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The Immediate Early Gene 1 Product of Human Cytomegalovirus Is Sufficient for Up-Regulation of Interleukin-8 Gene Expression

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We previously observed that human cytomegalovirus (CMV) infection induced a massive production of a chemokine with potent neutrophil chemotactic activity, interleukin-8 (IL-8). Hence, we examined the effect of CMV immediate early (IE) gene products on IL-8 production by the human astrocytoma cell line, U373MG. Transient or stable transfection with a CMV IE1 gene expression vector, but not with a IE2 gene expression vector, significantly augmented IL-8 protein secretion and IL-8 mRNA expression. Luciferase activity was enhanced in U373MG cells when the cells were cotransfected with CMV IE1 and chimeric firefly luciferase reporter genes driven by the transcriptional regulatory region of the human IL-8 gene. Moreover, IE1 gene-mediated enhancement of luciferase activity was abolished by the introduction of mutations into the AP-1 or NF-κB factor binding elements in the regulatory region of the IL-8 promoter. Furthermore, electrophoretic mobility shift assays demonstrated that CMV IE1 gene products induced the formation of NF-κB or AP-1 complexes. Finally, Western blotting analysis demonstrated that the CMV IE1 gene product increased the amount of NF-κB complexes translocated into the nucleus. Collectively, CMV IE1 gene expression may be sufficient to activate AP-1 and NF-κB, resulting in IL-8 gene expression.

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus, ordinarily existing in the host without causing any sequelae (1), but present as a lifelong latent infection. However, latently infected CMV is frequently activated in immunocompromised individuals (e.g., AIDS patients and those individuals who have received organ and bone marrow transplants), thereby causing severe morbidity and eventual mortality (2). Several lines of evidence suggest that the immune and inflammatory reactions directed against the virus can greatly modulate the course of CMV infection, particularly the latent infection. CMV genes are expressed in a temporally regulated manner after infection of permissive cells. The immediate-early (IE) 1 gene is transcribed from the major IE region under the control of the major IE promoter (3). The IE1 protein can activate transcription from both homologous and heterologous viral and cellular promoters including several cytokine genes (4, 5).

Accumulating evidence indicates an essential role for interleukin 8 (IL-8) in neutrophil-mediated tissue injuries (6, 7). Moreover, IL-8 enhanced infectious virion production and replication of CMV (8). Furthermore, IL-8 could attenuate the antiviral activities of interferon, particularly, type I interferon (9). These observations would imply that endogeneously-produced IL-8 may be an important mediator of the virus infection process.

Various viruses and viral products have been demonstrated to transactivate the IL-8 gene (10, 11); in most cases by acting concurrently at NF-κB and AP-1 sites in the IL-8 promoter. We previously observed that CMV infection also induced IL-8 gene transcription by enhancing NF-κB and AP-1 complex formation in the human monocytic cell line, THP-1 (12). In addition, an

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antisence oligonucleotide complementary to CMV IE mRNA significantly suppressed CMV-induced upregulation of IL-8 expression at the transcriptional and protein levels (13). In this manuscript we explore the effect of CMV IE gene products on IL-8 production and provide evidence that CMV IE1 is mainly responsible for IL-8 gene transcription by concurrently activating NF-κB and AP-1.

MATERIALS AND METHODS

Reagents. Pica Gene obtained from Toyo Ink Co., Ltd. (Tokyo, Japan) was utilized for luciferase assays. Rabbit antisera against human cRel, p65, p50 and RelB were kindly provided by Dr. Nancy Rice (National Cancer Institute, Frederick, MD) (14).

Cell culture and determination of IL-8 protein levels. A human astrocytoma cell line, U373MG, (15) was cultured in Dulbecco's modified Eagle minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Z. L. Bocknek Lab., Ontario, Canada), L-glutamine (0.3 mg/ml), gentamicin (50 mg/ml), and ammphotericin B (2.5 μg/ml). The cell line was mycoplasma-free as determined by 6-methylpurine deoxyriboside analysis (Mycotect Kit, Gibco, Grand Island, New York). IL-8 levels in the culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) specific for human IL-8 (16).

Plasmids. The pIE1/2 was constructed by subcloning the 7.1-kb *Eco*RI-*Sal*I fragment of CMV (Towne strain) DNA into pUC18. This fragment consists of the entire IE1 and IE2 genes and the major immediate early promoter (MIEP). The plasmid pIE1 was constructed by deleting the IE2-encoding sequence between the Sse8387I site in polylinker at the 3′ end of the insert and the SmaI site in exon 5 from pIE1/2. Plasmid pIE2 was constructed by deleting the 1.4-kb *Acc*I-*Acc*I fragment containing the IE1-encoding region from pIE1/2. Plasmids pIE1, pIE2 and pIE1/2 express the 72-kDa IE1, 86-kDa IE2, and both the 72-kDa IE1 and 86-kDa IE2 proteins, respectively. Luciferase expression vectors linked with various mutated 5′-flanking regions of the IL-8 gene promoter were prepared as described previously (12, 17). All plasmids were prepared utilizing Qiagen tip 500 (Qiagen Gmbh, Dusseldorf, Germany) according to the manufacturer's protocols.

Transfection. U373MG cells in a subconfluent state were collected after trypsinization, resuspended in DMEM medium supplemented with 5% FCS and cultured at a density of 2×10^5 in 3 ml/dish at 24 h prior to the transfection. Cells were transfected with 10 μg/dish of plasmid DNA (pIE1, pIE2, pIE1/2, or pUC18) by the calcium phosphate coprecipitation method as modified by Chen and Okayama (18). Total cellular RNA was prepared for Northern blotting analysis of IL-8 mRNA expression and culture supernatants were collected for determination of IL-8 protein expression by ELISA. Northern blotting analysis was performed using ^{32}P-labeled human IL-8 or 18S ribosomal RNA as a probe as described previously (12).

Establishment of U373MG cell lines stably expressing the IE proteins. pIE1, pIE2, or pIE1/2 plasmids were cotransfected with pSV2Neo (Clontech Laboratories, Inc.) into U373MG cells by the calcium phosphate method. Transfectants were selected in medium containing 0.5 mg/ml of G418 sulfate (Gibco BRL) beginning on the third day after the transfection. G418-resistant clones were expanded, and cells were analyzed for expression of IE proteins by Western blot analysis and indirect immunofluorescent analysis. Clones expressing high level of each IE protein were propagated and used for further studies.

Luciferase assay. U373MG cells (2×10^6 cells) were cotransfected with 10 μg of the luciferase expression vector DNA and 1 μg each of pIE1, pIE2 or pIE1/2, by the calcium phosphate coprecipitation method as modified by Chen and Okayama (18). After 48 h, the cell lysates were prepared using Pica Gene (Toyo Ink Co., Japan) according to the manufacturer's instructions. Protein concentrations were measured utilizing the PIERCE protein assay kit (Rockford, IL). Light intensity was measured on 10 μg of cell lysates using a Lumat model LB950 luminometer (Berthold, Germany). In some experiments, luciferase activities were also determined in U373MG cells expressing stably IE proteins. These cells were transiently transfected with 10 μg of the different luciferase expression vectors (19).

Electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted from the U373MG cell lines stably expressing IE proteins according to the method described by Dignam *et al.* (19). Five micrograms of nuclear protein was processed for EMSA using either the AP-1 binding site (−130 to −116 bp; GTGATGACTCAGGT) or the NF-κB binding site (−83 to −68 bp; CGTGGAATTTCCTCTG) of the IL-8 promoter as previously described (12, 17). In some experiments, nuclear protein was preincubated with either a 100-fold molar excess of unlabeled oligomers or 1 μl of rabbit antisera against human p65, p50, cRel, RelB (14) before radiolabeled probe and poly(dI-dC) were added.

Western blot analysis. Nuclear and cytosol proteins were extracted according to the method described by Dignam *et al.* (19). The resultant proteins were separated by electrophoresis on a 7.5% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). Proteins were then transferred to a nitrocellulose membrane (BA85, Schleicher & Schuell) using 20 mM Tris, 150 mM glysine, pH 8.3 in 20% methanol. Membranes were incubated for 1 h at room temperature with the blocking reagent (5% skim milk, Tris buffered saline-0.5% Tween 20 pH 7.6 (TBS-T)), and then incubated for 1 h at room temperature with primary antibody diluted 1/2,000 in TBS-T. Membranes were washed three times in TBS-T and incubated with peroxidase-conjugated second antibody diluted 1/20,000 in TBS-T for 0.5 h at room temperature. After washing three times in TBS-T, immune complexes were detected by the ECL plus system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The Effects of Transient Expression of IE Gene Products on IL-8 Gene Expression

We observed that a human astrocytoma cell line, U373MG, could produce a massive amount of IL-8 upon CMV infection or LPS stimulation. Moreover, ultraviolet-irradiated CMV or virus-free filtrates of culture supernatants from a CMV-infected human embryonic fibroblast cell line (8, 12), failed to induce IL-8 production by U373MG cells (data not shown).

The failure of ultraviolet-irradiated CMV to induce IL-8 production in U373MG cells suggests that CMV replication is indispensable for CMV-induced IL-8 production. Hence, we transfected U373MG cells transiently with IE gene expression vectors to examine the effects IE gene expression on IL-8 mRNA and protein production. Transfection with pUC18 failed to augment significantly IL-8 production by U373MG cells, compared with untreated cells (Fig. 1A). In contrast transfection with either pIE1 or pIE1/2 increased IL-8 production, compared with pUC18 transfection, whereas that with pIE2 did not. Northern blotting analysis demonstrated that the transfection with pUC18 enhanced IL-8 mRNA accumulation in U373MG cells (Figs. 1B and 1C). The transfec-

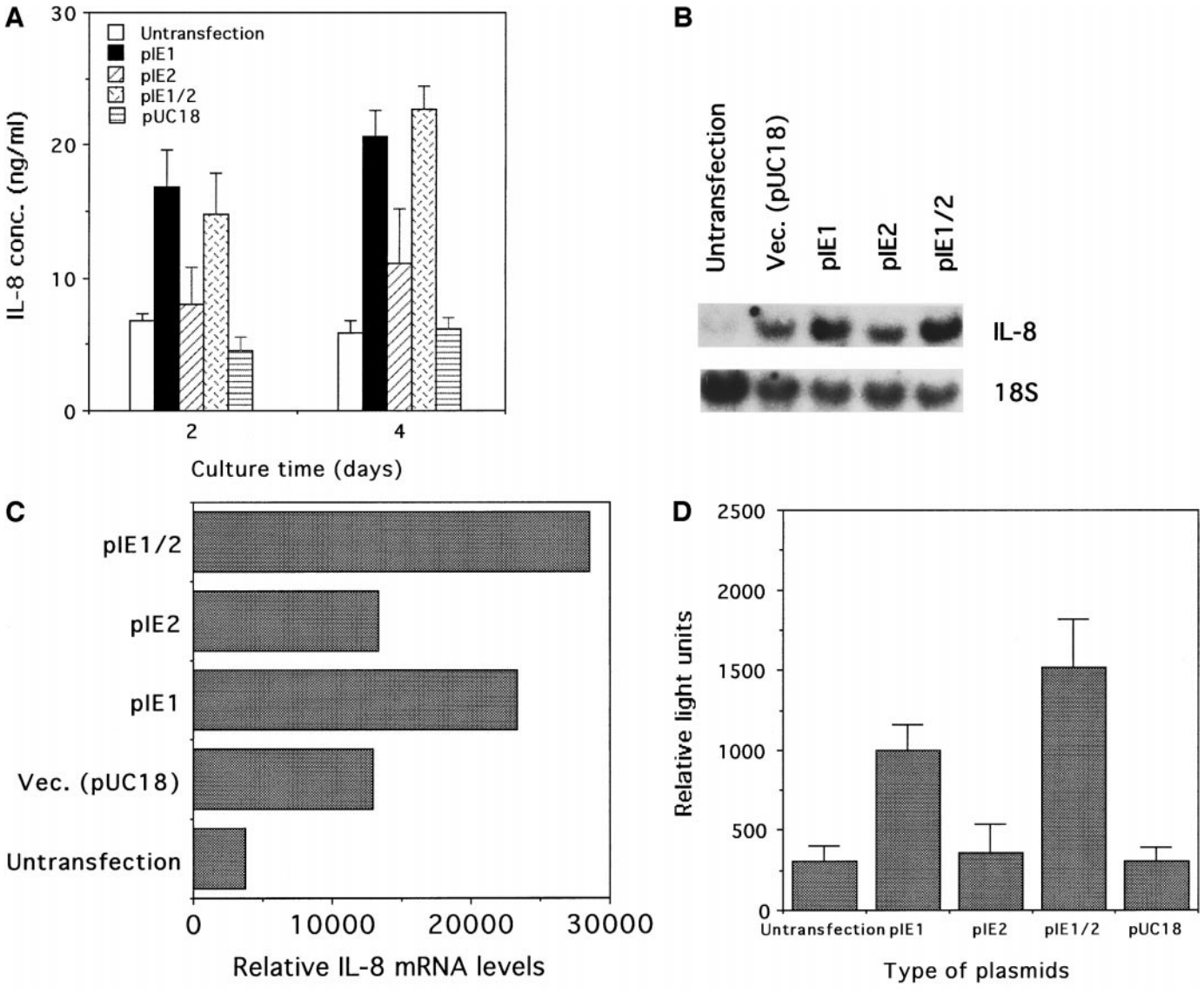

FIG. 1. Effect of transient transfection of CMV IE gene products on IL-8 production by U373MG cells. (A) Effect of CMV IE gene transfection on IL-8 production from 1×10^6 transfected cells as measured by ELISA. Data are expressed as mean ± standard error from three independent experiments. (B) Northern blotting analysis on total RNA extracted from CMV IE gene transfected U373MG cells. Northern blotting was performed as described in the Materials and Methods. (C) Relative rate of IL-8 transcription in CMV IE gene transfected U373MG cells. The bands in panel B were quantitated by densitometric analysis, and values are normalized to 18S rRNA. (D) The effect of CMV IE gene transfection on IL-8 gene promoter activity. U373MG cells were co-transfected with the luciferase expression vector containing the basic IL-8 promoter region (−133-luc) and various CMV IE gene expression vectors as described under Materials and Methods. The results are representative of three independent experiments and error bars indicate standard error.

tion with either pIE1 or pIE1/2 but not with pIE2 further increased IL-8 mRNA expression in U373MG cells. In order to determine whether IE gene products induced IL-8 gene expression at the transcriptional level, we co-transfected U373MG cells with various IE gene expression vectors and a luciferase expression vector driven by the basic promoter region of the IL-8 gene (−133 to +44 bp). This region of the promoter is sufficient to confer responsiveness of the IL-8 gene to various stimuli, including CMV infection (12, 17). Cotransfection with either pIE1 or pIE1/2 but not pIE2 or the control vector, enhanced luciferase activities in U373MG cells significantly (Fig. 1D). Collectively, transient expression of the CMV IE1 gene alone can transactivate the IL-8 gene although a contribution of the CMV IE2 gene cannot completely be excluded.

The Effects of Stable IE Gene Expression on IL-8 Gene Activation

In order to elucidate in more detail the molecular mechanisms of CMV IE gene-mediated IL-8 gene acti-

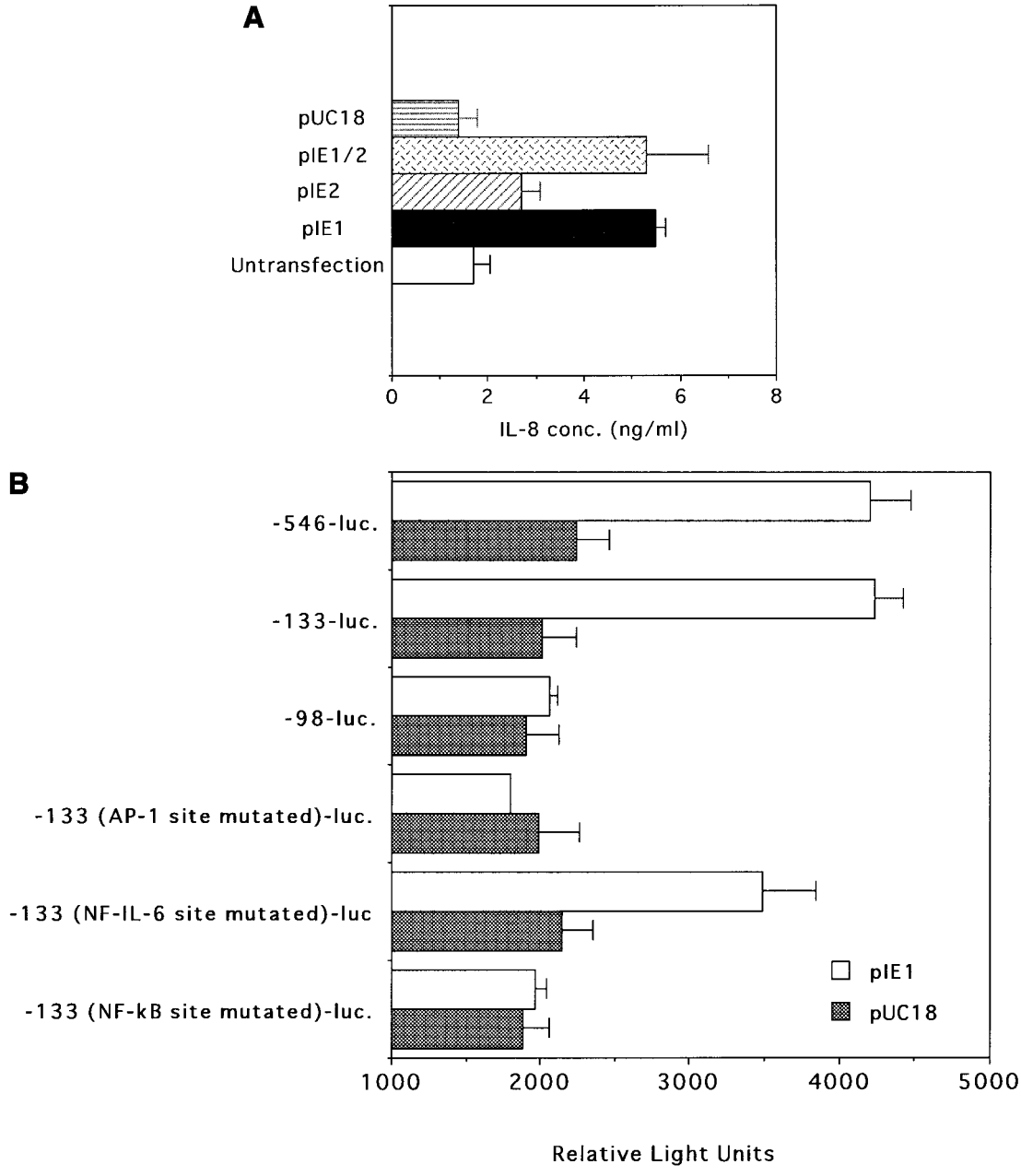

FIG. 2. IL-8 protein production and gene transcription activity in U373MG cell lines stably expressing IE proteins. (A) Comparison of IL-8 production by various cell lines, as measured by ELISA. Data are expressed as mean ± standard error from three independent experiments. (B) Determination of CMV IE1 gene responsive elements in the IL-8 promoter. Various chimeric IL-8 promoter luciferase (luc) reporter genes were transfected into cell lines stably expressing IE1 antigen (pIE1), or a control cell line (pUC18). Luciferase activity was measured as described under Materials and Methods. The results are representative of three independent experiments and error bars indicate standard error.

vation, we established U373MG cells stably expressing the IE1 or IE2 IE gene products. The constitutive expression of either IE gene product had negligible effects on morphology, cell viability, and proliferation rates of the resultant transfectants (data not shown). Even in the absence of stimuli, cells expressing either IE1 or IE1/2 gene produced significantly larger amounts of IL-8 than parental cells or cells transfected with either pIE2 or a control vector (Fig. 2A). IE1 gene expression has been reported to induce the production of IL-1β, which is a potent inducer of IL-8 in various types of cells (20, 21). IL-8 protein was only detected in the cells that expressed the CMV IE1 antigen in the whole nuclear region. Of interest is that IL-8 protein was localized in the perinuclear or cytosol regions (our unpublished data). Furthermore, the immunostaining

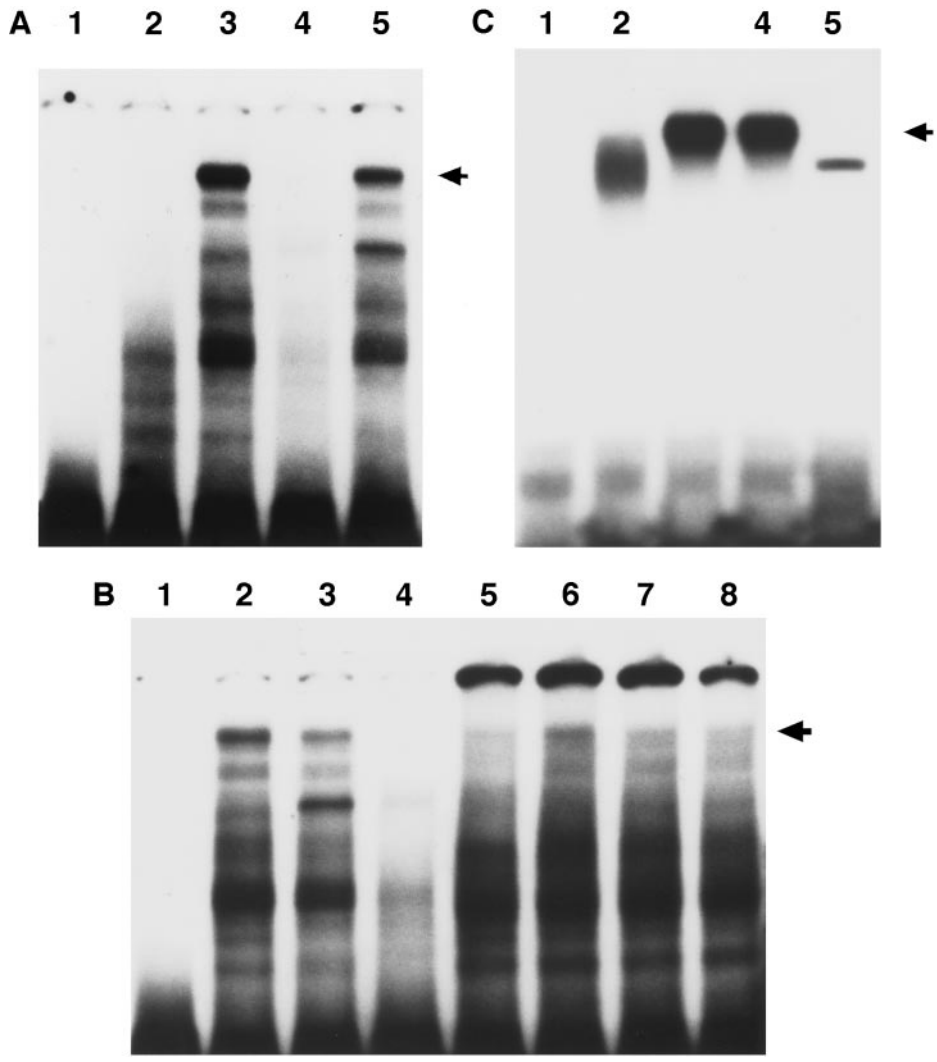

FIG. 3. EMSA using nuclear proteins extracted from U373MG cell lines stably expressing IE proteins. (A) Characterization of nuclear proteins bound to the NF-κB binding site in the IL-8 promoter. Nuclear proteins were extracted from a cell line stably expressing IE1 (lanes 3 to 5), or a vector control cell line (lane 2). Lane 1 represents the control without nuclear proteins. EMSA was performed on nuclear extracts preincubated with no reagents (lanes 1 to 3), an NF-κB oligomer (lanes 4), or an AP-1 oligomer (lanes 5), and the NF-κB binding site of the IL-8 promoter was used as the labeled oligomer probe as described in the text. (B) Immunochemical characterization of nuclear proteins bound to the NF-κB binding site in the IL-8 promoter. Nuclear proteins were extracted from the cell line expressing IE1 (lanes 2 to 8). Lane 1 represents the control without nuclear proteins. Nuclear proteins were preincubated with no reagents (lanes 1 and 2), an AP-1 oligomer (lane 3), a NF-κB oligomer (lane 4), or anti-p65 (lane 5), anti-p50 (lane 6), anti-cRel (lane 7), or anti-RelB (lane 8) antiserum, and the NF-κB binding site of the IL-8 promoter was used as a labeled oligomer probe. (C) Characterization of nuclear proteins bound to the AP-1 binding site in the IL-8 gene promoter. Nuclear proteins were extracted from U373MG cells expressing IE1 (lanes 3 to 5), a vector control transfected cell (lane 2), or without nuclear proteins (lane 1). Nuclear proteins were preincubated with no reagents (lanes 1 to 3), a NF-κB oligomer (lanes 4), or an AP-1 oligomer (lanes 5). The AP-1 binding site of the IL-8 gene promoter was used as a labeled probe as described in the text.

intensity with an anti-IL-8 antibody correlated well with that of anti-IE1, suggesting a direct relationship between the levels of IE1 and the levels of induced IL-8 (our unpublished data). These results raise the possibility that IE1 can directly induce IL-8 production. However, it can not be completely excluded that IE1 gene transfer induced IL-1β, which, in turn, induced IL-8 production in an autocrine mechanism.

Luciferase activities were significantly enhanced in cells expressing IE1 but not control transfectants when the cells were transduced with −546-luc and −133-luc, both of which contain the minimal essential promoter region of the IL-8 gene (Fig. 2B). Moreover, a deletion below −98 bp, or mutations in either the AP-1 or NF-κB sites, abrogated the inducibility of the luciferase activities in the cells expressing IE1, whereas the luciferase vector with a mutation in the NF-IL-6 site enhanced the luciferase activity in the same cell line. These results demonstrate that both AP-1- and NF-κB-binding sites are involved in CMV IE1 gene-induced

IL-8 gene activation. This result is consistent with our previous data demonstrating IL-8 induction upon CMV infection (12). Yurochko *et al.* reported that the simple binding of CMV to human monocytes is sufficient to transactivate NF-κB (22). However, ultraviolet-irradiated CMV, which could bind the target cells, failed to induce IL-8 production in several types of cells, including THP-1, U373MG, and a MRC-5 (our unpublished data). Furthermore, we also observed that IE1 gene transduction activated NF-κB. Collectively, our data suggests that NF-κB might be transactivated by CMV only after CMV enters the target cells and IE gene expression occurs.

In EMSA, nuclear proteins from control gene transfectants gave rise to a faint band when the NF-κB binding site was used as an oligomer probe. In contrast strong complex formation was observed when nuclear extract from the pIE1 transfectant was utilized (Fig. 3A). The complex formation was inhibited by the corresponding unlabeled oligomer but not an unrelated oligomer (Fig. 3A). Moreover, antisera to p65, p50, cRel and RelB supershifted the complexes (Fig. 3B), indicating that the induced complexes were composed of p65, p50, cRel and RelB as observed in the NF-κB complexes in CMV infected cells (12). In addition, AP-1 complex formation was also enhanced in the pIE1 transfectant, and the AP-1 complexes were inhibited by a specific oligomer but not by an unrelated oligomer (Fig. 3C). Our data suggests that CMV infection activates a nuclear binding protein complexes critical for IL-8 gene expression. We recently observed that IL-8 could inhibit anti-viral activities of interferon (9), thereby enhancing virus replication. Thus, it is tempting to speculate that CMV IE1-mediated IL-8 production may facilitate CMV infection by inhibiting endogenously-produced interferon.

NF-κB is retained in the cytosol in association with its inhibitor, IκB, in a resting state. Upon exposure to various stimuli, IκB is sequentially phosphorylated, ubiquitinized, and degradated by proteasomes. Liberated NF-κB translocates into nucleus and binds the cis-acting element in target genes, thereby causing their transcription. Thus, we evaluated the effects of IE gene expression on the subcellular localization of NF-κB p65. Immunofluorescence analysis demonstrated that p65 was mainly localized in the nucleus of the IE1 transfectant in contrast to its cytosol localization in control cells (data not shown). Moreover, the amount of cytosol p65 decreased in the IE1 transfectant, compared with control or the IE2 transfectant, while the levels of nuclear p65 increased in the IE1 transfectant (Fig. 4). These results suggest that IE1 but not IE2 expression induced nuclear transport of NF-κB. Several independent groups observed that CMV infection increased p65 and p50 mRNA expression at the transcriptional level (5, 23). However, our Western blotting analysis demonstrated that the total amount of NF-κB p65 remained unchanged in the IE1 transfected cells.

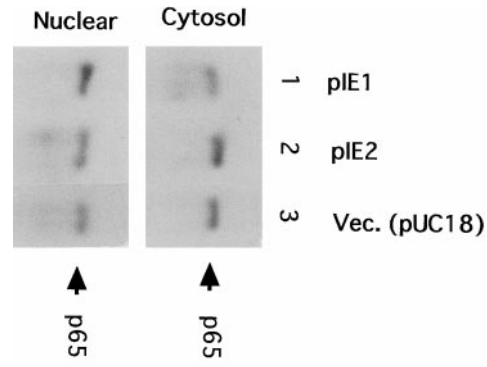

FIG. 4. Western blotting analysis using antibody against NF-κB p65. SDS–PAGE was performed on 8 μg of cytosol proteins, or 10 μg of nuclear proteins extracted from U373MG cell lines stably expressing CMV IE protein. (lane 1) IE1 expressing cell; (lane 2) IE2 expressing cell; and (lane 3) control vector transfected cell. After electrophoresis, immunoblotting was performed as described under Materials and Methods.

Hence, it is more likely that the IE1 protein activates NF-κB by inducing its nuclear translocation, rather than increasing the p65 mRNA levels. Shibutani *et al.* reported that CMV infection stimulated the arachidonic acid cascade, generating reactive oxygen species and thereby activating NF-κB (24). However, the involvement of reactive oxygen species in IE1-mediated NF-κB activation remains to be investigated.

The reactivation of persistent or latent infection causes overt CMV infection in AIDS patients and immunocompromised organ transplants recipients (1, 11). Recently, Sôderberg-Naucler *et al.* reported that long-term culture of latently CMV-infected cells with allogenic mononuclear cells could reactivate CMV infection (25). They also provided evidence that adherent monocytes were mainly responsible for this process and that IE gene expression was the first event in this reaction of CMV infection. Thus, IE1 reexpression may induce IL-8 production at the early phase of CMV reactivation. As IL-8 could enhance *in vitro* CMV replication in fibroblasts (8), IE1-mediated IL-8 production may, in turn, reactivate latent CMV infection by augmenting its replication. If so, more detailed analysis of the mechanisms involved in IE1-mediated IL-8 gene expression may lead to the development of therapeutic or preventive measures against a potentially hazardous opportunistic CMV infection. Thus, IL-8 may represent an important new target for the control of CMV replication.

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