JOURNAL OF VIROLOGY, Feb. 2006, p. 1321–1331 Vol. 80, No. 3
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.3.1321–1331.2006

Epstein-Barr Virus Protein Can Upregulate Cyclo-Oxygenase-2 Expression through Association with the Suppressor of Metastasis Nm23-H1

Rajeev Kaul, Subhash C. Verma, Masanao Murakami, Ke Lan, Tathagata Choudhuri, and Erle S. Robertson*

Department of Microbiology, University of Pennsylvania School of Medicine, and Tumor Virology Program, Abramson Comprehensive Cancer Center, Philadelphia, Pennsylvania 19104

Received 11 October 2005/Accepted 15 November 2005

Previous studies have demonstrated the interaction between the Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA3C) and the metastatic suppressor Nm23-H1 both in vitro and in vivo (C. Subramanian, M. A. Cotter II, and E. S. Robertson, Nat. Med. 7:350–355, 2001). EBNA3C can reverse the ability of Nm23-H1 to suppress migration of Burkitt's lymphoma and breast carcinoma cell lines in vitro. EBNA3C contributes to EBV-associated human cancers by regulating transcription of a number of cellular and viral promoters and by targeting and altering the transcription activities of the metastasis suppressor Nm23-H1. Cyclo-oxygenase-2 (COX-2), an inducible enzyme important in inflammation, is overexpressed in a variety of cancers and can influence cell migration. In this report we show that Nm23-H1 and EBNA3C can modulate expression of COX-2 in the context of EBV infection and transformation. The levels of COX-2 were consistently higher in EBV-positive cells than in EBV-negative cells. Additionally, we show that Nm23-H1 can upregulate the COX-2 promoter element in luciferase reporter assays, whereas EBNA3C alone did not affect the level of response but clearly contributed to an additive increase when coexpressed with Nm23-H1. The downstream effect of COX-2 expression was also evaluated and showed that prostaglandin E_2 levels increased with Nm23-H1 and that there was some level of cooperativity in the presence of EBNA3C. The majority of this response was mediated through the cyclic AMP response element and NF-κB sites. These studies suggest a potential role for COX-2 in EBV-associated human cancers.

Epstein-Barr virus (EBV) is a human gammaherpesvirus which predominantly targets B cells and epithelial cells and is associated with a number of human cancers, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease as well as AIDS-associated and transplant-associated immunoblastic lymphomas and controversially, invasive breast carcinoma (9, 26, 56). In vitro infection of B cells with EBV gives rise to lymphoblastoid cell lines (LCLs), which express a subset of 12 latent viral transcripts (56). These 12 transcripts encode six EBV nuclear antigens (EBNAs), three latent membrane proteins (LMPs), two EBV early RNAs, and the BARF transcripts (56). Recent studies have shown that EBNA3C, one of the six nuclear antigens, interacts with the suppressor of cell migration and metastasis, Nm23-H1 (69). This interaction between Nm23-H1 and EBNA3C has been shown to result in an increase in transcriptional activity on a responsive promoter (70). Nm23-H1 tethered to DNA by a Gal4 DNA binding domain can activate transcription from a basal promoter at relatively low levels. However, when EBNA3C was introduced, the transactivation activity was shown to be substantially increased (70). These results suggest that Nm23-H1 may possess transcriptional regulatory activities independent of a possible role in directly binding to DNA or through its interaction with EBNA3C (69). Interestingly, the presence of EBNA3C mediates the cellular translocation of Nm23-H1 from a mostly cytoplasmic to a predominantly nuclear signal. Moreover, EBNA3C can reverse the antimigratory effects of Nm23-H1 in vitro (69).

The nm23 gene family is highly conserved among a wide variety of eukaryotic species. Eight genes have been identified in humans: Nm23-H1 (59), nm23-H2 (68), nm23-H3 (76), nm23-H4 (38), nm23-H5 (40), nm23-H6 (37, 74), nm23-H7 (75), and nm23-H8 (50). Members of the nm23 gene family are structurally and functionally conserved and consist of four to six identically folded subunits, enclosing a large (25-Å) central cavity (81). Expression of nm23 genes has been linked to suppression of tumor metastasis, differentiation, apoptosis, proliferation, and DNA mutation rate (14); expression of the genes is also associated with nucleoside diphosphate kinase activity (7, 42, 79), serine phosphorylation (8, 20, 34, 41), histidine protein kinase activities (78), as well as transcriptional stimulatory activities (3, 6, 53, 54).

The human Nm23-H1 gene product is the best-characterized member of this family of proteins. It is 152 amino acids in length with leucine repeats and alpha-helical and basic domains (56). Nm23-H1 is 88% identical to nm23-H2 and maps 4 kb apart at position q21.3 on chromosome 17 near the BRCA1 locus, known to be associated with early onset of familial breast and ovarian cancer (5). The Nm23-H1 gene product is more closely associated with the inhibition of metastasis and signal transduction, has an acidic pI, and is identical to the A subunit of nucleoside diphosphate kinase (17). Importantly, in a number of human cancers, there is an inverse relationship between Nm23-H1 expression and metastasis (14,

* Corresponding author. Mailing address: Department of Microbiology, University of Pennsylvania School of Medicine, 201E Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104. Phone: (215) 746-0116. Fax: (215) 898-9557. E-mail: erle@mail.med.upenn.edu.

36). In neuroblastoma, mutations in the leucine zipper motif of Nm23-H1 directly correlates with increased metastatic potential (19, 30).

The mechanism by which Nm23-H1 regulates metastasis is not fully understood; however, the experimental data accumulated thus far strongly implicates Nm23-H1 in the regulation of metastasis in a diverse number of human cancers (35). Recently, Nm23-H1 has also been identified as a granzyme A-activated, apoptosis-inducing DNase which forms a part of the SET complex (16). The SET complex translocates to the nuclei of target cells within minutes of GzmA loading or cytotoxic T-lymphocyte attack (16). Although the normal function of the SET complex is not known, its constituent proteins have been highly implicated in tumorigenesis. They have been linked to diverse functions, including chromatin modification, transcriptional activation of proto-oncogenes, DNA repair, and mRNA stability (10, 15, 33, 63, 65). The SET complex has also been suggested to be involved in repair and transcriptional activation in response to oxidative stress (16). All these observations indicate that translocation of Nm23-H1 from the cytoplasm to the nucleus may be an important event that may result in modulation of expression of cellular genes important in tumorigenesis.

Prostaglandin (PG) G/H endoperoxidase synthase, also known as cyclo-oxygenase (COX), is a key enzyme in the syntheses of prostanoids (PG and thromboxanes) (66). Of the two isoforms of COX, COX-1 is the constitutive form and COX-2 is the inducible form (67). The PG products derived from COX-1 activity are thought to facilitate many physiological processes (67). In contrast, COX-2 is highly induced in a variety of inflammatory diseases and in response to cytokines, growth factors, and other tumor promoters (21, 31, 48). Elevated levels of COX-2 are found in many premalignant lesions and epithelial cancers (27, 49, 84). Overexpression of COX-2 has been reported in various tumors, such as colon cancer (60), lung cancer (82), breast cancer (22), gastric cancer (58), esophageal cancer (88), and head and neck cancers (12), suggesting that COX-2 may be involved in carcinogenesis. Upregulation of COX-2 in cancer cells has also been linked to increased angiogenesis and metastasis (39, 52, 62). COX-2 can be regulated at both transcriptional and posttranscriptional levels (13). Transcriptional activation of COX-2 in particular has been studied in depth because of its roles in inflammation, immune responses, and carcinogenesis (11). The transcriptional activation of COX-2 is mediated by the binding of inducible transcriptional factors to *cis*-acting elements in the COX-2 promoter. Binding sites for the regulatory elements, including nuclear factor κB (NF-κB), nuclear factor for interleukin-6 (NF-IL-6), cyclic AMP response element (CRE), PEA-3, SP-1, activator protein-2 (AP-2), and T-cell factor 4 (TCF-4), have been identified in the 5′-flanking region of the COX-2 gene (2, 72). Transcription factors that activate COX-2 expression are elevated in a number of disease states and emergency responses, such as infection, are therefore potential targets. Possible involvement of the NF-κB, NF-IL-6, and CRE binding (CREB) transcription factors has been shown by deletion experiments using a luciferase reporter assay system (87).

Here we present evidence to suggest that Nm23-H1 upregulates COX-2 expression. This upregulation is further enhanced in the presence of the EBV essential latent protein EBNA3C. We also show that Nm23-H1 upregulates COX-2 expression through CRE and NF-κB binding sites of the COX-2 promoter. This suggests that EBV protein EBNA3C might have a role in upregulation of COX-2 through Nm23-H1, which promotes the growth and survival of EBV-associated tumor cells.

TABLE 1. Mutations in the COX-2 promoter constructs

Promoter site	Sequence[a]	Reference
NF-κB (−223/−214)		
Wild-type	GGGACTACCC	
Mutated	cccgggACCC	
NF-IL-6 (−132/−124)		
Wild-type	TTACGCAAT	23
Mutated	TTggtaccT	
CRE (−59/−53)		
Wild-type	TTCGTCA	
Mutated	TTgagCt	

[a] Mutant nucleotides are shown as lowercase letters.

MATERIALS AND METHODS

Constructs and cell lines. The T-antigen-transformed human embryonic kidney cell line 293T (HEK293T) was obtained from Jon Aster (Department of Pathology, Brigham and Women's Hospital, Harvard Medical School). The HEK293T cell line used in transient-transfection experiments was maintained in Dulbecco's modified Eagle's medium supplemented with 7% bovine growth serum (BGS), 2 mM glutamine, 25 U/ml of penicillin-streptomycin, and 20 μg/ml of gentamicin. The EBV-negative BJAB cell line was obtained from Elliott Kieff (80). The BJAB cell line was maintained in RPMI 1640 medium (Invitrogen/Gibco-BRL, Rockville, MD) supplemented with 7% BGS, 2 mM glutamine, and 25 U/ml of penicillin-streptomycin (Gemini Bioproducts, Inc., Carlsbad, CA).

The COX-2 promoter constructs (promoter construct consisting of nucleotides −327 to +59 of the COX-2 promoter [−327/+59]) cloned upstream of the luciferase reporter gene (23) were obtained from Hiroyasu Inoue (Department of Food Science and Nutrition, Nara Women's University, Nara, Japan). Specific mutations are shown in Table 1. The KBM construct is the −327/+59 COX-2 promoter construct in which the NF-κB site (−223/−214) was mutagenized, ILM is the −327/+59 COX-2 promoter construct in which the NF-IL-6 site (−59/−53) was mutagenized, and CRM is the −327/+59 COX-2 promoter construct in which the CRE site (−59/−53) was mutagenized (see Fig. 4). KBM+CRM represents the −327/+59 COX-2 promoter construct in which the both NF-κB and CRE sites were mutagenized, KBM+ILM represents the −327/+59 COX-2 promoter construct in which both the NF-κB and NF-IL-6 sites were mutagenized, ILM+CRM represents the −327/+59 COX-2 promoter construct in which both the NF-IL-6 and CRE sites were mutagenized, and KBM+ILM+CRM represents the −327/+59 COX-2 promoter construct in which the NF-κB, NF-IL-6, and CRE sites were mutagenized (23) (see Fig. 5). EBNA3C and Nm23-H1 expression construct used in these experiments were as described earlier (44, 69). EBNA3C mutant construct (amino acids 621 to 675 deleted) used was generated by PCR amplification and deletion of the region encoding 621 to 675 amino acids in the EBNA3C open reading frame and cloned in frame in the pA3M expression vector Myc tagged at the carboxy terminus (4).

Transfection. BJAB and HEK293T cells were transfected by electroporation with a Bio-Rad Gene Pulser II electroporator. Ten million cells were harvested in exponential phase, collected, and washed in phosphate-buffered saline and then resuspended in 400 μl of RPMI 1640 medium or Dulbecco's modified Eagle's medium with DNA for transfection. Resuspended cells were transferred to a 0.4-cm cuvette and electroporated at 975 μF and 220 V. The electroporated cells were then transferred to 10 ml of complete medium, followed by incubation at 37°C and 5% CO_2. Transfected cells were harvested after 24 h and assayed for activity.

Luciferase assay. HEK293T cells were collected at 70% confluence; 10 million cells were resuspended, along with plasmid DNA, in 400 μl of medium. The cells were transfected by electroporation with the Bio-Rad Gene Pulser II at 210 V and 975 μF. Transfected cells were transferred to 100-mm plates in 10 ml of Dulbecco's modified Eagle's medium with 7% BGS. The plates were incubated at 37°C with 5% CO_2 for 20 h. BJAB cells were collected at 5×10^5 cells/ml; 10 million cells were transfected at 220 V and 975 μF. At 20 h, cells were harvested

and washed once with phosphate-buffered saline (Invitrogen-Gibco, Inc., Bethesda, MD). The cells were subsequently lysed with 200 μl of reporter lysis buffer (Promega, Inc., Madison, WI); 40 μl of the lysate was mixed with 100 μl of luciferase assay reagent. Luminescence was measured for 10 s by the Opticomp I luminometer (MGM Instruments, Inc., Hamden, CT). The lysates were also tested at various dilutions to ensure that luciferase activity was within the linear range of the assay. The results shown represent experiments performed in triplicate.

Electrophoresis mobility shift assays (EMSAs). Two double-stranded DNA probes, one containing the NF-κB binding site sequence from the COX-2 promoter sequence (5′-GGGAGAGTGGGGACTACCCCCTCTGC-3′) and the other containing the CRE binding site sequence (5′-GAACAGTCATTTCGTCACATG GGCTT-3′), were prepared. ^{32}P-labeled probes were synthesized via the Klenow fill-in reaction and purified with Select-D with G-25 columns (Shelton/IBI, Inc., Peosta, IA). Radioactive probes were diluted in water to a final concentration of 80,000 cpm/μl. DNA binding reactions were performed in a manner similar to that described previously (26). Proteins from nuclear extracts were mixed with 1 μg of poly(dI-dC) (Sigma-Aldrich, Inc., St. Louis, MO) in 1× DNA binding buffer for 5 min at room temperature; 1 μl of labeled probe was added to each reaction mixture, and the tubes were incubated at room temperature for 15 min. DNA-protein complexes were resolved via nondenaturing 6% polyacrylamide gel electrophoresis. The gel was run in 0.5× Tris-borate-EDTA buffer at a constant voltage of 150 V. Following electrophoresis, the gel was transferred to Whatman paper and dried for 1 h at 80°C. Dried gels were exposed to a PhosphorImager screen for 12 h (Amersham Biosciences, Inc., Piscataway, NJ) and scanned by a PhosphorImager (Molecular Dynamics, Piscataway, NJ).

Real-time quantitative PCR. Total RNA from EBV-positive and -negative B-cell lines was collected using Trizol reagent (Invitrogen, Inc., Carlsbad, CA) following the manufacturer's instructions. cDNA was made using a Superscript II reverse transcription kit (Invitrogen, Inc., Carlsbad, CA) following the manufacturer's instructions. The specific primers for COX-2 used were as follows: sense, 5′-GATACTCAGGCAGAGATGATCTACCC-3′; antisense, 5′-AGACCAGGC ACCAGACCAAAGA-3′. PCR amplification yielded a 92-bp product. For β-actin, the primers were as follows: sense, 5′-GCTCGTCGTCGACAACGGCTC-3′; antisense, 5′-CAAACATGATCTGGGTCATCTTCTC-3′. PCR amplification yielded a product 352 bp in length. The cDNA was amplified using SYBR green real-time mastermix (MJ Research, Inc., Waltham, MA), 1 mM each primer, and 1 μl of the cDNA product in a total volume of 20 μl. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94°C, 1 min at 56°C, and 30 s at 72°C), followed by 7 min at 72°C, were performed in an MJ Research Opticon II thermocycler (MJ Research, Inc., Waltham, MA). Each cycle was followed by two plate readings, with the first at 72°C and the second at 85°C. A melting curve analysis was performed to verify the specificity of the products, and the values for the relative quantitation were calculated by the $\Delta\Delta C_t$ method. The experiment was performed in triplicate.

PGE_2 ELISA. The concentration of prostaglandin E_2 (PGE_2) in cell culture supernatant was estimated by using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (R&D Systems, Minneapolis, MN). The ELISA was performed in duplicate, and results are shown as means ± standard errors of the means (SEMs).

Western blot analysis. Protein was extracted from cultured cells, and the concentration of protein was calculated. Samples (100 μg) of cultured cell lysates were electrophoresed and transferred onto a nitrocellulose membrane. Equal loading of samples was confirmed with Ponceau S staining of the membrane in all cases. COX-2 and Myc-tagged Nm23-H1 or EBNA3C were analyzed as reported with the use of rabbit anti-human COX-2 polyclonal antibody (Cell Signaling Technology, Beverly, MA) and anti-Myc antibody (77), respectively.

RESULTS

COX-2 levels are induced in EBV-positive lymphoblastoid cells. Previous studies have reported that COX-2 levels are increased in a variety of tumors and that higher levels of COX-2 in human tumors are related to poor prognosis (27, 49, 84). To determine the effect of EBV on the level of expression of COX-2 in cancer cells, we investigated whether or not there was any difference in the level of COX-2 expression between known EBV-positive cancer cells and EBV-negative cancer cells. These cell lines were assayed by real-time quantitative PCR to determine the level of COX-2 mRNA. The values were normalized with the β-actin mRNA level for each cell line. The results of real-time quantitative PCR for EBV-positive cell lines (LCL2, Raji, and LCL1) showed that the levels of COX-2 mRNA were higher in these cell lines than in EBV-negative cell lines (Loukes, DG75, and Ramos) (Fig. 1A). The results indicated that COX-2 mRNA expression levels in EBV-positive cells were approximately 200-fold higher than the levels in EBV-negative cells (compare Raji with DG75 [Fig. 1A]).

To determine whether mRNA levels corresponded to protein levels, we fractionated proteins from cell lysates and analyzed them by Western blotting to detect COX-2 protein. The results from Western blot analysis showed that EBV-positive cells had higher levels of COX-2 than EBV-negative cells did (Fig. 1B). Interestingly, the levels in the recently transformed cell lines LCL1 and LCL2 were greater than that seen in Raji cells, a long-term established cell line. Raji cells are known to have at least two major deletions in the EBV genome, one of which is located within the open reading frame of the essential EBV latent antigen, EBNA3C (1). However, all the LCLs were expressing latent antigens compared to Raji cells, which expressed predominantly EBNA1 and low levels of LMP proteins (1). This suggested that possibly other EBV latent proteins also play some role in COX-2 modulation. LMP1 has previously been shown to upregulate COX-2 expression in nasopharyngeal carcinoma cells (46). Incidentally, the level of COX-2 protein in Raji cells (Fig. 2B) was significantly lower than in other two LCLs, although it was significantly higher than the level in EBV-negative cells.

To test whether long-term and stable expression of EBNA3C plays any role in the regulation of COX-2 expression, we also determined the levels of COX-2 mRNA transcripts in BJAB cells stably expressing EBNA3C. The levels of COX-2 at both the mRNA and protein levels in these cells (BJAB E3C-10 and BJAB E3C-07) were significantly higher than in negative-control BJAB cells (Fig. 1D), indicating some role for EBNA3C in modulation of COX-2 expression. Also, the levels of PGE_2, a downstream product of COX-2 metabolism, in supernatant from cell lines were also determined by an ELISA. PGE_2 is one of the COX-2-mediated metabolic products of arachidonic acid (45). Indomethacin, a prostaglandin synthesis inhibitor, was added to all samples at approximately 10 μg/ml at time of sample collection. The results of the ELISA showed that the EBV-positive cell lines produced higher levels of PGE_2 than EBV-negative cell lines did (Fig. 1C). The mean PGE_2 concentration in EBV-positive cell lines was approximately 512 pg/ml compared to 88 pg/ml in EBV-negative cell lines (Fig. 1C).

Nm23-H1 upregulates transcription of the COX-2 promoter in reporter assays. To study the effect of the EBV latent antigen EBNA3C on COX-2 expression levels, we performed reporter assays using a COX-2 promoter construct cloned in pGL2 luciferase reporter vector (Fig. 2A). The effect of EBNA3C on the COX-2 promoter construct (pHPES2 −327/+59) inserted into the pGL2 basic vector was investigated by luciferase assay. We also studied the effect of Nm23-H1, a known metastatic suppressor of the COX-2 promoter. pA3M-EBNA3C and pA3M-Nm23-H1 were transiently transfected along with pHPES2, the reporter plasmid containing the COX-2 promoter, into HEK293T cells. Total transfected DNA was balanced with empty vector, and the transfection efficiency

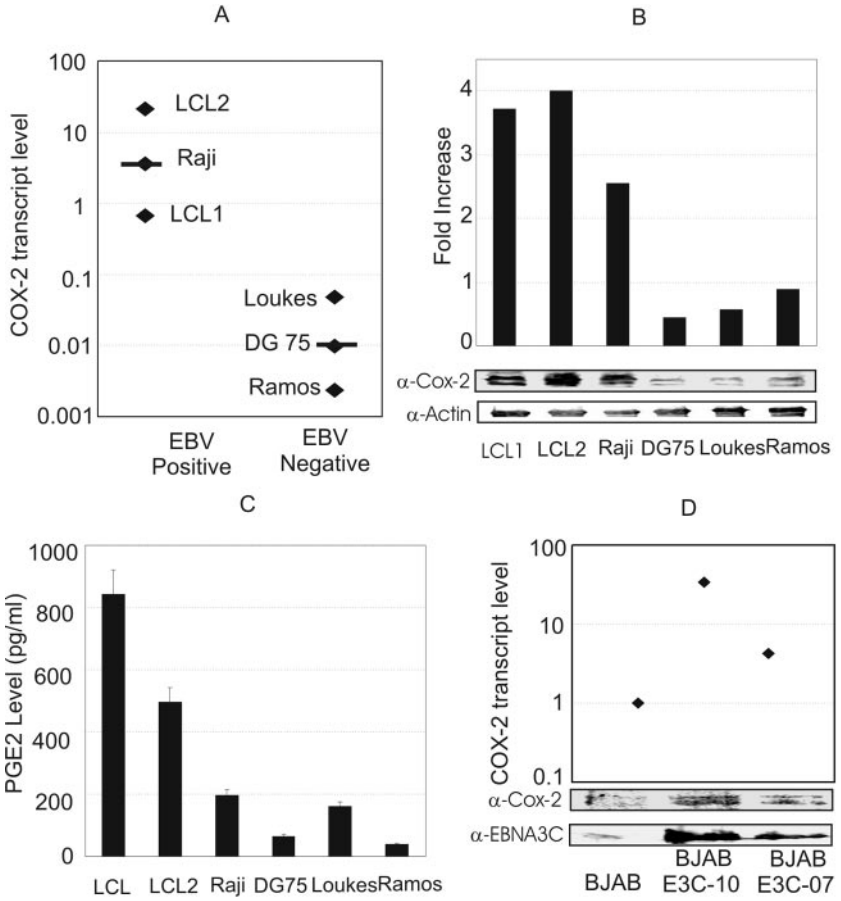

FIG. 1. (A) COX-2 mRNA expression levels in EBV-positive (LCL1, LCL2, and Raji) and EBV-negative (Loukes, DG75, and Ramos) cell lines. The expression level of COX-2 was normalized with the β-actin gene expression level. The level of expression in EBV-positive cell lines was significantly higher than in EBV-negative cell lines. The bar denotes the median gene expression level. (B) COX-2 protein expression levels in EBV-positive and -negative cell lines as detected by Western blotting. Total protein was quantified in cell lysates, and equal amounts were loaded in all lanes. Anti-COX-2 (α-Cox-2) and antiactin (α-Actin) antibodies were used. (C) PGE_2 level detected in supernatants from different EBV-positive and -negative cell lines. Cells were seeded at a concentration of 1 million cells per ml, and the supernatant was harvested after 24 h. (D) The level of COX-2 mRNA expressed in BJAB cells stably expressing EBNA3C protein is significantly higher than in control BJAB cells.

was monitored by the using pEGFP and counting transfected cells for green fluorescent protein (GFP) fluorescence. The results of the assay demonstrated that, individually, Nm23-H1 activated the COX-2 promoter to levels approximately fourfold higher than those of the vector alone in a dose-dependent manner. However, EBNA3C alone did not seem to have any major effect on the activation or repression of the COX-2 promoter (Fig. 2B).

EBNA3C cooperates with Nm23-H1. Previous studies in our lab have shown that Nm23-H1 interacts with a specific domain of EBNA3C which lies between the proline- and glutamine-rich regions (69). Interestingly, the presence of EBNA3C results in a change in the localization of Nm23-H1 from primarily cytoplasmic to primarily nuclear (69, 70). To study the implications of this event, we also determined whether EBNA3C can affect the Nm23-H1-driven upregulation of the COX-2 promoter. The results showed that cotransfection of pA3M-EBNA3C and pA3M-Nm23-H1 cooperated in the COX-2 promoter to increase the level of COX-2 approximately eightfold over the level with EBNA3C alone or at least twofold over that seen with Nm23-H1 alone (Fig. 2C). Importantly, the levels increased in a dose-responsive manner when either molecule was kept constant with increasing amounts of the second molecule. Thus, the changes were consistent at least in the reporter assays. These results suggested that EBNA3C might have some role in increasing the upregulation of the COX-2 promoter by Nm23-H1. After multiple experiments the results consistently demonstrated that the presence of EBNA3C increases the upregulation of the COX-2 promoter by Nm23-H1 in a dose-dependent manner (Fig. 2C). EBNA3C did not have any effect on the COX-2 promoter, but the presence of an increasing amount of Nm23-H1 activated the COX-2 promoter in a dose-dependent manner to up to eightfold higher over the level with the vector alone (Fig. 2C). In the presence of a fixed amount of Nm23-H1, the increasing amount of EBNA3C also had a similar effect in activating the COX-2 promoter in a dose-dependent manner (Fig. 2C).

Nm23-H1 cooperates with EBNA3C in enhancing COX-2 activity in vitro. To further corroborate the effect of EBNA3C on Nm23-H1-driven upregulation of the COX-2 promoter, we

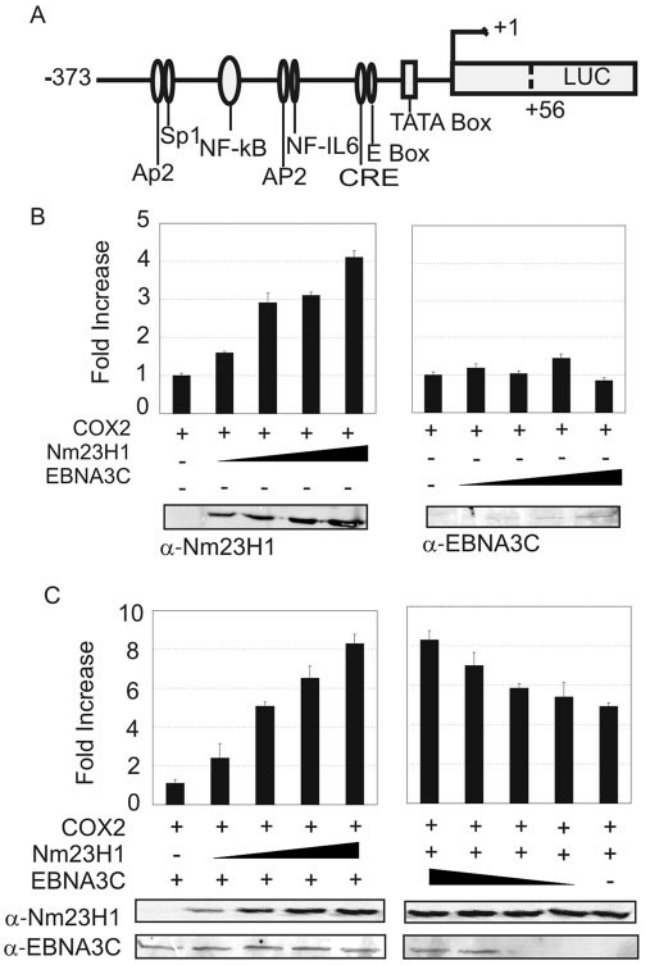

FIG. 2. Identification of the role of EBV latent protein EBNA3C in Nm23-H1-dependent modulation of the COX-2 promoter. (A) The 5′-flanking region of COX-2 showing the relative positions of promoter element binding consensus sequences. LUC, luciferase. (B and C) The relative luciferase activity obtained from transcriptional activity of the COX-2 promoter construct in 293T cells. The 90% confluent 293T cells were transfected (+) with 5.0 μg of COX-2 reporter vector pHPES2 (−327/+59) (COX2) with either pA3M-Nm23-H1 (Nm23H1) or pA3M-EBNA3C (EBNA3C) or with both. The results are expressed as the means ± SEMs (error bars) of three separate experiments performed in duplicate or triplicate. Anti-Nm23-H1 (α-Nm23H1) and anti-EBNA3C (α-EBNA3C) antibodies were used.

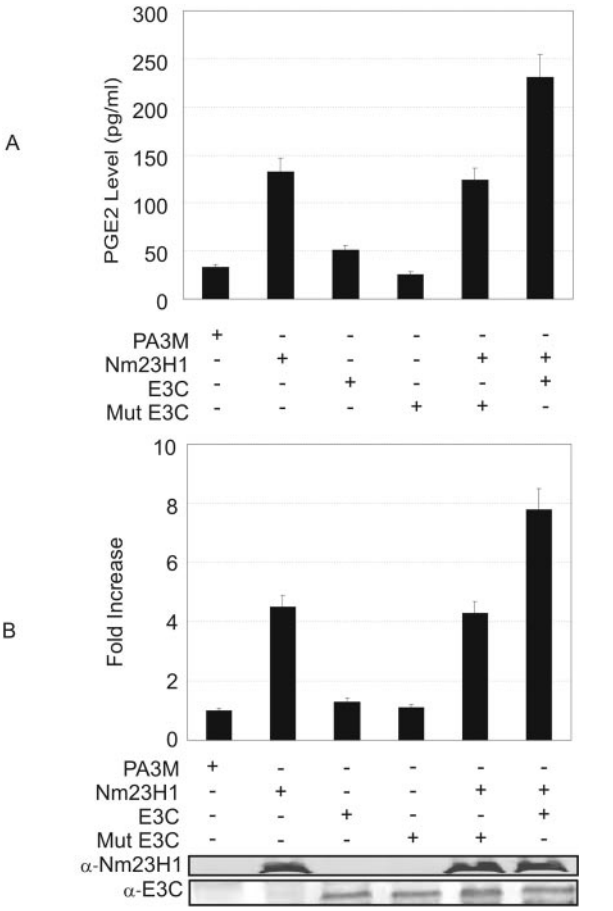

FIG. 3. Interaction of EBV protein EBNA3C with Nm23-H1 increases the upregulation of COX-2 expression by Nm23-H1. (A) Effect of wild-type EBNA3C or mutated EBNA3C (the Nm23-H1 binding domain deleted) on Nm23-H1 induction of COX-2 (−327/+59) firefly luciferase reporter construct. The 90% confluent 293T cells were transfected with 5.0 μg of COX-2 reporter vector pHPES2 (−327/+59) along with pA3M-Nm23-H1 (PA3M) and with either pA3M-EBNA3C (E3C) or pA3M-Mut-EBNA3C (Mut E3C) or both. The results are expressed as the means ± SEMs (error bars) of three separate experiments performed in duplicate or triplicate. (B) Interaction of EBV protein EBNA3C with Nm23-H1 increases the upregulation of the functional level of COX-2 by Nm23-H1. Transfected 293T cells were induced by lipopolysaccharide (final concentration of 1 μg/ml), and the PGE_2 levels in supernatant harvested after 24 h were determined by an ELISA. Anti-Nm23-H1 (α-Nm23H1) and anti-EBNA3C (α-E3C) antibodies were used.

tested whether this upregulation translates to increased COX-2 activity. HEK293T cells were transfected with pA3M-EBNA3C and pA3M-Nm23-H1, and the PGE_2 levels in cell culture supernatant were determined after 24 h by ELISAs. The results showed that the PGE_2 level after EBNA3C transfection did not appear to change. However, on Nm23-H1 transfection, PGE_2 levels increased approximately five- to six-fold (150 pg/ml) over the level with vector alone (25 pg/ml) (Fig. 3A). Cotransfection of EBNA3C and Nm23-H1 consistently increased the PGE_2 levels up to 10-fold (250 pg/ml) over vector alone (Fig. 3A). Importantly, an EBNA3C mutant lacking the Nm23-H1 interacting domain showed little or no upregulation in this assay.

The Nm23-H1 binding site is critical for EBNA3C to cooperate with Nm23-H1 in upregulating the COX-2 promoter. Previous studies have demonstrated that EBNA3C interacts with Nm23-H1 through amino acid sequences located between the glutamine- and proline-rich domains and can also cooperate in the activation of transcription (69). The Nm23-H1 binding domain on EBNA3C has been mapped within the region comprising amino acids 637 to 675 (69). To determine whether this binding site plays any role in activation of transcription of COX-2 by Nm23-H1, we used a deletion mutant of EBNA3C in which the Nm23-H1 binding domain had been deleted. The mutant EBNA3C was transfected with Nm23-H1 and the reporter vector pHPES2 in HEK293T cells and tested for lucif-

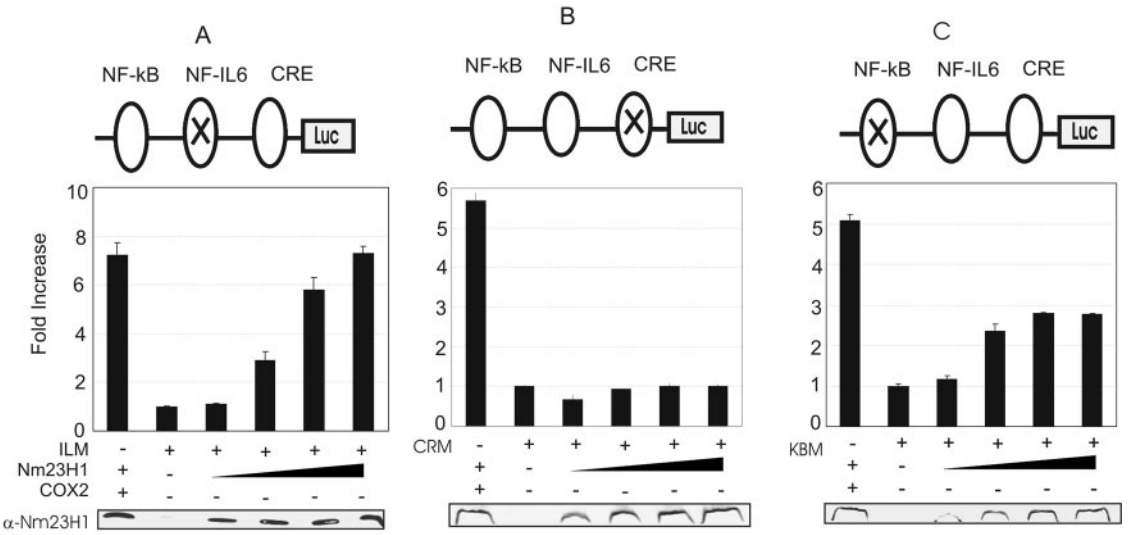

FIG. 4. Identification of *cis*-acting elements in Nm23-H1-induced COX-2 (−327/+59) firefly luciferase (Luc) reporter constructs containing wild-type CRE (−59/−53), NF-IL-6 (−132/−124), and NF-κB (−223/−214) *cis*-acting elements or their respective site-directed mutants. The results are expressed as the means ± SEMs (error bars) of three separate experiments performed in duplicate or triplicate. Constructs ILM, CRM, and KBM are described in Materials and Methods. Anti-Nm23-H1 (α-Nm23H1) antibody was used.

erase activity in cell lysates. The results of the luciferase assays demonstrated that the presence of mutant EBNA3C has little or no effect on upregulation of the COX-2 promoter. However, changes were seen with the wild-type EBNA3C molecule (Fig. 3B). Thus, these changes were similar to that seen with the ELISA above and corroborate the result, suggesting that the interaction of EBNA3C with Nm23-H1 cooperates to upregulate the COX-2 promoter.

Cellular transcription factors CRE and NF-κB are important for Nm23-H1 upregulation of the COX-2 promoter. NF-κB, CRE, and NF-IL-6 have been shown to be important in transcriptional regulation of the COX-2 promoter (24, 25, 86). Therefore, we wanted to determine whether any of these known transcription factors may be involved in Nm23-H1-mediated induction of COX-2 expression. We used the COX-2 promoter mutant constructs cloned into the pGL2 luciferase vector in which NF-κB, CRE, and NF-IL-6 binding sites had been mutated (23). These constructs were transfected into HEK293T cells along with increasing amounts of Nm23-H1 and assayed for luciferase activity. The results of these assays showed that mutations of the NF-IL-6 binding site did not cause any change (Fig. 4A), whereas mutations in the CRE binding site resulted in loss of the ability of Nm23-H1 to upregulate the COX-2 promoter (Fig. 4B). Mutation of the NF-κB binding site similarly resulted in reduction in upregulation by approximately 50%, indicating that both CRE and NF-κB are important for Nm23-H1-mediated upregulation of COX-2 (Fig. 4C). The assays with the double and triple mutant constructs of the COX-2 promoter indicated that only the construct with mutated NF-κB and NF-IL-6 sites showed some upregulation but only to about 75% of the level with the wild-type promoter. However, when constructs were mutated in the CRE site within either the triple or double mutant, all activities returned to the baseline level or the level with reporter alone (Fig. 5). As expected, Nm23-H1 activated the

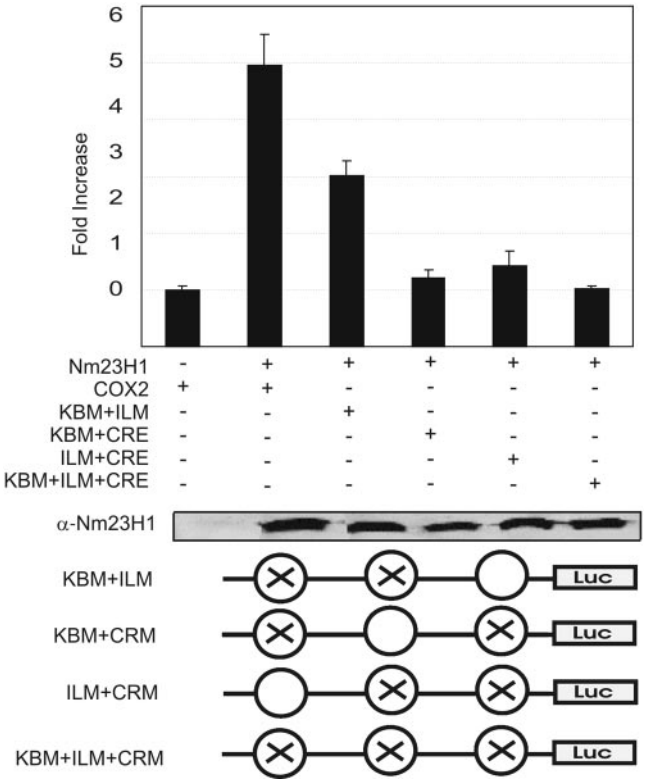

FIG. 5. Identification of *cis*-acting elements in Nm23-H1-induced COX-2 (−327/+59) firefly luciferase (Luc) reporter constructs containing wild-type CRE (−59/−53), NF-IL-6 (−132/−124), and NF-κB (−223/−214) *cis*-acting elements or their respective site-directed double or triple mutants. The constructs are described in Materials and Methods. The results are expressed as the means ± SEMs (error bars) of three separate experiments performed in duplicate or triplicate.

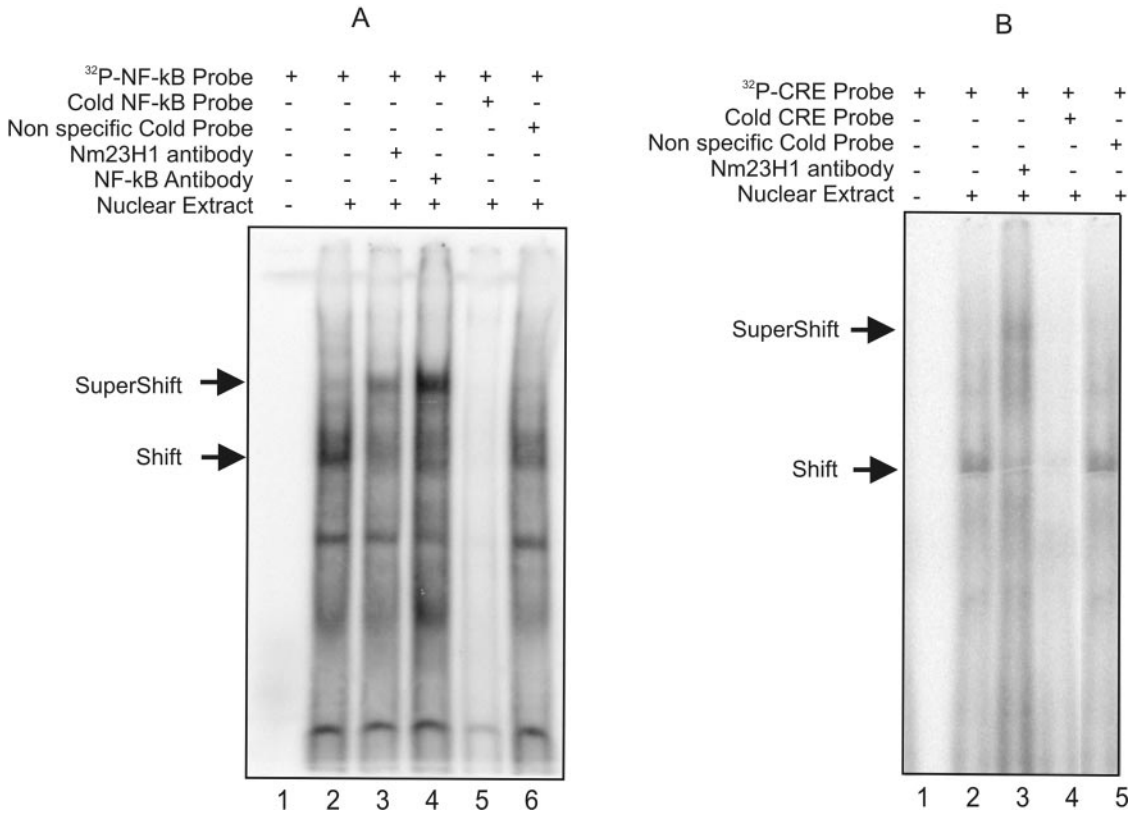

FIG. 6. (A) NF-κB is involved in induction of the COX-2 promoter by Nm23-H1. Nuclear extracts of 293T cells transfected with pA3M-Nm23-H1 were mixed with ^{32}P-labeled NF-κB probe and analyzed by electrophoretic mobility shift assay (lanes 1 to 4). Excess nonlabeled NF-κB (100×) was used as a competitor (lane 5). (B) CRE is involved in induction of the COX-2 promoter by Nm23-H1. Nuclear extracts of 293T cells transfected with pA3M-Nm23-H1 were mixed with ^{32}P-labeled CRE probe and analyzed by electrophoretic mobility shift assay (lanes 1 to 3). Excess nonlabeled CRE (100×) was used as a competitor (lane 4).

promoter about fivefold. Together, these data strongly suggest an important role for CRE and NF-κB in upregulation of the COX-2 promoter by Nm23-H1.

Nm23-H1 interacts with NF-κB and CRE bound to their respective cognate sequences within the COX-2 promoter. The results obtained with the luciferase reporter promoter mutants indicated that both the CRE and NF-κB binding sites within the COX-2 promoter are critical for Nm23-H1-mediated upregulation of COX-2. Therefore, we wanted to test whether Nm23-H1 was regulating COX-2 through transcription factor-mediated DNA binding. The probes for the EMSAs consisted of the NF-κB and CRE binding sites from the COX-2 promoter, including sequences of 11 bases on the 5′-flanking region and 11 bases on the 3′-flanking region of the target sequence. Double-stranded DNA probes for the NF-κB binding site and CRE binding site were labeled and tested for binding to their respective proteins from nuclear extracts generated from HEK293T cells transiently transfected with the Nm23-H1 expression construct.

The results of the experiment show that a specific NF-κB shift was observed in nuclear extracts from HEK293T cells overexpressing Nm23-H1, and the specificity of the shift was verified through its disappearance in the presence of specific unlabeled competitor (Fig. 6A, compare lanes 2 and 5). No effect was seen in the presence of nonspecific competitor (Fig. 6A, lane 6). The presence of Nm23-H1 in the complex was verified by additional supershifted complexes in the presence of an anti-Nm23-H1 antibody (Fig. 6A, lane 3). This probe was also supershifted by anti-p50 antibodies, indicating its presence in the complex (Fig. 6A, lane 4).

To determine whether Nm23-H1 formed a complex with CRE bound to DNA, an EMSA was also performed as described above. The results showed a specific CRE shift when nuclear extract was included with the probe (Fig. 6B, lane 2). The specificity of the shift was verified through its disappearance in the presence of specific cold competitor (Fig. 6B, compare lanes 2 and 4), with no effect seen in the presence of nonspecific competitor (Fig. 6B, lane 5). The probe was clearly supershifted in the presence of anti-Nm23-H1 antibodies (Fig. 6B, lane 3). These results suggest that Nm23-H1 may regulate the COX-2 promoter through binding to the NF-κB and CRE transcription factors bound to their sequence-specific binding sites.

DISCUSSION

Earlier studies have revealed that COX-2 is overexpressed in a number of different tumors (12, 22, 58, 60, 82, 88). There is also evidence to show striking upregulation of COX-2 in various human cancers as well as murine cancer models (32, 71). Interestingly, selective COX-2 inhibitors have been found to

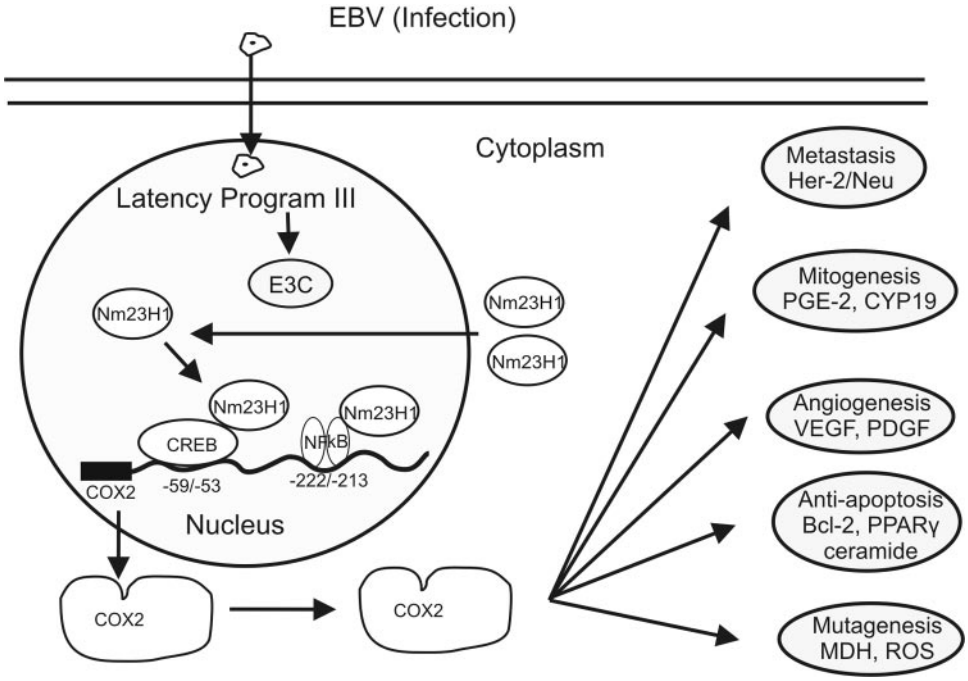

FIG. 7. Schematic model for the interaction of EBNA3C and Nm23-H1 resulting in regulation of COX-2 expression. The presence of EBNA3C results in a change in the localization of Nm23-H1 from primarily cytoplasmic to primarily nuclear. Nm23-H1 upregulates COX-2 through NF-κB and CRE promoter elements, which play a role in increasing carcinogenesis and metastasis through various factors. Abbreviations: E3C, EBNA3C; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; PPARγ, peroxisomal proliferator-activated receptor gamma; MDH, methanol dehydrogenase; ROS, reactive oxygen species.

reduce tumorigenesis or tumor cell growth (55, 57, 64). Together, these observations suggest a strong correlation between COX-2 and carcinogenesis. More importantly, upregulation of COX-2 in cancer cells has also been shown to be linked with increased angiogenesis and metastasis (39, 62). Recently, overexpression of COX-2 has also been shown to be closely linked with lymph node metastasis in nasopharyngeal carcinoma (52). COX-2 has been found to be overexpressed in various B-cell lymphoma cell lines (83). EBV is associated with a number of human lymphoid and epithelial tumors. The EBV antigen EBNA3C has been shown to interact with the known metastasis suppressor Nm23-H1, and the binding domain for Nm23-H1 has been mapped on EBNA3C (637 to 675 amino acids) (70). The interaction between EBNA3C and Nm23-H1 is of interest, as it presents a potential manner where the interaction of this essential viral oncoprotein and the known cellular metastasis suppressor and regulator of cell proliferation can lead to reversion of cell migration in vitro and affect cell proliferation and metastasis in EBV-positive tumors (69). Nm23-H1 has been shown to localize to the cytoplasm; however, in the presence of EBNA3C, its localization has been shown to shift to an almost exclusive nuclear signal (69). This change in localization strengthens the possibility that Nm23-H1 may function as a transcriptional regulator. Therefore, we tested the possibility that the EBNA3C–Nm23-H1 interaction might also contribute to metastasis through induction of COX-2.

Our results clearly show that expression of COX-2 in EBV-positive cell lines is significantly higher than in EBV-negative cell lines, suggesting that EBV plays a role in modulation of COX-2 expression. This was confirmed at transcription and translation and by the activity levels of COX-2; the results indicated a role of EBV essential latent antigens in modulation of COX-2, which is well-known to play a important role in both carcinogenesis and metastasis. EBNA3C is one of the EBV proteins that are expressed only in type III latency during EBV infection. Type III latency is commonly associated with AIDS-associated lymphomas and posttransplant lymphoproliferative disorders (73). At least in one study, two cell lines derived from EBV-positive gastric carcinoma tissue have been shown to express the type III latency program, indicating that EBNA3C may also be expressed in tumors of epithelial origin (43). In addition, EBV transformation of B lymphocytes requires a subset of six latent proteins, including EBNA3C.

Our results clearly show that EBNA3C on its own does not have any effect on COX-2 modulation. This is not surprising, considering that EBNA3C has been shown to function as a transcriptional activator or repressor, depending on its interaction with specific cellular factors (70). However, the upregulation of COX-2 expression by increased expression of Nm23-H1 is surprising, considering that Nm23-H1 is known to be antimetastasis. The primary localization of Nm23-H1 in B cells is in the cytoplasm (59). As a part of the SET complex, it has been shown to translocate to the nuclei of target cells within minutes of GzmA loading or cytotoxic T-lymphocyte attack (16). The presence of EBNA3C may in essence have a similar effect (69). Therefore, the translocation of Nm23-H1 from cytoplasm to nucleus appears to be an important event for modulation of Nm23-H1 functions. Our data also clearly show that upregulation of COX-2 by overexpressed Nm23-H1 is further enhanced in the presence of EBNA3C in a dose-

dependent manner. The upregulation of the COX-2 promoter also translated to an increase in COX-2 activity as evident by an increase in the PGE_2 level detected by an ELISA. However, the differences in the COX-2 expression levels of EBV-positive cell lines and EBV-negative cell lines (Fig. 1A) also suggest the possible role of other EBV latent antigens in transcriptional control of COX-2. EBV protein LMP1, which is expressed in both type II and type III latencies, has previously been shown to upregulate COX-2 expression in nasopharyngeal carcinoma cells (46). Our studies clearly indicate that interaction of EBNA3C with Nm23-H1 is an important component of COX-2 upregulation by Nm23-H1 and EBNA3C in EBV-positive cells. However, interaction between EBNA3C and Nm23-H1 might only partially contribute to modulation of COX-2 upregulation, although this may be one of the predominant controlling molecules.

It has been well established that NF-κB, NF-IL-6, and CREB/AP-1 are the main transcription factors responsible for the regulation of COX-2 expression (51). Here, we tested which of these may be important for Nm23-H1-modulated upregulation of the COX-2 promoter. Our reporter assay studies with mutated promoters revealed that NF-κB and CRE may be critical for Nm23-H1-mediated upregulation of COX-2 but that CRE may have a dominant role. Recently, Nm23-H1 has been shown to bind NF-κB and modulate transcription of a targeted promoter (28). Whether Nm23-H1 can directly bind to DNA has not been demonstrated despite our numerous attempts. Our EMSA data also provide evidence that Nm23-H1 forms a complex by binding to NF-κB and its cognate sequence at the COX-2 promoter. The EMSA data also show that Nm23-H1 forms a complex with CRE bound to its cognate sequence. Although studies have shown that CREB/ATF-2 and AP-1 interact with the CRE of the COX-2 promoter, further studies will be required to determine which of these complexes are formed with Nm23-H1 on the COX-2 promoter (18, 85).

In uninfected cells, Nm23-H1 has been detected in the nucleus at low levels (29, 47), suggesting that a cellular partner may play a role in its translocation from cytoplasm to nucleus or that Nm23-H1 itself may be posttranslationally modified to mediate this event. However, increased expression of Nm23-H1 results in increased levels in the nucleus as evident by Western blot detection of Nm23-H1 in nuclear extracts from transfected cells (data not shown). This results in upregulation of the COX-2 promoter. The presence of EBNA3C, which results in a change in the localization of Nm23-H1 to primarily nuclear, thus leads to increased upregulation of COX-2.

It is interesting to note that EBV infection has been experimentally shown to suppress COX-2 expression in isolated monocytes from healthy individuals (61). It has been suggested that this might be an additional strategy utilized by EBV to disturb the immune response and promote viral replication during primary infection (50). However, the upregulation of COX-2 by EBV proteins during latency when a limited number of viral proteins are expressed could represent a process which is initiated or at least includes COX-2 upregulation, ultimately resulting in the tumorigenic phenotype (Fig. 7). LMP1, which is expressed in type III and type II latency, has also been shown to upregulate COX-2 expression and is likely to work through a different mechanism independent of Nm23-H1 (46). Our data suggest that interaction between EBNA3C and Nm23-H1 also leads to upregulation of COX-2 expression. The COX-2 protein, which is downregulated at the time of primary infection possibly to promote viral survival and establishment of latent infection, is later upregulated as the infection is established, and only a few of the latent viral proteins are expressed. This reiterates the complexities associated with understanding the role of EBV in tumorigenesis in view of the fact that different sets of viral proteins are expressed in different stages of infection.

ACKNOWLEDGMENTS

This work was supported by grants from the Leukemia and Lymphoma Society of America and Public Health Service grants from the NCI (CA072510, CA108461, and CA091792) and from the NIDCR (DE01436) (to E.S.R.). E.S.R. is a Scholar of the Leukemia and Lymphoma Society of America.

We are thankful to Hiroyasu Inoue (Department of Food Science and Nutrition, Nara Women's University, Nara, Japan) for providing the wild-type and mutant constructs of the COX-2 promoter.

REFERENCES

1. **Allday, M. J., and P. J. Farrell.** 1994. Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G_1-arrested cells. J. Virol. **68:**3491–3498.
2. **Araki, Y., S. Okamura, S. P. Hussain, M. Nagashima, P. He, M. Shiseki, K. Miura, and C. C. Harris.** 2003. Regulation of cyclooxygenase-2 expression by the Wnt and Ras pathways. Cancer Res. **63:**728–734.
3. **Arcinas, M., and L. M. Boxer.** 1994. Differential protein binding to the c-myc promoter during differentiation of hematopoietic cell lines. Oncogene **9:**2699–2706.
4. **Aster, J. C., E. S. Robertson, R. P. Hasserjian, J. R. Turner, E. Kieff, and J. Sklar.** 1997. Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jκ or nuclear localization sequences retain the ability to associate with RBP-Jκ and activate transcription. J. Biol. Chem. **272:**11336–11343.
5. **Backer, J. M., C. E. Mendola, I. Kovesdi, J. L. Fairhurst, B. O'Hara, R. L. Eddy, Jr., T. B. Shows, S. Mathew, V. V. Murty, and R. S. Chaganti.** 1993. Chromosomal localization and nucleoside diphosphate kinase activity of human metastasis-suppressor genes NM23-1 and NM23-2. Oncogene **8:**497–502.
6. **Berberich, S. J., and E. H. Postel.** 1995. PuF/NM23-H2/NDPK-B transactivates a human c-myc promoter-CAT gene via a functional nuclease hypersensitive element. Oncogene **10:**2343–2347.
7. **Biggs, J., E. Hersperger, P. S. Steeg, L. A. Liotta, and A. Shearn.** 1990. A *Drosophila* gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. Cell **63:**933–940.
8. **Bominaar, A. A., A. D. Tepper, and M. Veron.** 1994. Autophosphorylation of nucleoside diphosphate kinase on non-histidine residues. FEBS Lett. **353:**5–8.
9. **Bonnet, M., J. M. Guinebretiere, E. Kremmer, V. Grunewald, E. Benhamou, G. Contesso, and I. Joab.** 1999. Detection of Epstein-Barr virus in invasive breast cancers. J. Natl. Cancer Inst. **91:**1376–1381.
10. **Brennan, C. M., I. E. Gallouzi, and J. A. Steitz.** 2000. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J. Cell Biol. **151:**1–14.
11. **Cao, Y., and S. M. Prescott.** 2002. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J. Cell. Physiol. **190:**279–286.
12. **Chan, G., J. O. Boyle, E. K. Yang, F. Zhang, P. G. Sacks, J. P. Shah, D. Edelstein, R. A. Soslow, A. T. Koki, B. M. Woerner, J. L. Masferrer, and A. J. Dannenberg.** 1999. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res. **59:**991–994.
13. **Cherukuri, D. P., A. C. Goulet, H. Inoue, and M. A. Nelson.** 2005. Selenomethionine regulates cyclooxygenase-2 (COX-2) expression through nuclear factor-kappa B (NF-κB) in colon cancer cells. Cancer Biol. Ther. **4:**175–180.
14. **de la Rosa, A., R. L. Williams, and P. S. Steeg.** 1995. Nm23/nucleoside diphosphate kinase: toward a structural and biochemical understanding of its biological functions. Bioessays **17:**53–62.
15. **Demple, B., T. Herman, and D. S. Chen.** 1991. Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. Proc. Natl. Acad. Sci. USA **88:**11450–11454.
16. **Fan, Z., P. J. Beresford, D. Y. Oh, D. Zhang, and J. Lieberman.** 2003. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell **112:**659–672.

17. **Gilles, A. M., E. Presecan, A. Vonica, and I. Lascu.** 1991. Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. J. Biol. Chem. **266:**8784–8789.
18. **Guo, Y. S., M. R. Hellmich, X. D. Wen, and C. M. Townsend, Jr.** 2001. Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. J. Biol. Chem. **276:** 22941–22947.
19. **Hailat, N., D. R. Keim, R. F. Melhem, X. X. Zhu, C. Eckerskorn, G. M. Brodeur, C. P. Reynolds, R. C. Seeger, F. Lottspeich, J. R. Strahler, and S. M. Hanash.** 1991. High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-*myc* gene amplification. J. Clin. Investig. **88:**341–345.
20. **Hemmerich, S., and I. Pecht.** 1992. Oligomeric structure and autophosphorylation of nucleoside diphosphate kinase from rat mucosal mast cells. Biochemistry **31:**4580–4587.
21. **Herschman, H. R.** 1996. Prostaglandin synthase 2. Biochim. Biophys. Acta **1299:**125–140.
22. **Hwang, D., D. Scollard, J. Byrne, and E. Levine.** 1998. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J. Natl. Cancer Inst. **90:**455–460.
23. **Inoue, H., T. Nanayama, S. Hara, C. Yokoyama, and T. Tanabe.** 1994. The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells. FEBS Lett. **350:**51–54.
24. **Inoue, H., C. Yokoyama, S. Hara, Y. Tone, and T. Tanabe.** 1995. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J. Biol. Chem. **270:**24965–24971.
25. **Kim, Y., and S. M. Fischer.** 1998. Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. J. Biol. Chem. **273:**27686–27694.
26. **Knight, J. S., M. A. Cotter II, and E. S. Robertson.** 2001. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus transactivates the telomerase reverse transcriptase promoter. J. Biol. Chem. **276:** 22971–22978.
27. **Kujubu, D. A., B. S. Fletcher, B. C. Varnum, R. W. Lim, and H. R. Herschman.** 1991. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. **266:**12866–12872.
28. **Kuppers, D. A., K. Lan, J. S. Knight, and E. S. Robertson.** 2005. Regulation of matrix metalloproteinase 9 expression by Epstein-Barr virus nuclear antigen 3C and the suppressor of metastasis Nm23-H1. J. Virol. **79:**9714–9724.
29. **Lacombe, M. L., X. Sastre-Garau, I. Lascu, A. Vonica, V. Wallet, J. P. Thiery, and M. Veron.** 1991. Overexpression of nucleoside diphosphate kinase (Nm23) in solid tumours. Eur. J. Cancer **27:**1302–1307.
30. **Leone, A., R. C. Seeger, C. M. Hong, Y. Y. Hu, M. J. Arboleda, G. M. Brodeur, D. Stram, D. J. Slamon, and P. S. Steeg.** 1993. Evidence for nm23 RNA overexpression, DNA amplification and mutation in aggressive childhood neuroblastomas. Oncogene **8:**855–865.
31. **Levy, G. N.** 1997. Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer. FASEB J. **11:**234–247.
32. **Liu, X. H., and D. P. Rose.** 1996. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res. **56:**5125–5127.
33. **Ma, D., Z. Xing, B. Liu, N. G. Pedigo, S. G. Zimmer, Z. Bai, E. H. Postel, and D. M. Kaetzel.** 2002. NM23-H1 and NM23-H2 repress transcriptional activities of nuclease-hypersensitive elements in the platelet-derived growth factor-A promoter. J. Biol. Chem. **277:**1560–1567.
34. **MacDonald, N. J., A. De la Rosa, M. A. Benedict, J. M. Freije, H. Krutsch, and P. S. Steeg.** 1993. A serine phosphorylation of Nm23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. J. Biol. Chem. **268:**25780–25789.
35. **MacDonald, N. J., A. de la Rosa, and P. S. Steeg.** 1995. The potential roles of nm23 in cancer metastasis and cellular differentiation. Eur. J. Cancer **31A:**1096–1100.
36. **Martin, K. K., and G. J. Pilkington.** 1998. Nm23: an invasion suppressor gene in CNS tumours? Anticancer Res. **18:**919–926.
37. **Mehus, J. G., P. Deloukas, and D. O. Lambeth.** 1999. NME6: a new member of the nm23/nucleoside diphosphate kinase gene family located on human chromosome 3p21.3. Hum. Genet. **104:**454–459.
38. **Milon, L., M. F. Rousseau-Merck, A. Munier, M. Erent, I. Lascu, J. Capeau, and M. L. Lacombe.** 1997. nm23-H4, a new member of the family of human nm23/nucleoside diphosphate kinase genes localised on chromosome 16p13. Hum. Genet. **99:**550–557.
39. **Molina, M. A., M. Sitja-Arnau, M. G. Lemoine, M. L. Frazier, and F. A. Sinicrope.** 1999. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. Cancer Res. **59:**4356–4362.
40. **Munier, A., C. Feral, L. Milon, V. P. Pinon, G. Gyapay, J. Capeau, G. Guellaen, and M. L. Lacombe.** 1998. A new human nm23 homologue (nm23-H5) specifically expressed in testis germinal cells. FEBS Lett. **434:**289–294.
41. **Munoz-Dorado, J., S. Inouye, and M. Inouye.** 1993. Eukaryotic-like protein serine/threonine kinases in *Myxococcus xanthus*, a developmental bacterium exhibiting social behavior. J. Cell. Biochem. **51:**29–33.
42. **Munoz-Dorado, J., S. Inouye, and M. Inouye.** 1990. Nucleoside diphosphate kinase from *Myxococcus xanthus*. II. Biochemical characterization. J. Biol. Chem. **265:**2707–2712.
43. **Murakami, M., Y. Hoshikawa, Y. Satoh, H. Ito, M. Tajima, K. Okinaga, Y. Miyazawa, T. Kurata, and T. Sairenji.** 2000. Tumorigenesis of Epstein-Barr virus-positive epithelial cell lines derived from gastric tissues in the SCID mouse. Virology **277:**20–26.
44. **Murakami, M., K. Lan, C. Subramanian, and E. S. Robertson.** 2005. Epstein-Barr virus nuclear antigen 1 interacts with Nm23-H1 in lymphoblastoid cell lines and inhibits its ability to suppress cell migration. J. Virol. **79:**1559–1568.
45. **Murakami, M., Y. Nakatani, T. Tanioka, and I. Kudo.** 2002. Prostaglandin E synthase. Prostaglandins Other Lipid Mediat. **68–69:**383–399.
46. **Murono, S., H. Inoue, T. Tanabe, I. Joab, T. Yoshizaki, M. Furukawa, and J. S. Pagano.** 2001. Induction of cyclooxygenase-2 by Epstein-Barr virus latent membrane protein 1 is involved in vascular endothelial growth factor production in nasopharyngeal carcinoma cells. Proc. Natl. Acad. Sci. USA **98:**6905–6910.
47. **Nakamura, G., N. Shikata, T. Shoji, T. Hatano, K. Hioki, and A. Tsubura.** 1995. Immunohistochemical study of mammary and extramammary Paget's disease. Anticancer Res. **15:**467–470.
48. **Needleman, P., J. Turk, B. A. Jakschik, A. R. Morrison, and J. B. Lefkowith.** 1986. Arachidonic acid metabolism. Annu. Rev. Biochem. **55:**69–102.
49. **O'Banion, M. K., H. B. Sadowski, V. Winn, and D. A. Young.** 1991. A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. J. Biol. Chem. **266:**23261–23267.
50. **Padma, P., A. Hozumi, K. Ogawa, and K. Inaba.** 2001. Molecular cloning and characterization of a thioredoxin/nucleoside diphosphate kinase related dynein intermediate chain from the ascidian, *Ciona intestinalis*. Gene **275:**177–183.
51. **Park, S. W., M. W. Sung, D. S. Heo, H. Inoue, S. H. Shim, and K. H. Kim.** 2005. Nitric oxide upregulates the cyclooxygenase-2 expression through the cAMP-response element in its promoter in several cancer cell lines. Oncogene **24:**6689–6698.
52. **Peng, J. P., H. C. Chang, C. F. Hwang, and W. C. Hung.** 2005. Overexpression of cyclooxygenase-2 in nasopharyngeal carcinoma and association with lymph node metastasis. Oral Oncol. **41:**903–908.
53. **Postel, E. H., S. J. Berberich, S. J. Flint, and C. A. Ferrone.** 1993. Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. Science **261:**478–480.
54. **Postel, E. H., and C. A. Ferrone.** 1994. Nucleoside diphosphate kinase enzyme activity of NM23-H2/PuF is not required for its DNA binding and in vitro transcriptional functions. J. Biol. Chem. **269:**8627–8630.
55. **Reddy, B. S., Y. Hirose, R. Lubet, V. Steele, G. Kelloff, S. Paulson, K. Seibert, and C. V. Rao.** 2000. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res. **60:**293–297.
56. **Rickinson, A. B., and E. Kieff.** 1996. Epstein-Barr virus, p. 2397–2446. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
57. **Rioux, N., and A. Castonguay.** 1998. Prevention of NNK-induced lung tumorigenesis in A/J mice by acetylsalicylic acid and NS-398. Cancer Res. **58:**5354–5360.
58. **Ristimaki, A., N. Honkanen, H. Jankala, P. Sipponen, and M. Harkonen.** 1997. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res. **57:**1276–1280.
59. **Rosengard, A. M., H. C. Krutzsch, A. Shearn, J. R. Biggs, E. Barker, I. M. Margulies, C. R. King, L. A. Liotta, and P. S. Steeg.** 1989. Reduced Nm23/Awd protein in tumour metastasis and aberrant *Drosophila* development. Nature **342:**177–180.
60. **Sano, H., Y. Kawahito, R. L. Wilder, A. Hashiramoto, S. Mukai, K. Asai, S. Kimura, H. Kato, M. Kondo, and T. Hla.** 1995. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res. **55:**3785–3789.
61. **Savard, M., C. Belanger, M. J. Tremblay, N. Dumais, L. Flamand, P. Borgeat, and J. Gosselin.** 2000. EBV suppresses prostaglandin E2 biosynthesis in human monocytes. J. Immunol. **164:**6467–6473.
62. **Sawaoka, H., S. Kawano, S. Tsuji, M. Tsujii, E. S. Gunawan, Y. Takei, K. Nagano, and M. Hori.** 1998. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. Am. J. Physiol. **274:**G1061–G1067.
63. **Seo, S. B., P. McNamara, S. Heo, A. Turner, W. S. Lane, and D. Chakravarti.** 2001. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the Set oncoprotein. Cell **104:**119–130.
64. **Sheng, H., J. Shao, S. C. Kirkland, P. Isakson, R. J. Coffey, J. Morrow, R. D. Beauchamp, and R. N. DuBois.** 1997. Inhibition of human colon cancer cell

growth by selective inhibition of cyclooxygenase-2. J. Clin. Investig. **99:**2254–2259.

65. **Shikama, N., H. M. Chan, M. Krstic-Demonacos, L. Smith, C. W. Lee, W. Cairns, and N. B. La Thangue.** 2000. Functional interaction between nucleosome assembly proteins and p300/CREB-binding protein family coactivators. Mol. Cell. Biol. **20:**8933–8943.
66. **Smith, W. L., D. L. DeWitt, and R. M. Garavito.** 2000. Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. **69:**145–182.
67. **Smith, W. L., R. M. Garavito, and D. L. DeWitt.** 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. **271:**33157–33160.
68. **Stahl, J. A., A. Leone, A. M. Rosengard, L. Porter, C. R. King, and P. S. Steeg.** 1991. Identification of a second human nm23 gene, nm23-H2. Cancer Res. **51:**445–449.
69. **Subramanian, C., M. A. Cotter II, and E. S. Robertson.** 2001. Epstein-Barr virus nuclear protein EBNA-3C interacts with the human metastatic suppressor Nm23-H1: a molecular link to cancer metastasis. Nat. Med. **7:**350–355.
70. **Subramanian, C., and E. S. Robertson.** 2002. The metastatic suppressor Nm23-H1 interacts with EBNA3C at sequences located between the glutamine- and proline-rich domains and can cooperate in activation of transcription. J. Virol. **76:**8702–8709.
71. **Tan, W. C., O. S. Privett, and M. E. Goldyne.** 1974. Studies of prostaglandins in rat mammary tumors induced by 7,12-dimethylbenz(a)anthracene. Cancer Res. **34:**3229–3231.
72. **Tazawa, R., X. M. Xu, K. K. Wu, and L. H. Wang.** 1994. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. Biochem. Biophys. Res. Commun. **203:**190–199.
73. **Thompson, M. P., and R. Kurzrock.** 2004. Epstein-Barr virus and cancer. Clin. Cancer Res. **10:**803–821.
74. **Tsuiki, H., M. Nitta, A. Furuya, N. Hanai, T. Fujiwara, M. Inagaki, M. Kochi, Y. Ushio, H. Saya, and H. Nakamura.** 1999. A novel human nucleoside diphosphate (NDP) kinase, Nm23-H6, localizes in mitochondria and affects cytokinesis. J. Cell. Biochem. **76:**254–269.
75. **van Golen, K. L., S. Risin, A. Staroselsky, D. Berger, M. A. Tainsky, S. Pathak, and J. E. Price.** 1996. Predominance of the metastatic phenotype in hybrids formed by fusion of mouse and human melanoma clones. Clin. Exp. Metastasis **14:**95–106.
76. **Venturelli, D., R. Martinez, P. Melotti, I. Casella, C. Peschle, C. Cucco, G. Spampinato, Z. Darzynkiewicz, and B. Calabretta.** 1995. Overexpression of DR-nm23, a protein encoded by a member of the nm23 gene family, inhibits granulocyte differentiation and induces apoptosis in 32Dc13 myeloid cells. Proc. Natl. Acad. Sci. USA **92:**7435–7439.
77. **Verma, S. C., and E. S. Robertson.** 2003. ORF73 of herpesvirus saimiri strain C488 tethers the viral genome to metaphase chromosomes and binds to *cis*-acting DNA sequences in the terminal repeats. J. Virol. **77:**12494–12506.
78. **Wagner, P. D., and N. D. Vu.** 1995. Phosphorylation of ATP-citrate lyase by nucleoside diphosphate kinase. J. Biol. Chem. **270:**21758–21764.
79. **Wallet, V., R. Mutzel, H. Troll, O. Barzu, B. Wurster, M. Veron, and M. L. Lacombe.** 1990. *Dictyostelium* nucleoside diphosphate kinase highly homologous to Nm23 and Awd proteins involved in mammalian tumor metastasis and *Drosophila* development. J. Natl. Cancer Inst. **82:**1199–1202.
80. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
81. **Webb, P. A., O. Perisic, C. E. Mendola, J. M. Backer, and R. L. Williams.** 1995. The crystal structure of a human nucleoside diphosphate kinase, NM23-H2. J. Mol. Biol. **251:**574–587.
82. **Wolff, H., K. Saukkonen, S. Anttila, A. Karjalainen, H. Vainio, and A. Ristimaki.** 1998. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res. **58:**4997–5001.
83. **Wun, T., H. McKnight, and J. M. Tuscano.** 2004. Increased cyclooxygenase-2 (COX-2): a potential role in the pathogenesis of lymphoma. Leukoc. Res. **28:**179–190.
84. **Xie, W. L., J. G. Chipman, D. L. Robertson, R. L. Erikson, and D. L. Simmons.** 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl. Acad. Sci. USA **88:**2692–2696.
85. **Xu, X. M., J. L. Tang, X. Chen, L. H. Wang, and K. K. Wu.** 1997. Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity. J. Biol. Chem. **272:**6943–6950.
86. **Yamamoto, K., T. Arakawa, N. Ueda, and S. Yamamoto.** 1995. Transcriptional roles of nuclear factor κB and nuclear factor-interleukin-6 in the tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J. Biol. Chem. **270:**31315–31320.
87. **Yamamoto, S., K. Yamamoto, H. Kurobe, R. Yamashita, H. Yamaguchi, and N. Ueda.** 1998. Transcriptional regulation of fatty acid cyclooxygenases-1 and -2. Int. J. Tissue React. **20:**17–22.
88. **Zimmermann, K. C., M. Sarbia, A. A. Weber, F. Borchard, H. E. Gabbert, and K. Schror.** 1999. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res. **59:**198–204.