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The expression of matrix metalloproteinase 9 is enhanced by Epstein–Barr virus latent membrane protein 1

TOMOKAZU YOSHIZAKI*†, HIROSHI SATO‡, MITSURU FURUKAWA*, AND JOSEPH S. PAGANO†

*Department of Otolaryngology, School of Medicine, and ‡Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920, Japan; and †University of North Carolina Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina, Campus Box 7295, Chapel Hill, NC 27599-7295

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ABSTRACT **Matrix metalloproteinases (MMPs) are frequently expressed in malignant tumor cells and are thought to play crucial roles in tumor invasion and metastasis. Here we report that expression of MMP9 is increased in Epstein-Barr virus (EBV)-infected type III latency lymphoma cell lines, but not in type I lines where latent viral gene expression is highly restricted. Type III cell lines express abundant EBV latent membrane protein 1 (LMP1), the principal EBV oncoprotein, as well as the other latency proteins including the transcriptional factor, EBV nuclear antigen 2, which is also required for cell immortalization. Transfection of an LMP1 expression plasmid in the C33A cell line increased MMP9 expression, whereas overexpression of EBV nuclear antigen 2 did not. Three motifs, homologous to the binding sites of NF-κB, SP-1, and AP-1 proteins, contribute to induction of the MMP9 promoter by 12-O-tetradecanoyl-phorbol-13-acetate and tumor necrosis factor α. Here we report that binding sites for NF-κB, SP-1, and AP-1 also contribute to induction of the MMP9 promoter by the viral protein, LMP1, mainly through the NF-κB and, to a lesser extent, the SP-1 and AP-1 sites. Moreover the AP-1 binding site is essential in that mutation of it abolished reporter gene induction by LMP1. The enhancement of MMP9 expression was blocked by cotransfection of an IκB expression plasmid. Thus in addition to its transforming properties, the oncoprotein LMP1 may contribute to invasiveness and metastasis of EBV-associated tumors such as nasopharyngeal carcinoma.**

Invasion into surrounding tissue is a characteristic of malignant tumors such as Epstein–Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC), and both invasion and metastasis involve sequential multistep processes. Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases that bring about the degradation of extracellular matrix during physiologic tissue remodeling such as wound healing, embryonic development, and immune cell migration (1–3). Each MMP has a different substrate specificity; for example, interstitial collagenase (MMP1) degrades type I, II, and III collagens, and stromelysin 1 (MMP3) has broad substrate specificity to degrade type III and IV collagens, proteoglycan, laminin and fibronectin (4–7). The 72-kDa type IV collagenase/gelatinase A and the 92-kDa type IV collagenase/gelatinase B (MMP2 and MMP9, respectively) are capable of degrading type IV and V collagens, laminin, fibronectin, and gelatin (1, 7–9). Several membrane-type MMPs that activate proMMP2 to active MMP2 have also been identified (10, 11). The uncontrolled expression of MMPs may be relevant to the pathogenesis of rheumatoid arthritis as well as tumor invasion and metastasis (12, 13). MMP activity is tightly regulated, mainly by the control of gene transcription, but also by proenzyme activation and by the action of specific tissue inhibitors of metalloproteinase (14, 15).

In the cascade of steps in metastasis, invasion of basement membranes by tumor cells is thought to be critical. Two type IV collagenases, MMP2 and MMP9, are often expressed by malignant tumor cells, but their expression is not always coordinated with that of MMP1 and MMP3 (16). The expression of MMP2 and/or MMP9 is thought to contribute to the invasive character of the producer cells through ability to degrade type IV collagen, which is a major component of basement membrane. The release of MMP2 and/or MMP9 has been correlated with metastasis in several systems (17–21). However, the expression of these two collagenases is not necessarily linked, which suggests an independent expression pattern for both proteinases, related to differences in cellular origin of the enzymes and transcriptional regulation (3, 12, 13, 20–23).

EBV, the causative agent of infectious mononucleosis and oral hairy leukoplakia of the tongue, is also associated with a variety of malignancies such as Burkitt lymphoma (BL) (24), as well as Hodgkin's disease (25), NPC (26), and lymphoproliferative disease in organ-transplant recipients and in patients with AIDS (27). The majority of tumor cells, regardless of type, are latently infected. Gene expression in latent infection is restricted to six nuclear antigens [EBV nuclear antigen 1 (EBNA1), -2, -3A, -3B, -3C, and LP), three latent membrane proteins (LMP1, -2A, and -2B), and two nonpolyadenylated EBV-encoded RNAs (EBER1 and 2)] (28–31). Latency is classified in three types, mainly based on the pattern of viral protein expression. Type I cells express only EBNA1; type II cells express EBNA1, latent membrane protein 1 (LMP1), -2A, and -2B; and type III cells express all six nuclear antigens and the three membrane proteins. All latently infected cells express EBER1, and -2 (32, 33). Some of the viral latency proteins such as LMP1 and EBNA2 are needed for transformation of cells (34–39). There are distinctive clinical features related to type of latency. BL, which exhibits type I latency, is characterized by localized growth or growth in regional lymph-nodes, whereas type III lymphomas associated with immunosuppression often disseminate to peripheral lymphoid tissue (34). In NPC (type II latency), cervical lymph-node metastasis is the first evidence of the malignancy in many patients (34). Therefore, we hypothesized that viral genes expressed in type II and type III but not in type I latency may influence the ability of tumor to invade. In this report, we show that the expression of MMP9, but not MMP2, is enhanced by the EBV oncoprotein, LMP1.

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Abbreviations: MMP, matrix metalloproteinase; EBV, Epstein–Barr virus; BL, Burkitt lymphoma; NPC, nasopharyngeal carcinoma; LMP, latent membrane protein; EBNA, Epstein–Barr virus nuclear antigen; CAT, chloramphenicol acetyltransferase; TNF, tumor necrosis factor.

MATERIALS AND METHODS

Cell Lines. SAV I is a type I latency cell line, and SAV III is type III; the two lines were cloned from the same parental BL cell line (40, 41). P^3HR-1 cells are derived from the Jijoye BL cell line (42). Although both lines are type III, P^3HR-1 virus is unable to induce cell immortalization due to deletion of the genes encoding EBNA2 and EBNA-LP. BL30 and BL41 are EBV genome-negative BL cell lines. BL30-P^3HR-1 and BL41-P^3HR-1 were established by single-cell cloning 2–4 weeks after infection with P^3HR-1 virus. BL30-B95–8 and BL41-B95–8 were obtained by infection with B95–8 virus (43, 44). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. C33A epithelial cells, derived from human papilloma virus-negative cervical carcinoma, were obtained from the ATCC and cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Plasmids. The LMP1 ORF was cut from pSV2 gptMTLM (38) and subcloned downstream of the cytomegalovirus immediate-early promoter in pcDNA3 (Invitrogen). An EBNA2 expression plasmid was constructed as described previously (45). The cytomegalovirus immediate-early promoter-driven IκB expression plasmid was obtained from Albert Baldwin (46). A series of 5′ flanking sequence of MMP9 were inserted upstream of the chloramphenicol acetyltransferase (CAT) reporter gene as described (16).

Transient Transfection and Conditioned Media. C33A cells were transfected with 6 μg of appropriate reporter and effector plasmids by using Lipofectamine (GIBCO/BRL) following the manufacturer's protocol. Transfected cells were cultured in DMEM with 10% fetal bovine serum overnight then in DMEM with neither fetal bovine serum nor antibiotics for 24 hr at 37°C. Lymphoid cells were cultured in AIM-V (GIBCO/BRL) for 48 hr to provide conditioned media for gelatin zymography. Transfection efficiency was monitored by cotransfection with a β-galactosidase reporter construct (67).

Gelatin Zymography. MMP2 and MMP9 were assayed by gelatinolytic activity by means of gelatin zymography as reported (47). The conditioned medium was mixed with SDS sample buffer [50 mM Tris·HCl (pH 6.8), 10% glycerol, 1% SDS, 0.01% bromophenol blue] in the absence of reducing agent to denature MMPs and to dissociate any complexes with tissue inhibitors of metalloproteinases. The mixture was then incubated at 37°C for 20 min, and SDS/PAGE containing gelatin at a final concentration of 0.1% was performed. After electrophoresis, the gel was rinsed in 2.5% Triton-X for 1 hr and then incubated for 24 hr at 37°C in 50 mM Tris·HCl (pH 7.6), 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3. The MMPs were identified following staining of the gel in 0.1% Coomassie blue R250 (Sigma) dissolved in 40% methanol, 10% acetic acid and destaining in the same solution, but without Coomassie blue. Gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin. This is the most sensitive method for identification of MMP2 and MMP9. MMP2 is detected by the clear band appearing at 72 kDa and MMP9 at 92 kDa (1–15, 17–19).

Western Blot Analysis. Protein extracts were obtained from 1.0×10^6 of lymphoid cells then mixed with 2× SDS sample buffer containing reducing agent. After SDS/PAGE, the proteins were transferred to Immobilon membranes (Millipore) with the Hoefer Semi-Dry transfer apparatus. Nonspecific reactivity was blocked by incubation overnight in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dried milk. The membrane was then incubated with primary antibody to LMP1 (Dako 1:50 dilution) and with antiserum to EBNA2 for 2 hr (44). Secondary antibody (1:3,000 dilution) was used to detect bound primary antibody. Reactive protein was detected by enhanced chemiluminescence (Amersham). For Western blotting for MMP9 conditioned medium was concentrated 20-fold by vacuum. MMP9 protein was detected with primary antiserum (Fuji Chemical, Takaoka, Japan).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from C33A cells transfected with an LMP1 expression plasmid (48). Synthetic oligonucleotides used for probes were identical to the NFκB or AP-1 nuclear factor binding sequences in the promoter region of MMP9 (16). The oligonucleotides were labeled with [α-^{32}P]CTP with the use of Klenow DNA polymerase. The unlabeled oligonucleotides were used for competition. Nuclear extracts were incubated in 12 mM Hepes buffer, 12% glycerol, 4 mM Tris·HCl (pH 7.9), 1 mM EDTA, and 3 μg poly(dI-dC) with 50,000 cpm labeled probe. The mixture was analyzed by 4.8% polyacrylamide gel in 0.5× TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3).

CAT Reporter Assay. CAT assays were performed with extracts of C33A cells after transient transfection. Construction of the MMP9 promoter reporter series has been described (16). Cells were incubated 48 hr after transfection, then harvested; acetylated [^{14}C] chloramphenicol was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS

MMP9 Activity in B-Lymphoblastoid Cell Lines. Assay of MMP gelatinolytic activity in lymphoblastoid cells growing in culture showed that MMP9 expression correlates with EBV infection and a form of latency EBV (type III) in which all of the EBV proteins needed for cell transformation are expressed. Specifically both MMP9 enzymatic activity (Fig. 1 *Top*) and protein (Fig. 1 *Middle*) are expressed in SAV III, BL30B95, BL41 B95, and Jijoye cells in which the EBV-encoded proteins, LMP-1, EBNALP, and EBNA-2 are expressed. Because the LMP-1 promoter is activated by EBNA2 (44), we determined LMP-1 protein expression. Western blot analysis showed that LMP-1 is expressed in these same cell lines (Fig. 1 *Bottom*). Neither MMP9 nor LMP-1 is expressed in the EBV-negative cells (BL 30, and BL 41), type I cells (SAV 1) or lines in which the EBNA2 gene is deleted and LMP-1 expression is low or not detectable (P^3HR1, BL30 P^3HRI, and BL41-P^3HR1). Therefore one or more of the EBV transforming proteins is necessary and perhaps sufficient for induction of MMP9 expression. In summary, expression of MMP9

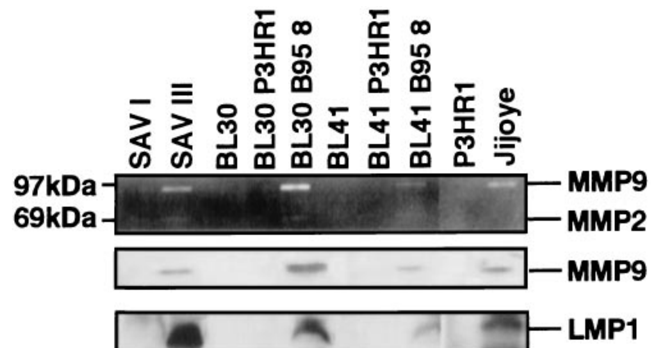


FIG. 1. MMP9 and MMP2 activity in EBV-infected type I and type III lymphoblastoid cells. SAV I is a type I BL line, and SAV III is a sister type III line. BL30 and BL 41 are EBV-negative BL lines. BL30-P^3HR-1, BL30-B95–8, BL41-P^3HR-1, and BL41-B95–8 lines are infected with P^3HR-1 virus (EBNA2 deleted) or B95–8 virus. Jijoye is the parental BL line of P^3HR-1 and has intact EBNA2 gene. (*Top*) Gelatinolytic activity; (*Middle*) Western blot of MMP9; (*Bottom*) Western blot of LMP1. Lymphoid cells (2.0×10^6) were cultured in 1 ml media for 48 hr. After centrifugation, supernatant fluid was prepared for zymography and the pellet for Western blot analysis as described in *Materials and Methods*.

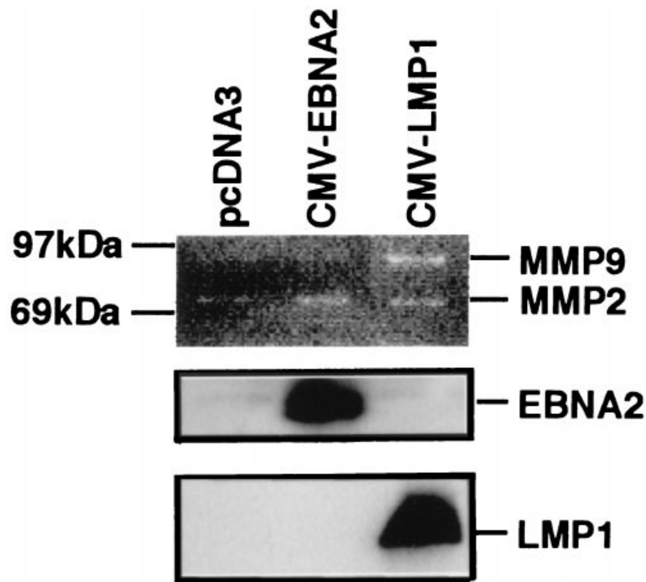
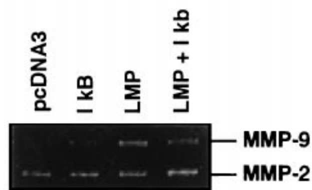


FIG. 2. MMP9 and MMP2 activity in EBV-negative cells transfected with EBV oncoprotein LMP1 or EBNA2. (*Top*) Gelatinolytic activity of C33A cells transfected with LMP1 and EBNA2. (*Middle* and *Bottom*) Immunoblots for LMP1 and EBNA2 protein. Cells (4.0×10^5) were placed in three sets of 60-mm culture plates 12 hr before transfection. Samples were prepared for zymography, and LMP1 and EBNA2 protein detection.

correlated with type III latency and, specifically, with expression of LMP1.

Low levels of 72-kDa MMP2 activity were also detected in SAV III, BL30-B95–8 and BL41-B95–8 cells, but not in Jijoye.



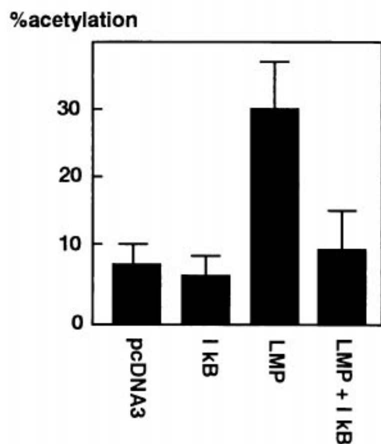


FIG. 3. IκB inhibits the enhancement of MMP9 expression by LMP1. (*a*) Gelatin zymography. Samples of C33A cells were prepared as described in *Materials and Methods*. (*b*) CAT assays. The effector plasmids were cotransfected with −670 CAT (full-length MMP9 promoter construct). The mean values and SDs represented were obtained from three experiments.

There was not a clear or consistent difference among these cell lines (Fig. 1). Therefore, EBV infection did not appear to induce MMP2 activity.

MMP2 and MMP9 Activity in Cells Transfected with EBV Latency Genes. Since some of the cell lines showing gelatinolytic activity have an EBNA2 gene deletion that also causes LMP1 downregulation, the effect of LMP1 and EBNA2 expression on MMP9 production was determined by transfection of either LMP1 or EBNA2 expression plasmids into EBV-negative C33A cells. Enhancement of 92-kDa MMP9 activity was detected in LMP1-transfected C33A cells, but not in EBNA2-transfected C33A cells (Fig. 2*a*). The expression of LMP1 and EBNA2 proteins was confirmed by Western blot (Fig. 2*b*). MMP2 gelatinolytic activity was not significantly affected by either viral protein (Fig. 2*a*). Thus, the enhanced MMP9 activity detected in type III cells appears to be due to expression of LMP1.

Inhibition of MMP9 Expression by IκB. LMP1 has been shown to activate the NF-κB pathway (50–53). Therefore, the inhibitory effect of IκB on LMP1-mediated MMP9 expression was examined. Cotransfection of IκB and LMP1 expression

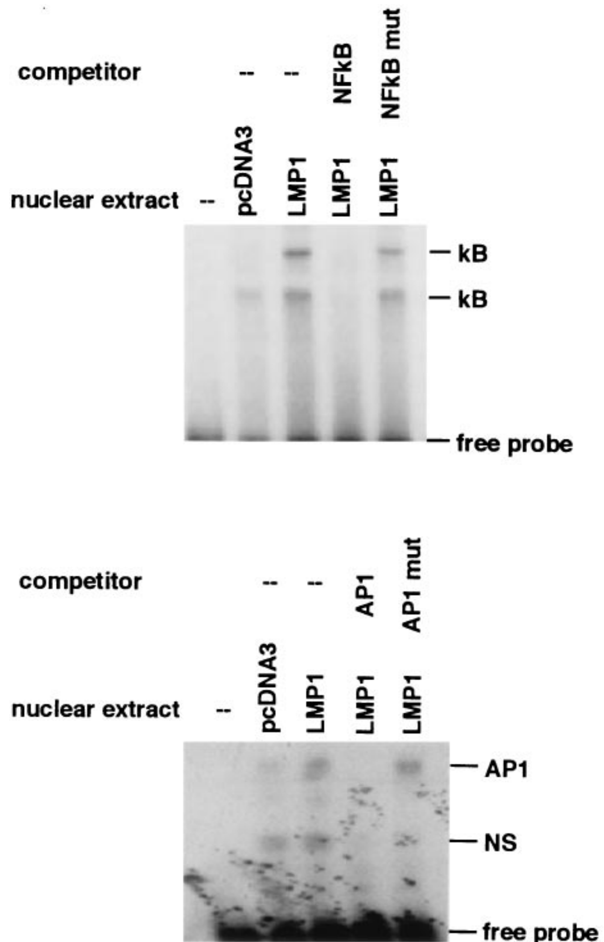


FIG. 4. Induction of NF-κB and AP-1 DNA-binding activity in LMP1-transfected cells. Nuclear extracts from C33A cells transfected either with or without LMP1 expression plasmid were mixed with either NF-κB or AP-1 ^{32}P-labeled probes. Excesses of nonlabeled NF-κB, NF-κB mutant, AP-1 and AP-1 mutant probes (×100) were used as competitors (NF-κB, 5′-GATCGGGTTGCCCCAGTGGAATTCCCCAGCCTT-3; NF-κB mut, 5′-GATCGGGTTGCCCCAGT**TT**AATTCCCCAGCCTT-3′; AP-1, 5′-GATCTTCTAGACCGGATGAGTCATAGCTG-3′; AP-1 mut, 5′-GATCTTCTAGACCGGAT**A**AG**G**CATAGCTG). Each of the mutant competitor oligonucleotides has two nucleotide substitutions as indicated by bold letters in binding sequence. Underlined letters indicate binding sequences in the promoter of the MMP9 gene. NS, nonspecific binding.

plasmids repressed the gelatinolytic activity of MMP9 whereas that of MMP2 was not affected (Fig. 3*a*). To confirm the effect of IκB, assays were also performed with the MMP9 promoter CAT reporter construct. Compared with activation produced by LMP1 alone, cotransfection of IκB repressed CAT activity (Fig. 3*b*). IκB by itself produced little effect. This result suggested that enhancement of MMP9 expression by LMP1 is, at least in part, mediated by the NF-κB signal pathway.

Induction of NFκB and AP-1 by LMP1 Transfection. LMP1 interacts with tumor necrosis factor (TNF) receptor-associated factors and activates several nuclear factors including NF-κB (50, 52, 53). Three such factors, NF-κB, SP-1, and AP-1, have been identified as transactivators of MMP9 when cells were treated with 12-O-tetradecanoyl-phorbol-13-acetate and/or TNFα (16). Therefore, we investigated the induction and binding of these nuclear factors to NF-κB, SP-1, and AP-1 binding sequences in the MMP9 promoter region by cotransfection with LMP1. Increases in nuclear factors binding to NF-κB binding sequence in the MMP9 promoter were detected in C33A cells transfected with LMP1 (Fig. 4). An increase in binding of nuclear factor to AP-1 sequence was also detected. Greater protein binding to SP-1 sequence was not detected (data not shown). Therefore, LMP1 induces the activation or availability of NF-κB and AP-1.

Role of Nuclear Factor Binding Elements in Promoter Activity. MMP activity is mainly regulated by the control of gene transcription, and also by posttranslational control (14, 15). Transcription of the MMP9 promoter is stimulated by TNFα through binding not only to NF-κB sites but also to AP-1 and SP-1 sites (16). To test the contribution of these binding sites, plasmids with deletions and point mutations in three sites in the MMP9 promoter were cotransfected with the LMP1 expression vector (Fig. 5). Deletion of the NF-κB site (−599 CAT) dramatically reduced but still retained some MMP9 promoter activity. The −90 CAT construct, which only contains the AP-1 binding site and TATA box, again decreased CAT activity but still retained some promoter activity. An additional deletion to include the AP-1 site (−73 CAT) abolished CAT activity. Mutational analysis of these binding sites revealed that point mutation of the NF-κB binding site (kb mut CAT) reduced CAT activity more dramatically than the deletional construct −599 CAT. Mutation of the SP-1 site also decreased CAT activity, but there was still 2-fold activation (SP-1 mut CAT). Mutation of the AP-1 site completely abolished CAT activity (AP-1 mut CAT) (Fig. 5). Therefore, the AP-1 binding site is essential for activation of the MMP9 promoter by LMP1, and the NF-κB site as well as the SP-1 site contribute to activation.

DISCUSSION

LMP1 is necessary for transformation of B lymphocytes and is the only EBV latency gene capable of transforming rodent fibroblasts (37, 38). Thus, LMP1 is thought to have a significant role in initiating EBV-associated lymphoproliferative disease and EBV-related malignancies (34–36, 40). Although LMP1 has until now been characterized mainly with respect of its primary oncogenic capacity, here we show that LMP1 may have an additional level of function, namely, the ability to induce cellular genes, specifically MMP9, that promote invasion and metastasis. LMP1 is expressed in at least 70% of NPC and all EBV-infected preinvasive lesions (54, 55). NPC, which exhibits type II latency, frequently disseminates clinically into regional lymph-nodes before forming a mass in the primary site, whereas BL (type I) is prone to form a localized mass before it disseminates (34). Moreover, LMP1 reduces expression of E-cadherin, another cell invasive factor, in a human keratinocyte cell line (56). Our results indicate therefore that LMP1 has a dual function, contributing not only to cell transformation but also invasiveness of EBV-associated tumors categorized as type II and III by enhancing MMP9 activity. Interestingly, the v-src oncogene not only promotes cellular transformation, but also transactivates expression of MMP9 (57). Finally, in head-and-neck squamous cell carcinomas originating in the oropharynx, which have not been associated with EBV infection, MMP1, -2, -7, -9, -11, and MT1-MMP are overexpressed (47, 58–61). Levels of these enzymes in NPC have not yet been reported.

Aggregation of LMP1 in the plasma membrane evokes activation of the TNF receptor downstream signaling pathway (50). LMP1 is comprised of 386 amino acids, of which the carboxy-terminal 200 amino acids are in the cytoplasmic domain of the protein. This portion of the protein contains two functional domains. One, the proximal domain (amino acids 187–231), interacts with TNF receptor-associated factors and mediates NF-κB activation (53). The other, the distal domain (amino acids 352–386) whose interaction partner has not been identified, mediates higher level NF-κB activation than the proximal domain (62, 63). Although the activation of the NF-κB signal pathway by LMP1 has been emphasized, the entire pathway has not been delineated. Induction of epidermal growth factor receptor is activated by LMP1 through a signal pathway distinct from the NF-κB pathway (52). There-

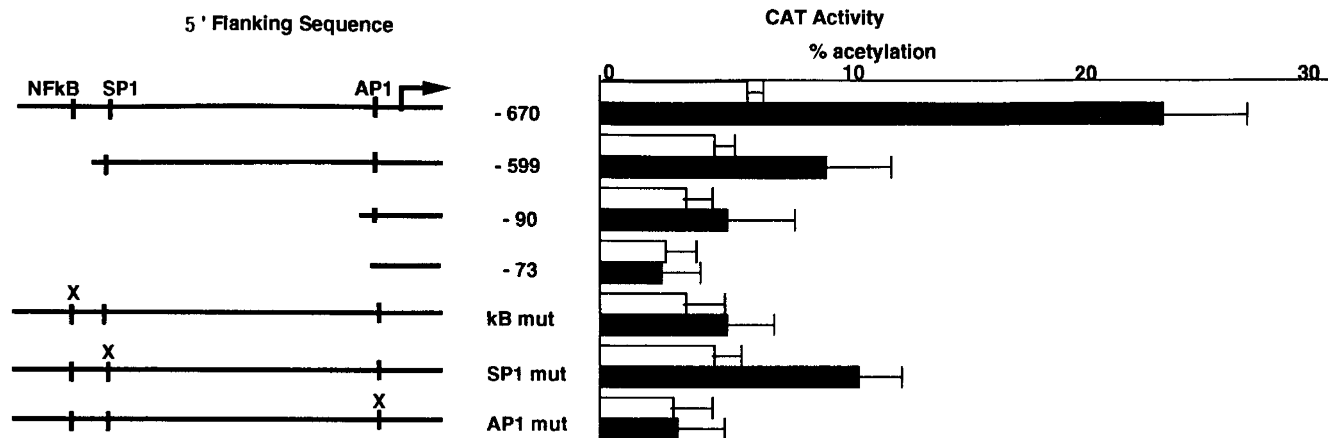


FIG. 5. Deletional and mutational analysis of the cis-elements required for LMP1-induced MMP9 promoter activity. Arrow indicates transcription start site and "X" the sites of mutation in the MMP9 5′ flanking sequence inserted upstream of the CAT gene. Open and solid bars represent CAT activity of pcDNA3 and cytomegalovirus-LMP1-transfected C33A cells, respectively. The mean values and error bars represent the results of two sets of three experiments.

fore, the elements responsible for the activation of MMP9 may differ from those that promote transformation and are being investigated.

Correlation between the production of type IV collagenases (MMP2 and MMP9) and spontaneous metastasis in a rat model (20, 21) has been reported. MMP9 is secreted by normal alveolar macrophages, granulocytes, osteoclasts, invading trophoblasts, and transformed cell lines, but not by fibroblasts. MMP2 is constitutively expressed in most fibroblast and some transformed cell lines (3, 12, 22). The promoter of the MMP9 gene contains NF-κB, SP-1, AP-1 binding sites, and a TATA box, whereas the MMP2 promoter contains AP-2 and SP-1 binding sites, but no TATA box (16, 22). The transcriptional factor Ets may also be involved in the transactivation of MMP9 in H-ras and c-myc-transformed rat cell lines (64). Finally, the fact that LMP1 did not induce MMP2 expression may be related in part to the lack of an NF-κB site in the MMP2 promoter (22).

EBNA2, which also activates NF-κB, did not significantly increase MMP9 expression. Chen *et al.* reported that the induction of NF-κB by EBNA2 was weaker than by LMP1 in EBV-negative cells (65). Our results indicate that the induction of NF-κB by EBNA2 alone is not sufficient to transactivate the MMP9 promoter to induce MMP9 enzyme activity.

Finally we show that the enhancement of expression of MMP9 by LMP1 is partially blocked by IκB. These results suggest that type II and type III malignancies such as NPC, Hodgkin disease (type II), and AIDS-related lymphoma (type III) may use MMP9 for metastasis. Therefore an agent that mimics IκB may be effective in preventing metastasis of these tumors (66).

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