Nasopharyngeal cancer (NPC) is a malignancy of the head and neck that affects a relatively young population with a median age of onset in the mid-1940s (1). NPC is endemic to certain geographic regions, such as Southeast Asia, and is associated with several environmental and genetic factors (2). NPC is also unique with its intimate association with the Epstein Barr virus (EBV) where over 80% of NPC patients worldwide harbor the EBV genome in their cancer cells (3).

The current treatment regimen for NPC is fractionated radiotherapy (radiation), and patients with advanced disease are also treated with chemotherapy, such as cisplatin (cisdiamminedichloroplatinum II) achieving an overall 5-year survival of ~65% (4). It has been repeatedly observed that plasma EBV gene copy number increases significantly immediately after treatment with radiation with or without chemotherapy (5), implying that either the NPC cells are undergoing apoptosis (6) or that the EBV genome might be switching from a latent to a lytic phase of gene expression. This latter possibility is the focus of our current investigation.

Agents that activate the cellular stress response, including radiation and chemotherapy, have been investigated with respect to their effects on the latent EBV within EBV-positive models, such as Burkitt’s lymphoma and lymphoblastoid cell lines, and have shown induction from a latent to lytic gene expression profile (7). This transition is mediated by the expression of one or both of the critical immediate-early lytic genes, BRLF1 and BZLF1. BZLF1 belongs to the extended fos/jun family of transcription factors, and through its interaction with specific activator protein 1–like BZLF1-binding motifs in the regulatory region of downstream lytic gene targets, it plays an important role in lytic gene expression (8). BRLF1 also plays an important role in the lytic phenotype where it binds directly to a GC-rich motif on its downstream targets (9). The regulation of these genes is complex, including self-regulatory feedback mechanisms, synergistic autoactivation of BRLF1 and...
BZLF1 processes (10), and regulatory roles of several other transcription factors (11). Despite these important experimental contributions, there remain many underlying mechanistic questions as to how clinically used modalities, such as radiation and/or chemotherapy (e.g., cisplatin), might affect EBV activation, particularly in NPC.

In this study, we identify nuclear factor-Y (NF-Y) as an important mediator in the regulation of BRLF1 and BZLF1 gene expression. NF-Y is a multisubunit transcription factor, composed of subunits NF-YA, NF-YB, and NF-YC, all of which are required for sequence recognition and subsequent transcriptional activation. The specific consensus binding sequence includes the CCAAT motif, which is estimated to be present in ~30% of human promoters (12). NF-Y is an important transcription factor that has been implicated as a critical regulator of genes in multiple biological processes, including parathyroid hormone activation (13) and multidrug resistance 1 expression (14). More recently, NF-Y has been shown to be important in the regulation of other genes involved in cellular stress response, such as GADD45 and HSP (15, 16). This current work characterizes the latent to lytic activation of EBV in response to clinically relevant stress agents in NPC, shown by an increase in BRLF1 and BZLF1 expression, mediated in part by NF-Y.

Materials and Methods

Cells, culture conditions, and tumor models. Cells and culture conditions were used as previously described (17). Briefly, the EBV-positive NPC cell line C666-1 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc.). All experiments were conducted when cells were in an exponential growth phase. Transfection experiments were done using LipofectAMINE 2000 (Sigma-Aldrich) with pDNA-NF-YA (kindly provided by R. Mantovani) and pNF-YA (Origene). Using the manufacturers’ protocol, a transfection efficiency of ~60% was achieved. Three NPC xenograft models were used: C15, C17, and C666-1; all propagated in the gastrocnemius muscle of severe combined immunodeficiency mice, as previously described (17). Briefly, the EBV activation, particularly in NPC.

Cisplatin and radiation treatment in vitro. To assess the effects of radiation, cells were irradiated at room temperature using a 137Cs unit (Gamma-cell 40 Exactor, Nordion International, Inc.) between 2 and 15 Gy (dose rate of 1.1 Gy/min). The chemotherapeutic agents (cisplatin, 0.0-50 µg/mL; Mayne Pharma) and paclitaxel (Taxol, 0.04 N HCl) was then added to all wells and mixed to dissolve the crystals formed by viable cells. Plates were then read-on a Bio-Rad 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay by seeding C666-1 cells in 96-well plates, and after approximately one doubling time or 24 h, cells were exposed to either cisplatin (25, 50, 100 µg/mL; Mayne Pharma), and paclitaxel (Taxol, 0.04 N HCl) was then added to all wells and mixed to dissolve the crystals formed by viable cells. Plates were then read-on a Bio-Rad 3350 microplate reader at 570 nm (Bio-Rad).

Quantitative real-time PCR. Gene expression in response to treatment was determined by quantitative real-time PCR (qPCR). After treatment, total RNA was extracted and quantified, and 1 µg was reverse-transcribed for qPCR analysis at the stated time points after treatment (Qiagen RNeasy; Taqman reverse transcription reagents, Applied Biosystems). The SYBR green protocol was used as per manufacturer’s instructions in a total reaction volume of 12.5 µL (SYBR green master mix, Applied Biosystems).

Unique primers were designed to amplify specific regions between 150 and 250 bp of the transcripts [BRLF1, BZLF1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NF-YA, NF-YB, NF-YC]. To assess changes in relative EBV genome level, genomic DNA was isolated (Qiagen DNeasy) and unique primers were used for the genomic region BamHI-W, as previously described by Lo et al. (20). Primer specificity for both mRNA and genomic DNA was confirmed by analysis of the fragment length using agarose gel electrophoresis and the presence of single-peak melting dissociation curves. Primer sequences used were as follows:

BamHI-W For: 5’CCCAACACTCTCACCCACACC’3, Rev: 5’TCTTAGGAGCTGTCCGAGGCG’3.
BRLF1 For: 5’AATTTCAGCGCAGGAGAGTG’3, Rev: 5’AGGCCGGTCTCTCCTACCCCGT’3.
BZLF1 For: 5’GGCTTACCAAGGAGCAAACACGC’3, Rev: 5’TTCAGATGTAGTTGGCAGCAG’3.
GAPDH For: 5’GAAGGTGAAGGTGATGGAGTC’3, Rev: 5’GAAGATGGGTAGTGAGGATTT’3.
NF-YA For: 5’TGGTGCATCTGGAACATCTGTA’3, Rev: 5’AGTTCGCCCAAAATCTGTGCAC’3.
NF-YB For: 5’AAAAGGTAATGTAGGAGCGCGGT’3, Rev: 5’CGATCTCGGGCAGGATTTC’3.
NF-YC For: 5’GGCGGCGGCAGAATTTTTATCA’3, Rev: 5’GCTCGGCGAGGATACAC’3.

qPCR was used as a Perkin-Elmer/ABI Prism 7900 sequence detection system (Applied Biosystems) using the following cycling profile: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. All experiments were done independently in triplicate, and qPCR was conducted on the same samples in duplicate.

Results were reported as mean fold change in gene expression, calculated using the ∆∆Ct method, where Ct values for the amplicon of interest were normalized to GAPDH expression compared with control or untreated cells.

Western blotting of NF-YA. Cell extracts were prepared in lysis buffer [0.1 mol/L Tris-Cl (pH 8.0), 0.1% SDS, 10 mmol/L EDTA, 2 mmol/L DTT, protease inhibitors] and protein concentrations were determined using the bicinchoninic acid protein assay (Bio-Rad). Immunoblotting was conducted as previously described (18). Briefly, samples containing equal amounts of protein were loaded onto a 4% to 12% SDS-PAGE gel, electrophoresed for 120 min at 100 V using the electrophoresis cell (Bio-Rad) and transferred onto nitrocellulose membranes using a transblot semidy cell (Bio-Rad). Membranes were blocked in 5% milk in PBST (0.1% Tween 20 in PBS) for 30 min at room temperature and then probed with 0.2 µg/mL of mouse monoclonal NF-YA/CBF-A (Santa Cruz Biotechnology) or GAPDH (Calbiochem), all in PBST containing 5% low fat milk. Blots were then washed with PBST and incubated with horseradish peroxidase–conjugated secondary antibodies detected using chemiluminescence (DuPont).

In vivo xenograft experiments. All animal experiments used severe combined immunodeficiency BALB/c mice obtained from the Ontario Cancer Institute, Animal Research Colony, and the experiments were conducted in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network. Female severe combined immunodeficiency mice of ages 6 to 8 weeks were injected i.m. (left gastrocnemius) with ~10^6 cells (C666-1, C15,
C17). After 2 to 4 weeks, tumor plus leg diameter reached 9 mm (0.3-0.4 g tumor), and the animals were then randomized into one of the following three groups: (a) control, no treatment; (b) cisplatin alone (4 mg/kg, i.p.); and (c) cisplatin (4 mg/kg, i.p.) followed immediately with radiation (15 Gy). For local radiation treatment, animals were immobilized in a lucite box with the tumor-bearing leg exposed to 100 kV at a dose rate of 10 Gy/min. Animals were euthanized, and tumor RNA was extracted at either 5 or 48 h posttreatment.

In silico promoter analysis. MatInspector Release Professional version 7.4 with Matrix Library version 5 (Genomatix) was used to analyze 2.8 kb upstream of the mRNA start site of BRLF1 from the B-98-5 EBV (HHV4) genomic sequence, NC_001345, 105183 to 10800 bp, (-) strand. Matrix families were used to identify potential binding sites of known transcription factors and weighed according to a matrix similarity algorithm. Three different matrices were used to identify potential NF-Y binding sites. Each matrix is weighted on a 15-bp sequence defined from compiled sequences identified experimentally.

ELISA. NF-Y activation was evaluated using an ELISA (TransAM, NF-YA transcription factor assay, Active Motif) as per manufacturer’s instructions. Briefly, C666-1 cells were treated with cisplatin (50 μg/mL) and radiation (15 Gy); at 0.1, 0.5, 1, 4, 8, and 24 h posttreatment, nuclear lysates were collected. Extracts of interest and the positive control cell line (Jurkat) were added at a concentration of 5 μg per well to a pretreated 96-well plate containing the NF-YA binding consensus oligonucleotide sequence. A monoclonal NF-YA primary antibody and rabbit horseradish peroxidase–conjugated secondary antibody (0.4 μg/mL) were used for colorimetric quantification.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done using the commercially available system as per manufacturer’s instructions (Active Motif). Briefly, cells were treated and fixed and cellular chromatin was isolated. Sonication shearing conditions were optimized to generate chromatin fragments between 200 and 1,000 bp (five pulses, 20 s with 30 s intervals on ice; 25% maximum intensity).

Immunoprecipitation was done using the monoclonal NF-YA (BD PharMingen) and NF-YB (Santa Cruz Biotechnology) antibodies at 4°C overnight. A monoclonal TFIIB antibody and IgG were used as negative controls. Input DNA was obtained from the same treated cells and underwent the entire protocol except the immunoprecipitation reaction. The resulting enrichment of chromatin bound to the NF-Y protein TFIIB and IgG was quantitated using qPCR. Primers specific for the three predicted NF-Y consensus regions in the BRLF1 promoter were designed and confirmed (data not shown):

NF-1 For: 5′GTCCTGAAAATTTCCCCATCC′3, Rev: 5′GCCGCCAG-TAGCATCTCTGTC′3.
NF-2 For: 5′TGTTAAGGGCCCTGTCGC′3, Rev: 5′ACTCCATG-AGCTTTAACAGC′3.
NF-3 For: 5′TGCTTCAAGCTATGAGAGCT′3, Rev: 5′ATGTGACAG-CCTGCTACAGC′3.

A negative control primer set for a region of genomic DNA between the GAPDH gene and the chromosome condensation-related SMC-associated protein (CNAP1) gene was used, which should not be enriched by any of the antibodies:

NEG For: 5′ATGGTGGCCACTTGCGGATC′3, Rev: 5′TGCCAAAGC-CTAGGGGAAGA′3.
GAPDH For: 5′GAAGGTGGAAGGATGGATT′3, Rev: 5′GAGATGAGT-GATGGGAGTTC′3.

Quantitation of the amplification of the enriched chromatin fragment was normalized to the raw Ct values of the input DNA and compared with values obtained under control conditions.

Statistical analysis. All data are reported as mean ± SE, unless otherwise stated. Statistical differences between groups were determined using a Student’s t test, one-way ANOVA test (Microsoft Excel).

Results

Dose-dependent stress activation of EBV. To assess the effect of different DNA-damaging agents on EBV activation, C666-1 cells were treated with increasing doses of 5-fluorouracil, Taxol, cisplatin, and radiation (Fig. 1A–E). qPCR was used to measure the level of BamH1-W expression, an indicator of EBV genome replication and copy number (21). There was no significant effect of 5-fluorouracil on EBV BamH1 expression at 48 h posttreatment (Fig. 1A). However, both Taxol and cisplatin alone induced a dose-dependent increase in BamH1 expression to a maximum of 3.0-fold and 5.2-fold over untreated controls, respectively (Fig. 1B and C). When C666-1 cells were exposed to increasing doses of radiation from 0 to 6 Gy, there was an associated modest increase in BamH1 detection with maximal levels observed at 1.4-fold, 24 h postradiation, which did not differ significantly at either 48 h or 72 h postradiation (Fig. 1D).

Because radiation and cisplatin are the mainstays of NPC clinical management, this combination was evaluated on EBV activation. Figure 1E again shows a dose-dependent increase in BamH1 expression with cisplatin alone at 48 h. Using 25 μg/mL cisplatin with 15 Gy of radiation, BamH1 increased 2.3-fold over untreated controls. When C666-1 cells were treated with a higher dose cisplatin (50 μg/mL) combined with 15 Gy, a 6.5-fold increase in BamH1 expression was observed. Hence, these data confirm an increase in relative EBV genome copy number in C666-1 cells in response to agents, such as cisplatin, radiation, and Taxol, demonstrating EBV activation in response to DNA damage.

Viability of C666-1 cells after treatment with cisplatin and radiation was assessed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. There is a dose-dependent decrease in cell viability with increasing doses of cisplatin and radiation (Fig. 2). At 48 h post–15 Gy, 85% of cells were viable; when combined with 25 or 50 μg/mL cisplatin, viability was further reduced to 41% and 47%, respectively. There was a time-dependent decrease in viability from 24 to 72 h after treatment, where cisplatin (25 μg/mL) and radiation (15 Gy) reduced viability to 77%, 40%, and 11% at 24, 48, and 72 h, respectively. At 48 h posttreatment, cisplatin (50 μg/mL) plus radiation (15 Gy) resulted in 47% cell viability, which were the doses chosen for the majority of the subsequent experiments.

Cisplatin and radiation treatment induce EBV lytic gene expression in vitro and in vivo. The switch from EBV latent to lytic gene expression profile is associated with the expression of two critical immediate-early genes BRLF1 and BZLF1. Figure 3A shows that when C666-1 cells are treated with cisplatin (50 μg/mL) and radiation (15 Gy), there is a significant time-dependent increase in expression of both BRLF1 and BZLF1, as assessed by qPCR. The expression of BRLF1 is significantly increased 8 h posttreatment and reaches a maximum level of 20-fold induction at 48 h over the paired time control group. BZLF1 expression also increases in a time-dependent manner to a maximum of ∼23-fold at 48 h with a significant increase apparent at 8 h posttreatment (Fig. 3A). In a separate experiment, a more detailed examination of gene expression immediately after treatment shows a significant increase in BRLF1 expression within 5 min postcisplatin and radiation, followed by a comparable increase in BZLF1.
expression observed at 1 h posttreatment (Fig. 3B), suggesting that BRLF1 might play a more dominant role in EBV stress activation in NPC.

The induction of *BZLF1* and *BRLF1* expression in response to cisplatin and radiation was also evaluated *in vivo* using several EBV-positive NPC xenograft models. Results of this analysis for the C666-1 xenograft model are shown in Fig. 3C and D. There was a trend observed for *BRLF1* induction by 1.7-fold with cisplatin alone, which increased further with cisplatin plus radiation (2.4-fold induction); respective *P* values were 0.30 and 0.13. Similarly, *BZLF1* induction was also observed with cisplatin (1.5-fold), which increased further with cisplatin and radiation (1.9-fold). The respective *P* values were 0.16 and 0.05, bordering on statistical significance with this last combination.

These data were very similar for the other two EBV-positive NPC xenograft models of C15 and C17 (data not shown). In summary, these experiments suggest that the increase in lytic
EBV gene expression (BRLF1 and BZLF1) in response to cisplatin and radiation treatment is observed both in vitro and in vivo for NPC.

**NF-Y interacts with regions of the BRLF1 promoter.** An in silico analysis (MatInspector) of the promoter regions of BRLF1 and BZLF1 was conducted to evaluate the possible mechanisms by which these genes are influenced in response to stress agents, leading to the identification of NF-Y as a potential mediator (Fig. 4A). NF-Y is a transcription factor that binds with high affinity and fidelity to CCAAT-containing consensus sequences and has also been described to be involved in cell cycle (22) and stem cell self-renewal (23).

The hypothesis that the NF-Y protein complex was binding directly to one or more of these sites and thereby influencing transcriptional activation was evaluated using chromatin immunoprecipitation, which examines protein-DNA interactions in a cellular context. Here, C666-1 cells were treated with cisplatin alone or cisplatin plus radiation and analyzed at 6 and 24 h posttreatment (Fig. 4B). After immunoprecipitation with either NF-YA or NF-YB antibodies, the extent of chromatin enrichment after treatment was quantified by qPCR of all three putative consensus sequences on the BRLF1 promoter. Figure 4B shows that the NF-Y trimeric transcription factor seems to be indeed binding to the BRLF1 regulatory region after cisplatin alone or cisplatin plus radiation (P < 0.05). This was observed early, at 6 h posttreatment, but not later at 24 h (data not shown), thereby documenting a physical interaction of NF-Y with the BRLF1 promoter soon after stress exposure with cisplatin or cisplatin plus radiation.

**NF-Y is activated after cisplatin and radiation treatment.** To assess the effect of NF-Y after stress, C666-1 cells were treated with cisplatin and radiation and an ELISA was done to assess NF-Y activation in a time-dependent manner (Fig. 5A). Functional NF-Y transactivation was evaluated by exposing nuclear extracts to an oligonucleotide containing the NF-Y consensus sequence, where positive activity would result after the successful interaction of the NF-Y protein with the oligonucleotide. The sequence specificity of this interaction was confirmed using competitive oligonucleotides corresponding to wild-type and mutant NF-Y consensus sequences. As shown in Fig. 5A, there is an immediate and significant 1.8-fold increase in nuclear activation of NF-Y as early as 5 min after treatment relative to untreated control cells. This activation continues for up to 48 h posttreatment. These results indicate that NF-Y protein and/or function increases rapidly and is sustained for up to 2 days in the nucleus of C666-1 cells after cisplatin and radiation treatment.

The NF-Y protein complex is composed of three independent subunits: NF-YA, NF-YB, and NF-YC; all of which are essential for its transcriptional activity. To assess the effect of cisplatin and radiation on NF-Y gene expression, transcript levels for each of the three subunits were assessed using qPCR. As shown in Fig. 5B, a significant time-dependent increase in NF-YA and BZLF1 expression was first observed at 8 h posttreatment and reached maximal levels of 10.8-fold and 6.3-fold at 48 h, respectively. NF-YC expression remained unchanged after cisplatin and radiation exposure. Detailed analysis of transcript levels immediately after treatment showed stable expression for all three subunits between 5 min and 2 h posttreatment (data not shown).

Western blot analysis confirmed the increase in NF-YA expression at the protein level. Both the 42 and 46 kDa isoforms of the NF-YA protein were observed to increase between 4 and 24 h posttreatment (Fig. 5C). This series of studies confirm an almost immediate nuclear activation of NF-Y after cisplatin and radiation, associated with an increase in NF-YA protein expression. This is followed subsequently by a delayed increase in the expression of NF-YA and NF-YB transcripts (Fig. 5B).

**NF-Y is an important regulator of BRLF1 and BZLF1 gene expression.** To assess the importance of NF-Y on BRLF1 and BZLF1 expression, a dominant-negative NF-YA plasmid was transiently transfected into C666-1 cells. Once the cells have re-attached as a monolayer, 12 h posttransfection, they were treated with cisplatin and radiation. Control cells were transfected with B-galactosidase plasmid. BRLF1 and BZLF1 gene transcript levels were determined at 24, 48, and 72 h posttreatment by qPCR (Fig. 6A). Induction of BRLF1 and BZLF1 expression were observed in B-galactosidase–transfected cells treated with cisplatin and radiation at 24, 48, and 72 h posttreatment. However, dominant-negative NF-YA–transfected cells showed a significant reduction in expression of both BRLF1 and BZLF1 genes, particularly at 72 h. Specifically, BRLF1 mean fold expression was decreased from 13.9-fold to 4.9-fold; BZLF1 expression also decreased from 12.8-fold to 8.1-fold, as a result of dominant-negative NF-YA.

This role of NF-Y in regulating BRLF1 and BZLF1 activity was examined conversely using transient transfection of the NF-YA plasmid with cisplatin and radiation. Figure 6B reveals an initial modest increase in BRLF1 expression in NF-YA over B-galactosidase–transfected cells after treatment with cisplatin and radiation, but by 72 h, this increased from 4.3 to 7.9 mean fold change in gene expression. Under these same experimental conditions, there was a similar trend in BZLF1 expression, with an increase from 4.0-fold to 7.5-fold observed at 72 h. Taken together, these data indicate an important role for NF-Y in regulating the transcription of the EBV lytic genes, BRLF1 and BZLF1.

**Discussion**

This report is the first detailed characterization of EBV lytic gene expression in NPC after cisplatin and radiation and
identification of NF-Y as an important mediator of this stress response. DNA-damaging agents, such as cisplatin and radiation, can induce EBV activation, associated with a significant induction in BRLF1 and BZLF1 lytic gene expression in a time-dependent manner. This, therefore, confirms the initiation of the EBV lytic gene expression profile after stress exposure, which is observed both in cells and in xenograft models in vivo. The modest induction in vivo might be partially explained by the observation that this induction is occurring only in a subpopulation of tumor cells, in that about 25% to 27% of cells expressed BZLF1 and BRLF1 72 h after cisplatin and radiation treatment in vitro (fluorescence-activated cell sorting data not shown). This subpopulation phenomenon has been observed in EBV-positive gastric (24) and B-cell models (7) after DNA-damaging treatments. The precise reason behind this subpopulation effect is unclear at the moment, but might be related to factors, such as the cell cycle phase.

Circulating levels of plasma EBV DNA is an established biomarker for monitoring NPC response to therapy (25); the precise etiology of these DNA fragments remains under investigation, but based on their short fragment sizes (~180 bp), they have been attributed to apoptotic death of NPC cells after exposure to chemotherapy and radiation (6). It is also observed that there is a “spike” in circulating EBV DNA levels immediately after the first treatment (5), suggesting an alternate hypothesis of the activation of EBV into a lytic phase. This hypothesis is supported by the detection of infectious EBV in NPC cells (26), the presence of the linear form of the EBV genome suggestive of a productive lytic cycle (27), and observations of BRLF1 expression in NPC biopsy samples along with circulating BRLF1 antibodies in NPC patients (28). Our current study supports the concept that EBV activation could contribute to the observed increased levels of circulating EBV after treatment.

One concern regarding this study might relate to the high radiation and cisplatin doses selected for evaluation given that radiation is usually given in a fractionated regimen, with 2 Gy per fraction, as opposed to 15 Gy as a single dose. In addition, the clinically used cisplatin dose of 100 mg/m² is significantly less than the 50 μg/mL used for these studies. Radiation alone has a modest effect on BamHI expression level (Fig. 1D); the combination, however, of radiation with cisplatin, even at 5 Gy radiation with 25 μg/mL cisplatin, did induce almost a 2-fold increase in BamHI, demonstrating that even at these lower doses of DNA-damaging agents, the EBV genome seems to be...
doubling, suggesting a switch in the EBV program from a latent to lytic phase.

BRLF1 and BZLF1 are two critical early-immediate genes activated upon initiation of the lytic program (29). Our data show that although both BRLF1 and BZLF1 are induced upon treatment, BRLF1 may play a more prominent role in EBV activation in NPC, given its more immediate and pronounced induction after cisplatin and radiation (Fig. 3B). Other groups have shown that BRLF1 is sufficient to disrupt latency in several EBV-positive human tumor cell lines (30). Moreover, Westphal et al. have shown that infection with adenoviral BRLF1 induces the expression of late lytic protein BMRF1 in another NPC cell line (31). Hence, BRLF1 may be a more dominant mediator of the latent to lytic transition in NPC, although there is a complex and intimate relationship with BZLF1 in this activation process, particularly with other stressors, such as phorbol esters and sodium butyrate, where both BRLF1 and BZLF1 are important (24).

Experimental work has shown that BZLF1 could be initiated by one of two promoters: the Zp promoter and the more distal Rp promoter. Transcription from Zp is the major contributor to the expression of a 0.9-kb transcript, whereas the Rp promoter is responsible for not only BZLF1 transcription but also the more proximal BRLF1 expression. The proximal region of Zp, -221 to +12 has been characterized to contain several responsive elements to extracellular stimuli through the interaction with cellular transcription factors, including regions of cAMP-responsive element binding

![EBV Genome](image)

**Fig. 4.** Interaction of NF-Y and the BRLF1 promoter in cells treated with cisplatin and radiation. A, schema depicting the genomic organization of the region containing BRLF1 and BZLF1 and their related promoters. Within the Rp regulatory region, three NF-Y consensus sequences denoted as NF1, NF2, and NF3 were identified. B, chromatin immunoprecipitation analysis of the BRLF1 promoter region. Specific primer sequences were designed and verified for three consensus regions NF1, NF2, and NF3. Cells were treated with cisplatin and radiation then analyzed for the potential interaction of the NF-Y protein with one or more of the NF consensus sequences at 6 h posttreatment. Data are analyzed as raw C, normalized to the input DNA (sample that did not undergo immunoprecipitation) and reported as a function of the untreated group (*, P ≤ 0.05).
Characterization of the proximal region of the Rp promoter has also revealed several sites for cellular transcription factor binding, including Sp1, YY1, and Zif268 (35, 36). Moreover, autoactivation of BRLF1 is achieved through two Sp1-binding sites at -279 and -45 relative to the transcriptional start site (37). Using 5'-deletion constructs of the BRLF1 promoter region, Glaser et al. examined up to -962 bp of the transcriptional start site, where they identified the distal NF1 site as being important for BRLF1 expression (38).

Our in silico analysis of the BRLF1 promoter was extended to -2.8 kb beyond the transcriptional start site, thereby identifying many more potential cellular transcription factor binding sites, including three CCAAT-containing consensus sequences for NF-Y (Fig. 4). NF-Y has been shown to be involved in viral gene expression with a role in latent gene expression from the EBV Cp promoter (39), and in other viral systems, such as hepatitis B (40), cytomegalovirus (41), adenovirus (42), and retroviral gene expression (43). There is emerging evidence of the role of NF-Y stress response further supporting its candidacy as a protein/activating transcription factor 1 binding, Sp1/Sp3, and a more distal YY1-negative element (32). In addition, the Zp promoter activity is regulated through chromatin structure, where upon external signaling, the inactive state maintained by histone deacetylation is released after phosphorylation of MEF-2D with subsequent recruitment of histone acetyltransferase, resulting in histone acetylation (33). An added level of regulation is achieved through an autoactivation mechanism mediated by the binding of the BZLF1 homodimer to the Zp promoter (34).

Fig. 5. NF-Y activation and increased expression after cisplatin and radiation treatment. A, NF-Y transcriptional activation after treatment (cisplatin, 50 μg/mL and radiation, 15 Gy) was determined using an ELISA and sequence-specific competitive oligonucleotides. Results are reported as mean fold activation normalized to the wild-type oligonucleotide (to ensure sequence specificity) compared with untreated cells. Jurkat nuclear lysates were used as a positive control. B, qPCR analysis of NF-YA, NF-YB, and NF-YC expression 2 to 48 h posttreatment. Columns, mean fold change in gene expression compared with that of matched untreated controls; *, P ≤ 0.05. C, Western blot analysis of NF-YA protein expression after cisplatin and radiation treatment. There is the expected increase in both the 42 and 46 kDa isoforms observed; GAPDH protein expression was used as the loading control.
potential mediator of EBV response to radiation and cisplatin treatment in NPC (15).

Our data show that cisplatin and radiation induce an immediate NF-Y nuclear translocation and activation that increases to maximal levels at 4 h posttreatment (Fig. 5C). This significant increase in nuclear transactivation suggests a nuclear localization of the NF-Y protein upon stress. This is supported by Kahle et al., who showed that the NF-Y complex,
which normally resides in the cytoplasm, rapidly translocates to the nucleus through a dual mechanism where NF-YA is transported in an importin β-dependent pathway, whereas the NF-YB and NF-YC subunits are translocated through a distinct pathway involving importin 13 (44). Our investigation of all three NF-Y subunits in response to cisplatin and radiation showed an increase in NF-YA and NF-YB transcript levels beginning at 8 h reaching maximal levels 48 h posttreatment (Fig. 5B). To our knowledge, this is the first report of a detailed kinetics evaluation of NF-Y expression after stress exposure.

Until recently, NF-Y target genes seemed to be regulated through NF-Y consensus sites located approximately -60 to -100 bp upstream of the transcriptional start site. However, recent evidence has demanded a broader definition in that growth hormone receptor (45), MHCII (46), and Hoxb4 (47) have all have been shown to be regulated through more distal NF-Y sites. In addition, a thorough chromatin immunoprecipitation on on-chip (microarray) analysis confirmed the exception to the position rule by demonstrating that NF-Y is not necessarily a promoter-specific factor. In fact 40% to 50% of NF-Y regulated genes containing CCAAT sites were located distant to the transcriptional start site (48).

Our experimental data support a model (Fig. 7) where, upon stress treatment with cisplatin and radiation, NF-Y localizes to the nucleus (44) and transcriptionally activates the expression of the EBV lytic genes BRLF1 and BZLF1 within 30 min. NF-Y activation of BRLF1 may be mediated by the physical interaction of NF-Y with one or a combination of the three consensus sequences within the distal promoter region. Alternatively, this interaction might also be occurring through participation of cofactors, such as Oct-1 (15) or Sp1 (39). Finally, NF-Y may also be exerting its effect indirectly through the up-regulation of the transcription factor Sp3 and its subsequent involvement in BRLF1 auto-activation (49), allowing for a positive feedback loop with further amplification of NF-Y–mediated lytic gene expression (Fig. 7).

EBV activation has been shown to have important potential therapeutic applications. Treatment with radiation, chemotherapy, or sodium butyrate can induce expression of an EBV late lytic gene BXLF1, a homologue of the herpes simplex thymidine kinase. BXLF1 is a potential sensitizer to ganciclovir, whereby BXLF1 could phosphorylate ganciclovir, resulting in the production of a nucleotide analogue thereby causing chain termination. This approach of ganciclovir, combined with radiation or chemotherapy, has already been shown to achieve greater cytotoxicity and tumor growth delay when compared with either radiation and chemotherapy alone (50). Similarly, a recent study had used bortezomib to activate EBV lytic gene expression, thereby facilitating the imaging of EBV positive malignancies in vivo (51).

In conclusion, for the first time, we have shown that the NF-Y transcription factor is an important mediator of EBV stress response in switching from latent to lytic gene expression in NPC models, occurring both in vitro and in vivo. This important observation not only offers biological insight but might also provide treatment opportunities, whereby NPC response could be potentially enhanced through manipulations of this regulatory pathway.

**Fig. 7.** Model of NF-Y–mediated expression of BRLF1/BZLF1 after radiation and cisplatin treatment. NF-Y localizes to the nucleus almost immediately after stress treatment either directly in response to stress or mediated by unknown factors (i). Upon nuclear localization, NF-Y mediates BRLF1 and BZLF1 expression through a direct interaction with the regulatory regions (ii) and/or with cofactors (iii), such as Oct-1 or Sp1. The relationship between NF-Y and BRLF1 may be further potentiated by the presence of BRLF1 consensus sites within the NF-YA regulatory region (iv).
Clinical Cancer Research

Nuclear Factor-Y and Epstein Barr Virus in Nasopharyngeal Cancer

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