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Identification and Characterization of a Novel GGA/C-Binding Protein, GBP-i, That Is Rapidly Inducible by Cytokines

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Immunosuppressive states with accompanying alterations in cytokine profiles have been postulated to play a vital role in the reactivation of viruses from latency. Cytokines regulate gene expression by activating transcription factors via well-characterized signal transduction pathways. In this study, we report the identification of a novel inducible protein, GBP-i, that binds to a double-stranded GGA/C-rich region of the transcriptional control region of the human papovavirus JC virus (JCV), specifically within the origin of viral DNA replication. GBP-i is distinct from previously characterized GC-box-binding proteins with respect to both its sequence specificity and its electrophoretic mobility on native and denaturing gels. GBP-i responds within 90 min to phorbol myristate acetate stimulation; however, unlike typical phorbol myristate acetate-inducible factors, this rapid induction is regulated primarily at the transcriptional level. Further, the induction of GBP-i appears to be widespread and mediated by many inflammatory cytokines, including interleukin-1β, tumor necrosis factor alpha, gamma interferon, and transforming growth factor β. Interestingly, the induced protein acts as a transcriptional repressor in its native context in the JCV_L promoter. However, when its binding sequence is transposed to a heterologous promoter, GBP-i appears to function as a transcriptional activator. The data presented here suggest a role for GBP-i in cytokine-mediated induction of viral and cellular genes.

JC virus (JCV) is a common neurotropic polyomavirus and is the etiological agent of the human demyelinating disease progressive multifocal leukoencephalopathy (PML) (34, 35). The clinical and pathological features of PML are attributed to the lytic viral infection of oligodendroglia, the myelin-producing cells of the central nervous system (reviewed in references 19 and 30).

Clinically, patients with PML exhibit an underlying state of immunodeficiency caused by neoplasia, chronic diseases, chemotherapy, or, more recently, AIDS (3, 9, 21, 31, 33). Immunosuppressive states represent a dysfunction of immune regulation and are thought to reflect an alteration in the relative levels of cytokines (42). Indeed, changes in the cytokine profile have been detected in human immunodeficiency virus (HIV)-positive patients during their progression to AIDS (14, 38). Analyses of cerebrospinal fluid from patients with AIDS have shown an elevation in the levels of interleukin-1β (IL-1β) and IL-6 but no changes in the level of IL-2 (20).

Cytokines can have a profound effect on the viral lytic cycle, via regulation of gene expression and viral replication (15, 17, 32, 36, 49). The best-characterized effector system involves the inducible transcriptional activator nuclear factor kappa B (NF-κB) (5–7). NF-κB represents a family of transcription factors defined by the ability to bind a well-defined DNA sequence, 5′-GGGACTTTCC-3′. The ability to activate NF-κB allows many cytokines, including tumor necrosis factor alpha/beta (TNF-α/β) and IL-1α/β, to modulate gene expression from several cellular and viral promoters.

JCV offers an excellent model system to study the effect of cytokines on the viral lytic cycle. Seroepidemiological studies indicate that a majority of the population (greater than 70 to 80%) become infected with the virus, generally during childhood (34, 46, 51). Once the individual is infected, the virus establishes a subclinical latency and is reactivated only in the context of chronic cellular immunosuppression, as documented for AIDS. In fact, PML shows a higher incidence in patients with AIDS than in those with other immunosuppressive conditions (30). The more pronounced impairment of cell-mediated immunity and/or an interaction between HIV and JCV may account for the increased frequency of PML in patients with AIDS.

Previously, we demonstrated that the HIV type 1 (HIV-1)-encoded *trans*-regulatory protein Tat is a potent activator of JCV late-gene expression (13, 45). Tat responsiveness is mediated by two distinct regions within the JCV late promoter that are located on either side of the transcription initiation start site (11). Tat directly interacts with the RNA transcript containing the downstream region, in a manner analogous to its interaction with HIV TAR (12). However, the mechanism of Tat-mediated activation from the upstream region is not clear. Tat does not bind directly to this region at the level of DNA or RNA (28a). It is suspected that Tat may mediate its effects on the upstream region indirectly, via an alteration in the expression of cellular genes. Tat has been previously demonstrated to increase the expression of several cytokines, including TNF-β, IL-6, and TGF-β (10, 15), which in turn have been shown to have profound effects on gene expression (15, 17). We hypothesized that the Tat-responsive upstream region would respond to cytokines and other activators of specific signal transduction pathways.

The upstream Tat-responsive region is also of particular interest to researchers studying the JCV lytic cycle, because it lies within the origin of DNA replication and overlaps with a viral T-antigen-binding site (Fig. 1). The upstream responsive region corresponds to nucleotide positions 5112 and +4 of the JCV genome and contains a GGA/C-rich sequence (GRS) (Fig. 1) (18). In this paper, we report the identification and

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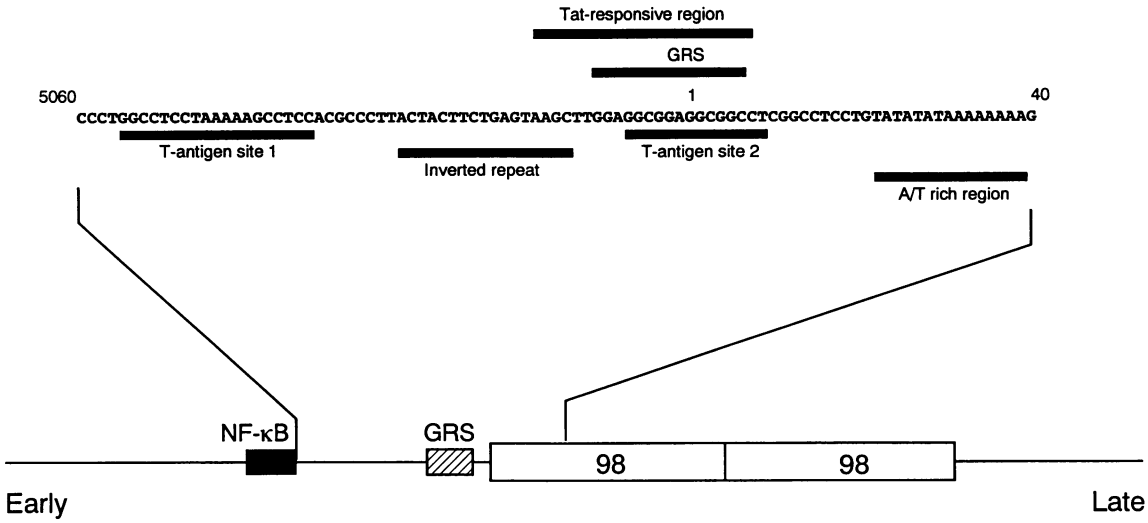

FIG. 1. JCV origin of DNA replication. The schematic depicts the structure of the JCV transcriptional control region, with its characteristic 98-bp repeats, the origin of DNA replication, and the NF-κB element. The highlighted sequence details the spatial relationship of several functional regions on the origin, including two T-antigen-binding sites, the HIV-1 Tat-responsive region, and the GRS. The numbers above the sequence reflect positions on the viral genome.

preliminary characterization of a novel inducible protein (GGA/C binding protein), GBP-i, that interacts with the GRS of the JCV regulatory region. GBP-i appears to function as a transcription factor, which reduces the activity of the JCV_L promoter. Interestingly, GBP-i appears to function as a transcriptional activator when its binding site is introduced into the context of a heterologous promoter. The induction of GBP-i is mediated by many cytokines in a wide spectrum of cells and may play a role in the reactivation of the virus from latency.

MATERIALS AND METHODS

Cells and tissue culture. The cell lines were all maintained in either RPMI 1640 or Dulbecco's minimal essential medium, supplemented with 10% (vol/vol) fetal calf serum (Gibco) and the antibiotics penicillin and/or streptomycin. U-87MG is a human glioblastoma cell line of astrocytic lineage (ATCC HTB14) that has been shown to support JCV replication.

Transfections and CAT assays. Transient-transfection assays were carried out by the calcium phosphate method, as previously described (23). Briefly, 5×10^5 cells were plated on a 60-mm plate and grown overnight. At 3 h prior to transfection, the cells were fed with new growth media. Transfections were carried out with 1 μg of reporter plasmids along with 19 μg of salmon sperm DNA. The precipitate was removed after 3 to 5 h and a glycerol shock was applied. At 24 h posttransfection, fresh medium was added to all plates; certain plates received phorbol myristate acetate (PMA) at a concentration of 50 ng/ml and were stimulated for the indicated period. At 48 h posttransfection, the cells were harvested, and a crude protein extract was made by repeated freeze-thaw cycles. The extracts were quantitated by the Bio-Rad Bradford assay, and equal amounts of protein were assayed for chloramphenicol acetyltransferase (CAT) activity. The fold transactivation was measured by scintillation counting of the spots cut from the thin-layer chromatography plate. Each experiment was repeated four or more times with different plasmid preparations. Transfection efficiency was monitored with a Rous sarcoma virus β-galactosidase control plasmid.

Nuclear extract preparation. Nuclear extracts were prepared by a modification of the miniextract protocol, as described by Schreiber et al. (41). These extracts were tested and found to exhibit a comparable binding activity to those prepared by the method of Dignam et al. (16). Briefly, 1 million cells were stimulated with PMA, at a concentration of 75 ng/ml, for the indicated periods. The cells were trypsinized, collected, washed once with medium and twice with phosphate-buffered saline, and transferred to an Eppendorf tube. The cells were then gently resuspended in cold hypotonic buffer [10 mM *N*-2-hydroxyethylpipezine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride] and allowed to swell on ice. Nonidet P-40 was added to a final concentration of 0.5% (vol/vol), and lysis was accomplished by vigorous vortexing. The nuclei were pelleted by centrifugation at 10,000 × *g*, resuspended in cold extraction buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and extracted at 4°C for 15 min on a rocking platform. The nuclear extract was centrifuged, and the supernatant was aliquoted and frozen at −70°C.

Activators and inhibitors. Optimal concentrations were determined for each of the following activators and inhibitors, as measured by the ability to increase or decrease PMA-induced GBP-i binding as well as by their effect on cell viability. Concentrations of the various inhibitors include 50 nM caphositin C, 4 μg of α-amanitin per ml, 20 μg of actinomycin D per ml, 100 μg of cycloheximide per ml, 6 μM H7, 100 μg of anisomycin per ml, 10 μM indomethacin, and 10 μM ibuprofen. Endogenous activators used include recombinant IL-2 (rIL-2; 10 to 10,000 U/ml), rIL-1α (1 to 10 U/ml), rIL-1β (1 U/ml), TNF-α (1 U/ml), TGF-β1 (0.1 ng/ml), TGF-β2 (1 ng/ml), gamma interferon (IFN-γ; 10 to 100 U/ml), and granulocyte-macrophage colony-stimulating factor (10 to 20 ng/ml). Exogenous activators include diacylglycerol (2 μM), phytohemagglutinin (5 to 10 μg/ml), concanavalin A (10 to 25 μg/ml), forskolin (1 to 2 μM), and phorbol dibutyrate (100 μM). In each case, glial cells were pretreated with the inhibitors for 45 min and then treated with PMA in the presence of

inhibitors for 45 min, unless otherwise described. Each set of experiments also contained untreated and PMA-treated samples in the absence of inhibitors as negative and positive controls of GBP-i induction. Initial experiments were also performed to test the ability of each inhibitor to induce GBP-i binding.

DNA-protein interactions. Oligonucleotides were prepared commercially by Oligos Etc., Guilford, Conn. The procedure for the band shift assay is essentially as previously described (47). GRS probes were end labeled with γ-^{32}P by using T4 polynucleotide kinase and then gel purified. A 100,000-cpm level of labeled GRS probe was incubated with 5 μg of nuclear extracts in a binding buffer containing 1.0 μg of the nonspecific competitor poly(dI-dC), 5 mM dGTP, 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, and 0.8 mM dithiothreitol. The reaction mixture was incubated at 4°C for 30 min to allow assembly of DNA-protein complexes. The complexes were resolved by electrophoresis on a low-ionic-strength (0.5× Tris-borate-EDTA [TBE]) 6% native polyacrylamide gel. The gel was then dried, and the complexes were detected by autoradiography at −70°C, with an intensifying screen. For the competition experiments, the extracts were preincubated at 4°C with the unlabeled competitor oligonucleotide for 10 min, before addition of the probe. The sequences of the various competitors from the promoters of HIV, myelin basic protein, and JCV are described below.

For the UV cross-linking assays, bromodeoxyuridine-incorporated probes were prepared as previously described (11). Reaction mixtures were set up with a fivefold increase in all components. Initial incubation was carried out at 4°C for 30 min, and then the reaction mixture was spotted onto Parafilm and exposed to UV light (254 nm) for 20 min. The cross-linked complexes were resolved on a 6% native 0.5× TBE gel and detected by autoradiography at 4°C. To analyze the molecular weight of each complex obtained in the electrophoretic mobility shift assay, gel slices corresponding to each complex were excised from the gel, weighed, and eluted with 2× sodium dodecyl sulfate (SDS) sample buffer for several hours at 37°C. The gel slice and buffer were then loaded on an SDS–12% polyacrylamide gel, electrophoresis was carried out at 60 V overnight, and the gel was fixed in 50% methanol–10% acetic acid, dried, and exposed for autoradiography.

Methylation interference assays. End-labeled oligonucleotides were alkylated with dimethyl sulfate as previously described (43). Binding-reaction mixtures were scaled up fivefold, with 500,000 cpm of the modified probe. The mobility shift assay was carried out as described above, and the wet gel was exposed overnight. Free and bound oligonucleotides corresponding to the GBP-i and GBP-b/c complexes were isolated from the gel and cleaved for 45 min at 95°C in 1 M piperidine (Sigma). Following four rounds of lyophilization, the products were electrophoresed and resolved on an 18% denaturing polyacrylamide gel.

Plasmid constructions. The construction of the pBLCAT$_2$ plasmids with the various GRS oligonucleotides cloned upstream of the *tk* promoter and the bacterial chloramphenicol acetyltransferase (CAT) gene has been described previously (11, 22).

Site-directed mutagenesis. PCR was used to generate specific site-directed mutations in the context of the viral late promoter. The mutation was based on the methylation interference data, and it targeted GGA/C motifs. One primer contains extensive mutations in a 12-bp region encompassing the GRS site followed by a 19-bp region of homology (underlined) with the 5′ end of the JCV$_L$ construct (5128 to +17): 5′-TTAAC TGGTC CACGG CCTCG GCCTC CTGTA T-3′.

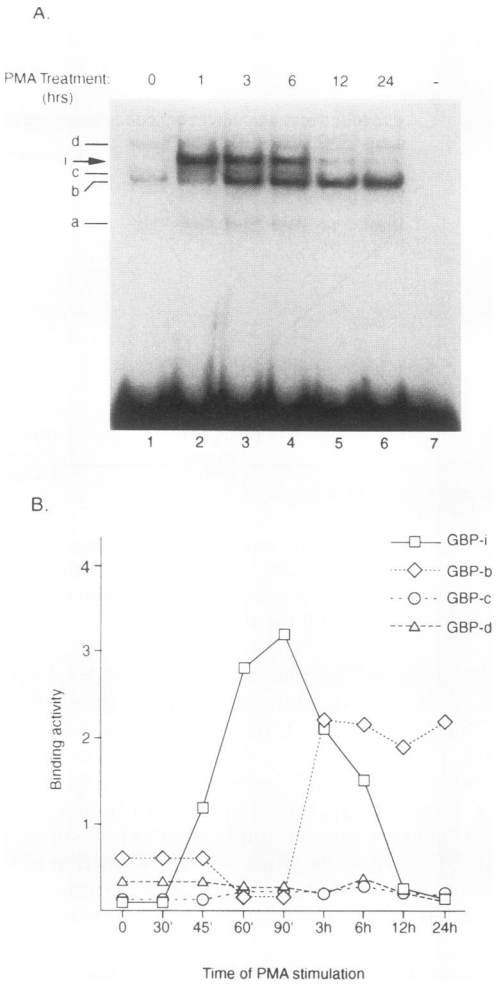

FIG. 2. Identification of an inducible GRS-binding complex. (A) A 5-mg portion of nuclear extracts derived from untreated (lane 1) and PMA-treated (lanes 2 to 6) glial cells were incubated with end-labeled double-stranded GRS oligonucleotide and then examined for binding by electrophoretic mobility shift assays, as described in Materials and Methods. The numbers above the lanes represent the various periods of PMA treatment before the extracts were prepared. The letters on the left denote designations for the different GBPs. (B) Specific bands corresponding to the various complexes were excised from a wet gel and quantitated by a scintillation counter. Binding activity is represented in arbitrary units shown on the *y* axis, and the time of PMA stimulation is shown on the *x* axis.

The second primer is a 19-bp oligomer which bears complete homology (underlined) to a region on the 3′ end of the late promoter construct and contains a *Bam*HI site: 5′-GGCTC GCAAA ACATG TTCC-3′. PCR was carried out with 10 ng of each primer and 100 pg of the JCV$_L$ template, in the presence of 50 mM $MgCl_2$. The 251-bp PCR product was directly ligated into a PCR-II vector (Invitrogen) and then transformed with blue-white selection. Selected clones were digested with the restriction enzymes *Hin*dIII and *Bam*HI; the 298-bp excised product was gel purified and ligated into a similarly digested pJCV$_L$ΔkB construct. This final construct, a pJCV$_L$ΔkB construct with a mutation in the GRS-binding site, was verified by sequencing.

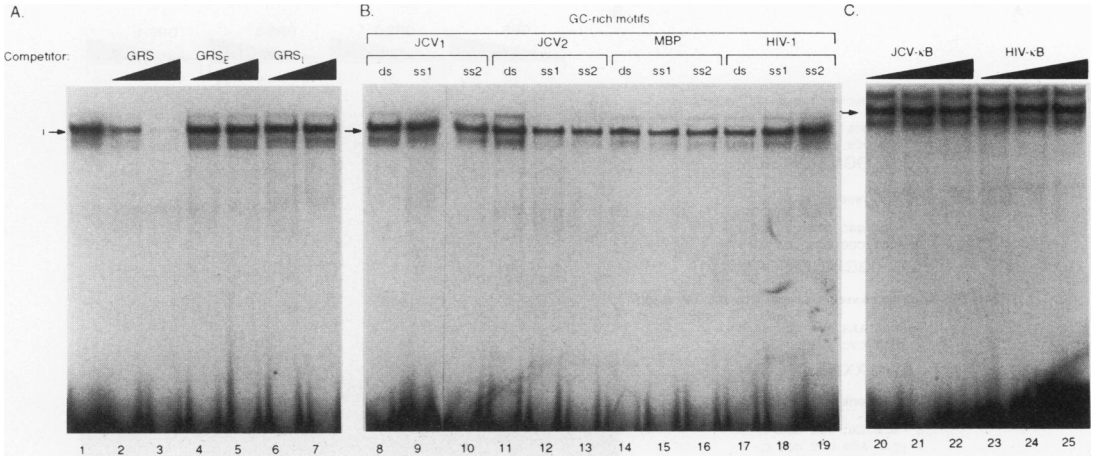

FIG. 3. Specificity of GBP-i interaction with the GRS. Competition mobility shift assays with PMA-treated nuclear extracts and labeled GRS probe were performed in the presence of cold unlabeled competitor, as described in Materials and Methods. (A) The binding specificity of the complexes was examined by competition with either 0 (lane 1) or 5 or 20 ng of homologous double-stranded GRS (lanes 2 and 3), as well as single-stranded GRS in early (lanes 4 and 5) and late (lanes 6 and 7) orientations. For comparison, the amount of labeled GRE probe is estimated at 100 pg per reaction. (B) Sequence specificity was examined by competition with 100 ng of single- and double-stranded G+C-rich motifs from JCV, myelin basic protein (MBP), and HIV promoters (lanes 8 to 19) with the following sequences: JCV1 ss1, 5′-GAAAGGGAAGGGATGG-3′; JCV2 ss1, 5′-TCGACGATGGCTGCCAGCCAAGCATGAT-3′; myelin basic protein ss1, 5′-AGGGAGGACAACACCTTCAAAGACAGGC CCTCTGA-3′; HIV ss1, 5′-CAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGGCGAG-3′. (C) Competition assays were performed to examine the relationship of GBPs to another PMA-responsive element, NF-κB. Three concentrations of consensus NF-κB-binding sites from JCV and HIV were used (lanes 20 to 25).

RESULTS

An inducible complex binds to the GRS. Since cytokines are postulated to mediate viral reactivation from latency, we investigated the possibility that cytokines could alter the binding of transcription factors to the control region of the JCV promoter spanning the GGA/C-rich element (GRS). The multipotent activator PMA, which has been shown to stimulate cellular secretion of cytokines, was used in our initial studies (32, 49). Nuclear extracts prepared from astrocytic glial cells, treated for different periods with PMA, were tested by electrophoretic mobility shift assays for binding to double-stranded oligonucleotides containing GRS.

A dramatic profile of PMA induction is seen, with the rapid appearance of a novel binding activity, GBP-i. This activity is undetectable in the unstimulated extract (Fig. 2A, lane 1) but is substantially induced within 1 h of PMA stimulation (lane 2). The induced binding activity remains at an elevated level for several hours but declines over longer periods (12 and 24 h) (lanes 2 to 6). The GBP-i activity remains above the basal level, even after 24 to 48 h of PMA stimulation. A more-detailed time course analysis reveals that the GBP-i activity begins to appear at 45 min after PMA treatment and peaks around 90 min, after which gradual decline in the binding activity is observed (Fig. 2B). Interestingly, cytoplasmic extracts prepared from glial cells stimulated for the same periods of PMA stimulation show a less dramatic yet similar induction profile in GBP-i binding (data not shown).

The uninduced glial extract shows four distinct complexes, labeled GRS-binding protein a (GBP-a), GBP-b/c, and GBP-d (Fig. 2A, lane 1). Complexes GBP-d and GBP-b/c represent 90- and 47- to 51-kDa binding activities, respectively, as previously reported, while GBP-a represents a variable, nonspecific DNA-binding activity (11). Interestingly, the GBP-b complex shows distinct induction kinetics, with an increase in binding activity at 3 h, that is maintained for the length of PMA stimulation (Fig. 2B). This biphasic pattern of induction of GRS-binding activity mirrors the induction profile of NF-κB-binding activity in the same glial cells (47). Further, no significant and reproducible change is observed with the formation of the other GBP complexes upon PMA induction.

Additionally, several oligonucleotides from the control region of the JCV promoter were tested for differences in DNA binding between untreated and PMA-treated extracts. As previously reported, the NF-κB-binding site showed an induced complex upon PMA treatment (39). However, no site on the 98-bp repeat of the JCV promoter showed any differences in binding upon PMA treatment for the same time intervals (data not shown). These data indicate that specific nucleoprotein complexes are induced upon PMA treatment.

GBP-i binds specifically to the GRS. PMA interacts with and stimulates the activity of many transcription factors. Therefore, we rigorously tested the specificity of the induced GBP-i-binding activity in the presence of excess unlabeled competitor oligonucleotides in mobility shift assays (Fig. 3A). As expected, the homologous competitor GRS was able to abolish binding of all the GBPs to the labeled GRS oligonucleotide (Fig. 3A, lanes 2 and 3). Single-stranded competitors in either orientation failed to inhibit the formation of any of the complexes (lanes 4 to 7), indicating that the GBP-i binds specifically to double-stranded DNA. Heterologous single-stranded and double-stranded competitors from G+C-rich regions of the promoters of JCV, myelin basic protein, or HIV failed to efficiently inhibit the binding of GBP-i (Fig. 3B, lanes 8 to 19). The uninduced and induced binding activities appear to share different sequence-specific characteristics, as evidenced by the ability of some but not all G+C-rich competitors from JCV and myelin basic protein (Fig. 3B, compare lanes 12 to 17 with lane 11) to inhibit GBP-b/c and GBP-d but not GBP-i binding. These competitors also represent established binding sites for distinct single-stranded and double stranded GC-binding transcription factors. The inability of these competitors to interfere with GBP-i binding also reflects their distinction from GBP-i.

Further competition experiments revealed that neither the induced nor the uninduced complexes were capable of binding

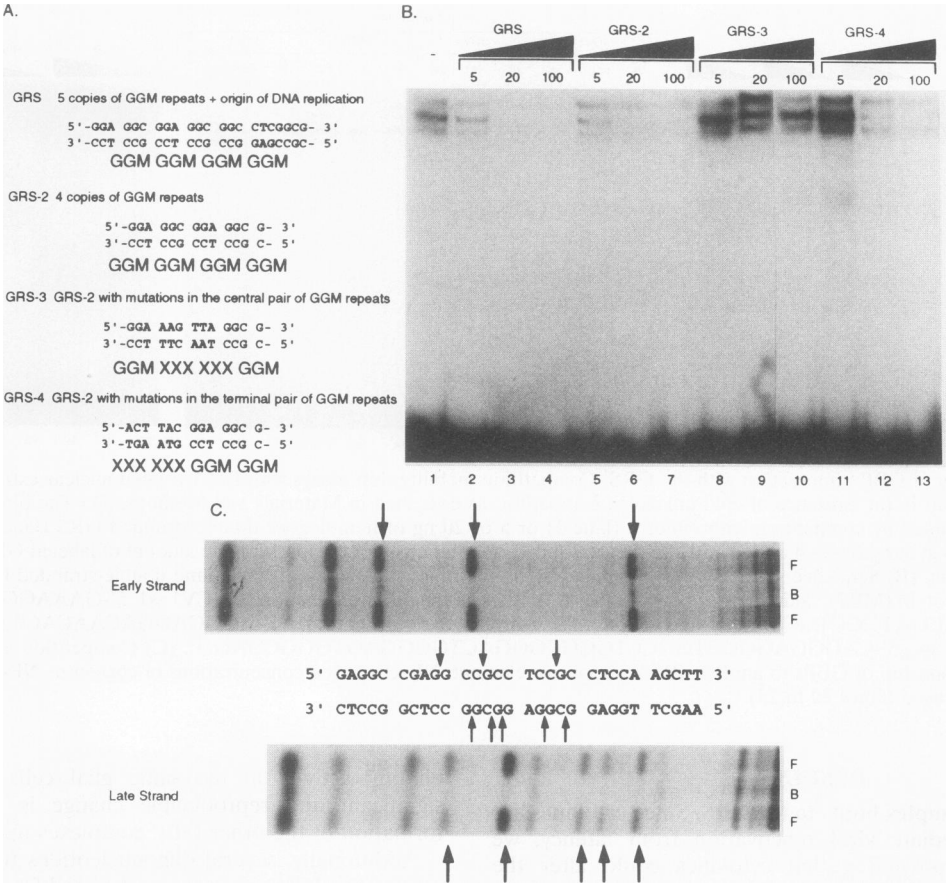

FIG. 4. Identification of the specific binding site for GBP-i. (A) Sequences of GRS mutant oligonucleotides (M is an IUB nomenclature for A or C). (B) Competition mobility shift assays were performed in the presence of three concentrations (5, 20, and 100 ng) of the homologous and various GRS mutant oligonucleotides. (C) Methylation interference assays were performed to identify the consensus binding site of the GBP-i complex. The early strand (top) and late strand (bottom) of the GRS were end labeled and methylated, and DNA-binding sites were identified after piperidine cleavage, as described in Materials and Methods. The arrows indicate protected residues seen in lane B (bound) when compared with lane F (free probe). The sequence of the GRS probe with its protected residues is shown in the middle panel.

to either of two consensus NF-κB sequences, one found in the HIV enhancer (5′-GGGACTTTCC-3′) and the other found in the JCV enhancer (5′-GGGAATTTCC-3′) (Fig. 3C, lanes 20 to 25). These data suggest that the induced complex is not a member of the NF-κB class of transcription factors, which are also induced by phorbol esters.

Binding of the GBPs requires at least two adjacent GGA/C sequences. Further characterization of GBP-i may elucidate its role in PMA responsiveness. The initial analysis involved identification of the minimal GBP-i-binding region within the GRS. GRS contains 22 nucleotides from the JCV promoter, including five copies of the GGA/C repeats and an additional seven nucleotides (Fig. 4A). GRS, as demonstrated above, efficiently inhibits the binding of the GBP complexes (Fig. 4B, lanes 2 to 4). An oligonucleotide, GRS-2, corresponding to residues 5115 to 5128 on the JCV genome, containing only four of the five GGA/C repeats (Fig. 4A), was able to inhibit the binding of the different GBP complexes to the GRS probe (Fig. 4B, lanes 5 to 7). The data suggest that the GBPs bind to the GGA/C repeats.

To define the minimal number of GGA/C repeats required for GBP binding, two additional oligonucleotides with point mutations altering distinct GGA/C repeats were used in the competition mobility shift assay. The GRS-3 oligonucleotide, which has alterations in the central pair of GGA/C repeats and individually isolates two flanking GGA/C motifs (Fig. 4A), fails to inhibit GBP-i binding to GRS, even at a 1,000-fold excess concentration (Fig. 4B, lanes 8 to 10). The uninduced but not the induced GBP complexes are inhibited by high concentrations of GRS-3 oligonucleotide, indicating a higher specificity of interaction between the induced complex and the DNA (lane 10). Interestingly, the GRS-4 oligonucleotide is able to inhibit the GBP complexes, although at a lower efficiency than GRS (lanes 11 to 13). The GRS-4 oligonucleotide exhibits alterations in the proximal two GGA/C repeats and leaves a distal GGAGGCG motif intact (Fig. 4C). The ability of GRS-4 but not GRS-3 to inhibit the induced complex suggests that the minimal contact site for GBP-i binding is two adjacent GGA/C repeats. Further, the lower efficiency of the GRS-4 inhibition indicates that sequences additional to the two adjacent GGA/C repeats may be required for optimal GBP-i binding.

To define the specific nucleotides involved in the interaction of GBP-i with GRS, methylation interference experiments were performed with both the GBP-b/c and GBP-i complexes. The protection pattern indicates that GBP-i binds to several nucleotides on both strands of the GRS, specifically to the GGA/C repeats (Fig. 4C). Strong protection was observed over the distal AGGCG region, with additional protection over a

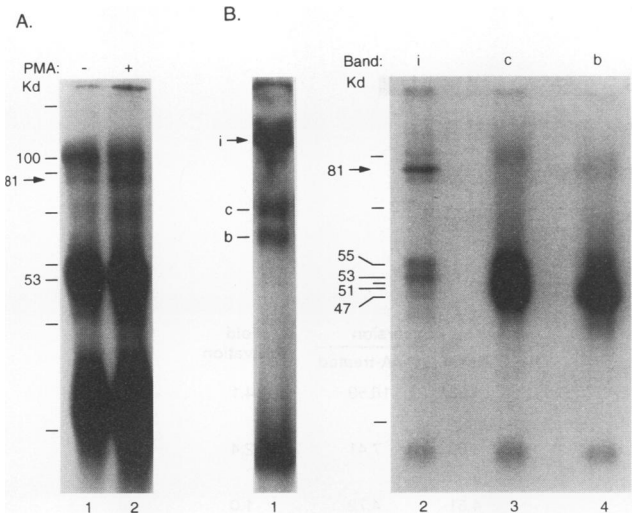

FIG. 5. Molecular mass of the GBP-i complex. UV cross-linking experiments were performed with bromodeoxyuridine-substituted probe to identify the sizes of the different DNA-protein complexes, as described in Materials and Methods. (A) Nuclear extracts from untreated (lane 1) and PMA-treated (lane 2) glial cells were cross-linked and resolved by SDS-PAGE. (B) Individual complexes were examined by two-dimensional UV cross-linking experiments. GBP-i, GBP-b, and GBP-c were excised from the native gel (lane 1), eluted, and then individually resolved by SDS-PAGE (lanes 2 to 4). The locations of molecular mass markers, along with the approximate sizes of the complexes, are shown on the side.

more proximal GCG region. The methylation interference data indicate that the optimum sequence for GBP-i binding is 5′-GCGNAGGCGNC-3′. These data support the inferences drawn from the competition experiments indicating that the nucleotide contact points for GBP-i lie within the four copies of the GGA/C repeats. Curiously, GBP-b binding did not exhibit any significant protection over these GGA/C repeats (data not shown). As indicated by previous competition experiments, GBP-b has a different sequence specificity and appears to bind to a more proximal and yet uncharacterized region of the GRS oligonucleotide.

GBP-i is an 81-kDa DNA-protein complex. To obtain information about the proteins involved in the GBP-i DNA-protein complex, UV cross-linking assays were performed. Nuclear extracts from untreated as well as PMA-treated glial cells were allowed to form complexes with labeled GRS probe, cross-linked by exposure to UV light, and the DNA-protein complexes were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Several cross-linked complexes could be seen (Fig. 5A), including a 100-kDa complex, an 81-kDa complex, and a broad complex around 53 kDa. The 81-kDa DNA-protein complex appears to be enhanced in the stimulated extract (Fig. 5A, compare lanes 1 and 2) and may represent the GBP-i-binding activity. By contrast, the other complexes do not appear to be markedly enhanced.

Further confirmation of the size of the GBP-i DNA-protein complex is derived from two-dimensional UV cross-linking gel electrophoresis (the first dimension is a native [PAGE] gel, and the second dimension is an SDS-PAGE gel of a specific excised band). Nuclear extracts from PMA-treated glial cells were incubated with the labeled bromodeoxyuridine-substituted GRS probe and UV cross-linked, and the complexes were resolved initially on a native 6% gel (Fig. 5B, lane 1). Prominent GBP complexes, corresponding to GBP-i, GBP-c, and GBP-b, were excised from the native gel and then resolved by SDS-PAGE. GBP-i shows a prominent 81-kDa DNA-protein complex and minor 55- and 53-kDa complexes (Fig. 5B, lane 2). GBP-i is likely to be a multiprotein complex of the 81-, 55-, and 53-kDa complexes, with the 81-kDa complex as the stoichiometrically dominant species. Although the 55- and 53-kDa species may represent contamination from the adjacent GBP-b/c complexes, repeated UV cross-linking experiments have consistently indicated the presence of these species in the induced complex. Regardless, the 81-kDa species is of further interest, because it appears to be differentially expressed in the induced extracts. The actual size of the induced protein must be smaller than 81 kDa, which is the size of DNA-protein complex.

Three complexes corresponding to the 47-, 51-, and 55-kDa species are seen in the GBP-c complex (Fig. 5B, lane 3), while the GBP-b complex shows only 47- and 51-kDa species, as previously demonstrated (11) (lane 4). Further, the GBP-b and GBP-c complexes clearly demonstrate a lack of the 81-kDa species. Thus, the GBP complexes may represent a combinatorial assembly of the various species, with their relative electrophoretic migration determined by the size of the various species. The apparent increase in intensity of the GBP-b/c complexes relative to the GBP-i complex in this study may reflect differences in their cross-linking efficiencies. The induction process could represent an activation of the 81-kDa species and the appearance of the GBP-i complex as a combination of three species: the 53-, 55-, and induced 81-kDa complexes.

GBP-i regulates transcription from the GRS. To examine whether the induction of GBP-i has a functional significance, a reporter construct with the GRS cloned upstream of the transcription initiation start site of the $pBLCAT_2$ plasmid was examined for responsiveness to PMA in the U-87MG cell line (Figure 6A). Transient-transfection assays consistently revealed a modest (2.5- to 4-fold) enhancement of the basal reporter activity upon a 90-min PMA stimulation, suggesting that PMA treatment induces a factor that enhances expression from a promoter containing GRS (Fig. 6B). By contrast, the $pBLCAT_2$ vector, with its thymidine kinase promoter elements, showed no change in the promoter activity.

To correlate the involvement of GBP-i with the observed PMA-induced transcriptional activity from the chimeric promoter, various mutant GRS oligonucleotides (GRS-2, GRS-3, and GRS-4) were cloned into $pBLCAT_2$ and used in transient-transfection assays in glial cells (Fig. 6A). PMA responsiveness was maintained, with constructs containing either the GRS-2 or the GRS-4 sequences, both of which contain adjacent copies of the GGA/C repeats (Fig. 6A). In fact, the $pBLCAT_2$ construct with GRS-2 showed a higher level of activation (five- to sevenfold) than did the construct with GRS. This observation is consistent with the methylation interference data showing that the GRS-2 oligonucleotide contains the GBP-i-binding site with no additional sequences. The construct with the GRS-4 oligonucleotide showed a smaller activation (two- to threefold) in response to PMA, consistent with previous data showing that the GRS-4 oligonucleotide represents a less than optimal binding site for GBP-i. Interestingly, the $pBLCAT_2$ construct with GRS-3 showed no responsiveness to PMA (Fig. 6B). The GRS-3 oligonucleotide lacks adjacent copies of the GGA/C repeat and does not represent a binding sequence for GBP-i. This pattern of PMA responsiveness correlates well with a model that requires GBP-i binding to the GRS to mediate PMA responsiveness. Further, the data presented here indicate a role for the PMA-induced GBP-i as a transcriptional activator.

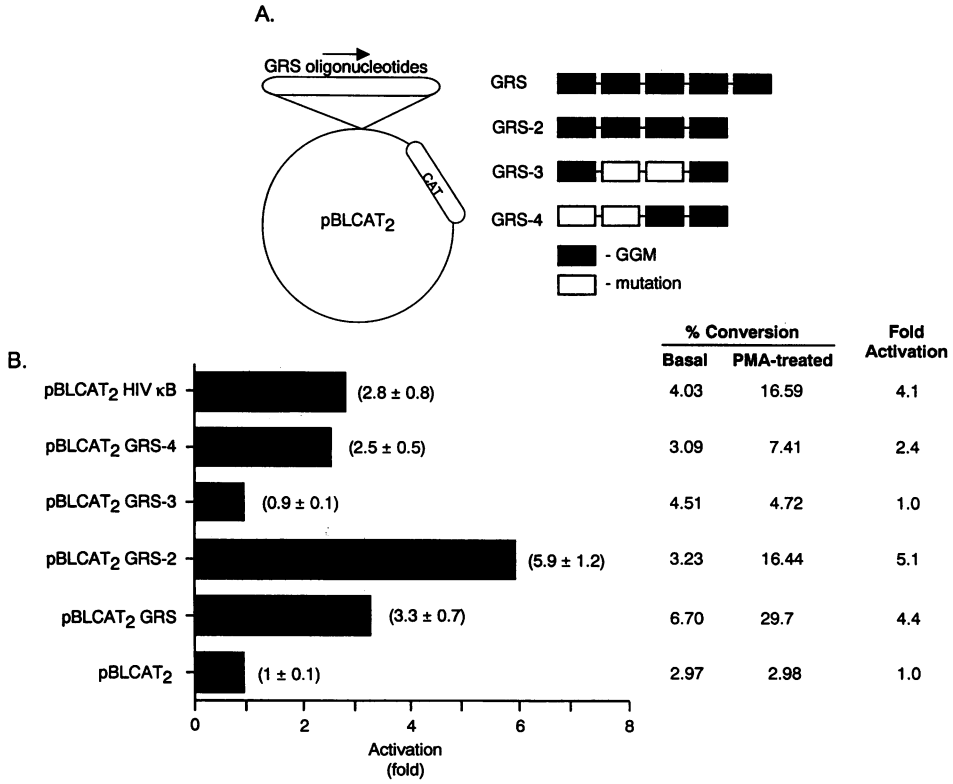

FIG. 6. Effect of GBP-i on the transcriptional activity of a heterologous promoter containing GRS. (A) A single copy of the GRS oligonucleotide and its mutant derivatives were separately cloned into the *Hin*dIII-*Sal*I sites of the pBLCAT$_2$ reporter plasmid. A schematic detailing the various oligonucleotides is also shown, with the solid boxes representing the original GGM repeats and the open boxes denoting mutated sequences as seen in Fig. 4A. (B) Each experiment was performed several times, and a summation of the data is shown on the left. The average fold activation over the basal level is graphed for each construct, with error bars indicating the standard deviation from the mean. Additionally, a representative experiment is tabulated to the right of the graph, with raw data showing the percent conversion of both the basal and PMA-treated samples.

PMA regulates the JCV promoter via the GRS site. The ability of GBP-i to activate transcription of a heterologous promoter containing the GRS led to the question of its biological significance on the JCV promoter. Previously, we had shown that PMA could enhance JCV$_L$ expression via an NF-κB element located upstream of the GRS site on the viral late promoter (39). Hence, we investigated the effect of PMA on JCV transcription through the GRS in the presence and absence of the NF-κB-binding site.

Reporter plasmids under the control of the JCV late promoter were transfected into glial (U-87MG) cells, and the cells were treated with PMA for 90 min. Figure 7A illustrates the anatomy of the JCV promoter and schematizes the structure of the various deletion constructs. The JCV$_L$ construct contains the NF-κB-binding site as well as the GGA/C repeats; the JCV$_L$ΔkB construct has the GGA/C repeats but lacks the NF-κB-binding site, while the JCV$_L$ΔGRS construct possesses neither the NF-κB binding site nor the GGA/C repeats. All the constructs contain the characteristic viral 98-bp repeats upstream of a CAT reporter gene.

The JCV$_L$ construct is activated five- to sevenfold by PMA (Fig. 7B). This observation is consistent with the demonstrated role for NF-κB binding sites as a mediator of PMA responsiveness. An HIV long terminal repeat construct bearing two NF-κB-binding sites exhibits a similar fold of activation in response to PMA in these cells (data not shown). Interestingly, reporter activity from the JCV$_L$ΔkB construct decreases three- to fourfold upon PMA treatment. Further, expression from the JCV$_L$ΔGRS deletion construct is enhanced two- to threefold by treatment with PMA. To further confirm the importance of the GBP-i-binding site on the JCV promoter, an additional construct, pJCVL ΔkB GRS^{mt}, with site-directed mutations in the GBP-i-binding site, was generated and tested for its responsiveness to PMA (Fig. 7A). The expression from this mutant reporter construct was enhanced two- to threefold upon PMA stimulation, thereby confirming the role of the GRS site as a target for inducible transcriptional repression. These data also indicate that the inducible GRS-binding protein, GBP-i, functions as a transcriptional repressor in the native context of the JCV promoter, in contrast to its role as a transcriptional activator in the context of the pBLCAT2 construct with its thymidine kinase promoter.

These data indicate the presence of at least three PMA-responsive elements on the JCV promoter: the NF-κB-binding site, the GRS, and a third, more-proximal and as yet unidentified region on the promoter. The NF-κB-binding site, as well as the proximal site, confers PMA inducibility to the viral promoter, while the GRS sequence appears to abrogate the response to PMA.

To determine whether the transcriptional response of the various constructs to PMA stimulation was restricted to glial cells, similar transient-transfection experiments with the GRS in a heterologous promoter context or in its native context in the JCV promoter were performed in several cell lines, includ-

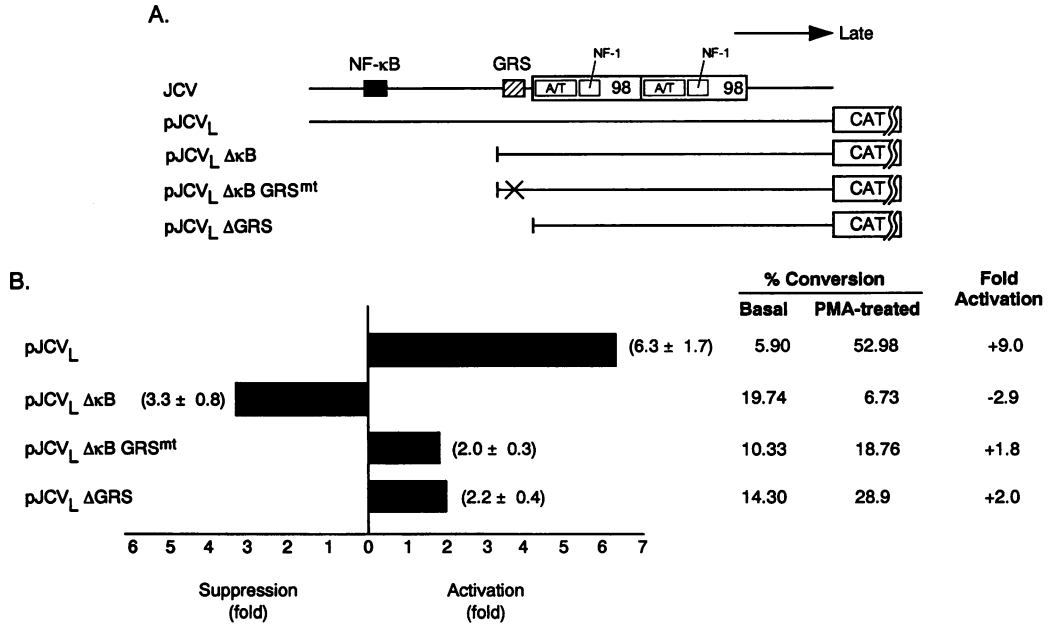

FIG. 7. Effect of GBP-i on transcriptional activity from the JCV promoter. (A) Schematic of the JCV promoter details the reporter constructs with special regard to the NF-κB- and GRS-binding sites. Basically, 1 μg of reporter constructs was transfected into U-87MG cells and then stimulated with PMA for 90 min, as described in Materials and Methods. (B) The average fold activation or suppression of the various constructs relative to the basal unstimulated level is illustrated in a representative experiment on the left. Again, a representative experiment is tabulated to the right of the graph, with raw data on the percent conversion.

ing HeLa, EAHY-926, and BJA-B. A similar pattern of transcriptional regulation mediated by GBP-i was observed (data not shown). These data suggest that the PMA-induced activity, GBP-i, is able to mediate its effects on promoters containing the GRS, without restriction to the cell type.

GBP-i induction requires de novo protein synthesis. The multipotent activator PMA has been shown to stimulate many signal transduction pathways, involving posttranslational modification or transcriptional activation. Traditionally, posttranslational modification involving various protein kinases is an immediate activation step that occurs within minutes, whereas activation at the transcriptional level often takes 6 to 24 h. In contrast, the induction kinetics of GBP-i shows a rapid (within 1 h) enhancement, which is neither immediate (within minutes) nor delayed (several hours).

To determine the mechanism involved in GBP-i activation, experiments were performed with specific inhibitors in the presence of PMA. Glial cells were pretreated with different inhibitors for 45 min and then stimulated with PMA for 90 min in the presence of optimal concentrations of specific inhibitors. None of the inhibitors alone was able to induce GBP-i over concentrations spanning several orders of magnitude and for the periods used (data not shown). Further, most inhibitors did not significantly alter the binding of GBP-b/c and GBP-d. Nuclear extracts made from cells treated with PMA in the presence of the different inhibitors were assayed for the induction of GBP-i by electrophoretic mobility shift assays. Cycloheximide, a protein translation inhibitor, was able to dramatically reduce the induction (Fig. 8A, lane 3), implicating at least a translation process involved in the induction of GBP-i. Actinomycin D, a transcription initiation inhibitor, was able to specifically block the GBP-i induction (lane 4). A similar inhibition of GBP-i activation was observed when cells were pretreated with α-amanitin (data not shown). This suggests that the rapid induction of GBP-i is regulated at the transcriptional level.

H7, a nonspecific protein kinase inhibitor, was able to decrease but not block the activation by PMA of the GBP-i binding (Fig. 8A, lane 5). Further experiments to detail the role of protein kinase C in this induction were performed with the more-specific inhibitor calphositin C, which showed an inability to block GBP-i induction by PMA (Fig. 8A, lane 6). Neither forskolin, an inhibitor of protein kinase A, nor herbimycin, a tyrosine kinase inhibitor, was able to block the GBP-i induction by PMA. Additionally, none of the GBP complexes appear to be hyperphosphorylated, as evidenced by a lack of change in the mobility of the complexes upon treatment with either alkaline or acid phosphatase (data not shown). The induction of GBP-i by PMA also appears to be unaffected by the presence of indomethacin or ibuprofen, both inhibitors of the prostaglandin synthesis pathway, which was previously shown to be induced by PMA (Fig. 8A, lanes 7 and 8). These data point strongly to the requirement for transcriptional activation as well as a role for an H7-sensitive pathway in the induction of GBP-i-binding activity.

To identify the roles of transcriptional activation and the H7-sensitive pathway in this rapid induction, an additional set of inhibitor studies were performed. Two different time points of 45 and 180 min of PMA stimulation in the presence of inhibition were used to simulate two distinct phases of the induction: the initial activation of GBP-i and the maintenance of the induced level. An additional set of cells were treated with PMA in the presence of inhibitor without pretreatment (Fig. 8B, lanes 2, 4, 6, and 8). Interestingly, cycloheximide blocked both phases of the induction (lane 2 to 5). The addition of H7 to the PMA-stimulated cells caused a slight decrease in the initial induction of GBP-i but resulted in no change over longer periods or in the levels of induction (lanes

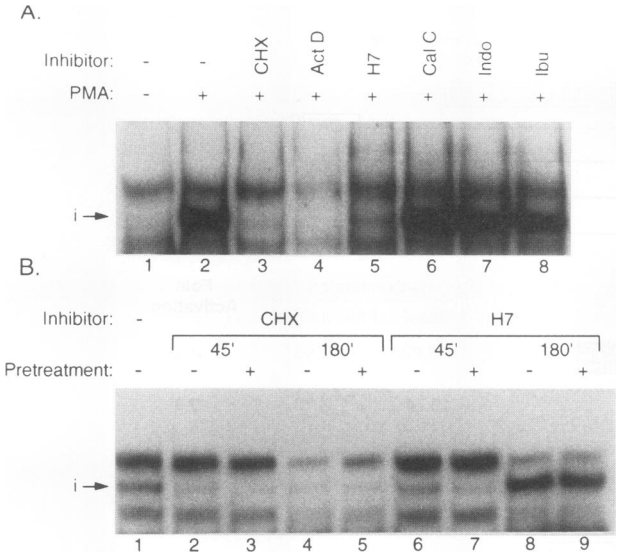

FIG. 8. GBP-i induction requires de novo protein synthesis. (A) Nuclear extracts were prepared from glial cells treated with PMA in the presence of inhibitors, whose optimal concentrations are detailed in Materials and Methods. Cells were preincubated with inhibitors for 45 min and treated with PMA in addition to the inhibitors, and extracts were prepared at 45 min posttreatment. Abbreviations: CHX, cycloheximide; Act D, actinomycin D; Cal C, calphositin C; Indo, indomethacin; Ibu, ibuprofen. (B) Nuclear extracts were prepared from cells treated with PMA in the presence of H7 or cycloheximide for 45 or 180 min, either without pretreatment (lanes 2, 4, 6, and 8) or after a 30-min pretreatment with the inhibitor (lanes 3, 5, 7, and 9).

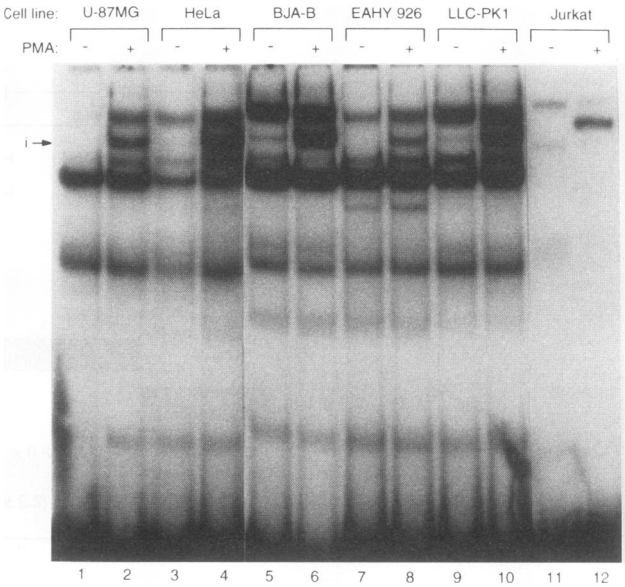

FIG. 9. Induction of GBP-i is widely observed. Nuclear extracts were prepared from several cell lines, either without treatment or after treatment with PMA for 90 min, and then tested for GBP-i induction by an electrophoretic mobility shift assay with the GRS probe.

6 to 9). GBP-i binding in the extract made after 180 min of PMA treatment in the presence of H7 (lanes 8 and 9) occurs, as expected, at a higher level than in extract made after 45 min of PMA stimulation (lane 1). These data indicate that the optimal initial activation of GBP-i binding requires a protein translation step and a posttranslational process but that only the protein translation step is absolutely required for activation.

GBP-i induction is ubiquitous. JCV exhibits a highly defined neurotropism and replicates efficiently in cell culture only in primary fetal glial cells (1, 33, 52). The tissue-specific restriction of viral gene expression is mediated by the transcriptional control region of the JCV genome (44). Previous data from our laboratory indicate tissue-specific differences in the complexes binding to the GRS (11). Hence, we asked whether GBP-i binding was induced in a cell-type-specific manner.

Unstimulated and PMA-stimulated nuclear extracts were prepared from several human cell lines derived from T lymphocytes (H9, Jurkat), B lymphocytes (BJA-B), kidney (CV-1, LLC-PK1), endothelium (EAHY 926), and cervical epithelium (HeLa). The uninduced extracts from these cell lines exhibit some differences in patterns of binding activity from those seen in glial cell lines (Fig. 9, lanes 1, 3, 5, 7, 9, and 11). Strikingly, the PMA-treated extracts show an induction of GBP-i binding in all these cell lines (lanes 2, 4, 6, 8, 10, and 12). Further experiments carried out with a more elaborate range of cell lines, including those from mice, rats, and hamsters, have consistently demonstrated the presence of the GBP-i complex upon PMA treatment (data not shown). The binding characteristics of GBP-i are identical in all cell lines tested, as verified by similar electrophoretic migration, its unique time course of induction, and a similar competition profile with the various mutant GRS oligonucleotides (data not shown). These data, in addition to the transient-transfection data, suggest that the induction of GBP-i activity is not restricted to a particular cell type and potentially indicate a role for GBP-i in reactivation of the virus from latency.

GBP-i is rapidly induced by several inflammatory cytokines. To examine the biological significance of GBP-i induction, we investigated the ability of immunoregulatory agents to induce GBP-i. Nuclear extracts were prepared from glial cells treated with optimized levels of the various cytokines for 90 min and assayed for GBP-i binding (Fig. 10). GBP-i activity is induced upon exposure to IL-1β (the binding activity is clearly visible on an autoradiogram subjected to a longer exposure) but not IL-2 (Fig. 10, lanes 3 and 4). IL-2 is unable to activate GBP-i binding over concentrations spanning 5 orders of magnitude. Interestingly, TNF-α, IFN-γ, TGF-β1, and TGF-β2 are able to activate GBP-i (lanes 5 to 8). The fold induction does not appear to be as dramatic as with the phorbol esters; however, the binding activity is clearly induced when compared with the basal level. The ability of all these inflammatory cytokines to rapidly induce GBP-i binding suggests a physiological role for GBP-i in cellular as well as viral gene expression. Correlations between the levels of TNF-α, IFN-γ, TGF-β1, IL-1β, TGF-β2, and immunosuppressive states implicate GBP-i in reactivation from latency of JCV.

Additional exogenous activators like phorbol dibutyrate, diacylglycerol, and ionomycin were able to activate GBP-i binding, albeit weakly. In contrast, the mitogen phytohemagglutinin was unable to induce GBP-i (data not shown). These data support the previous observations implicating an H7-sensitive pathway and de novo protein synthesis in the GBP-i induction. The ability of these various agents, both endogenous and exogenous, to induce GBP-i binding to GRS also points to a common signal transduction pathway that rapidly triggers de novo protein synthesis and GBP-i complex formation.

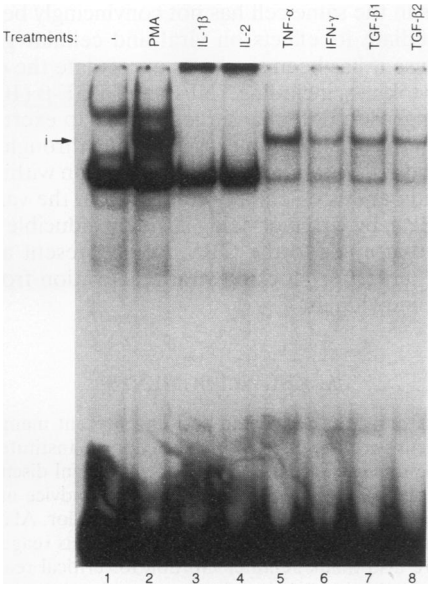

FIG. 10. Cytokines induce GBP-i. Nuclear extracts were prepared from U-87MG cells after treatment with optimized concentrations of several cytokines for 90 min and assayed for GBP-i binding by electrophoretic mobility shift assay with the GRS probe.

DISCUSSION

The immune system can rapidly mobilize an effective physiological response to a stimulus, by careful orchestration of the expression of selected genes. Coordination and regulation of gene expression requires cell-cell communication, involving cytokines. Previous studies have identified a role for several transcription factors, including NF-κB, JAK-STAT, and NF-IL6, in mediating the effect of cytokines on gene regulation (36). In this study, we report the identification and characterization of a novel transcription factor, GBP-i, that appears to be rapidly responsive to various immunomodulators.

GBP-i binds specifically to double-stranded GGA/C repeats and forms a ~81 kDa complex. GBP-i has a high affinity for the GRS, as shown by the lack of competition by a 1,000-fold excess of closely related G+C-rich sequences. GBP-i appears to be distinct from previously characterized transcription factors that bind to similar sequences. Sp1 (95 to 100 kDa), PuF (17 kDa), another GC-binding factor (GCF) (90 kDa), and Pur factor (28 kDa) all differ from GBP-i by virtue of their molecular weights and their consensus DNA-binding sequence, thereby identifying GBP-i as a novel factor (8, 26, 28, 37).

The induction kinetics of GBP-i represent an intriguing system: GBP-i binding begins to appear at 45 min poststimulation, attains a peak around 90 min, and then decreases over time. The activation of GBP-i is rapid yet conforms to neither the pattern of almost instantaneous activation by posttranslational modification nor the much slower stimulation (several hours) at the transcriptional level, like that seen for GBP-b. The rapid induction kinetics would favor posttranscriptional control, but precedents exist for a swift response at the transcriptional level, as seen for the GCF transcription factor (27). Studies performed with specific inhibitors indicate that the GBP-i induction requires a rapid de novo protein synthesis, with the primary regulation at the transcriptional level. The ability of H7 to decrease the initial but not later induction of GBP-i suggests that posttranscriptional processes may be involved but not required for the initial induction step.

A model showing the effect of an extracellular signal on the different signal transduction pathways (Fig. 11) illustrates three classes of pathways, depending on their kinetics of activation. The immediate-early class involves posttranslational modification, often a protein kinase-dependent phosphorylation step, and activation occurs within 15 min. In contrast, the late class requires several hours before activation and may involve a transcriptional process (29). The emerging early class, which includes GBP-i and GCF, is distinct in its requirement for a transcriptional process and an activation process that occurs within 90 min.

The inducible activity, GBP-i, appears to function as a silencer in the context of the JCV promoter. The ability of a G+C-rich binding transcription factor to function as a transcriptional repressor has been previously documented for GCF (28). The transcriptional repression function of GBP-i, as well

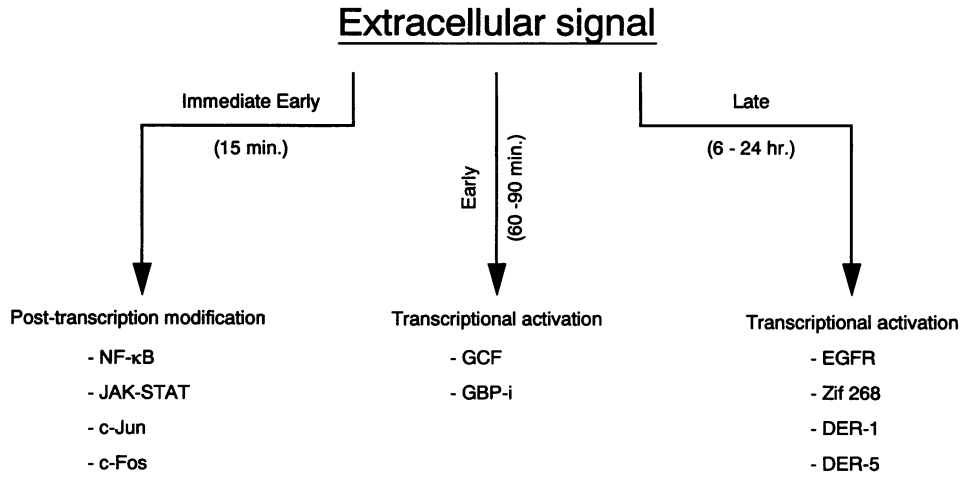

FIG. 11. Model for GBP-i activation. The model depicts three classes of the signal transduction pathway, based on the process and speed at which the activation takes place. The proposed name of each pathway in response to an extracellular signal is denoted on the arrow, along with its described time course. The process required for activation of the specific gene product is shown at the end of the arrow, along with representative factors affected by each process.

as the induction profile at the RNA level, strongly suggests that GBP-i and GCF belong to the class of rapidly inducible transcription repressors. GBP-i is distinct from GCF in both its consensus binding sequences and its molecular mass.

Interestingly, GBP-i also acts as a transcriptional activator in the context of a herpes simplex virus thymidine kinase promoter or in the absence of a defined promoter sequence. GBP-i could function as weaker transcriptional activator than the constitutive GBP-binding proteins in the context of the viral promoter and thus could function as a transcriptional repressor. The potential interaction between various GBPs and their common target sequence could represent an important control step in the regulation of gene expression. This duality of function could also involve interaction with either the basal transcriptional machinery or other transcription factors (reviewed in reference 24). The JCV promoter, with its ample registry of regulatory factors, provides many potential candidates, including the third PMA-responsive site on the viral late promoter, for interaction with GBP-i. This model also allows different cytokines to modulate gene expression of JC virus differently through the GRS, as the interaction of GBP-i with other cytokine-induced proteins could determine the activation status of the viral promoter.

The rapid induction of GBP-i is mediated by many inflammatory cytokines, like IL-1β, IFN-γ, TGF-β1, and TGF-β2, whose levels are also elevated in immunosuppressive states. These cytokines are known to activate other transcription factors within a short period, as is the case for the NF-κB and JAK-STAT families of transcription factors (6, 40). Even though a different signal transduction pathway is used for the activation of these transcription factors, the nuclear milieu upon stimulation contains all these various factors. The net effect of the cytokines on any promoter containing the binding sites for the various transcription factors must reflect their combinatorial interactions. In support of this hypothesis, recent studies in the laboratory have indicated that TGF-β1 activates transcription from the JCV late promoter through both the GRS and the NF-1 sites (37a). The effect of the various inflammatory cytokines on the viral late-gene expression is currently under investigation in the laboratory.

Although the neurotropism of the virus is clearly established, the functional sites of latency of JCV are still unclear. In healthy seropositive individuals without PML, JCV has not been clearly demonstrated in the brain. B lymphocytes and the kidneys have been shown to be reservoirs of JCV in healthy seropositive individuals, but it remains to be proven if reactivation of virus at these sites can trigger PML (2, 25, 48, 50). GBP-i appears to be widely induced in glial as well as in many nonglial cells, including T, B, and kidney cells. GBP-i could play an important role in mediating JCV reactivation at all of these suspected sites of latency, especially given the potential for different cytokines to induce its binding.

The GBP-i-binding site, GRS, overlaps with the binding site (GAGGC) of the JCV large T antigen and is contained in the origin of viral DNA replication (18). Interestingly, GBP-i binding to GRS is disrupted by JCV T antigen, and its effects on the viral late promoter are ablated in the presence of the T-antigen (37b). Characterization of the role of GBP-i in DNA replication, especially in the context of JCV T antigen, is under way in the laboratory and could potentially reveal its role in viral pathogenesis.

Previous studies in our laboratory have indicated an interaction between HIV-1 Tat and the JCV promoter. Most studies indicate that JCV and HIV infect different cell populations in the brain (4), oligodendroglia for JCV and monocytes/macrophages for HIV. Coinfection and the presence of both viruses in the same cell has not convincingly been shown. Tat may mediate its effects on viral and cellular genes indirectly, because it has been shown to upregulate the expression of several cytokines, including TNF-β and TGF-β (10, 15). Our data here suggest a potential target for Tat to exert its effects indirectly on the JCV promoter, specifically through the GRS.

Finally, the GRS is a highly conserved region within the JCV control region and is essentially conserved in the various JCV isolates. GBP-i, by virtue of being a widely inducible transcription factor that binds to the GRS, may represent a potential therapeutic target for blocking viral reactivation from latency in high-risk individuals.

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