Roles of Polypyrimidine Tract Binding Proteins in Major Immediate-Early Gene Expression and Viral Replication of Human Cytomegalovirus

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Received 21 November 2008/Accepted 5 January 2009

Human cytomegalovirus (HCMV), a member of the β subgroup of the family Herpesviridae, causes serious health problems worldwide. HCMV gene expression in host cells is a well-defined sequential process: immediate-early (IE) gene expression, early-gene expression, DNA replication, and late-gene expression. The most abundant IE gene, major IE (MIE) gene pre-mRNA, needs to be spliced before being exported to the cytoplasm for translation. In this study, the regulation of MIE gene splicing was investigated; in so doing, we found that polypyrimidine tract binding proteins (PTBs) strongly repressed MIE gene production in cotransfection assays. In addition, we discovered that the repressive effects of PTB could be rescued by splicing factor U2AF. Taken together, the results suggest that PTBs inhibit MIE gene splicing by competing with U2AF65 for binding to the polypyrimidine tract in pre-mRNA. Intron deletion mutation assays and RNA detection experiments (reverse transcription [RT]-PCR and real-time RT-PCR), we further observed that PTBs target all the introns of the MIE gene, especially intron 2, and affect gene splicing, which was reflected in the variation in the ratio of pre-mRNA to mRNA. Using transfection assays, we demonstrated that PTB knockdown cells induce a higher degree of MIE gene splicing/expression. Consistently, HCMV can produce more viral proteins and viral particles in PTB knockdown cells after infection. We conclude that PTB inhibits HCMV replication by interfering with MIE gene splicing through competition with U2AF for binding to the polypyrimidine tract in MIE gene introns.

Human cytomegalovirus (HCMV) is a leading cause of birth defects and transplantation failures, especially in individuals with compromised immunity (28). The viral genome is about 235 kbp long (which is variable among different strains or due to serial propagation in the laboratory in cell culture) and putatively encodes about 200 proteins that are produced sequentially (8, 10, 30, 51). In the presence of protein synthesis inhibitors, HCMV-infected cells express the first viral genes, i.e., the immediate-early (IE) genes. Among them, IE1 and IE2 are the most abundant, leading to their being named the major IE (MIE) genes (46). IE1 and IE2 encode two phosphorylated proteins, IE72 and IE86, respectively; these transcripts result from the differential splicing of the pre-mRNA. MIE genes consist of five exons and four introns (48). The first exon contains the initiation site but does not encode any amino acids. In order for the exons to fuse and produce IE1 and IE2, the introns must be spliced out of the pre-mRNA; the resultant genes share exons 2 and 3. In both transfection with the entire MIE gene construct and infection by HCMV in cell culture, IE1 is always produced at much higher levels than IE2 (an intriguing fact that is the basis for our interest in MIE gene splicing) at an early stage of infection. The virus must use the cellular splicing machinery, and viral-gene splicing must also be regulated by cellular splicing regulation factors.

In eukaryotic cells, mRNA maturation in the nucleus requires three major steps: capping, polyadenylation, and pre-mRNA splicing. The mature mRNA is exported to the cytoplasm for translation. Most transcripts of mammalian cells contain introns and exons, and the introns must be removed before the nuclear export of mRNA for translation. Alternative splicing of pre-mRNA is a common process in mammalian cells by which one gene can produce multiple gene products (23, 38). This is also true for some viral-gene expression, because viruses have a limited genome size and use cellular pre-mRNA-processing machinery. The CMV MIE gene can yield at least five distinct proteins by splicing: IE1, IE2, IE18, IE19, and IE55 (and possibly IE9). IE1 and IE2 share the same promoter, the MIE promoter, and the first three exons and introns. Their gene structures and regulation of gene expression have been intensively investigated (2, 16, 22, 48, 50, 52). However, the splicing regulation of MIE genes remains unclear, and studies of this topic are limited.

Pre-mRNA splicing takes place within a highly congregated site in the nucleus called the spliceosome, a large molecular complex composed of four small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4/U6, and U5) and about 50 to 100 non-snRNP splicing factors (3, 13, 20, 25, 64). Many cellular factors are involved in RNA processing. snRNP complexes are essential to define exons and introns and to enhance both alternative splicing and polyadenylation; therefore, they are splicing enhancers (12, 24, 57). U2 auxiliary factor (U2AF) is essential in defining the splicing site by recognizing a polypyrimidine (Py) tract nonconsensus sequence near the 3’ splice...
site. Spliceosome assembly follows an ordered sequence of events that begins with the recognition of the 5′ splice site by the U1 snRNP and the binding of U2AF to the Py tract and the 3′ splice site. Human U2AF is a heterodimer composed of a 65-kDa subunit (U2AF65), which binds to the Py tract, and a 35-kDa subunit (U2AF35), which interacts with the AG dinucleotide at the 3′ splice site (56, 64). Assembly of U2AF with the pre-mRNA requires an interaction with the U1 snRNP and is important for the subsequent recruitment of the U2 snRNP to the spliceosome (29).

Conversely, most hnRNPs (heterogeneous nuclear ribonucleoproteins) are splicing repressors. Recently, Py tract binding protein (PTB), also called hnRNPI, has been widely studied. It has been reported to be involved in the repression of the pre-mRNA splicing of many important genes, including exons in actin, tropomyosin, troponin, c-src, fibronectin, and FGF receptors 1 and 2 (26, 36, 41, 44). PTB represses gene splicing by competing in the Py tract in introns with U2AF65 and/or interacting with U2AF65 to inhibit the function of U2AF65 (44). The Py tract for binding with splicing factors in yeast consists of the same nucleotide sequence for all genes. However, in mammalian genes, the Py tract sequences are different for each individual intron (18). The idea that the procedures for viral-gene and cellular splicing might be the same is based on the fact that viruses use the cellular machinery for pre-mRNA splicing. The Py tracts in the individual introns of the viral genes should be different from each other, which provided a specific target for studies leading to the production of some small molecules that could specifically inhibit viral-gene splicing and production.

This study marks the first time that the effects of different PTB isoforms on MIE gene splicing, expression, and production have been investigated. In so doing, we found that PTBs are gene-splicing inhibitors, inhibiting HCMV MIE gene expression and hence viral-protein production, as well as blocking virus production by competing with U2AF, a splicing enhancer.

**MATERIALS AND METHODS**

**Cell culture and viruses.** HEP-2 (ATCC) and 293-T (ATCC) cells and human foreskin fibroblasts (HFF) (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. To add green fluorescent protein (GFP) to HEp-2 cells, and 293-T cells, 

For immunohistochemistry to detect PTB, HEP-2–kidPTB cells were seeded on coverslips and 24 h later were washed twice with PBS (PBS), fixed in 1% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.2% Triton X-100 on ice for 20 min. Primary antibody (anti-PTB; 1:500) was added and incubated for 30 min at room temperature. The cells were then washed twice with PBS. Either anti-rabbit or anti-mouse immunoglobulin G (IgG) secondary antibody labeled with Texas Red or Fluorescein isothiocyanate (green) was added and incubated for an additional 30 min at room temperature. After a final wash with PBS, the cells were stained with Hoechst 33258. The cells were examined with a Leica TCS SP2 confocal laser scanning system.

**Immunoblot analysis.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting, as described below.

**Transfection.** Transfection was performed using Metafectene Pro (Biontex, Martinsried/Planegg, Germany) according to the manufacturer’s instructions. Briefly, the cells were washed with MEM (without serum and penicillin-streptomycin) when they were 75% confluent, and the DNA-lipid complexes formed at room temperature were added to the cells and incubated at 37°C in a CO2 incubator for 2 h. Finally, the supernatant of the cells was replaced with complete MEM (with 10% fetal bovine serum and 1% penicillin/streptomycin) and incubated at 37°C in a CO2 incubator overnight.

**Immunocomplementation (co-IP) assay.** Nuclear extracts were obtained essentially as described previously (34). Antibodies were coupled to protein G-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) overnight, according to the manufacturer’s instructions. After being blocked with PBS-0.1% bovine serum albumin, the beads were incubated overnight at 4°C with clarified nuclear extracts, washed repeatedly in 0.1% bovine serum in buffered saline, and resuspended in a mixture of phosphate-buffered saline (PBS) and 2 x Laemmi buffer. After being heated at 95°C for 5 min, the beads were isolated by centrifugation; proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting, as described below.

**Immunohistochemistry.** For immunohistochemistry to detect PTB, HEP-2–kidPTB cells were seeded on coverslips and 24 h later were washed twice with PBS (PBS), fixed in 1% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.2% Triton X-100 on ice for 20 min. Primary antibody (anti-PTB; 1:500) was added and incubated for 30 min at room temperature. The cells were then washed twice with PBS. Either anti-rabbit or anti-mouse immunoglobulin G (IgG) secondary antibody labeled with Texas Red or Fluorescein isothiocyanate (green) was added and incubated for an additional 30 min at room temperature. After a final wash with PBS, the cells were stained with Hoechst 33258. The cells were examined with a Leica TCS SP2 confocal laser scanning system.

**Immunoblot analysis.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20 μg loaded in each lane) transferred to nitrocellulose membranes (Amersham Pharmacia Biotech AB, Uppsala, Sweden), according to the manufacturer’s instructions. After being blocked with PBS-0.1% bovine serum albumin, the beads were incubated overnight at 4°C with clarified nuclear extracts, washed repeatedly in 0.1% bovine serum in buffered saline, and resuspended in a mixture of phosphate-buffered saline (PBS) and 2 x Laemmi buffer. After being heated at 95°C for 5 min, the beads were isolated by centrifugation; proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting, as described below.
according to standard methods. The membranes were stripped with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8), washed with PBS-0.1% Tween 20, and used to detect additional proteins. The following antibodies (and dilutions) were used: anti-IE1/2 (MAB810; Chemicon, Temecula, CA, 1:2,000), polyclonal antibody anti-HCMV IE1 (from H. Zhu at the university of Medicine and Density of New Jersey 1:2,000), monoclonal antibody anti-tubulin (Sigma-Aldrich, St. Louis, MO: 1:1,000), monoclonal antibodies anti-HCMV pp65 and anti-MCP (from W. Britt at University of Alabama; 1:1,000), monoclonal antibody anti-GFP and anti-U2AF65 (MC3) (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000); anti-PTB monoclonal antibody (Abcam, Cambridge, MA; 1:1,000), and anti-nPTB polyclonal antibody (from D. Black at the University of California—Los Angeles; 1:1,000). To quantitatively analyze the relative increase or decrease of HCMV proteins or PTB, we compared the intensities of the specific bands using densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Richmond, CA).

RNA isolation, treatment with DNase I (RNase free), reverse transcription (RT)-PCR, and real-time RT-PCR. Total RNA was isolated using Tri reagent (Ambion, Inc., Austin, TX) and treated with DNase I (RNase free; Invitrogen catalog no. 18047-019) according to the manufacturer’s instructions; the DNase I was inactivated by extraction of the RNA sample with phenol and chloroform. RT was carried out using a kit (Invitrogen, Carlsbad, CA) and a specific primer (pRev (5'-CAT CCT CCC ATC ATA TTA-3')) according to the manufacturer’s protocol. PCR was performed using three different forward primers and a reverse primer (pRev). The forward primers were 5'-ATG TCG CAG AAC-3' (pX3S, from the 3' terminus of exon 3), 5'-GAC GTT CCT GCA GAC-3' (pXL3, from the 5' terminus of exon 3), and 5'-GAC CCT GAT AAT CCT CT-3' (pX2, from the middle of exon 2).

To detect mRNA levels in total RNA samples from the pgfpIE1-transfected Hep-2-κdPTB and Hep-2–pLKO cells, real-time RT-PCR was undertaken using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). A total of 1 μg of total RNA and 0.2 μM of sense and antisense primers—pX3X4 (CCT CCA AGG TGC CAC GGC CCA G), which can amplify only cDNA as it transcribes the two exons, exon 3 and exon 4, and pRev—were used in a final 25-μl master mixture volume. An RT step of 20 min at 50°C was included prior to PCR. The PCRs consisted of 50 cycles with optimal conditions as follows: 94°C for 20 s, 50°C for 30 s, and an optimized data collection step of 80°C for 5 s. Fluorescence captured at 80°C was determined to be lacking signal generated by primer dimers. All samples were run in triplicate; data were collected and recorded by iCycler IQ software (Bio-Rad) and expressed as a function of the threshold cycle (Ct), which represented the number of cycles at which the fluorescence intensity of the SYBR green dye was significantly greater than the background fluorescence. The Ct is directly correlated with the log10 copy number of the RNA standards. RNA copies were extrapolated from standard curves (Ct versus the log10 copy number) representing at least seven point serial dilutions of standard RNA (102 to 104 copies/μl). RNA standards were used as calibrators for the relative quantitation of product generated in the exponential phase of the amplification curve for real-time RT-PCR. The results for standard curves with correlation coefficients greater than 0.95 were accepted. A melting-temperature curve analysis was obtained by measuring the fluorescence during a period of warming from 60°C to 95°C, after the amplification cycles.

Plaque assay. To detect the viral growth curve, Hep-2–κdPTB or Hep-2–pLKO cells were infected with BADD/UL131 virus (with a repaired UL131 open reading frame) at a multiplicity of infection of 1 PFU/cell. For infection in Hep-2 cells, a multiplicity of infection of 1 PFU/cell was needed because Hep-2 cells cultures could not be maintained for the extended period of time needed for an analysis of a low-multiplicity infection. Media and cells from infected cultures were collected on different days p.i., and virus was obtained by three freeze-thaw cycles of the collected culture. Virus titers were determined on HFF after the PFU were analyzed. Student’s t test was used to statistically analyze the difference between the two groups; a P value of <0.05 indicated a significant difference.

Infection and transfection efficiency assays. For detection of infection efficiency, cells were infected for 12 h with virus, washed with PBS, and stained with anti-GFP antibody and secondary antibody-fluorescein isothiocyanate (FITC). For detection of transfection efficiency, cells were transfected with plasmid (pgfpIE1) and then trypsinized and suspended in PBS. The cells were then analyzed with a FACSCalibur system with two lasers and four channels (Becton Dickinson) to detect the total cell number and cells with fluorescence. Uninfected or nontransfected cells were prepared during the same experiments as background controls.

RESULTS

The splicing of pre-mRNA is regulated by different nuclear proteins, including PTBs (which are splicing inhibitors) and U2AFs (splicing enhancers). PTB proteins consist of four isoforms, PTB1, -2, and -4 and nPTB, which result from different splicing. nPTB is more abundantly produced in neural cells (7, 34, 44, 45). PTB binds to single-stranded Py RNA, with a high preference for UCUU, CUCU, and UUCU in introns. U2AF consists of two subunits, U2AF65 and U2AF35. U2AF65 binds to Py, and U2AF35 binds to the 3' splice site of the intron; the binding of U2AF35 to the 3' end of the intron strengthens the binding of U2AF65 to Py (40). Both PTB and U2AF are abundant nuclear proteins. Their existence in the nucleus is critical in order to maintain the balance of cellular-gene expression. Since viruses use the cellular machinery for gene expression, it is reasonable to speculate that PTBs could repress the expression of viral genes that are to be spliced; however, this has never been tested on HCMV MIE genes.

Overexpression of PTB (PTB1, PTB2, or PTB4) inhibits the production of IE1/2. In this study, we set out to test whether HCMV MIE genes could be regulated by gene-splicing regulators, i.e., PTBs. The HCMV MIE gene comprises four introns that need to be spliced before the MIE pre-mRNA is processed into mRNA and the mRNA is exported to the cytoplasm for translation. If the production of IE1/2 is affected negatively by the overexpression of PTB, MIE gene regulation at the splicing level would be suggested. We cotransfected an IE1/2-GFP (IE1/2 tagged with GFP in front of exon 2) plasmid (pgfpSVH), together with a PTB-expressing plasmid (PTB1-Xpress, PTB2, or PTB4-Xpress), into Hep-2 cells. A Western blot assay was used to detect the production of IE1/2 and PTB, using anti-GFP antibody to label IE1/2 proteins and anti-PTB antibody to label PTB proteins (Fig. 1, left). It was clear that IE1/2 production was strongly repressed when PTB was overexpressed. We could see two bands in both PTB1 and PTB4 because PTB1 and PTB4 were tagged with Xpress. To further demonstrate our observations, we repeated the cotransfection of pgfpSVH with different doses of PTB-expressing plasmid. We found that this inhibition was PTB plasmid DNA dose dependent (Fig. 1, right), suggesting that HCMV IE1/2 gene expression is regulated by splicing inhibitors. In the Western blot assay results, the nonspecific bands (which could also be seen in the “Mock” lanes) were kept for the control of sample loading. To quantitatively analyze the relative repression of IE1/2 by PTB or relative overexpression of PTB, we compared the intensities of the specific bands using densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Richmond, CA). First, we compared the intensities of the IE1/2 or PTB bands (in pgfpSVH with or without pPTB) with their own nonspecific bands for normalization. Then, the normalized IE1/2 (from pgfpSVH without PTB) was compared to normalized IE1/2 (from the cotransfected group); the ratio was the amount of repression of IE1/2 by PTB [ratio of IE1/2 from pgfpSVH alone to that from pgfpSVH and pPTBs = (intensity of IE1/2 in pgfpSVH without PTB/its nonspecific band)/(intensity of IE1/2 in pgfpSVH with PTB/its nonspecific band)]. Similarly, we obtained the amount of PTB overexpression [PTB overexpression = (intensity of PTB in pgfpSVH with pPTB/its
nonspecific band)/(intensity of PTB in pgfpSVH without pPTB/its nonspecific band).

The inhibition of HCMV IE1/2 production by PTB was rescued by U2AF65. At least three different models have been proposed to explain the inhibitory effect of PTB on splicing. The first and most accepted model is that PTB simply competes with the splicing factor U2AF65 for binding to Py. A second possibility is that the repressed exons loop out as a result of the binding of PTB to flanking sites. Structural analysis of PTB bound to RNA suggests that PTB monomers can induce loops, but recent studies have indicated that repression by PTB involves more than just binding to RNA (19, 44, 45, 59, 62). A third model suggests that PTB can interact with U2AF65 so that U2AF65 can be isolated from binding to Py (39, 45). In order to determine which of these models fits with our observation that PTB inhibits HCMV IE1/2 gene expression, we cotransfected HEp-2 cells with U2AF and PTB, together with IE1/2 plasmids. A Western blot assay (Fig. 2A) showed that the cotransfection of the PTB1 plasmid with the U2AF expression plasmid could rescue the repression of IE1/2, which implies that the inhibitory effect on HCMV IE1/2 gene expression by PTB results from either competition or interaction with U2AF65. We noticed that there were two bands for U2AF65 when there was higher expression of IE1/2, which might have resulted from modification by IE1/2; we will further investigate this interesting observation.

It has been reported that PTB interacts with U2AF65, and we wondered whether the interaction is mediated by RNA. An RNA-mediated interaction of PTB with U2AF65 could suggest that the activity of U2AF65 on the inhibitory effect of PTB on MIE gene expression is due to competition with PTB for the binding site in pre-mRNA rather than segregation of PTB by direct interaction. It can be detected by adding RNase to treat the nuclear extract both before and during the co-IP assay. As
shown in Fig. 2B for the co-IP assay, we used both anti-PTB and anti-U2AF65 antibodies to pull down the nuclear extract of HEp-2 cells with (bottom) and without (top) RNase treatment. RNase activity was detected before the assays by running aliquots: one was treated with RNase, and the other was not treated. Antibodies (mouse anti-PTB and anti-U2AF65) and pre-immune mouse IgG were used to react with the nuclear extracts for 2 h, and then Sepharose-protein G (after being washed) was added to the reaction mixture for another 2 h at 4°C, and the samples were analyzed by Western blot (WB) assay with anti-PTB. 

A. 

10kDa anti GFP 

8kDa anti PTB 

56kDa anti U2AF65 

8kDa anti PTB 

56kDa anti U2AF65 

B. WB: anti-PTB 

anti PTB 

anti-PTB 

anti-PTB 

anti-PTB 

anti PTB 

anti-PTB 

anti PTB 

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anti PTB 

anti-PTB 

anti PTB 

anti-PTB 

Fig. 2. The repressive effect of PTB can be rescued by U2AF. (A) The IE1/2-expressing plasmid pgfpSVH was transfected into HEp-2 cells with the DNA amount control plasmid pcDNA3 (lane 2 from left) or together with pPTB1 (lanes 3, 4, and 5) or pPTB1 and pU2AF65 (lane 6) for 24 h. Whole-cell lysates were collected, and Western blotting was performed to detect IE1/2 (using anti-GFP antibody), PTB (using anti-PTB antibody), U2AF65 (with anti-U2AF65 antibody), and tubulin (as a sample-loading control). (B) Co-IP. The nuclear extracts from HEp-2 cells were prepared and divided into two aliquots: one was treated with RNase, and the other was not treated. Antibodies (mouse anti-PTB and anti-U2AF65) and pre-immune mouse IgG were used to react with the nuclear extracts for 2 h, and then Sepharose-protein G (after being washed) was added to the reaction mixture for another 2 h at 4°C, and the samples were analyzed by Western blot (WB) assay with anti-PTB.

Intron 2 of the MIE gene is involved in the repressive effect of PTB on IE1 gene production. If the repressive effects of PTB on MIE gene products are a result of competing with U2AF65, as we found (described above), it is highly likely that the cis elements for those PTBs that are on pre-mRNA are located in introns. We searched the sequences of the MIE introns and noticed that there were Py-like sequences (UCUC, UCCU, or CUCC) in all of them. Therefore, we wondered whether PTBs act on all the introns or on only some of them. The sequences between exons 4 and 5 contain both the 3′-terminal untranslated region of IE1 and the intron of IE2; it is difficult to interpret the activity of PTB on the sequence in this area. To simplify the experiments, we removed exon 5 and kept only the IE1 gene intact, resulting in pgfpIE1 (Fig. 3A), which has only three introns. Intron 1 is the most complicated because it overlaps with other open reading frames, such as UL124, UL125, and UL126. We mutated pgfpIE1 by removing intron 2 only (fusing exons 2 and 3; pgfpIE1_dN2), intron 3 only (fusing exon 3 with exon 4; pgfpIE1_dN3), both introns 2 and 3 (fusing exons 2, 3, and 4; pgfpIE1_dN2N3), and all three introns (fusing exons 1, 2, 3, and 4; pgfpIE1_dN1N2N3) (Fig. 3A). 

After cotransfecting the IE1-expressing plasmids with the introns deleted with PTBs, we detected IE1 production using a Western blot assay. We observed that intron 3 was not necessary for the repression by PTB of IE1 production (Fig. 3B), since reductions in IE1 production by PTBs were the same in pgfpIE1_dN3 as in pgfpIE1. However, upon the removal of either intron 2 (alone) or both introns 2 and 3, we observed that the repressive effects of PTBs had been reduced; however, it should be noted that PTBs still reduced IE1 production. This information implies that intron 2 rather than intron 3 is important for the inhibitory effects of PTBs. Intron 1 could also be involved in the effects of PTBs on MIE gene splicing because PTBs lost their repressive effects on MIE gene expression when introns 1, 2, and 3 were all removed. Investigations to identify the minimum length of the sequences in introns that interact with PTB will also be undertaken. As before, the nonspecific band was employed for controlling sample loading.
while, we also observed that PTB could still be detected by Western blotting, even though it was not detectable by immunofluorescence.

To eliminate the possibility that the constructed (HEp-2–kdPTB) cells would have a higher infection or transfection efficiency than the control (HEp-2–pLKO) cells, we performed infection and transfection efficiency assays using FACSCalibur. We transfected pgfpIE1 into both types of cell overnight, and the cells were trypsinized and directly applied to detect total cell numbers and GFP-positive cell numbers, and the non-transfected cells were used as controls. For detection of infection efficiency, we infected cells with HCMV for 12 h, after which the cells were fixed and stained with anti-IE1/2 antibody and FITC-labeled secondary antibody. The cells were sus-pended in PBS and used to detect the total number of cells and the total number of FITC-positive cells; uninfected cells were used as controls. As can be seen in Fig. 4D, there was no significant difference between the infection and transfection efficiencies in the two cell lines. Therefore, our constructed cells were appropriate for use in the studies.

So far, we have demonstrated that PTBs have repressive effects on MIE gene products that are all at the protein production level. It is also necessary to demonstrate that PTBs have effects on MIE gene splicing. MIE gene splicing should be enhanced when the PTB gene is knocked down, which would result not only in higher gene expression and production, but also in a higher ratio of mRNA to pre-mRNA than in non-knockdown cells. RT-PCR and real-time RT-PCR were undertaken to test this speculation. First, we performed RT-PCR. The total RNA isolated from two different groups of cells (pgfpIE1-transfected HEp-2–kdPTB and pgfpIE1-transfected HEp-2–pLKO) was used to do the RT-PCR, using a specific primer (pREV) in exon 4. Then, the RT products were used for PCR, using the same reverse primer as was used for the RT-PCR; the forward primer was as indicated in Fig. 5B, top. For the RT-PCR assay, we designed three different pairs of primers, using the same reverse primer (which allowed the transverse of the introns). By doing so, we were able to amplify cDNA from both pre-mRNA and mRNA. Since the primers rest in different sites, the amplified cDNAs reverse transcribed from pre-mRNA and mRNA had different lengths. The designing, sites, and directions of the primers are indicated in

![FIG. 3. Effects of PTBs on different IE1 gene-expressing constructs. (A) Diagram describing the mutants made in this study. pgfpSVH (top) shows all the introns and exons of the MIE gene, as indicated. UTR, untranslated region. (B) Western blot to detect the production of IE1 and PTBs, as indicated. The samples are the whole-cell lysates made 24 h after transfection in HEp-2 cells. Above the IE1 band there is a nonspecific band, which can also be seen in the mock sample. The nonspecific band could be used as a sample-loading control.](http://jvi.asm.org/)
The diagram (Fig. 5A), pgfpIE1 DNA was used as a positive control for the PCR, which produced bands of the same size as those of cDNAs from pre-mRNA. As shown in Fig. 5B, bottom, in which pIE1cDNA was transfected into the two cell lines, the RT-PCR results showed that there were no differences in the production of cDNA. Taking the data together, we showed that the amount of IE1 mRNA was greater in HEp-2–kdPTB cells than it was in HEp-2–pLKO.
FIG. 5. Detection of IE1 gene expression at the RNA level. (A) Diagram of the IE1 gene containing exon 2 (X2), exon 3 (X3), and exon 4 (X4); introns are between the exons. The positions of primers pX2, pX3L, pX3S, pX3X4, and pRev are shown. (B) RT-PCR. Total RNAs from HEP-2-kdPTB (pgfpIE1-transfected) and HEP-2-pLKO (pgfpIE1-transfected) cells (top) or from HEP-2-kdPTB (pgfpIE1-cDNA-transfected) and HEP-2-pLKO (pgfpIE1-cDNA-transfected) cells (bottom), as described previously, were transcribed using a specific primer, pRev, with an RT kit (Invitrogen). The cDNAs were then amplified using different pairs of primers, as indicated. pgfpIE1 DNA was used as a control (as it produces a product the same size as the cDNAs from pre-mRNA), and the total RNA (treated with RNase-free DNase I) was used as a negative control for the PCR. The bands below single asterisks represent cDNA amplified from pre-mRNA; the bands above double asterisks represent the cDNA amplified from spliced RNA. (C) Real-time RT-PCR. Total RNA (1 μg) isolated from HEP-2-kdPTB (pgfpIE1-transfected) and HEP-2-pLKO (pgfpIE1-transfected) cells, as described previously, were analyzed by one-step real-time RT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). Water was used as a negative control for subtraction of the background. The three curves in each sample represent triplicate experiments.
cells, which suggests that PTB is involved in the splicing of the IE1 gene.

To further detect the effects of PTB on MIE gene expression at the mRNA level, we employed real-time RT-PCR, the method of choice in the quantitative detection of mRNA. The same amount of HEp-2-kdPTB or HEp-2-pLKO cells was seeded in a six-well plate and transfected with the same amount of pgfpIE1 when the cells were at 75% confluence. Total RNA was isolated at 20 h posttransfection. One microgram of the total RNA was used for real-time RT-PCR. The forward primer, pX3X4 (Fig. 5A) (5'-H11032-GTC CTG GCA GAA CTC GTC AA-3', with the first 15 nucleotides from the end of exon 3 and the last 5 nucleotides from the beginning of exon 4), can amplify only cDNA from mRNA. As shown in Fig. 5C, the fluorescence absorption symbolizing the PCR products in HEp-2-kdPTB cells was approximately two to three times stronger than it was in HEp-2-pLKO cells, which is consistent with our observations of protein production by Western blot assay.

Knockdown of PTB enhances HCMV gene production and viral replication but has no effect on permissiveness. Transfection systems can detect protein function only on specific cis elements; it still remained unclear whether the PTBs could affect HCMV infection in vitro. It was reasonable to speculate that PTB has a repressive effect on HCMV infection, because PTB interferes with HCMV IE1/2 gene splicing and represses IE1/2 production; to test this, we needed to infect the HEp-2-kdPTB and HEp-2-pLKO cell lines with HCMV. The laboratory strains of HCMV, including Towne, AD169, and Toledo, are not able to infect endothelial or epithelial cells (Fig. 6C); therefore, we used the repaired HCMV, vDW215-BA'DrUL131 virus (60, 61), in this study. By comparing viral-
protein production levels using the Western blot assay, we showed that the knockdown of PTB could increase the production of different HCMV proteins (Fig. 6A). To detect viral replication, we infected the HEp-2–kdPTB and HEp-2–pLKO cell lines with HCMV, vDW215-BADUL131 virus, at a multiplicity of infection of 1 for up to 8 days. Viruses were collected every day after infection. The collected virus samples were frozen and thawed three times, and the supernatants were analyzed by PFU assay in HFF. We observed a three- to sevenfold increase of viral particles by PFU assay (Fig. 6B) when PTB was knocked down. We also noticed in the viral growth curve that the viral titer went down after 6 days p.i., which might be because PTB knockdown cells died earlier after viral infection. However, the knockdown of PTB did not influence the permissiveness of epithelial cells regarding HCMV; the laboratory strains failed to infect HEp-2–kdPTB cells (Fig. 6C).

Rescued expression of PTB resulted in repressive effects in PTB knockdown cells. To demonstrate that the effects of PTB on MIE gene expression (shown in PTB knockdown cell lines) were specific to PTB, we constructed the PTB1 expression plasmid pcDNA3PTB-reverse, which could produce PTB in HEp-2–kdPTB cells, by mutating the nucleotides corresponding to the shRNA sequence (though the amino acid sequences remained the same) (Fig. 7A). We transfected the HEp-2–kdPTB cells with pIE1 (no GFP fusion), together with pcDNA3 (as a control), pcDNA3PTB, or pcDNA3PTB-reverse, and performed Western blotting to detect IE1 production in order to determine whether expression of PTB in the HEp-2–kdPTB cells could rescue the repressive effects of PTB on IE gene expression. As can be seen in Fig. 7B, pcDNA3PTB-reverse expressed PTB in the knockdown cells and pcDNA3PTB did not. In addition, it is also shown (lane 4) that rescued expression of PTB in HEp-2–kdPTB repressed IE gene expression.

DISCUSSION

The studies discussed in this article are the first to show that PTB represses HCMV MIE gene splicing, IE1/2 gene expression and production, and, subsequently, viral-protein production and replication. Since the MIE gene products IE1 and IE2 are both critical for HCMV replication, study of their regulation might provide a new clue leading to the design of a strategy to specifically target HCMV infection. This is the first step in our ongoing project to determine HCMV gene-splicing regulation. Previous studies by Adair et al. (1) found that HCMV infection can induce a response from cellular splicing factors, including PTB, and implied that PTB might be involved in the interaction of HCMV and cells. Other studies of PTB showed that it has positive effects on viral infection by activating the internal ribosome entry site (4, 42). Therefore, PTBs are a group of multifunctional proteins and are important in maintaining the equilibrium of gene regulation.

The fact that PTB has a clear repressive effect on HCMV replication (as shown in this study) and that the PTB level changes after HCMV infection (demonstrated by Adair et al. [1]) implies that PTB might be one of the factors activated during the cellular defense response to viral infection. In recent years, intrinsic defenses against viral infection have been extensively studied, and several different cellular proteins have been identified as defensive molecules. Proteins related to PML bodies congregate in the nuclei, and many viruses evolve measures to destroy these congregates in order to induce a productive infection; the proteins studied include PML, Daxx, SP100, and HP1 (6, 11, 14, 35, 37, 53–55). Histone deacetylases are a second group of gene expression suppressors that have inhibitory effects on CMV infection, which is evidenced by the fact that histone deacetylase inhibitors can enhance virus production (27, 31, 33, 54, 58). All the defensive cellular proteins investigated previously inhibit virus production, either by repressing gene expression or by inducing cell apoptosis. In this study, we report that PTBs negatively affect MIE gene expression, and hence HCMV replication, by interfering with splicing. Therefore, PTBs may be another type of defensive cellular protein, one that acts at the gene-splicing level.

Regulation of alternative splicing is mediated by RNA-binding proteins, including SR (serine/arginine-rich) and hnRNP family proteins (5). The hnRNPs have mostly been considered splicing inhibitors. PTB (also called hnRNPI) is a widely expressed protein (59) that has been found to be involved in
much gene-splicing regulation (44). In nonneural cells, PTB exists in three different isoforms, PTB1, PTB2, and PTB4, all of which result from alternative splicing. They differ by the insertion of 19 to 26 amino acids (63). In addition to having repressive effects on splicing, PTB is also involved in 3′-end processing, internal initiation of translation, and RNA localization, in which PTBs exert positive effects (9, 15, 17, 21). We were curious whether PTB could also be involved in MIE gene-splicing regulation, since the MIE gene is essential for HCMV replication. A general cotransfection study (previously described) showed that PTB can strongly repress MIE gene expression. This study also demonstrated that all three PTB isoforms appear to have the same repressive effect (Fig. 1). Interestingly, this finding differs from what has been observed in other gene-splicing experiments, such as those done with α-tropomyosin (63), in which PTB4 had a stronger effect than either PTB1 or PTB2. Consistently, when PTB was knocked down, MIE gene expression was greatly promoted (Fig. 4, 5, and 6).

PTB is, in general, a splicing inhibitor and could affect the expression of many other cellular genes, implying that the effects of PTB on MIE gene expression might also be indirect. However, RT-PCR showed that PTB affected MIE gene splicing in that the ratio of mRNA to pre-mRNA was greater when PTB was depleted (Fig. 5). This is the first evidence supporting the idea that PTB might have a direct effect on MIE gene splicing.

In further studies, we deleted either intron 2 or 3, both introns 2 and 3, or all three introns in pgpIE1. The mutation studies revealed that intron 2 was involved in interaction with PTB because the repressive effect was much less when both introns 2 and 3 or all three introns were removed, but not when only intron 3 was removed (Fig. 3). Taken together, our results support the conclusion that the effect of PTB on HMCV MIE gene splicing is a direct interaction between PTB and the cis elements in the pre-mRNA of the MIE gene. We are currently using an in vitro RNA-protein interaction assay and an in vitro splicing assay to further determine the interaction of PTB and U2AF65 with MIE gene introns.

Currently proposed models for gene-splicing suppression by PTB are threefold, as described in Results. Our results suggest that the activity of PTB on HCMV is through competition with PTB. In addition, HCMV is an evolved pathogen and has adopted different measures against cellular defensive mechanisms; we will explore what kind of strategy is used by HCMV to evade the repressive effect of PTB.

ACKNOWLEDGMENTS

This study was supported by the Research Center for Minority Institutes (RCMI) program (grant G12RR003050) of the Ponce School of Medicine and a startup fund from the Ponce School of Medicine to Q.T. We thank W. Britt, D. Black, M. Carmo-Fonseca, R. Stenberg, G. Maul, T. Shenk, and H. Zhu for reagents. We acknowledge Bob Ritchie for English editing. We also thank Ann Campbell and Julie Berry for their critical reviews of the article before publication and Richard J. Noel, Jr., Pablo Lopez Colón, and Omayra De Jesus Matos for technical assistance.

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