marked by *vertical arrows* marked *A1*, *B2*, *C2*, *X2*, and *X1*). After cotransfection with pbel1s, the four individual NFI protein-DNA complexes gave rise to an additional set of complexes with lower mobility (*vertical arrow*s in Fig. 4*A*, marked *A1*+ *Bel1*, *C2*+ *Bel1*, *X2* +*Bel1*, and *X1*+ *Bel1*). The partial shifts were concomitant with a slight decrease in intensity of the DNA-Bel1 complexes. The Bel1-DNA-NFI-X2 and -X1 complexes migrated as comparatively intense bands (*lanes 11* and *13*). In contrast, the NFI-B2-DNA complex became detectable only after longer exposure; however, a band corresponding to a Bel1-DNA-NFI-B2 complex was not observed (*lane 7*). Two additional bands observed in nuclear extracts from transfected cells are considered to be unspecific (*lane 2*) as reported previously (10, 23). These results indicate that the different NFI-A1, -C2, -X2, and -X1 proteins bound the core Bel1-IP.BRE DNA (−166 to −116) together with Bel1. To assess the presence of other potential protein-IP.BRE complexes, different conditions with varying buffers and copolymers were used. No evidence for any other complexes than those detected before and shown in Fig. 4*A* was found.

To confirm the specificity of the IP.BRE interaction with Bel1 and NFI proteins, a polyclonal Bel1-specific antibody and a monoclonal directed against the HA epitope of the four mNFI proteins was used in supershift assays (Fig. 4*B*). The intense bands of the Bel1-DNA complexes are marked by *short arrows* in *lanes 3*, *4*, *9*, and *10*. The HA and Bel1 antibodies unrelated to each other served as specificity controls (*lanes 4* and *8*). The EMSAs shown in Fig. 4*B* indicate that in the presence of nuclear extracts from 293T cells transfected with Bel1, the two Bel1-oligonucleotide complexes reacted with antibody directed against Bel1 that resulted in a complete supershift to three bands (*lane 5*, *short arrows* marked Bel1 +αBel1, Fig. 4*B*) as reported previously (10, 23). Incubation with antibody against the HA epitope located at the N terminus of the NFI-X2 protein resulted in a supershift of the original NFI-X2-oligonucleotide complex to positions marked by the *long arrow* NFI-X2+αHA (*lane 7*, *inset*, *lane 9 versus 10*).

The mobilities of the NFI-X2-DNA complexes (*lanes 6*, *8*, and *9*) were slightly slower than the Bel1-DNA complexes in *lanes 3*, *4*, *9*, and *10*). In contrast, the NFI-X2-DNA complex resulted in a partial shift to a new position after cotransfection of the Bel1 expression plasmid (*inset*, *lane 9*). Clearly, the ternary NFI-X2-DNA-Bel1 complexes were shifted by addition of either anti-HA or anti-Bel1 antibodies (*inset*, *lanes 9*, *10*, and *11*). Thus, we defined the *upper band* (*lane 9*) as a Bel1-DNA-NFI-X2 complex. The *uppermost band* in *lane 11* likely presents a DNA complex of NFI-X2 and Bel1-anti-Bel1 that migrated more slowly than the DNA complex of anti-HA-NFI-X2 and Bel1 (*lane 10*).

Similar EMSA evidence of NFI-Bel1-DNA ternary complexes was obtained when the mNFI-C2 expression was used for transfection instead of mNFI-X2 (data not shown).

To confirm the specificity of the EMSA assay and anti-HA and antiBel1 antibody supershifts, we carried out EMSA and supershift experiments with a ^{32}P-labeled FIB2.6 oligodeoxynucleotide. FIB 2.6 was reported to bind specifically to NFI proteins with very high affinity (31). The results of the binding assays in Fig. 4*C* clearly show that with nuclear extracts from 293 cells cotransfected with Bel1 alone, FIB2.6 was incapable of binding to Bel1 (*lanes 3–5*). Expression of Bel1 did not lead to any new band (*lanes 9–11*) even after reaction with Bel1-specific antibodies (*lane 11*). Both proteins, mNFI-X2 and Bel1, were expressed as confirmed by Western blotting (data not shown). The absence of any band shift in *lanes 9* and *11* indicates that direct NFI-Bel1 protein interactions are not detected.

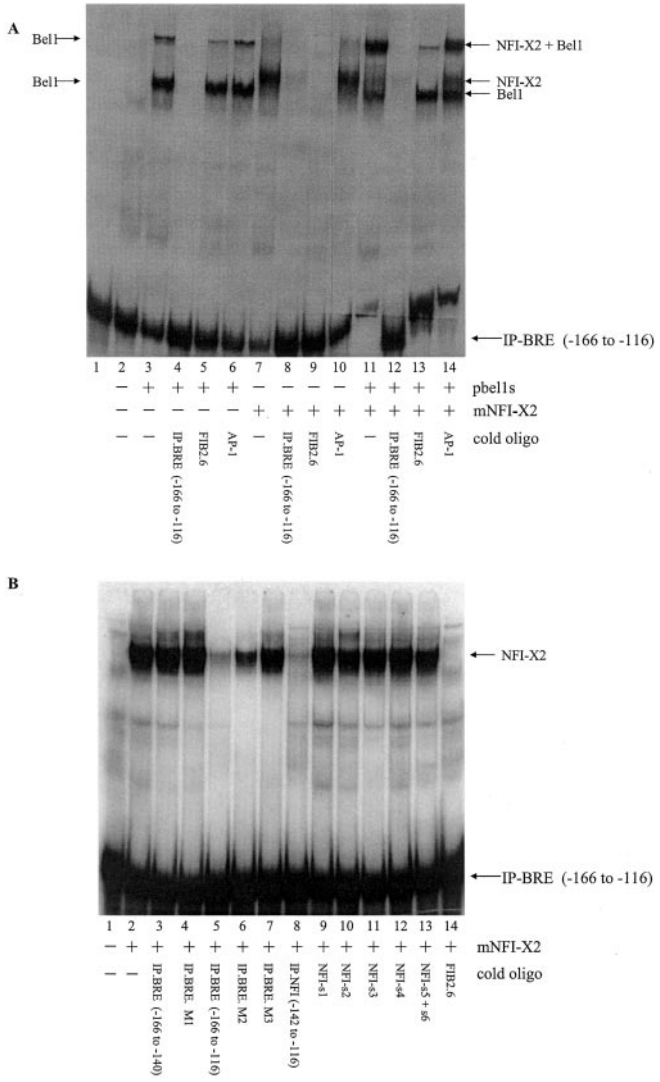


FIG. 5. **EMSA of nuclear extracts from NFI- and Bel1-transfected cells and reacted with various oligonucleotides as competitors for binding to the core IP.BRE and NFI sites.** *A*, the oligonucleotide IP.BRE −166 to −116 was labeled with [γ-^{32}P]ATP and used as probe. The probe was incubated with 2 μg of nuclear extract and 1000 molar excess of unlabeled competitors IP.BRE −166 to −116 (*lanes 4*, *8*, and *12*); the strongly NFI-binding oligonucleotide FIB2.6 (*lanes 5*, *9*, and *13*) and an unrelated AP-1 oligonucleotide (*lanes 6*, *10*, and *14*). *Arrows* mark the different DNA-nuclear protein complexes. *B*, The IP.BRE oligonucleotide from −166 to −116 was labeled with [γ-^{32}P]ATP and used as probe. Nuclear extracts from mNFI-X2-transfected 293T cells were treated as described in *A* and incubated with excess of various unlabeled oligonucleotides as competitors as indicated (sequences in Fig. 1*B*).

Competition EMSAs of Nuclear Extracts from NFI-X2-transfected Cells Reacted with Various Double-stranded Oligodeoxynucleotides as Competitors for Binding to the Core IP.BRE—To determine whether distinct oligonucleotides were able to act as competitors for the formation of the different nuclear protein-IP.BRE complexes, the following oligonucleotides were used: unlabeled core IP.BRE (−166 to −116), FIB2.6 (Fig. 1*B*) shown previously to strongly bind NFI *in vitro* (31) and an unrelated AP-1 site (sequence under "Experimental Procedures") in the presence of excess of poly(dA-dT)·poly(dA-dT). In nuclear extracts from Bel1-transfected cells one major and a minor DNA-Bel1 complex were formed (Fig. 5*A*, *lanes 3*, *5*, and *6*). The data shown in Fig. 5*A* revealed that unlabeled

core IP.BRE (−166 to −116) functioned as effective competitor for both the major and minor DNA-Bel1 complexes (*lanes 4* and *12*) almost as well as for the DNA-NFI-X2 complexes (*lanes 8* and *12*). It is noteworthy that the unlabeled 26-bp FIB2.6 oligonucleotide effectively competed for the NFI-X2-DNA complex (*lanes 9* and *13*) but not for Bel1-DNA complex (*lane 5*). Interestingly, the proposed ternary NFI-X2-Bel1-DNA complex was effectively competed out by FIB2.6 (Fig. 5*A*, *lane 13 versus lane 11*). FIB2.6 partially blocked the formation of the minor IP.BRE-Bel1 complex (*lanes 5* and *13*). These results indicate that most or all of the upper band in *lane 11* contained a NFI protein. By analogy, the modest decrease in intensity of the slowest migrating DNA-Bel1* complex suggests that an endogenous NFI protein may be part of this complex (*lanes 5* and *13* compared with *lanes 3* and *6*). These data are in agreement with the results obtained in the supershift assay (Fig. 4*B*, *lane 10*). The unrelated AP-1 oligonucleotide did not act as competitor for either of the nuclear protein-DNA complexes as anticipated. The data argue for a specific interaction of the NFI DNA binding sites s1 and s2 of the IP.BRE with NFI proteins.

In an effort to further delineate the contributions of individual NFI DNA binding sites to DNA-NFI complex formation, we used an unlabeled 27-bp-long IP.NFI DNA from −142 to −116 as competitor. In addition, three IP.BRE mutants, DNAs of five different NFI sites, and two IP.BREs were used tested for competition (for sequences, see Fig. 1, *A* and *B*). The core IP.BRE was used as labeled probe. As shown in Fig. 5*B*, *lane 6*, the core IP.BRE was capable of inhibiting formation of the DNA-NFI-X2 complex. In contrast, the minimal IP.BRE (−166–140), IP.BRE mutant 1, and oligonucleotides representing the NFI DNA binding sites s1–s6 did not block formation of the DNA-NFI-X2 complexes (*lanes 4*, *9–13 versus 3*). Intriguingly, the IP.NFI (*lane 8*) was the more effective competitor pointing to an essential role of NFI-s2 or another site that resides in the flanking sequences. A role of NFI-s2 was revealed by the ability of IP.BRE.M2 (*lane 6*) to compete with the IP.BRE. IP.BRE.M2 contains a mutation in the NFI-s2, which resulted in a detectable competition although slightly less effective than that of the native core IP.BRE (Fig. 5*B*). To more precisely determine the roles of the potential NFI sites s2 and s7, a double mutant, IP.BRE mutant 3 that contains an additional mutation at s7, was used as competitor (Fig. 1*B*). It is revealing that the double mutant had almost completely lost its ability to act as competitor (Fig. 5*B*, *lane 7*) when compared with IP.BRE −166 to −116 (*lane 5*). We conclude that the NFI-s2 and -s7 sites play more important roles than the NFI-s1 DNA binding site.

To determine the *in vivo* relevance of NFI-dependent repression, the infectious pHSRV13 DNA plasmid (12) was transfected with and without the hNFI-X1 expression plasmid into two different cell lines. Immunoblotting of cellular extracts revealed that pHSRV13-transfected cells expressed the viral structural proteins Gag and Pol (Fig. 6, *A* and *B*). The viral Gag proteins p70 and p68 migrated as a characteristic double band (marked by the *arrow* in Fig. 6*A*) as reported previously (11, 14), and their expression levels decreased upon cotransfection with the NFI-X1 expression plasmid. The known HFV Pol proteins p127 and p65 (11) were detected by an anti-RNase H serum (marked by *two arrows* in *B*). In extracts from cells cotransfected with hNFI-X1, the relative amounts of these Pol proteins were substantially decreased (as marked by *arrows* in *lanes 2*, *4*, and *6*). Importantly, the cellular lysates expressed the 36-kDa Bel1 protein after transfection with pHSRV13 as determined with an anti-Bel1/Bet serum (Fig. 6*C*, *lanes 1* and *3*).

In contrast, in cellular extracts from 293T cells cotransfected

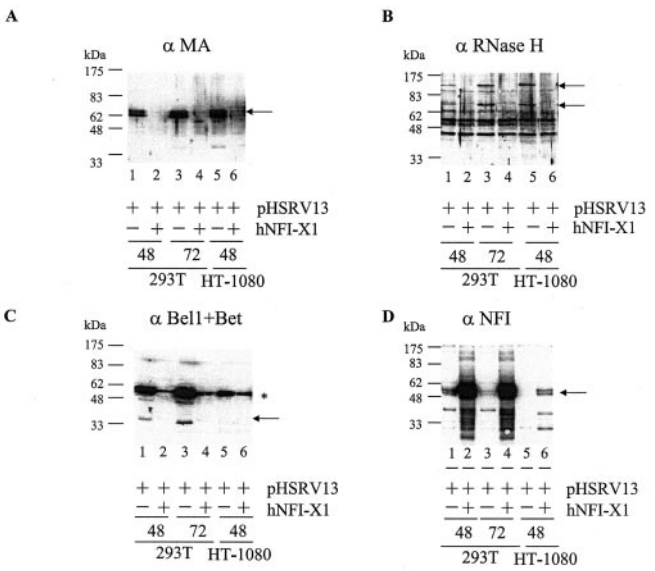


FIG. 6. **Repression of Bel1 and viral structural protein expression by transfection of the infectious pHSRV13 DNA and cotransfection with the NFI-X1 expression plasmid into two cell lines as determined by immunoblotting.** *A*, Western blot analysis of HFV Gag expression after transfection of pHSRV13 into either 293T cells (*lanes 1–4*) or HT-1080 cells (*lanes 5* and *6*). Cellular lysates were harvested after 48 or 72 h after transfection with pHSRV13 (*lanes 1–6*) or cotransfection with hNFI-X1 (*lanes 2*, *4*, and *6*) as indicated. All antisera were used at a dilution of 1:1000. Polyclonal anti-Matrix serum was reacted with the nuclear extract; *arrow* marks the characteristic double HFV Gag band. *B*, Western blotting as described in *A* except that a polyclonal anti-RNase H serum was used. *C*, Western blotting as described in *A* except that a polyclonal anti-Bel1/Bet antiserum was used; *arrow* marks the 36-kDa Bel1 protein and *asterisk* the abundantly expressed viral Bet protein of 56 kDa. *D*, Western blotting as described in *A* except that a polyclonal antibody against human NFI-X was used. *Arrow* marks the NFI-X1 proteins.

with hNFI-X1 the relative amount of Bel1 protein strongly decreased (Fig. 6*C*, compare *lanes 1* and *3* with *lanes 2* and *4*). Taken together, the results indicate that in cell cultures that overexpress NFI-X1 proteins, expression of Bel1 was downregulated in agreement with our transfection data.

DISCUSSION

The present report indicates that NFI-mediated repression of Bel1-driven transactivation and gene expression involves the interaction of NFI transcription factors with the retroviral internal promoter core IP.BRE. This report is the first that describes the identification of a cellular transcription factor that negatively regulates Bel1-mediated transactivation of the core IP.BRE *in vitro* and in cell cultures. This promoter is transactivated by Bel1, the key regulator of viral expression (7, 11–14). In transiently transfected 293T cells, different NFI proteins specifically repressed the level of Bel1-driven transactivation as assessed by luc expression assays. The level of activation and repression was dependent on the size of the promoter region. To gain insight into the mechanism of repression, we focused on the core IP.BRE region from −166 to −116 that allowed us to define the roles of NFI and Bel1 proteins in DNA binding by carrying out EMSAs, supershifts, and competition EMSAs. Several groups have reported that Bel1 binds its target sequence directly (10, 16, 23). The minimal IP.BRE of 27 bp seems to be the one with a high DNA binding affinity compared with the corresponding LTR BRE (10). Our data show that the minimal IP.BRE of 27 bp was transactivated by Bel1 to a limited extent, but it did not efficiently bind NFI proteins. Accordingly, the levels of NFI-mediated repression were relatively low when the minimal IP.BRE was used. In

contrast, the core IP.BRE possessed both higher transactivation activity and stronger NFI binding activity as assessed by luc assays and competition EMSAs. Thus, the functional and DNA binding properties of NFI are consistent for both IP.BREs. Apparently, the minimal region for optimal NFI repressor activity resides within the core IP.BRE.

It is well known that NFI proteins can act as either activators or repressors depending on the transcriptional context. The complex roles of the NFI gene products in transcription and development have been reviewed (33, 34). Expression of NFI proteins can modulate transcription by either transactivation or repression. To account for the multiple *in vivo* functions of NFI proteins, several models have been put forward (33). As NFI proteins act as dimers, the models include (i) recruitment of coactivators, corepressors, or RNA polymerase components, (ii) displacement of activators, repressors, or nucleosomes, and (iii) cooperative recruitment by specific NFI isoforms of adjacent DNA binding proteins. Here we will focus the discussion on repression. NFI proteins contain a highly conserved ~200-residue N-terminal DNA binding and dimerization domain. The C-terminal domains of the proteins harbor both repression and transactivation domains including a Pro-rich domain (33).

There are multiple potential models to address the formation of the different NFI-Bel1 complexes. The first is that IP.BRE DNA binds Bel1 and NFI-X2 proteins cooperatively so that the transcriptional factors affect each other; *i.e.* once NFI protein is bound to its DNA target site, it then affects subsequent Bel1 binding in a way that repression of the Bel1-mediated activation is achieved. This would likely require that the two binding proteins interact directly with each other. Thus, the repressive effect of NFI would be due to affects on Bel1 protein binding. However, our data provided no evidence for any direct NFI-Bel1 interaction.

An alternative hypothesis is that the core IP.BRE DNA binds the Bel1 and dimeric NFI-X2 proteins directly but independently at distinct but partially overlapping sites leading to a partial displacement of the Bel1 activator, particularly when NFI proteins were overexpressed. The presence of three different NFI half-sites within the core IP.BRE is in agreement with this assumption as it is well known that NFI proteins binds to half-sites as either homo- or heterodimers (35). However again, our data did not indicate that NFI-X2 could displace Bel1 from the full or minimal IP. BRE. A third model would predict that NFI-X2 binds to the promoter in the presence of Bel1 and recruits corepressor molecules that abolish Bel1 activation of the promoter. This model proposes as yet unidentified corepressors that NFI-X2 might recruit to the promoter. The identification of such corepressors will be an important future task. Our data also revealed that one of the DNA-Bel1 complexes might contain unknown endogenous nuclear proteins as well as endogenous NFI proteins (Fig. 5*A*). In supershift assays, this complex resulted in a partial supershift to a slowly migrating NFI-DNA-Bel1 complex indicating the composite nature of the DNA complexes and the possible presence of both activator and repressor proteins. The influence of other known or unknown cellular transcription factors on Bel1 activation remains to be determined. We can, however, rule out a role for C/EBPα that neither activated nor repressed Bel1-mediated transactivation of the IP.BRE.[3]

Further studies are needed to determine the complete complement of endogenous transcription factors that influence Bel1-mediated activation of the IP.BRE.

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REFERENCES

1. Yu, S. F., Baldwin, D. N., Gwynn, S. R., Yendapalli, S., and Linial, M. L. (1996) *Science* **271,** 1579–1582
2. Linial, M. L. (2003) *Curr. Top. Microbiol. Immunol.* **277,** in press
3. Meyering, C. D., and Linial, M. L. (2001) *Clin. Microbiol. Re.* **14,** 165–176
4. Heneine, W., Switzer, W. M., Sandstrom, P., Brown, J., Vedapuri, S., Schable, C. A., Khan, A. S., Lerche, N. W. Schweizer, M., Neumann-Haefelin, D., Chapman, L. E., and Folks, T. M. (1998) *Nat. Med.* **4,** 403–407
5. Linial, M. L. (1999) *J. Virol.* **73,** 1747–1755
6. Cullen, B. C. (1991) *J. Virol.* **65,** 1053–1056
7. Coffin, J. M., Hughes, S. H., and Varmus, H. E. (1997) *Retroviruses*, pp. 225–230, Cold Spring Harbor Laboratory Press, New York
8. Rethwilm, A. (1995) *Curr. Top. Micobiol. Immunol.* **195,** 1–24
9. He, F., Blair, W. S., Fukushima, J., and Cullen, B. C. (1996) *J. Virol.* **70,** 3902–3908
10. Kang, Y., Blair, W. S., and Cullen, B. R. (1998) *J. Virol.* **72,** 504–511
11. Löchelt, M., and Flügel, R. M. (1995) in *The Retroviridae* (Levy, J. A., ed) pp. 239–292, Plenum Press, New York
12. Löchelt, M., Muranyi, W., and Flügel, R. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7317–732113
13. Löchelt, M., Flügel, R. M., and Aboud, M. (1994) *J. Virol.* **68,** 638–645
14. Löchelt, M., Yu, S. F., Linial, M., and Flügel, R. M. (1995) *Virology* **206,** 601–610
15. Meyering, C. D., Rubio, C., May, C., and Linial, M. L. (2001) *J. Virol.* **75,** 6547–6557
16. Zou, J. X., and Luciw, P. A. (1996) *Proc. Natl. Acad. Sci.* **93,** 326–330
17. Keller, A., Partin, K. M., Löchelt, M., Bannert, H., Flügel, R. M., and Cullen, B. C. (1991) *J. Virol.* **65,** 2589–2594
18. Bodem, J., Löchelt, M., Zemba, M., and Flügel, R. M., (1998) *Virology* **251,** 22–27
19. He, F., Sun, S. D., Garrett, E. D., and Cullen, B. C. (1993) *J. Virol.* **67,** 1896–1904
20. Löchelt, M., Zentgraf, H., and Flügel, R. M. (1991) *Virology* **184,** 43–54
21. Chang, J., Lee, K. J., Jang, K. L., Lee, E. K., Baek, G. H., and Sung, Y. C. (1995) *J. Virol.* **69,** 801–808
22. Winkler, I., Bodem, J., Haas, L., Zemba, M., Delius, H., Flower, R., Flügel, R. M., and Löchelt, M. (1997) *J. Virol.* **71,** 6727–6741
23. Kido, K., Doerks, A., Löchelt, M., and Flügel, R. M. (2002) *J. Biol. Chem.* **277,** 12032–12039
24. Venkatesh, L. K., Yang, C., Theodorakis, P. A., and Chinnadurai, G. (1993) *J. Virol.* **67,** 161–169
25. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
26. Goyal, N., Knox, J., Gronostajski, R. M. (1990) *Mol. Cell. Biol.* **10,** 1041–1048
27. Soto, U., Das, B. C., Lengert, M., Finzer, P., zur Hausen, H., and Rösl, F. (1999) *Oncogene* **18,** 3187–3198
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419–6420
30. Chaudhry, A. Z., Vitullo, A. D., and Gronostajstaski, R. M. (1999) *J. Biol. Chem.* **274,** 7072–7081
31. Gronostajski, R. M. (1986) *Nucleic Acids Res.* **14,** 9117–9131
32. Apt, D. Liu, Y., and Bernhard, H.-U. (1994) *Nucleic Acids Res.* **22,** 3825–3833
33. Gronostajski, R. M. (2000) *Gene* **249,** 31–43
34. Chaudhry, A. Z., Lyons, G., and Gronostajstaski, R. M. (1997) *Dev. Dyn.* **208,** 313–325
35. Gounari, F., De Francesco, R., Schmitt, J., van der Vliet, P. C., Cortese, R., and Stunnenberg H. (1990) *EMBO J.* **9,** 559–566

[3] K. Kido, H. Bannert, R. M. Gronostajski, and R. M. Flügel, unpublished observations.