JOURNAL OF VIROLOGY, Feb. 1991, p. 887–896
0022-538X/91/020887-10$02.00/0

Vol. 65, No. 2

Human Cytomegalovirus *ie2* Negatively Regulates α Gene Expression via a Short Target Sequence Near the Transcription Start Site

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Received 6 September 1990/Accepted 19 November 1990

Repression of human cytomegalovirus α (immediate-early) gene expression is under the control of the viral *ie2* gene. Here we show that ie2 negatively regulates gene expression directed by the strong cytomegalovirus enhancer via a specific 15-bp target sequence (which we term *cis* repression signal [*crs*]). This *crs* is located between −14 and +1 relative to the transcription start site and will function in an orientation-independent fashion, consistent with repression occurring at the transcriptional level. Repression is dominant over transactivation by *ie1* gene products. The *crs* (5′-CGTTTAGTGAACCGT-3′) does not contain previously recognized binding sites for cellular transcription factors, and a precise copy is not found elsewhere in the human cytomegalovirus genome. The position of the signal near the transcription start site appears to be important in function; addition of the *crs* near the transcription start site of a heterologous promoter, from the thymidine kinase gene of herpes simplex virus type 1, conferred cytomegalovirus *ie2*-dependent repression upon this promoter. Thus, we propose that an *ie2* gene product or an induced cellular protein mediates repression by binding to *crs*. Negative regulation of α gene expression may be important during viral replication or latency.

Human cytomegalovirus (human herpesvirus 5; CMV), a herpesvirus causing disease primarily in immunocompromised patients, carries over 200 genes whose expression is coordinately regulated and sequentially ordered during viral growth (35, 53). Three regions of α (immediate-early) gene expression have been mapped on the CMV genome (21, 26, 47, 50, 51, 62, 63), with one much more abundantly expressed than the others in infected human fibroblast (HF) cells (10, 21, 59, 61, 63). This prominent α region maps between 0.738 and 0.762 on the CMV genome and includes genes that encode a family of nuclear phosphoproteins (16, 17, 27, 33). Two genes within this region, *ie1* and *ie2*, that carry out regulatory functions (8, 11, 12, 19, 20, 29, 39) are expressed via differential splicing from a single transcription start site (7, 47, 51; Fig. 1). Expression of *ie1* and *ie2* is under control of a strong enhancer (1, 4, 59) and is subject to both positive and negative regulation by factors acting on the promoter-enhancer region (8, 19, 20, 29, 37, 39, 42, 44, 45, 48, 49, 54).

Transient expression experiments have demonstrated that the product of the most abundantly expressed α gene, *ie1*, a 491-amino-acid (aa) protein referred to as the major immediate-early protein (17, 53), participates in enhancer activation via the 18-bp repeat element (8). This positive autoregulation by ie1 is mediated through the cellular transcription factor, NFκB, which binds to a site within the 18-bp repeat element (36, 42). Although *ie1* transcript levels peak between 5 and 8 h postinfection, protein products continue to accumulate throughout infection (47; our unpublished data). One predominant *ie2* gene product 579 aa in size (also called 86K on the basis of electrophoretic mobility) and one less abundant 425 aa in size are expressed immediately after infection from transcripts that are 5′ coterminal and share three exons with *ie1* (47, 51; Fig. 1). At late times, one abundant *ie2* gene product (302 aa in size) is expressed from a promoter that maps just downstream of the *ie1* polyadenylation signal (47, 51). Spliced *ie2* transcripts structurally similar to those expressed immediately after infection continue to persist throughout infection (47).

The transactivating and repressing properties of *ie2* products have been reported. In combination with *ie1*, the *ie2* region is capable of complementing an adenovirus mutant defective in E1a (56, 57). In transient assays, *ie2* gene products can transactivate a variety of heterologous target promoters: herpes simplex virus type 1 (HSV-1) glycoprotein D (12); adenovirus E2 (20, 58); human immunodeficiency virus type 1 long terminal repeat (9); and HSV-1 ICP4 (IE175), simian virus 40 early, adenovirus E3, and β interferon (39). The presence of *ie1* gene products generally increases transactivation of these promoters in the presence of the *ie2* gene, presumably through increasing the levels of expression of *ie2* gene products. Significantly, at least four CMV β (delayed-early) promoters can be transactivated by the *ie2* products in conjunction with *ie1*: the promoter of the β gene encoding the 2.2-kb transcript (46), the promoter of the DNA polymerase gene (11), the promoter of the β gene encoding the predominant 2.7-kb transcript (25), and the multiple promoters of the β gene encoding the 1.7-kb transcript (6). The work of Pizzorno et al. (39) has suggested that important ie2 transactivating functions are contained within the amino-terminal sequences shared between ie1 and either the 579- or 425-aa species of ie2. Further work on the 579-aa protein has implicated both amino- and carboxyl-terminal sequences in transactivation (48).

In addition to the transactivating properties of the *ie2* products, Pizzorno et al. (39) were the first to show that these products may negatively autoregulate the α promoter-enhancer. The target construct that was repressed in their transient expression assays contained CMV α promoter-

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enhancer sequences from −760 to +10 (relative to the transcription start site) fused to an indicator gene. Using effector plasmids with deletions in *ie*1 and *ie*2 in transient cotransfection assays, they demonstrated that the repression function resided in a region distinct from the transactivation domain, i.e., that the repression function, unlike the transactivating function, lies outside of the sequences shared by *ie*1 and *ie*2 gene products, within the *ie*2 gene (exon 5) of the α region. This work has been recently confirmed and extended by others (19, 48) who have shown that a carboxyl-terminal domain of this ie2-specific exon is involved in negative regulation.

In this report, we show that the *cis* target site through which ie2 negatively regulates the α promoter-enhancer is a 15-bp sequence located from −14 to +1 (relative to the transcriptional start site). Repression is dominant over transactivation by *ie*1 gene products. This sequence will function in an orientation-independent fashion, consistent with repression occurring at the transcriptional level. The results predict a direct mechanism for ie2-mediated repression that may involve blocking formation of an active transcription complex.

MATERIALS AND METHODS

Cell culture, viruses, and transient assays. HF cells, Vero cells, and CMV (Towne) were grown as described previously (44). Plasmids were introduced into HF or Vero cells by DEAE-dextran-mediated transfection (40, 43, 44). Following transfection, β-galactosidase (β-gal) synthesis directed from these plasmids was measured by adding 4-methylumbelliferyl-β-D-galactoside (Sigma) to the medium between 48 and 72 h posttransfection, at which time the cleavage product was measured on a Dynatech Microfluor fluorimeter that excites at 365 nm and reads emission at 450 nm (14). All transient assays were performed at least four times, with all critical assays performed at least 10 times. The effector-to-target ratio in our experiments was 2:1, a ratio which does not result in enhancer competition effects (8; data not shown). In most experiment, 10^6 HF cells were transfected with 10 μg of target and 20 μg of effector plasmid.

Plasmid constructs. Plasmids pXbaE, pON308, and pON303 have been described elsewhere (44, 60). pON303ΔAcc, which is similar to plasmids constructed by others (20, 39), was constructed by digesting pON303 with *Acc*I (Fig. 1) and subsequently religating. Indicator plasmids pON249, pON284, pON283, pON2044, and pON2046 have been described previously (8, 14, 44). pON249 contains the α promoter-enhancer sequences from −1138 to −14 fused to *lacZ*. pON284 contains the α promoter-enhancer sequences from −672 to −14 fused to *lacZ*. The 5′ endpoints of the plasmids are as follows: pON284, −672; pON283, −219; pON2044, −103; and pON2046, −75. All of these constructs extend 3′ to −14 (all numbers are relative to the transcription start site). pON239 contains α promoter-enhancer sequences from −1138 to +913 (including the first exon and first intron of the α locus) fused to *lacZ* (44). pON249crs was constructed by digesting pON249 at the unique *Hin*dIII site (polylinker site at −5 relative to the transcription start site) and inserting the double-stranded synthetic −14 to +9 oligonucleotides 5′-AGCTT**CGTTTAGTGAACCGTCAGA TCGCA**-3′ and 5′-AGCTT**GCGATCTGACGGTTCACTAA ACGA**-3′ (CMV DNA sequences shown in bold). Clones of the pON249crs substitution mutation series were all constructed in the same way as pON249crs except that a synthetic double-stranded *cis* repression signal (*crs*) oligonucleotide carrying a specific substitution mutation was inserted in place of the wild-type −14 to +9 sequence (see Fig. 6). pON2200 was constructed by digesting pON249 at the unique *Sna*BI site (at −242) and inserting the wild-type −14 to +9 oligonucleotide after filling in the *Hin*dIII ends with Klenow polymerase (30). pON239*crs*E was constructed by ligating together three fragments to create a plasmid that contained the α promoter-enhancer sequences from −1138 to +1 fused to *lacZ*. The first was a 7-kbp *Hin*dIII-*Sal*I fragment generated from pON239 that contained *lacZ* and pBR322 sequences; the second was an 1,124-bp *Sac*I-*Sal*I fragment from pON239 that contained α promoter-enhancer sequences from −1138 to −14; and the third was double-stranded synthetic oligonucleotide containing the sequences 5′-CGTTTAGTGAACCGTA-3′ and 5′-AGCTTACGGTTC ACTAAACGAGCT-3′ (5′ *Sac*I end and a 3′ *Hin*dIII end) representing α promoter sequences from −14 to +1. The enhancer deletion plasmids were constructed as follows: parent *lacZ* indicator plasmids (8) with various amounts of enhancer sequences, pON283 (containing −219 to −14), pON2044 (containing −103 to −14), and pON2046 (containing −75 to −14), were digested with *Hin*dIII, and the −14 to +9 *crs* oligonucleotide was inserted into the *Hin*dIII site of each plasmid, resulting in pON283crs, pON2044crs, and pON2046crs. pON2201 was constructed by inserting the 240-bp *Pst*I HSV-1 β thymidine kinase (TK) promoter fragment from TK/LS −6/−16 (32) into pON3 (31) which had been digested with *Pst*I. pON2202 was constructed by digesting pON2201 with *Bam*HI, filling in with Klenow enzyme, and ligating to the filled-in double-stranded *crs* oligonucleotide; the sequence was cloned in the natural 5′-to-3′ (−14 to +9) orientation. pRB201 contains the HSV-1 *Hin*dIII HM junction fragment with the HSV-1 α0, α4, and α27 genes.

RNA isolation and blot hybridization. Whole-cell RNA was isolated by the guanidium isothiocyanate method (30). A 10-μg sample of each RNA was electrophoresed on a formaldehyde–1% agarose gel, and the RNA was transferred to nitrocellulose and probed as previously described (8, 13, 14). Equivalent amounts of RNA were loaded on each lane, as indicated by identical rRNA bands following ethidium bromide staining.

Immunofluorescence assays. Transfected HF cells were grown on glass coverslips, and 72 h posttransfection the cells were washed in medium and fixed in cold acetone for 3 to 5 min. They were then incubated with 100 μl of a mixture of mouse monoclonal antibody (MAb810; Chemicon) directed against an epitope encoded by the third exon of the CMV α gene locus (27; Fig. 1) and a polyclonal anti-β-gal antibody (Cappel) at final dilutions of 1:100 and 1:60, respectively, for 30 min at 20°C. After the cultures were washed in medium, the cells were exposed to a mixture of tetramethylrhodamine isothiocyanate (TRITC)-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G (IgG) and fluorecein isothiocyanate (FITC)-conjugated goat IgG anti-rabbit IgG (both from Cappel) at final dilutions of 1:100. After further washing in medium, the coverslips were mounted in *p*-phenylenediamine on a microscope slide and examined under both phase-contrast and epifluorescence microscopy with a Zeiss photomicroscope. Cells were observed under a 500-fold magnification with TRITC as well as FITC filters to determine the number of cells expressing α gene products or β-gal per approximately 10,000 cells examined.

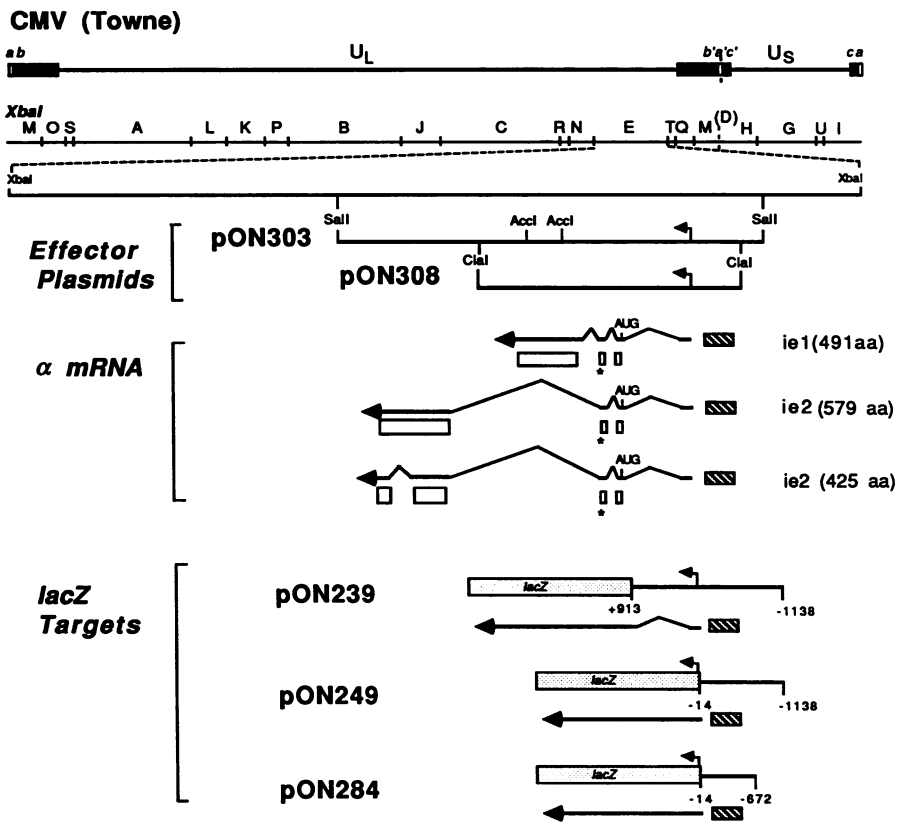

FIG. 1. Organization of the human CMV genome. The 230-kbp human CMV genome is depicted on the top line, with the large boxes indicating inverted repeats (*ab* and *ca*) flanking the unique (U) regions of the L and S components. An *Xba*I restriction map is shown. The *Xba*I E fragment with effector plasmids pON303, carrying the *ie*1 and *ie*2 genes, and pON308 (44), carrying *ie*1, are depicted in the expanded region along with the splicing pattern of the predominant α mRNAs. α protein coding sequences are represented as open boxes, and the epitope for MAb810 is indicated with an asterisk. *lacZ* target constructs pON239 (44), pON249 (14), and pON284 (8) are depicted. The *lacZ* cassette (stippled box) contains a simian virus 40 early polyadenylation site (44). The arrowhead (◄) indicates transcription start sites, and the striped box represents the CMV enhancer on plasmid constructs.

RESULTS

ie2-mediated repression of α gene expression. In previous studies of the effect of α gene products on the α promoter-enhancer, we had observed an activation by *ie*1 products (8, 42). Others (19, 39, 48, 49) reported negative effects of *ie*1 or *ie*2 gene products. The effector plasmids used in these different studies were similar; however, the enhancer-promoter targets varied with respect to indicator gene chosen, chloramphenicol acetyltransferase or *lacZ*, as well as with respect to the point of fusion to the indicator gene. To ascertain the influence of *ie*2 gene products without regard to incidental target gene structure, we examined the autoregulatory influence of this gene directly on α gene expression. We included for comparison in our experiments previously characterized α promoter-enhancer–*lacZ* fusion constructs.

Cotransfection experiments were performed with effector *ie*1 and *ie*2 constructs, and expression of α gene products was monitored by indirect immunofluorescence. Effector plasmids (diagramed in Fig. 1) pON303 (carrying *ie*1 and *ie*2), pON308 (carrying *ie*1), and pON303ΔAcc (carrying *ie*2) each expressed gene products readily detected with murine monoclonal antibody MAb810 (Chemicon) as previously established (27). We observed that the presence of *ie*2 decreased overall expression of α proteins in transient expression assays (Table 1). Figure 2 shows an example of immunofluorescence demonstrating the predominant nuclear localization of ie1 and ie2. All three effector plasmids expressed the epitope reactive with MAb810 (Table 1). Importantly, and consistent with previous reports obtained by using similar plasmids (20, 27, 39), ie2 was expressed from pON303ΔAcc (Table 1). The number of cells expressing α

TABLE 1. Immunofluorescence analysis of gene expression by effector and target constructs

Plasmid(s) transfected	No. of cells[a] expressing:		% cells expressing ie1 and ie2 also expressing β-gal
	α gene products	β-Gal	
Exp 1			
284	0	8	0
284 + 308 (*ie*1)	423	78	19
284 + 303 (*ie*1 + *ie*2)	177	73	41
284 + 303ΔAcc (*ie*2)	30	5	17
Exp 2			
284	0	4	0
284 + 308	133	39	29
284 + 303ΔAcc + 308	39	23	59
284 + 303ΔAcc	13	4	30

[a] Per approximately 10,000 HF cells scanned at 72 h posttransfection.

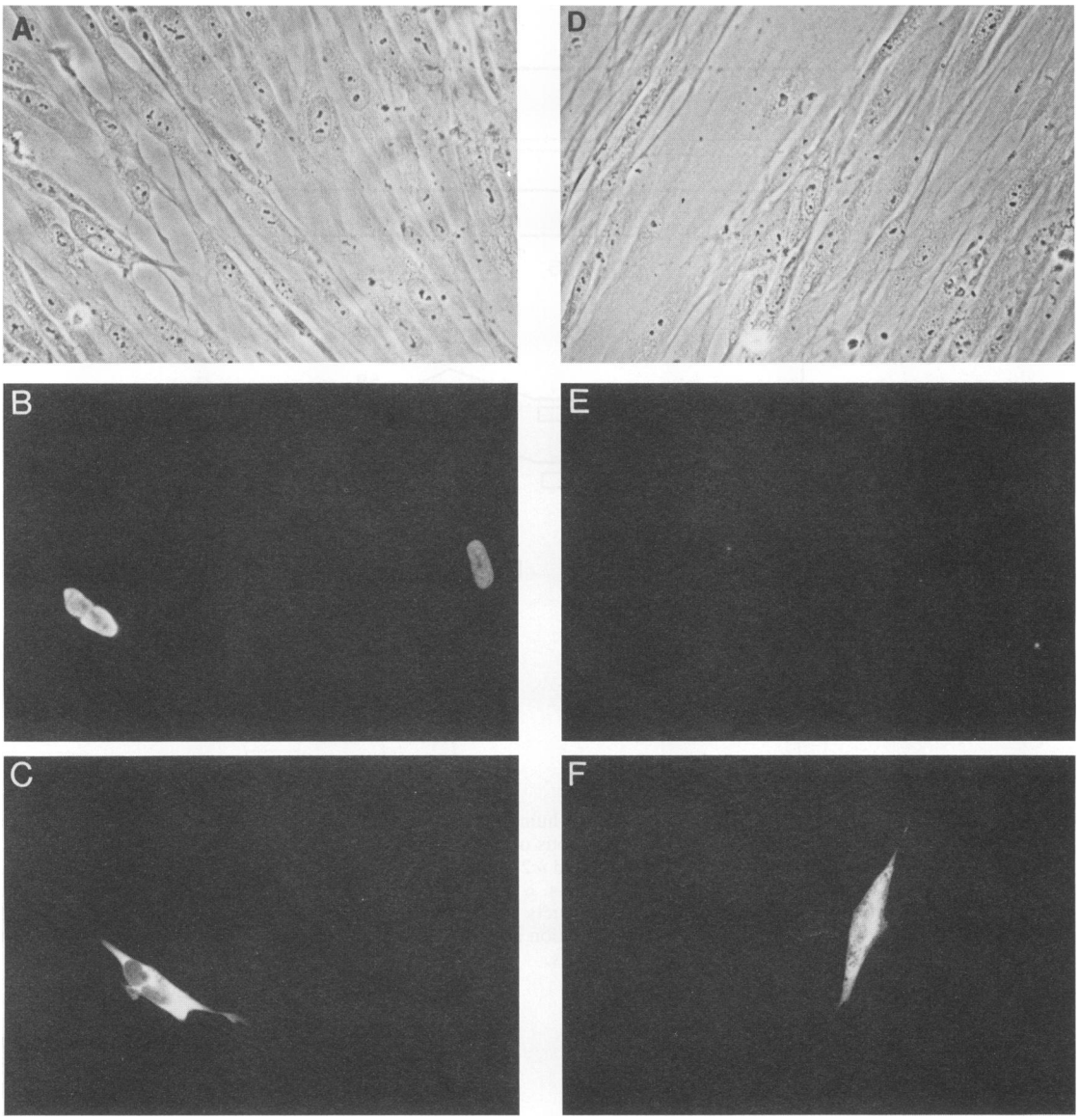

FIG. 2. Double-label immunofluorescence staining on HF cells 72 h posttransfection with either pON284 and pON308 (A to C) or pON284 alone (D to F). (A and D) Phase-contrast photomicrographs showing HF cell monolayers; (B and E) staining with MAb810 and goat $F(ab')_2$ fragments directed against mouse IgG conjugated with TRITC; (C and F) staining with rabbit anti-β-gal antibody and goat IgG anti-rabbit IgG conjugated with FITC. Magnification, ×500.

gene products from pON303 was dramatically reduced compared with pON308, suggesting that the presence of *ie*2 products had a negative effect on expression. When *ie*2 was introduced in *trans* by using pON303ΔAcc, expression from pON308 was also lower than in cells receiving pON308 alone (Table 1). This finding is consistent with reports of others (19, 39, 48) using indicator gene constructs as targets and, in addition, demonstrates that the presence of *ie*2 products clearly had a negative effect on the expression of the intact α gene cluster. This observation supports a role for repression in the biology of CMV.

Because this result appeared to contrast with our previous findings demonstrating that plasmid constructs carrying *ie*1 plus *ie*2 or *ie*1 alone were equally capable of transactivating the α promoter-enhancer (8), we included a *lacZ* target construct in the assay. Inclusion of this indicator construct did not influence the level of expression of α gene products from any of the effector plasmids or the negative effect of *ie*2 on the effector plasmids (unpublished data). Each of these constructs was cotransfected with target pON284 (carrying the *lacZ* gene under control of a 658-bp α promoter-enhancer, from −672 to −14 relative to the transcriptional start site [8; Fig. 1]). Double-label indirect immunofluorescence was performed with MAb810 to detect ie1 and ie2 expression and with a rabbit antibody to detect β-gal expression. Figure 2 illustrates the pattern of expression observed following immunofluorescence staining on an HF culture 72 h after cotransfection with pON284 and pON308. Figure 2B shows two cells expressing *ie*1 and *ie*2 gene products, and one of these cells also showed expression of β-gal (Fig. 2C), indicating transactivation of the α promoter-enhancer by ie1. A small number of cells transfected with pON284 alone ex-

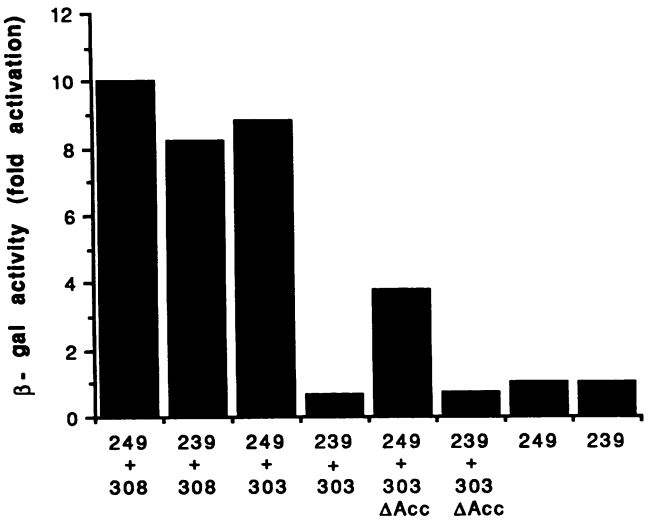

FIG. 3. Repression of expression from the α promoter-enhancer by *ie*2 gene products. The target construct pON249 or pON239 (10 μg) was cotransfected with 20 μg of effector construct pON308 (carrying *ie*1), pON303 (carrying *ie*1 and *ie*2) or pON303ΔAcc (carrying *ie*2) into 3 × 10^6 HF cells. β-Gal activity was measured at 48 and 72 h after cotransfection on duplicate dishes (8). β-Gal levels are expressed as fold activation measured after cotransfection with an effector clone compared with transfection of pON249 or pON239 alone; the values represent averages of β-gal activities measured from 12 separate transfection experiments. The β-gal activities ranged no more than 10% above or below the indicated values.

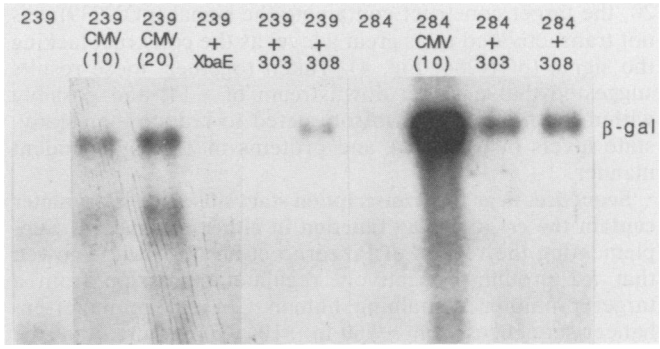

FIG. 4. Blot hybridization analysis to determine steady-state RNA levels in transfected cells. Target plasmids pON239 and pON284 were individually transfected into HF cells (lanes 1 and 7), transfected into cells that were subsequently infected with CMV (lanes 2, 3, and 8), or cotransfected together with effector plasmid pXbaE (lane 4), pON303 (lanes 5 and 9), or pON308 (lanes 6 and 10). Transfections were performed by using 10 μg of target plasmid and 20 μg of effector plasmid. CMV infections were performed by using a multiplicity of infection of 10 (lanes 2 and 9) or 20 (lane 3) PFU per cell. Whole-cell RNA was harvested at 24 h postinfection and 48 h posttransfection; 10 μg of each sample was loaded into a lane on a formaldehyde–1% agarose gel. Following electrophoretic separation and transfer to nitrocellulose, the blot was hybridized with a ^{32}P-labeled RNA probe complementary to the 3′-terminal 199 bp of *lacZ*, which detected a 3.6-kb *lacZ* transcript (indicated by β-gal at the right).

pressed β-gal (Fig. 2F and Table 1) apparently as a result of enhancer activity in the absence of any viral regulatory functions. Consistent with our previous results, immunofluorescence analysis showed that cotransfection of either effector plasmid with pON284 resulted in a similar number of cells in which β-gal expression had been transactivated (Table 1). We interpret this finding to mean that although α gene expression from the two effector plasmids themselves was decreased by the presence of *ie*2, the number of cells that contained sufficient α gene products to activate β-gal expression remained the same (Table 1). Taken together, these results predicted that a *cis*-acting signal was present on the natural genes carried by effector plasmids but absent from our *lacZ* target constructs. This signal was apparently responsible for ie2-mediated repression of α gene expression. These results also suggested that even though it is subjected to shutoff, pON303 makes sufficient α gene products to transactivate the α promoter-enhancer on the *lacZ* target construct.

The major difference between *lacZ* target constructs (pON284 and pON249) and the natural *ie*1 and *ie*1-*ie*2 constructs (pON308 and pON303) was the fact that the target constructs carried enhancer-promoter sequences only to −14 relative to the transcription start site. To determine whether sequences downstream of this site were important in ie2-mediated repression of gene expression, we cotransfected *ie*1 and *ie*1-*ie*2 effector plasmids with a *lacZ* target construct pON239 (44) containing an α gene region from −1138 upstream of the enhancer to +913 bp (i.e., through the first exon and first intron). In transient assays, pON239 was compared with pON249 or pON284, which included upstream sequences extending only to −14. We previously showed that ie1 alone or ie1 and ie2 together transactivated pON284 or pON249 to similar levels (8) and thus have used these two target plasmids interchangeably in our investigation of the regulation of the α locus. In agreement with our previous results, ie1 expressed from pON308 transactivated both pON239 and pON249 to equivalent levels and pON249 was highly transactivated by ie1 and ie2 expressed from pON303 (Fig. 3). pON249 was also transactivated by pON303ΔAcc, suggesting that ie2 can activate expression from the CMV enhancer when the repression signal is absent. In contrast, ie1 and ie2 expressed from pON303 (and ie2 expressed from pON303ΔAcc) failed to transactivate pON239, suggesting that *ie*2 products repressed β-gal expression from pON239 in a manner similar to what was observed with the full-length *ie*1 and *ie*2 constructs. Indeed, levels of expression from pON239 in the presence of ie2 (pON303 or pON303ΔAcc) were consistently below those of pON239 alone (Fig. 3), suggesting that basal expression by the CMV α promoter-enhancer was also repressed by ie2. Thus, the target sequence for ie2-mediated repression was apparently contained within a 927-bp region from −14 to +913 relative to the transcription start site.

To investigate whether this difference in β-gal activity in these cotransfections was a result of a difference in transcriptional levels, an RNA blot analysis was conducted (Fig. 4). Steady-state levels of *lacZ* RNA from either target construct containing (pON239) or lacking (pON284) the target signal were similar when transactivated by pON308, as shown previously (8); however, RNA levels were significantly lower in cells cotransfected with the *ie*1-*ie*2 effector pON303 plus pON239 than in to those cotransfected with pON303 plus pON284. Furthermore, when cotransfection was performed with pXbaE, which carries *ie*1 and *ie*2 plus additional flanking sequences not present in pON303, pON239 expression was repressed (Fig. 4). Finally, when cells transfected with pON284 or pON239 were superinfected with CMV at a multiplicity of infection of 10 or even

20, the target construct containing the signal (pON239) was not transactivated to as great a level as the construct lacking the signal (pON284; Fig. 4). Taken together, these results suggested that a signal downstream of −14, and possibly within the first exon or intron, acted to reduce the steady-state levels of α mRNA and proteins in an ie2-dependent manner.

Sequences near the transcription start site of the α promoter contain the *crs* and may function in either orientation. Supplementing the results of Pizzorno et al. (39), who showed that *ie2* products negatively regulate expression from a target promoter containing human CMV α promoter-enhancer sequences from −760 to +10, we predicted that the *crs* should be contained within the sequences between −14 and +10. To determine whether this sequence alone was sufficient for repression, a double-stranded oligonucleotide representing −14 through +9 was synthesized, inserted into target indicator plasmids, and tested in transient cotransfection assays for the ability to confer ie2-mediated repression. This oligonucleotide was cloned in both orientations (pON249crs and pON249crs'; Fig. 5) into a position such that the expected transcription initiation site in the sense construct (pON249crs) would be approximately 12 bp upstream of its normal position (14, 59). Following cotransfection with pON303, only constructs containing this oligonucleotide were repressed, thereby showing that the *crs* was contained within this −14 to +9 region (Fig. 5). Effector pON303, expressing iel and ie2, but not effector pON308, repressed β-gal expression. Again, as for the previously tested target constructs, basal expression of these target constructs was repressed in the presence of the *ie2* construct pON303ΔAcc (unpublished data). Given the demonstration that this sequence functions as a repression signal independent of orientation, we believe that repression is being mediated at the level of transcription.

Oligonucleotides containing substitution mutations throughout the −14 to +9 region were synthesized and cloned into the *Hin*dIII site of pON249, maintaining the spacing as in pON249crs (Fig. 6). These target constructs were then cotransfected with pON303, and β-gal activity was measured 48 h later. Sequences within the 5′ portion of the signal were crucial for repression by ie2 (pON249*crs*A, pON249*crs*B, and, to a lesser extent, pON249*crs*C were no longer repressed by pON303); however, a mutation that disrupted the extreme 3′ end of the −14 to +9 oligonucleotide (pON249*crs*D) was not repressed by pON303 at all (Fig. 6). An oligonucleotide representing the sequences from −14 to +1 (pON249*crs*E) was repressed to a level equivalent to that measured from pON239 or pON249crs. In all cases of the mutant *crs* constructs, the *crs* was cloned in both orientations and subsequently assessed for function in transient expression assays; repression was consistently found to be independent of the orientation of the signal (unpublished data).

In the pON249crs substitution mutation series, the transcription start site of the α promoter would be expected to have shifted approximately 12 nucleotides upstream of its normal position. To ensure that the −14 to +1 signal would function to mediate repression in its precise natural location within the α promoter, pON239*crs*E was constructed. This plasmid was similar to pON239 except that it contained a deletion of nucleotides +2 to +913, thus removing the first exon and first intron of the α gene locus. pON239*crs*E responded exactly as pON239 when cotransfected into cells with pON303: β-gal expression was repressed from both targets (unpublished data). Furthermore, β-gal expression

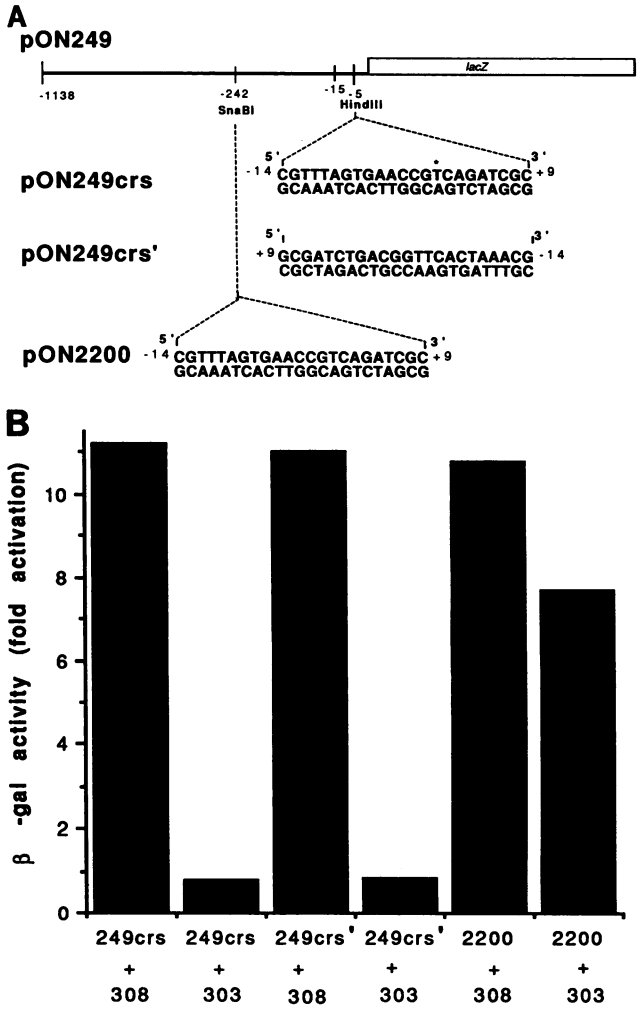

FIG. 5. Occurrence of ie2-mediated repression via a region −14 to +9 spanning the transcription start site. (A) *lacZ* target constructs. pON249 contains enhancer sequences from −1138 to −14 fused to *lacZ* (14). pON249crs contains the same upstream sequences as pON249 plus an insertion of the double-stranded *crs* oligonucleotide in positive orientation (5′ −14 to +9 3′) at the unique *Hin*dIII site at −5 (relative to the transcription start site). pON249crs' contains the *crs* oligonucleotide cloned in opposite orientation (5′ +9 to −14 3′) into the same *Hin*dIII site. pON2200 shares enhancer sequences with pON249 but contains the *crs* oligonucleotide cloned (in positive orientation) into the *Sna*BI site at −242. The asterisk depicts the transcriptional start site (+1) for the *crs* (−14 to +9) sequence when present in its natural setting within the viral genome (59). (B) Repression of *crs* when positioned near the transcription start site. The target constructs containing the *crs* in sense (pON249crs) or antisense (pON249crs') orientation near the transcription start site or upstream in the enhancer (pON2200) were cotransfected with pON303 or pON308 as indicated. β-Gal activity was assayed as described for Fig. 3.

from pON239*crs*E was repressed in an ie2-dependent manner just as it had been from target pON249*crs*E. These data confirmed that the sequence −14 to +1 of the α promoter was functional as a *cis* repression element when placed in its natural position within the α promoter-enhancer and that the results obtained from the pON249crs substitution mutation series accurately reflected the natural setting. Notably, this sequence is not repeated elsewhere in the CMV genome; the

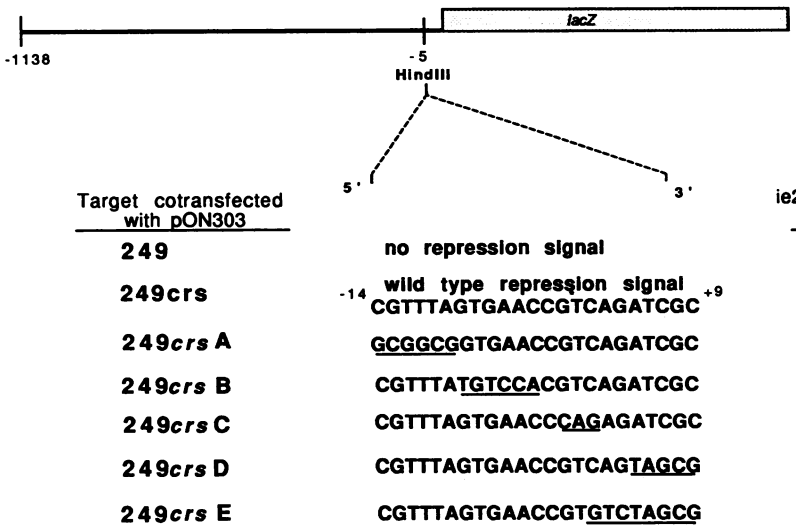

FIG. 6. Location of the *crs* for ie2-mediated repression upstream (between −14 to +1) of the natural transcription start site. Substitution mutations within the *crs* oligonucleotide were synthesized and cloned into the *Hin*dIII site of pON249, shown as constructs 249*crs*A through 249*crs*E. Nucleotides within the −14 to +9 region that have been substituted are underlined. These mutant constructs (10 μg) were cotransfected with pON303 (20 μg) into 3×10^6 HF cells and assayed for β-gal activity 48 and 72 h posttransfection as described for Fig. 3. Results were identical when 4×10^5 cells were transfected with either 2 or 4 μg of effector plasmid and 1 μg of target plasmid. Similar results were obtained from transfection of Vero cells (data not shown).

closest homologies require three mismatched base pairs and are not located near known α genes (unpublished data).

The *crs* does not function when positioned far from the start site of transcription; complex enhancer sequences are not required to mediate repression. To determine whether the *crs* needed to be positioned near the transcription start site to function, we cloned the signal at −242 in pON249 (pON2200). Following transfection, pON2200 was not repressed by *ie2* (Fig. 5), suggesting that proximity to the promoter may be critical for *crs* function.

There were at least two possibilities for the observed results: either the *crs* must be present very close to the transcription start site or the signal requires additional enhancer sequences to be functional. To distinguish between these two possibilities, we first constructed the pON249crs enhancer deletion series. These constructs (pON283crs, pON2044crs, and pON2046crs) carry deletions in upstream enhancer sequences and contain the −14 to +9 signal near the transcriptional start site of the CMV α promoter at the same unique *Hin*dIII site used to construct pON249crs (8). Parental *lacZ* constructs (pON249, pON283, pON2044, and pON2046) do not carry the repression signal. Following cotransfection with pON303, all constructs carrying the *crs* were repressed in an ie2-dependent manner even though the enhancer repeat motifs and consensus binding sites for a variety of cellular transcription factors were successively deleted (Table 2). Therefore, the *crs* was able to function on a 75-bp α promoter containing a TATA box, an Sp1 site, a CAAT box, and one 19-bp repeat element with its associated ATF binding site. Repression by ie2 via the *crs* was not dependent on the highly complex enhancer.

ie2-mediated repression of a heterologous promoter. Given the requirement that the *crs* be placed near the transcription start site to function in ie2-mediated repression, the signal may require other elements surrounding this region. To determine whether the *crs* could confer ie2-mediated repression when present near the start site of a promoter other than the CMV α promoter, we inserted the this signal near the transcription start site of the HSV-1 TK promoter. As expected from the HSV literature (12, 15, 38), the HSV TK promoter without the *crs* (pON2201) was very well activated by a plasmid (pRB201) carrying the *Hin*dIII HM junction fragment with the HSV α0, α4, and α27 genes (Fig. 7). Addition of pON303 did not alter the ability of the HSV α proteins to transactivate the TK promoter and had only a small positive effect on expression from pON2201 in the absence of pRB201. When the *crs* was used to replace sequences near the transcription start site of the HSV-1 TK promoter (pON2202), transactivation by HSV-1 α proteins was not affected (Fig. 7). To determine whether ie2 was able to repress expression from pON2202, we conducted transfection experiments with pRB201, pON2202, and pON303. pON303 repressed HSV-1 α protein-activated expression of pON2202 (Fig. 7). Furthermore, pON303ΔAcc, which expressed functional *ie2* gene products, was able to replace pON303 in this assay and mediate repression (unpublished data). In the absence of pRB201, pON303 had no effect on expression from pON2202. Thus, ie2-mediated repression can be conferred on a heterologous promoter by *crs* if the promoter is transcriptionally active.

TABLE 2. Results showing that delections removing enhancer elements are still subject to ie2-mediated repression via *crs*

Target plasmid	No. of:				Fold activation upon cotransfection with 303
	21-bp repeats	19-bp repeats	18-bp repeats	16-bp repeats	
249	2	4	5	3	10
249crs	2	4	5	3	0.8
283	0	2	2	1	5.4
283crs	0	2	2	1	0.8
2044	0	1	2/3	0	3.2
2044crs	0	1	2/3	0	0.8
2046	0	1	0	0	2.8
2046crs	0	1	0	0	0.9

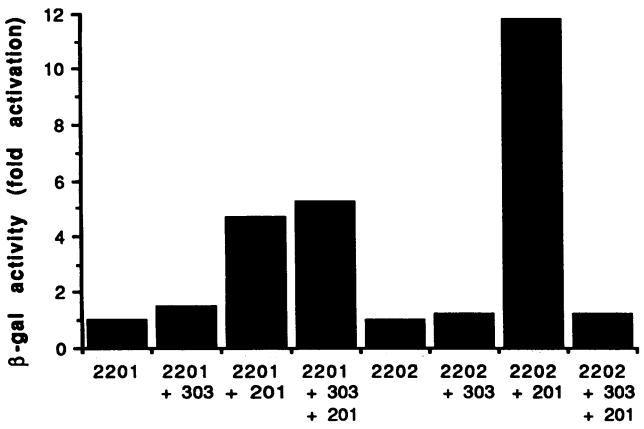

FIG. 7. Demonstration that the −14 to +9 *crs* confers ie2-mediated repression upon a heterologous promoter. A 10-μg sample of target constructs pON2201 (an HSV-1 TK promoter–*lacZ* fusion) or pON2202 (a TK promoter–*lacZ* fusion with the *crs* oligonucleotide inserted at −16 relative to the transcription start site) was cotransfected with 20 μg of effector construct pON303 (carrying CMV *ie*1 and *ie*2) alone, pRB201 (carrying HSV-1 α0, α4, and α27 genes) alone, or both pON303 and pRB201 together. β-gal activity was assayed as described for Fig. 3.

DISCUSSION

We have demonstrated that CMV *ie*2 proteins negatively regulate the α promoter-enhancer through a *cis* signal, *crs*, contained in the sequence 5′-CGTTTAGTGAACCGT-3′, which naturally overlaps the transcriptional start site (between −14 and +1) of the CMV α promoter-enhancer. Our primary interest has been the ability of ie2 to inhibit activation of the α gene by ie1; however our data indicate that the gene expression is repressed even in the absence of enhancer transactivation. The signal may be moved to a heterologous promoter and confer ie2-dependent repression on that promoter. Steady-state RNA levels from *crs*-containing promoters are reduced in the presence of ie2, and the *crs* is functional in either orientation when placed near but upstream of the transcription start site, features that strongly suggest that repression occurs at the transcriptional level.

While a great deal has been learned over the past several years concerning mechanisms of activation of eucaryotic gene expression, little is known about negative regulation, or repression, of transcription. Although the list of eucaryotic repressors is quite short, a number of mechanisms for their function have been proposed (28). Our current concept of repression of eucaryotic gene expression is largely derived from work conducted in procaryotic systems where the overriding theme demonstrates a repressor protein binding to DNA at or near the transcriptional start site such that the interaction of general transcription machinery, including RNA polymerase, with the promoter is blocked (24). Examples of direct repression of gene expression via target signal have been presented for bovine and human papillomaviruses, in which the E2 protein can mediate shutoff by binding to sites near a transcription start site (41, 52). Direct binding to DNA was initially believed to be responsible for simian virus 40 T-antigen repression of early gene expression; however, recently indirect mechanisms have been proposed (18, 34). During the regulation of several drosophila homeobox proteins, a related type of direct repression occurs through competition for the same or a similar DNA-binding site upstream of a promoter that relies on activator binding and activity for expression (22); repressor binding prevents activator binding, resulting in repression.

Our results suggest that ie2-mediated repression occurs directly or via an induced cellular protein, by blocking access of RNA polymerase to the promoter or somehow interfering with assembly or processivity of the transcription complex. The *ie*2 proteins have not as yet been shown to possess any sequence-specific DNA-binding activity or to contain regions of significant homology to other transcriptional regulators; therefore, it is possible that ie2-mediated repression involves an interaction with a cellular protein. Clues to the function of ie2 in repression may come from its well-established role as a heterologous transactivator (58) similar to adenovirus E1A (3). In the absence of the *crs*, ie2 functions as a transcriptional activator of a variety of promoters, including the α promoter itself (6, 8, 9, 11, 12, 20, 25, 39, 46, 58; this work). A complete analysis of *cis* targets responsive to ie2 transactivation has not been completed; however, it appears that ie2 may be mediating transactivation indirectly via a range of cellular transcription factors along the same lines as adenovirus E1A. Although cellular transcription factors appear to be activated by viral proteins like E1A and ie2, possibly certain cellular factors induced by ie2 act to downregulate transcription through the *crs*.

In light of the above observations, it is interesting that ie2 acts as a dominant transcriptional repressor even in the presence of the activator protein ie1 as long as the *crs* is present at the target promoter. This dominant repression by ie2 appears to extend to other heterologous systems such as the TK gene activated by HSV α gene products. This finding suggests that ie2 may exert its repression function via one of at least two mechanisms depending on the concentration or availability of other viral proteins and cellular factors with which it may interact. One possible mechanism would have ie2 itself or an ie2-induced cellular protein binding to the *crs* and preventing transcription initiation. The data presented here suggest that ie2-mediated repression occurs in a relatively straightforward way, by preventing transcription through direct or indirect occupation of the *crs*, a mechanism reminiscent of classical procaryotic repression. Whether ie2-mediated repression occurs by steric hindrance as has been demonstrated for a number of procaryotic repressors, including *Escherichia coli lac* (2), P22 *mnt* and P22 *arc* (64), and λ *cro* and λ cI (23, 24), or by affecting the function of bound transcriptional machinery remains an open question. Second, ie2 itself or an ie2-induced protein may repress transcription by blocking some component of the transcription apparatus which is particularly sensitive to the presence of *crs* between the TATA box and the transcriptional start site. A similar type of mechanism has been proposed to explain how the yeast *GAL1* gene is repressed by negative regulatory factors bound between the upstream activator sequence and the TATA region (5, 55) and for the function of bovine papillomavirus E2 (41).

The CMV *ie*2 proteins possess a variety of functions: they transactivate homologous and heterologous promoters and repress their own expression as well as the expression of ie1. Indeed, in the context of the intact virus, transcription of *ie*1 and *ie*2 peaks early in infection and decreases thereafter, consistent with repression occurring at this locus (47, 49, 51). The multiple functions of the *ie*2 proteins are likely to be critical elements of both productive and latent infections. Furthermore, their diverse functions will probably involve interactions with accessory cellular proteins, and further

work should determine the role of these factors in ie2-mediated repression and activation of α gene expression.

ACKNOWLEDGMENTS

We thank Maria Kirichenko for cell culture and Bernard Roizman, Saul Silverstein, and Steve McKnight for plasmids.

This work was supported by grant MV230 from the American Cancer Society and by Public Health Service grants AI20211 and HL33811 from the National Institutes of Health. E.L.K. was supported by grant DK41143 from the National Institutes of Health. J.M.C. was a registered graduate student in the Department of Microbiology at the University of Minnesota during completion of this work.

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