Role of Interferon Regulatory Factor-1 in the Induction of Biliary Glycoprotein (Cell CAM-1) by Interferon-γ*

(Received for publication, June 17, 1996, and in revised form, August 6, 1996)

Chang-Jui Chen, Tsiu-Ti Lin, and John E. Shively‡

From the Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010

Biliary glycoprotein (BGP), also known as C-CAM-1, has been shown to be down-regulated in colon and prostate tumors. Previously, we demonstrated that BGP mRNA is up-regulated by interferon-γ (IFN-γ) in colon cancer cell lines (Takahashi, H., Okai, Y., Paxton, R. J., Hefta, L. J. F., and Shively, J. E. (1993) Cancer Res. 53, 1612–1619). We now show that the BGP promoter contains an interferon-sensitive response element (ISRE) that is specifically protected in *in vitro* footprints. Interferon regulatory factor-1 (IRF-1) was identified as the ISRE-binding factor by electrophoretic mobility shift assays. The induction of IRF-1 mRNA by IFN-γ in HT-29 cells reaches a maximum at 6 h and is superinduced by cycloheximide. Four mRNA species for BGP are induced by IFN-γ, the major band of which is inhibited by cycloheximide. Transfection of HT-29 cells with an IRF-1 expression plasmid (pAct-1) transactivates a BGP promoter reporter gene containing wild-type (but not mutant) ISRE. Electrophoretic mobility shift assay analysis of a second footprint reveals the binding of Sp1, an Sp1-like protein, and upstream stimulatory factor. The Sp1-like complex was also induced by IFN-γ treatment of HT-29 cells and may be a second point of transcriptional control for the BGP gene.

Human biliary glycoprotein (BGP) is a member of the CEA gene family, a gene cluster on chromosome 19, including CEA, BGP, nonspecific cross-reacting antigen, and pregnancy-specific glycoprotein (1–5). BGP was first described as a CEA cross-reacting substance in bile (6) and was localized to the cell surface of bile canaliculi in the liver and gallbladder mucosa (7). Molecular cloning studies on BGP (8, 9) revealed that it exists in alternatively spliced isoforms from a single gene (9). BGP has a transmembrane domain and, depending on alternative splicing, a long (71 amino acids) or short (10 amino acids) cytoplasmic domain. The long form can be phosphorylated by protein kinases (10) after activation by antibody binding (11) or activation of the insulin receptor (12). The rat homologue of BGP was identified as a cell adhesion molecule, C-CAM-1 (13). The mouse and human forms of BGP have been shown to act as Ca2+-dependent cellular adhesion molecules (14, 15). Murine BGP also serves as receptor for murine hepatitis virus (16). The expression of BGP is down-regulated in human colon (17) and prostate (18) cancer. Similarly, murine BGP is down-regulated in carcinogen-induced colon cancer in mice (19). Transfection of the rat BGP gene into the human tumorigenic prostate cell line PC-3 reverts the cells to non-tumorigenic in nude mice (20). Conversely, transfection of a gene expressing antisense BGP renders non-tumorigenic prostate cells tumorigenic (20). Thus, BGP appears to be a product of terminally differentiated cells, has a polar expression profile on the luminal side of epithelial cells, and is down-regulated in tumors of epithelial origin. The tumor suppressor properties of BGP have prompted its use in preliminary gene therapy experiments (21). However, this approach is complicated by the need to transfect all tumor cells with the BGP gene. Another approach may be to determine the mechanism of BGP gene regulation and to identify factors capable of BGP gene up-regulation.

We have previously shown that type II interferon, interferon-γ (IFN-γ), is capable of up-regulating both the BGP and CEA genes, but by different mechanisms (22). CEA is slowly induced by IFN-γ over a period of 24–72 h, requiring new protein synthesis, while BGP is rapidly induced from 4–24 h, without significant inhibition by cycloheximide (22). Rapid induction of gene expression by IFN-γ is known to be mediated by GAS (for a review, see Ref. 23), found in a variety of genes, including interferon regulatory factor-1 (IRF-1). IRF-1, in turn, can activate a large number of genes by binding to the interferon-stimulated response element (ISRE). ISRE was previously identified for genes activated by type I interferons, IFN-α and IFN-β. Thus, gene activation by IRF-1 is a major crossover pathway for gene activation by type I and II interferons. The mRNA for IRF-1 has a short half-life (30 min) (24) and is rapidly down-regulated by IRF-2 by competing for the same ISRE-binding site (25, 26). Because of their potent effects on cell growth, IRF-1 has been termed a tumor suppressor gene and IRF-2 an oncogene (27).

In this report, we show that the BGP promoter has an ISRE that is specifically activated by IRF-1 after treatment of colorectal or HeLa (cervical) cells with IFN-γ. IRF-1 mRNA is superinduced in these cells by cycloheximide, and a major species of BGP mRNA is inhibited by cycloheximide. We also show that both USF and Sp1 bind to another footprint in the BGP promoter and that an Sp1-like protein is induced by IFN-γ in HT-29 cells.

**MATERIALS AND METHODS**

**Cell Culture and Treatment**

The colon carcinoma cell lines HT-29 and SW403 and the cervix carcinoma cell line HeLa were obtained from American Type Culture Collection. The cells (0.5 × 10⁶ cells/ml) were suspended in 20 ml of medium in 75-cm² flasks, allowed to reach confluence (3 days), and
treated with recombinant human IFN-γ (Sigma) at 500 units/ml for up to 2 days at 37°C in 5% CO2. In some experiments, cycloheximide (50 μg/ml) was added 10 min before the addition of IFN-γ.

Oligonucleotide and DNA Probes

Oligonucleotide probes were synthesized by the City of Hope Oligonucleotide Synthesis Core Facility. Probes for gel mobility shifts were purified by gel electrophoresis, electroeluted, annealed, end-labeled with [γ-32P]ATP with T4 polynucleotide kinase (New England Biolabs Inc.), and purified from free ATP on spin columns (Microcon-3, Amicon, Inc.). The sequences of these probes are shown in Table I. PCR primers (see Table I) were purified by gel electrophoresis.

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared either by a freeze-thaw method (28) as described previously (29) or by a modification of the 0.4 M NaCl extraction method of Digman et al. (30). All buffers contained sodium vanadate (0.1 mM), EDTA (0.1 mM), EGTA (0.1 mM), dithiothreitol (1 mM), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), and aprotinin (33 μg/ml), phenylmethylsulfonyl fluoride (0.25 mM), L-1-tosylamido-2-phenylethyl ketone (0.1 mM), and N′-p-tosyl-L-lysine chloromethyl ketone (0.1 mM). Mobility shifts were performed with 50–100 fmol of rabbit IgG and SuperSignal CL-HRP (Pierce).

Nuclear extracts were incubated with bromodeoxyuridine-labeled probes (see Table I) and run on native gels as described for the EMSAs. The bands were excised, exposed to UV light for up to 2 days at 37°C in 5% CO2. In some experiments, cycloheximide (50 μM), leupeptin (10 μg/ml), and aprotinin (33 μg/ml) were added to allow quantitative comparison to BGP and IRF-1 bands. The gel bands were quantitated on a Bio-Rad 963 scanning densitometer. Similarly, a 263-bp probe corresponding to the coding sequence of IRF-1 was used to quantitate IRF-1 mRNA. The specific activity of the labeled GAPDH probe was lowered to allow quantitative comparisons to BGP and IRF-1 mRNAs, which were in lower abundance.

In Vivo Footprinting by LM-PCR

In vivo methylation and DNase I digestion of cells were performed according to Pfeifer et al. (32, 33). LM-PCR was performed according to Garrity and Wold (34) as described (35). The three nested sets of primers for the 5′- and 3′-ends of BGP promoter region, −411 to −7 (numbering from the translation start site) and the annealing temperatures are shown in Table I. After 21 cycles of PCR, [γ-32P]ATP-labeled P3 was added, and PCR was continued for two more cycles. PCR products were analyzed on 6% urea, 8% polyacrylamide gels.

RNase Protection Assays

A 190-bp probe corresponding to the cytoplasmic domain/3′-untranslated region (positions 1435–1625) of the BGP cDNA was subcloned into Bluescript, and an antisense riboprobe was synthesized with the MAXiscript kit (Ambion Inc.) and labeled with α-32P-UTP. The antisense probe was hybridized with total RNA isolated from cells along with a 316-bp GAPDH probe (control); digested with RNases A and T1; and run on 6% urea, 8% polyacrylamide gels. The gel bands were quantitated on a Bio-Rad 963 scanning densitometer. Similarly, a 263-bp probe corresponding to the coding sequence of IRF-1 was used to quantitate IRF-1 mRNA. The specific activity of the labeled GAPDH probe was lowered to allow quantitative comparisons to BGP and IRF-1 mRNAs, which were in lower abundance.

Transfection Assays

BGP promoter fragments (−308 to −2 and −623 to −2) were subcloned into POGH (Nichols Institute) to create POGH/BGP307 and POGH/BGP622. POGH/BGP907N, which contained six base pair changes in the ISRE motif (AGAAAGGAAAGAGAAAGTA to AGAACGACCTATCAAGT), was generated by PCR. The vectors were transfected into HeLa, SW403, and HeLa by calcium phosphate precipitation. The luciferase reporter plasmids were cotransfected with the eukaryotic expression vector pMIR-IFN-γ (500 units/ml). Transient assays were performed with 0.5 μg of pACT-1 or pACT-2 (36), expression vectors for murine IFN-1 and IFN-2, respectively.

RESULTS

In Vivo Footprint Analysis of the BGP Promoter—Cells (HT-29, SW403, and HeLa) were treated with IFN-γ for up to 72 h, removed at intervals, and treated with either DNase I or DMS. LM-PCR was performed on the isolated DNA using the three sets of nested primers shown in Table I, corresponding to BGP promoter region, −411 to −7 (numbering from the translation start site). The DNase I results (Fig. 1) show two footprints.
previously identified for BGP, FP-I (−167 to −137) and FP-II (−305 to −280), and an IFN-γ-inducible footprint, IRE (−230 to −210). The IRE identified corresponds to the ISRE consensus sequence AGTTTCNNTTTCN(C/T). The degree of protection increases with the time course of IFN-γ treatment and the protected region is flanked by hypersensitive sites HS-II and HS-III. Hypersensitive sites also flank FP-I. New hypersensitive sites (HS-I–III) are observed at the boundaries of FP-I and IRE after IFN-γ treatment of SW403 and HeLa cells. HT-29 cells that constitutively produce small amounts of BGP exhibit protection for FP-I and FP-II prior to IFN-γ treatment; however, no footprints are observed for HeLa cells until after treatment with IFN-γ. The results for DMS-treated cells (Fig. 2) over the region of FP-I are in accordance with the DNase I footprints. The Sp1 site (−153 to −161, GGGCCG, non-coding strand) is specifically protected in HT-29 cells before IFN-γ treatment, and the degree of protection increases with time of treatment (Fig. 2A). The same site in HeLa cells shows protection only after IFN-γ treatment (Fig. 2B). The USF site (−148 to −143, CACATG) is partially protected from DMS methylation after IFN-γ treatment of HT-29 and HeLa cells. The IRE site (Fig. 2C) shows two G nucleotides (−223 and −217) protected from DMS treatment after 6 h of IFN-γ treatment.

Electrophoretic Mobility Shift Assays of IRE—A 27-bp end-labeled probe (Table I) was synthesized based on the boundaries of the IRE identified in Fig. 1 and incubated with nuclear extracts produced from HT-29 and HeLa cells during the time course of IFN-γ treatment, and binding was assessed by EMSAs on native polyacrylamide gels. EMSAs for both cell lines (Fig. 3A) show the appearance of five complexes (A–E), with complex C appearing as the most intense band for HT-29 and HeLa cells (arrow), absent at 0 h, increasing to a maximum at 1–6 h, and decreasing to lower levels by 24 h. All five complexes were competed with a 50-fold excess of unlabeled probe (data not shown). The DNA-protein complex C was supershifted with antibodies to IRF-1, but not with anti-ISGF3-γ antibody (the 48-kDa component of ISGF3) or anti-IRF-2 antibody (Fig. 3B). Although the IRF-2 complex was detected by antibody supershifting (Fig. 3B), the amount of the complex detected at all time points was low compared with IRF-1. Anti-IRF-1 antibody also supershifted the less intense complex A (Fig. 3A). The lower migration of complex A is consistent with the possibility that complex A contains other proteins in addition to IRF-1 (e.g. ISGF3). Since cells that respond to IFN-α are known to make a bona fide ISGF3 complex, this possibility was tested by treating HeLa cells with IFN-α. The results (data not shown) are similar to those obtained for HeLa cells treated with IFN-γ alone or in combination with IFN-α. Since none of the A complexes were supershifted with anti-ISGF3-γ antibody, it was concluded that while the higher complex does contain IRF-1, it is not a bona fide ISGF3 complex (i.e. complex A contains no ISGF3-γ and is not induced by IFN-γ). In addition, we have shown that complex A is not supershifted with antibodies to Sp1 (see below). Complex B is present in the EMSAs for all three cell lines and does not change with IFN-γ treatment. IFN-γ-induced IRF-1 complex was inhibited by treatment of HT-29 cells with cycloheximide (data not shown).

Western and Southwestern Blots and UV Cross-linking of IRF-1—Nuclear extracts from HT-29 cells treated with IFN-γ were run on SDS gels, transferred to nitrocellulose membranes, and probed with anti-IRF-1 antibodies. The results (Fig. 4A) show the induction of a major band at 52 kDa. The reported molecular mass for IRF-1 (ISGF2) as measured by SDS gel electrophoresis is 56 kDa (37). When nuclear extracts from the 0- and 3-h time points were transferred to nitrocellulose and probed with the double-stranded IRE probe, a major band at 46 kDa and a minor band at 52 kDa were observed (Fig. 4B). Both bands were faint before IFN-γ treatment. The BGP IRE probe was substituted with bromodeoxyuridine, mixed with nuclear extracts from HT-29 cells before and after IFN-γ treatment, and run on EMSAs. When the complexes (A–E; see Fig. 3A)
were excised, irradiated with UV light, and run on SDS gels, a major cross-linked protein at ~50 kDa was shown to increase binding to the probe after IFN-γ treatment (Fig. 5). The cross-linked complexes A and C show the greatest increase in intensity. This is consistent with the results shown above (Fig. 3), where both complexes A and C were supershifted by anti-IRF-1 antibody.

**Time Course of IRF-1 and BGP mRNA Induction**—Analysis of the time course of IRF-1 mRNA induction in IFN-γ-treated HT-29 cells by RNase protection assays (Fig. 6) reveals that the peak of induction for HT-29 cells is 3–6 h (8-fold induction from 0 to 3 h) and that levels are reduced by 24 h (6-fold induction). The addition of cycloheximide caused superinduction of IRF-1 mRNA at all time points after IFN-γ treatment (12-fold from 0 to 3 h). BGP mRNA showed four bands, one major and three minor. The major band peaked at 3–6 h and was inhibited by cycloheximide. There was a 2-fold increase in the major band from 0 to 3 h or from 0 to 6 h, which was reduced to 1.9- and 1.4-fold by cycloheximide at 3 and 6 h, respectively. The -fold increase for the major band was 1.5 from 0 to 24 h, reduced to 0.75 by cycloheximide. One of the minor bands was superinduced by cycloheximide (2-fold). SW403 and HeLa cells were also analyzed (data not shown), exhibiting similar kinetics for the induction of BGP mRNA; but in the case of HeLa cells, BGP mRNA is produced at ~10% of the highest levels seen for HT-29 and SW403 cells. Thus, all three cell lines show BGP mRNA at all time points after IFN-γ treatment (12-fold from 0 to 3 h). BGP mRNA showed four bands, one major and three minor. The major band peaked at 3–6 h and was inhibited by cycloheximide. There was a 2-fold increase in the major band from 0 to 3 h or from 0 to 6 h, which was reduced to 1.9- and 1.4-fold by cycloheximide at 3 and 6 h, respectively. The -fold increase for the major band was 1.5 from 0 to 24 h, reduced to 0.75 by cycloheximide. One of the minor bands was superinduced by cycloheximide (2-fold). SW403 and HeLa cells were also analyzed (data not shown), exhibiting similar kinetics for the induction of BGP mRNA; but in the case of HeLa cells, BGP mRNA is produced at ~10% of the highest levels seen for HT-29 and SW403 cells. Thus, all three cell lines show BGP mRNA kinetics that correlate with IRF-1 mRNA kinetics and the formation of the BGP IRE/IRF-1 complex.

**Time Course of IRF-1 Gene Activation**—To determine if the time course of IRF-1 gene activation corresponded to its binding the BGP IRE, we synthesized a 30-bp probe corresponding to the GAS element of the IRF-1 gene (~142 to ~112) and tested it in EMSAs with nuclear extracts from IFN-γ-treated HT-29 cells. The results (Fig. 7) show that an increase in the Stat-1-GAS complex (arrow) is detected as early as 60 min, reaching a maximum at 6 h, with no decrease up to 24 h (data not shown). Inhibition of the complex formation with anti-Stat-1 antibodies demonstrates that the complex contains Stat-1. Previous studies on HeLa cells show that the formation of the Stat-1-GAS complex reaches a maximum at 1 h and correlates with the production of IRF-1 mRNA (38, 39). In the case of HT-29 cells (shown here), the time course of Stat-1-GAS complex formation is somewhat longer. We considered the possibility that the complex has shifted from Stat-1c-GAS to Stat-1p-GAS, a shift that would result in the inactivation of the IRF-1 gene [40, 41]. This possibility was checked by performing Western blotting with anti-Stat-1 antibodies on nuclear extracts from IFN-γ-treated HT-29 cells. The results (data not shown) show the absence of Stat-1β at all time points with a steady increase of Stat-1 through 24 h.

**Reporter Assays with the BGP Promoter**—Previous results have shown that a 307-bp fragment of the BGP promoter is fully active in a reporter assay [42]. Both 307- and 622-bp fragments were inserted before the hGH gene (P0GH/BGP307 and P0GH/BGP622) and transiently transfected into HeLa, SW403, and HT-29 cells. When the cells were assayed for hGH production before and after treatment with IFN-γ (Table II), a 2–3-fold increase in production was observed for HeLa and HT-29 cells and a 1.5-fold increase for SW403 cells. No difference was observed for the two size fragments (data not shown for the larger fragment), suggesting that no additional cis-acting elements were present in the larger fragment. When the cells were cotransfected with an IRF-1-expressing plasmid and the BGP/hGH reporter gene, a 2–4-fold increase in hGH production was observed, demonstrating that IRF-1 is able to transactivate the BGP promoter. Cotransfection with pAct-2 (an IRF-2 expression vector) or control plasmids (minus insert) does not transactivate P0GH/BGP307. When a mutant IRE form of the reporter plasmid (P0GH/BGP307m) was tested in the cotransfection experiments, no transactivation was observed.

**EMSAs of BGP FP-I**—Since the in vivo footprinting results

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**FIG. 2. In vivo DMS footprint analysis of the BGP promoter in HT-29, SW403, and HeLa cells treated with IFN-γ.** Cells, before and after treatment with IFN-γ (500 units/ml), were treated with DMS. Total genomic DNA was isolated, cleaved with piperidine, and used as a template for in vivo footprint analysis using LM-PCR with a sense primer set covering the BGP promoter from −112 to −357. Open circles, bases protected from DMS methylation; solid circles, bases hypersensitive to DMS methylation. Results are shown for the noncoding strand, 5′ to 3′ and top to bottom. A, time course of treatment of HT-29 cells with IFN-γ (untreated control of HeLa cells shown for comparison); B, time course of treatment of HeLa cells with IFN-γ; C, identification of the BGP IRE in SW403 and HeLa cells treated with IFN-γ using antisense primer set −7 to −64 (Table I).
show that an Sp1 site in FP-I is protected in HeLa cells after IFN-γ treatment and is enhanced in HT-29 cells, we investigated the possibility that Sp1 was directly involved in the BGP gene response to IFN-γ treatment. A double-stranded probe was synthesized corresponding to BGP FP-I (−2176 to −2133), incubated with nuclear extracts from HT-29 cells treated with IFN-γ at various times, and run on EMSAs. The results (Fig. 8) show that Sp1 does form a complex with BGP FP-I and that there is a slight increase in the intensity of the complex during the time course of IFN-γ treatment. Sp1 was positively identified by inhibition with anti-Sp1 antibody and competition with an unlabeled Sp1 consensus double-stranded inhibitor. Similarly, the complex just below Sp1 was identified as USF by inhibition with anti-USF antibodies. The complex just below USF was tentatively identified as an Sp1-like protein by its competition with the Sp1 consensus sequence (ATTCTGATCGGGCGGCGCGAC), but was not supershifted by anti-Sp1 antibodies. This complex (X) appears to be more strongly induced during the time course of IFN-γ treatment.
Previously, we have shown that the BGP message is rapidly induced within 4 h after IFN-γ treatment (22). In this report, we extend our studies to the analysis of the promoter region by in vivo footprinting, an approach that reveals protein-DNA interactions in live cells treated with IFN-γ. Two footprints, previously identified for the BGP gene by Hauck et al. (42), FP-I (−167 to −137) and FP-II (−305 to −280), were observed in our in vivo DNase I footprint analysis, suggesting that the two sites previously identified by in vitro footprinting are involved in BGP gene regulation in vivo. In addition to these two footprints, a distinct footprint, labeled IRE (−230 to −210) in Fig. 1, was located between FP-I and FP-II. Unlike FP-I and FP-II, IRE is induced only after IFN-γ treatment. The IRE identified corresponds to the ISRE consensus sequence AGTTG(T/C)T, previously identified for other genes induced by IFN-γ (23). ISRE is a motif to which the ISGF3 complex binds and mediates gene activation induced by type I interferons (IFN-α/β). The ISGF3 complex includes Stat-1, Stat-2, and ISGF3-γ, a 48-kDa transcription factor (43). In addition to the ISGF3 complex, a group of transcription factors including IRF-1 (39, 44) and IRF-2 (26) have been shown to bind to ISREs. While IRF-1 is known to stimulate gene transcription, IRF-2 inhibits transcription. The time course of activation of some genes by IFN is extremely rapid (<30 min) and involves the translocation of Stat-1 and Stat-2 to the nucleus (23). The other group contains genes activated only after prolonged treatment with IFN-γ (6–24 h), requiring de novo protein synthesis. For the majority of these genes, IRF-1 or IRF-1-like proteins are thought to be involved. For example, IRF-1 has been shown to mediate the gene activation of guanylate-binding protein (45), IFN-α and IFN-β (46, 47), major histocompatibility class I antigen (48), and inducible nitric oxide (49, 50). In the case of BGP gene activation, we have shown here that IFN-γ first induces IRF-1 expression via Stat-1, and then IRF-1 induces BGP expression. The effect of IRF-1 on the BGP promoter was confirmed by transactivation with an IRF-1 expression plasmid plus a BGP reporter gene, while cotransfection with the BGP reporter gene with a mutant IRE results in no transactivation (Table II).

The time course of BGP mRNA in HT-29 cells after IFN-γ treatment correlates with the induction of IRF-1 mRNA and protein and the BGP IRE-IRF-1 complex as measured by RNase protection assays, Western blotting, and EMSAs (Figs. 4, 6, and 7). Since the induction of the IRF-1 gene depends on the formation of the Stat-1-GAS complex, we also examined the time course of its formation and found that this complex persists even through 24 h of IFN-γ treatment (Fig. 7). It is also known that Stat-1 contains a truncated (Stat-1a) and an untruncated (Stat-1b) form and that Stat-1a stimulates while Stat-1b inhibits IRF-1 transcription (41). Western blotting with anti-Stat-1 antibodies revealed that the amounts of Stat-1a steadily increased during the time course of IFN-γ treatment, while little or no Stat-1b was produced. Thus, the time course of Stat-1-GAS complex formation in HT-29 cells agrees with the induction of Stat-1 protein by IFN-γ, and there is no role for Stat-1b. The fact that a minor form of BGP mRNA is superinduced by cycloheximide suggests that an additional mechanism is operative (Fig. 6). The induction of the Sp1 and Sp1-like protein complexes within FP-I of the BGP promoter is a candidate.

Low levels of BGP mRNA are constitutively produced in HT-29 cells. This is consistent with the in vivo footprint results, where it is seen that FP-I and FP-II are apparent prior to IFN-γ treatment (Fig. 1). However, in the case of HeLa cells, where no BGP is made until after IFN-γ treatment, no footprints are observed until after treatment with IFN-γ. Thus, it appears that the binding of IRF-1 to the BGP IRE in cells with a silent BGP gene is sufficient to cause binding of all the additional factors identified in FP-I and FP-II. Since the expression levels of BGP in HeLa cells treated with IFN-γ never reach those in SW403 and HT-29 cells, it is likely that the optimal transcription complex is not fully formed in HeLa cells. A search for a silencer element by Hauck et al. (42) revealed no such element within the first 3 kilobases of the BGP promoter.

We were also concerned that other transcription factors may play a role in the induction of BGP by IFN-γ, especially in light of the recent reports by Sanceau et al. (51), who showed that IRF-1 can cooperate with Sp1 and NF-xB in monocytic cells treated with IFN-γ, and by Neish et al. (52), who showed that NF-xB and IRF-1 form a complex in tumor necrosis factor-α-treated endothelial cells. In our in vivo footprints, we observed a strong FP-I containing Sp1 and USF, adjacent to IRE in the BGP promoter. The USF site (CACATG, −148 to −143) is
Role of IRF-1 in BGP Regulation

In the transfection protocol, HT-29 and SW403 cells were trypsinized for 20 min before transfection. Cells (3 × 10^5) were transfected with a lipofectin (6 μg) and reporter DNA (1 μg) mixture for 48 h and then treated with IFN-γ overnight. The values are the average of six experiments and show the range.

![Graph](image)

**Fig. 8. Identification of FP-I-binding factors by electrophoretic mobility shift assays.** Nuclear extracts were prepared by the NaCl extraction method (30). Nuclear extracts (2–3 μg) from HT-29 cells, before and after IFN-γ (500 units/ml) treatment, were incubated with 32P-labeled double-stranded oligonucleotide corresponding to FP-I. The time course of treatment with IFN-γ is shown, along with inhibition by unlabeled probe as indicated (50-fold) and antibody supershifts with antibodies to Sp1, Stat-1/β, and USF.

TABLE II

| Cells          | Constructa | -IFN-γ | +IFN-γ | -Fold  | +pAct-1b | -Fold  | +pAct-2b | -Fold  |
|---------------|------------|--------|--------|--------|----------|--------|----------|--------|
| HeLa          | PØGH/BGP307 | 810 ± 116 | 1580 ± 359 | 2.0 | 1226 ± 144 | 1.5 | 687 ± 62 | 0.9 |
| PØGH/BGP307m  | 257 ± 68 | 258 ± 76 | 1.0 | 293 ± 56 | 1.1 | ND      | ND     |
| PØGH          | 329 ± 15 | ND     | ND     | ND     | ND       | ND     | ND       | ND     |
| PXGH          | 6179 ± 381 | ND     | ND     | ND     | ND       | ND     | ND       | ND     |
| HT-29         | PØGH/BGP307 | 529 ± 113 | 1334 ± 291 | 2.5 | 1157 ± 107 | 2.2 | 567 ± 78 | 1.1 |
| PØGH/BGP307m  | 386 ± 43  | 353 ± 58  | 0.9 | ND     | ND       | ND     | ND       | ND     |
| PØGH          | 334 ± 15  | ND     | ND     | ND     | ND       | ND     | ND       | ND     |
| PXGH          | 9685 ± 1290 | ND     | ND     | ND     | ND       | ND     | ND       | ND     |
| SW403         | PØGH/BGP307 | 1755 ± 392 | 2702 ± 319 | 1.5 | 5380 ± 77 | 3.1 | ND       | ND     |
| PØGH          | 330 ± 20  | ND     | ND     | ND     | ND       | ND     | ND       | ND     |
| PXGH          | 8134 ± 665 | ND     | ND     | ND     | ND       | ND     | ND       | ND     |

a PØGH/BGP307 contains BGP promoter −308 to −2 (BamHI); PØGH/BGP307m contains a mutant IRE as described under “Materials and Methods.”

b Cotransfection experiments were performed with 1 μg of BGP reporter plasmid plus 0.5 μg of IRF-1 expression vector. DNA/Lipofectin ratio was 1:4.

c ND, not detected.

partially protected from DNase I digestion (Fig. 1) and DMS methylation (Fig. 2). An Sp1-like complex located just below the USF complex in EMSAs (Fig. 8) was induced by IFN-γ and represents a second point of transcriptional control. This complex may explain why some of the BGP mRNAs are inhibited while others are superinduced by cycloheximide (Fig. 6).

Comparison of the CEA and BGP promoters reveals that the position of FP-I relative to the start of translation is the same for both genes and that both contain Sp1 and USF elements. The position of FP-II in BGP is shifted relative to FP-II in CEA, involving different elements (AP-2-like versus Sp1). In the place of FP-II in CEA, BGP has an IRE that was identified only after treatment of cells with IFN-γ. Thus, the two highly homologous genes have undergone subtle changes upstream of FP-I to allow different modes of regulation, including the fact that CEA is expressed in colon only and is not transcriptionally responsive to IFN-γ, while BGP is expressed in a wider variety of tissues and is transcriptionally responsive to IFN-γ. It is also noteworthy that CEA possess a transcriptional silencer (FP-V) that is absent in the BGP gene.

Since the function of neither CEA nor BGP has been definitively established, the differential role of IFN-γ in inducing these two genes may provide important clues as to function. Most genes with ISREs have been implicated in immune or antiviral responses. We have previously speculated that CEA may be a first-line defense molecule for protection of epithelial cells against bacterial attack (53–55). Supporting this hypothesis, CEA is found on the luminal side of the colon (56) and binds bacteria via their fimbriae (53). Other members of the CEA gene family including BGP are found on luminal surfaces (57) or, in the case of activated neutrophils, are mobilized to the cell surface during bacterial activation (11). Since production of IFN-γ by T-cells is a primary response to bacteria invasion, it may be advantageous for epithelial cells exposed to bacteria to up-regulate BGP.

Other studies suggest a role for CEA and BGP in homotypic cell adhesion, a role that seems, at first glance, to be incompatible with their luminal orientation. However, the ability of BGP to mediate cell adhesion in vitro may affect its behavior in tumorigenesis. Results to date suggest that down-regulation of BGP correlates with tumor progression. If the expression of BGP is associated with a terminally differentiated cell, incapable of further cell division, then its down-regulation would be expected to correlate with tumor progression. Such a function would fit in well with the effect of IFN-γ on cells, where IFN-γ is known to induce cytostasis. Since one of the main mediators of this effect is IRF-1, the role of IRF-1 in inducing BGP is reasonable. Based on these ideas, we speculate that when BGP is engaged by ligand (homotypic adhesion with other BGP molecules or heterotypic adhesion with bacteria), it may directly induce cytokinesis, and when other components of the immune system are engaged (e.g. T-cells), up-regulation of BGP would augment these effects.

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