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The RR1 Gene of Herpes Simplex Virus Type 1 Is Uniquely *trans* Activated by ICP0 during Infection

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As has been demonstrated for herpes simplex virus type 2, we show in this report that the herpes simplex virus type 1 ribonucleotide reductase large subunit (RR1) gene is *trans* activated in transient transfection assays by VP16 and ICP0 but not by ICP4. Deletion analysis demonstrated that responsiveness to induction to VP16 resides in an octamer/TAATGARAT sequence of the RR1 promoter and that the TATA box alone is sufficient to provide induction by ICP0. The induction of the RR1 gene by ICP0 but not by ICP4 suggested that it might be possible to identify the *cis*-acting element(s) responsive to ICP4 in an ICP4-inducible promoter. To this end, a series of chimeric promoters containing various portions of the regulatory sequences of the RR1 promoter and thymidine kinase (TK) promoter were constructed. The TK promoter is *trans* activated by both ICP0 and ICP4 in transient transfection assays and by ICP4 in infection. The data show that replacing the RR1 TATA region with the TK TATA region permits ICP4 inducibility even if the rest of the RR1 promoter elements remain intact. To test whether the RR1 gene is induced by ICP0 during infection, four mutant viruses were constructed. (i) TAATGARAT$^+$ has the wild-type RR1 promoter driving chloramphenicol acetyltransferase (CAT) and the RR2 promoter driving the *lacZ* gene. The RR2 gene codes for the small subunit of the ribonucleotide reductase and is expressed as a β gene. (ii) TAATGARAT$^-$ has a triple-base change in the octamer/TAATGARAT element which renders it unresponsive to VP16 *trans* activation, eliminating that portion of the activation of the RR1 gene. (iii) TAATGARAT$^-$Δα0 has a deletion of the α0 gene. (iv) TAATGARAT$^-$Δα4 has a deletion of the α4 gene. Infections were carried out in Vero cells at a multiplicity of infection of 10 per cell; cells were assayed for CAT and β-galactosidase (β-Gal) activities and for virus yields. The first two infections gave strong CAT and β-Gal activities and high yields of progeny virus. Infection with the third virus showed no CAT activity but did produce high levels of β-Gal activity and virus progeny. The fourth infection resulted in strong CAT activity but no β-Gal activity or progeny virus. The data demonstrated that the RR1 promoter was activated in the absence of ICP4 but not in the absence of ICP0 in these infections. We conclude that, in the absence of VP16 induction, ICP0 is the primary activator of the RR1 gene promoter in infection.

The more than 70 genes of herpes simplex virus type 1 (HSV-1) are classified into three major kinetic groups on the bases of the timing and manner of regulation of their expression after the onset of infection (45, 46). The five α, or immediate-early, genes are transcribed soon after infection in the presence of cycloheximide and, thus, are expressed in the absence of de novo protein synthesis. Expression of α genes is initiated by the interaction of VP16, a virion tegument protein (also called αTIF, Vmw65, ICP25, and U_L48), with the Oct-1 cellular transcription factor, leading to recognition of the octamer/TAATGARAT sequences in the regulatory regions of the α gene promoters (9, 37, 54, 59, 70, 77). ICP4, the product of the α4 gene, *trans* activates the expression of early (or β) and late (or γ) genes (19, 21, 22, 25, 34, 35, 71, 72, 79). The expression of both early and late genes is also modulated by ICP27, the product of the α27 gene (26, 63, 66, 80, 81, 84, 88). True late genes also require viral DNA synthesis for expression (42). During primary infection, virus passes into nerve endings and is transported to neuronal bodies in sensory ganglia, in which latency is established (32, 82, 90). Latency persists for the life of the host, with periodic reactivation resulting in production of infectious virus and the characteristic herpetic lesions.

Ribonucleotide reductase (RR) plays a major role in replication by reducing ribonucleotides to the corresponding deoxyribonucleotides, thus providing precursors for DNA synthesis (94). HSV-1 and HSV-2 both encode a heterodimeric enzyme which, like mammalian and bacterial RRs (24), is active in an $\alpha_2\beta_2$ configuration (5, 16, 23, 31, 47, 49, 78, 92). The large subunit, designated ICP6 or RR1 (45, 46), with a molecular weight of 140,000, is encoded by a 5.0-kb mRNA derived from the unique long region of the viral genome (U_L39) at coordinates 0.562 to 0.597 (4, 65). The small subunit, RR2 (molecular weight, 38,000), is encoded by a 1.2-kb transcript, the 3′ end of which is shared with the transcript of the large subunit (4, 65). Viruses mutated in the RR1 gene replicate normally in cell culture at 37°C. The viral reductase is not essential for virus replication at this temperature because RR activity is complemented by the cellular enzyme. The cellular enzyme is temperature sensitive at 41°C, and viral reductase activity is then essential for productive virus growth (39). In addition to its role in RR activity, the RR1 proteins of both HSV-1 and HSV-2 have an intrinsic protein kinase domain within a 300-amino-

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acid segment of the NH_2-terminal region of the molecule (2, 3, 12, 17). The NH_2-terminal region of the HSV-1 and HSV-2 RR1 protein contains a 360-amino-acid stretch that is absent from the large subunits of mammalian and other viral reductases (3, 36, 69, 92).

The induction of expression of the RR1 gene appears to be unique among the HSV genes. RR1 transcripts are made very quickly after the onset of infection (45, 46) and are synthesized in the presence of cycloheximide (98), which is characteristic of the kinetics of expression of the α class of HSV genes. Indeed, Wymer et al. (100) have shown that the promoter of the RR1 gene of HSV-2 contains an octamer/TAATGARAT-like element typical of all HSV α genes and that this element is responsive to induction in transient transfection assays by VP16, the virion protein which *trans*-activates α genes. This interaction probably accounts for the induction of RR1 message with α gene characteristics during infection. Both RR1 message and protein are synthesized in infections with either ICP4- or ICP0-defective mutants (21, 61, 65, 85). In addition, the promoters of the RR1 genes of HSV-1 and HSV-2 are activated in transient assays by ICP0 but not by ICP4 (39, 93, 100). The kinetics of induction of reductase enzyme activity is the same as that of other enzymes involved in viral DNA synthesis (55). The expression of the small subunit, RR2, is typical of the β class of proteins (96); as a consequence, reductase activity shows β class kinetics.

In this report, we examine the hypothesis that the RR1 gene of HSV-1, like that of HSV-2, is induced in a bipartite manner by both VP16 and an α gene product. We find that the HSV-1 RR1 promoter is induced by VP16 in transient assays and that the induction is dependent on the presence of an octamer/TAATGARAT element in the promoter. In addition, the HSV-1 RR1 gene is also *trans* activated by ICP0, but not by ICP4, in transfection experiments. Most importantly, we present genetic evidence that in infection, in the absence of VP16-octamer/TAATGARAT induction, ICP0 rather than ICP4 is the primary activator of the RR1 gene. As far as we are aware, the RR1 gene is the only gene among the β and γ genes of HSV which is activated by ICP0.

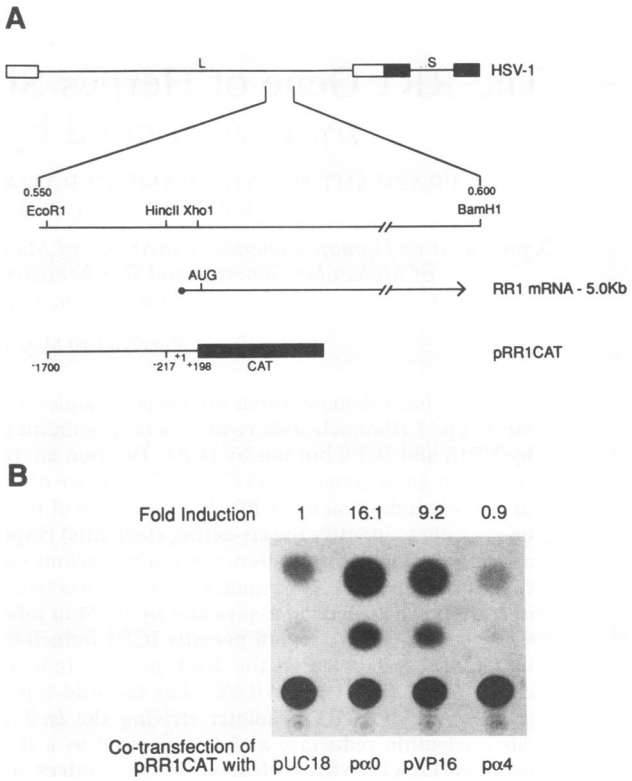

FIG. 1. *trans*-induction of the RR1 promoter in cotransfection assays. (A) The top two lines show the HSV-1 genome and an enlarged segment from map units 0.550 to 0.600, encoding the RR1 gene. Indicated are relevant restriction enzyme sites. The third line shows the extent and position of the 5.0-kb RR1 message. The fourth line shows the RR1 upstream sequence fused to the CAT gene in pRR1CAT. (B) pRR1CAT (5 μg) was cotransfected into Vero cells together with an equimolar quantity of plasmids which code for the ICP0, VP16, or ICP4 gene product. Cells were harvested 48 h after transfection, and the CAT activity present in these extracts was determined. The result of one such representative experiment is shown. Quantitation was done by Betascan analyses. CAT activity is presented as fold induction relative to cotransfection with pUC18 plasmid DNA. The data presented are the averages of at least three independent transfections, each of which was carried out in duplicate.

MATERIALS AND METHODS

Cells and viruses. The virus strain used was HSV-1 KOS. Vero cells were grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. Stocks of this virus were prepared by low-multiplicity passage in Vero cells. The mutant dlx 3.1, kindly provided by Priscilla Schaffer, is deleted for most of the transcriptionally regulatory signals and coding sequences of the α0 gene (85). Mutant d120 is deleted for most of the coding sequence of the α4 gene and is grown in the E5 Vero cell line, which has been transformed for a functional α4 gene and which complements d120 (21). The d120 virus and E5 cells were kindly provided by Neal DeLuca.

Construction of RR1 deletion plasmids. The RR1 gene sequences used to construct the various RR1 promoter-containing plasmids were derived from pSG124, which contains the *Eco*RI A fragment of HSV-1 (38). A 2.2-kb *Xho*I fragment from pSG124 was cloned into pUC18 at the *Xho*I site, which had been inserted into the *Sma*I site in the polylinker. The *Nru*I site at −1700 (relative to the start of transcription of the RR1 gene [33]) in this fragment was converted into an *Eco*RI site. The RR1 sequence spanning −1700 to +198 was obtained by cutting with *Eco*RI and *Xho*I and ligated into pUC18, which had been previously cut with *Eco*RI and *Xho*I. This plasmid was named pRR1. The chloramphenicol acetyltransferase (CAT) open reading frame and poly(A) sequence derived from pSV0CAT-BH (89) as a *Bgl*II-*Hin*dIII fragment were cloned into pRR1 at the *Bam*HI-*Hin*dIII sites to give pRR1CAT (Fig. 1A).

To generate a series of 5′ nested deletions in the RR1 promoter region, pRR1 was digested with *Hin*cII at −217 and the linearized molecules were digested with exonuclease III for various times to produce deletions starting at −217 and extending downstream and then were treated with S1 nuclease and blunt-end ligated with 10-bp oligonucleotide *Xba*I linkers. *Xba*I-*Xho*I fragments from these plasmids were purified from agarose gels and cloned into the *Xba*I-*Xho*I sites of pRR1XCAT, which is identical to pRR1CAT except that the *Hin*cII site at −217 has been changed to an *Xba*I site. Cutting pRR1XCAT with *Xba*I and *Xho*I removes the RR1 sequence between −217 and +198 and permits the substitution of the 5′ deleted fragments. The deletion plasmids are named according to the sequence deleted from the

RR1 promoter. Thus, pRR1Δ−217/−157 is deleted from −217 to −157. pRRΔ+17/+198 was constructed by cloning a *Hin*cII to *Bam*HI fragment, spanning from −217 to +17 (see below), into pRR1 previously digested with the same restriction enzymes to remove the sequences from −217 to +198. The CAT coding sequence, obtained as a *Bgl*II-*Hin*dIII fragment from pSV0CAT-BH (89), was then cloned into this plasmid after digestion with *Bam*HI and *Hin*dIII. DNAs from these plasmids were sequenced to determine the precise end points of the deletions by using Sequenase (U.S. Biochemicals) according to the procedure described by the manufacturer.

Construction of pRR1-LS-CAT. For the construction of pRR1-LS-CAT, a plasmid carrying a 3′ deletion of the RR1 promoter region spanning from −81 to +198 (see below) and a plasmid carrying a 5′ deletion from −217 to −58 were used. The 5′ deletion plasmid was digested with *Xba*I, blunt ended, and then cut with *Hin*dIII to release a fragment spanning from −58 to +198. This fragment was gel purified and cloned into the 3′ deletion plasmid, which was cut first with *Bam*HI, blunt ended, and then cut with *Hin*dIII. The blunt ends were ligated to 8-bp *Sal*I linkers. This plasmid contains the RR1 sequences from −438 to −218 and from −58 to +198 separated by 22 bp of linker sequence and is designated pRR1-LS. The *Hin*cII (−217) to *Xho*I (+198) fragment from pRR1 was replaced with the homologous fragment from pRR1-LS, and the CAT gene was then cloned into this vector as described above to give pRR1-LS-CAT.

Construction of plasmids containing RR1/TK chimeric promoters. As a first step in construction of the RR1/thymidine kinase (TK) chimeric promoters driving CAT, a 1.63-kbp *Bgl*II-*Bam*HI fragment containing the CAT gene from pSV0CAT-B was cloned into the *Bgl*II site of the pUC18 plasmid. This plasmid was called pUCAT1. A 0.839-kbp *Hin*dIII-*Bgl*II fragment containing the TK promoter regions (−198 to +52) from a number of differently positioned *Bam*HI linker-scanner (LS) mutant plasmids (kindly provided by S. McKnight and D. Coen) was ligated into the pUCAT1 plasmid. The LS mutants used were LS−119/−109, LS−105/−95, LS−79/−69, LS−56/−45, and LS−7/+3 (15). The resulting plasmids had different TK LS mutant promoters placed upstream of the CAT gene.

A plasmid containing the RR1 promoter region from nucleotide positions −438 to +198 was linearized by digestion with *Pst*I and *Xho*I, both of which cut in the multicloning site of the plasmid. The linear DNA was digested from the *Xho*I end with exonuclease III, and the single-strand ends were removed with S1 nuclease. This construct was blunt-end ligated to 10-bp *Bam*HI linkers, circularized (pRR1B), and then digested with *Eco*RI and *Bam*HI, and the resulting 1.683-kbp fragment containing the promoter region of RR1 was ligated into the *Eco*RI-*Bam*HI site of pUC18. The construct was digested with *Bam*HI and *Pst*I and progressively deleted unidirectionally from the *Bam*HI end with exonuclease III; deletion was followed by S1 nuclease digestion. The panel of deletions were separately blunt-end ligated to 10-bp *Bam*HI linkers and circularized, resulting in a series of RR1 promoter deletion mutants ranging from nucleotide −17 to nucleotide −145 upstream of the transcription initiation site. The precise end points of these deletions were determined by DNA sequencing.

The RR1/TK chimeras were produced through the following manipulations. The TK/CAT plasmids were linearized with *Hin*dIII, and the ends were filled in with the Klenow fragment (GIBCO BRL, Gaithersburg, Md.) of *Escherichia coli* DNA polymerase I. The linear plasmids were digested at their respective LS sites with *Bam*HI. The result was a series of linear DNA molecules of different lengths, with a 5′ *Bam*HI end upstream of the TK promoter region and a 3′ blunt end. The positions of the *Bam*HI ends of the constructs used for subsequent fusions varied from −109 to +3 with respect to the TK transcription initiation site. The RR1 promoter regions from each of the deletion mutants were excised separately by digestion with *Sna*BI and *Bam*HI and ligated separately to the *Bam*HI-*Hin*dIII-digested TK/CAT plasmids, resulting in a library of constructs containing RR1/TK promoter chimeras driving the CAT gene (see Fig. 4). The chimeras were arranged so as to maintain the normal spacing of the promoters as much as possible. The fusions included RR−145/TK−109, RR−112/TK−109, RR−94/TK−95, RR−69/TK−69, RR−37/TK−46, and RR−17/TK+3. It should be evident that the RR1 and TK promoter sequences are separated by a *Bam*HI linker in all the chimeric promoters. The viral nucleotide sequences surrounding the *Bam*HI linker were confirmed by DNA sequencing for each chimeric promoter.

Construction of mutant viruses. To facilitate the introduction of RR1 promoter constructs into the virus genome, an expression cassette in which the RR1 promoter drove the expression of the CAT gene and the RR2 promoter drove the *lacZ* gene was constructed. As a consequence of cotransfection between the plasmid carrying the expression cassette with viral DNA, recombinant progeny virus can be detected by the production of a blue plaque representing β-galactosidase (β-Gal) activity.

A 4.4-kb *Bgl*II-*Hpa*I restriction enzyme fragment derived from pSG124 (38) was cloned into the *Bgl*II-*Hin*cII sites of pUB (pUC18 with a *Bgl*II linker inserted at the *Sma*I site). The 4.4-kb fragment encodes the RR2 promoter, the RR2 open reading frame, and the polyadenylation signals for both the RR1 and RR2 genes; this plasmid was designated pRC1. pRC1 was digested with *Nco*I, and the ends were filled in and then partially digested with *Hin*cII to delete 1 kb between the *Nco*I site and the first 3′ *Hin*cII site. The ends of the deleted plasmid were ligated with 10-bp *Xho*I linkers (pRC2). The *Nco*I site overlaps the RR2 translation initiation codon. The *Xho*I-restricted *lacZ* gene from pSC8 (10; kindly provided by B. Moss) was cloned into this *Xho*I site so that expression of *lacZ* initiates at the RR2 ATG (pRC3). The CAT gene derived as a *Bgl*II-*Bam*HI fragment from pSV0CAT-B (89) was then cloned into the *Bgl*II site of pRC3, resulting in pRC4. pRC4 contains the CAT gene and the wild-type RR2 promoter driving the *lacZ* gene. This expression cassette was then removed as a *Bgl*II-*Hin*dIII fragment and cloned into pRR1 by using the *Bam*HI and *Hin*dIII sites in the vector. This plasmid was named pRRZ.

Marker transfer of the CAT-LacZ expression cassette in pRRZ into the wild-type KOS viral genome was carried out as previously described (44). pRRZ contains approximately 1.8 to 2 kb of HSV-1 flanking sequences on either side of the cassette to facilitate recombination into the viral genome. After cotransfection of linearized pRRZ and viral DNA into Vero cells, the viral titer was determined and plaques were scored for β-Gal activity. Blue plaques were picked and plaque purified by limiting dilution. DNA from cells infected with mutant virus was subjected to Southern blot analyses to confirm the presence of the expression cassette. This virus was designated TAATGARAT$^+$ (see Table 3).

Three additional viruses were constructed, each of which carried base changes in the octamer/TAATGARAT sequence of the RR1 promoter. Mutation of the octamer/TAATGARAT element was induced by using a substituted

oligonucleotide and the gapped-DNA technique (30). The oligonucleotide 5′ AGGCCAAGGGATAC 3′ was used to create three nucleotide substitutions in the wild-type sequence ATGCAAATGGATAC. pRR1 was restricted either with *Eco*RI or with *Hin*cII and *Xho*I, and the DNAs were denatured and then annealed to create double-stranded molecules with a single-stranded region between −217 and +198. The mutant oligonucleotide was annealed to the gapped DNA, which was then filled in with Klenow protein and then ligated. Positive bacterial clones were identified by colony hybridization with the α-^{32}P-labelled mutant oligonucleotide (99). The presence of the mutations was confirmed by the creation of a new *Hpa*I site in the RR1 promoter as a consequence of the nucleotide changes. The CAT-LacZ expression cassette was cloned from pRC4 as described above for pRR1. This plasmid was designated pTMZ and contained a 3-bp mutation in the octamer/TAATGARAT element of the RR1 promoter which drives CAT and the wild-type RR2 promoter which drives *lacZ*.

pTMZ was used in cotransfections (as described above) with three different viral DNA molecules to produce three additional mutant viruses (see Table 3). The first transfection was with wild-type KOS DNA and gave a virus designated TAATGARAT$^-$, which is identical to TAATGARAT$^+$ except for the presence of the triple-base substitution in the RR1 promoter. The second transfection was with mutant dlx 3.1 DNA, which carries a deletion of most of the α0 gene (85). This mutant virus was designated TAATGARAT$^-$ $\Delta\alpha$0. The final transfection was with mutant d120 DNA, which carries a deletion of the α4 gene (21). This mutant was designated TAATGARAT$^-$ $\Delta\alpha$4.

CAT assays. Two different CAT assays were employed. For the data in Fig. 1 and 3 and Table 1, assays were performed by the method of Gorman et al. (40) as described by Shapira et al. (89). The data in Tables 2 and 3 were acquired by the method of Seed and Sheen (87). The data are presented as fold increase over control.

β-Gal assay. β-Gal activity was assayed essentially as described by Maniatis et al. (60). Briefly, 30 μl of cell extract was added to a solution containing 3 μl of 100× Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), 66 μl of 1× ONPG (4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml in 0.1 M sodium phosphate, pH 7.5), and 201 μl of 0.1 M sodium phosphate (pH 7.5). Reaction mixtures were incubated at 37°C for 30 min, and reactions were terminated by adding 500 μl of 1 M Na_2CO_3. Optical densities were read at 420 nm.

RESULTS

Transient induction analysis of the HSV-1 RR1 promoter. Initial studies were carried out to phenotypically characterize the RR1 promoter with respect to its response to virally encoded *trans* activators in transfection assays. A fragment of the upstream region of the RR1 gene spanning from −1700 to +198 was fused to the structural gene for CAT. This plasmid, pRR1CAT (Fig. 1A), was cotransfected into Vero cells together with an equimolar quantity of pUC18, as a negative control, or with plasmids that encode either ICP0 (map units 0.778 to 0.813 [34]), VP16 (map units 0.685 to 0.712 [kindly provided by Peter Weber]), or ICP4 (map units 0.809 to 0.868 [79]). The results of one representative series of assays are shown in Fig. 1B. CAT activity was induced by cotransfection with plasmids that express ICP0 or VP16 but not with pUC18 or the plasmid that encodes ICP4. These results are similar to those reported by Wymer et al. (100) for the HSV-2 RR1 promoter. The stimulation of CAT expression by VP16 may explain the immediate-early expression of the RR1 gene during infection. Deletion of sequences from −1700 to −218, by using available restriction sites, did not alter the response of the RR1 promoter to *trans* activation as judged by transient assays (data not shown).

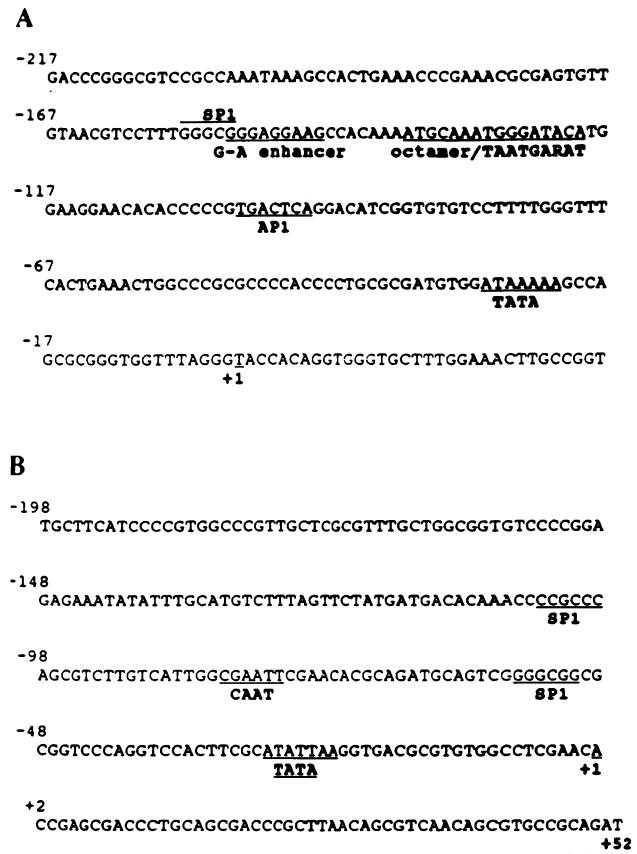

FIG. 2. Promoter sequences of the HSV-1 RR1 gene (64) (A) and TK gene (97) (B). The recognizable *cis*-acting transcription signals are underlined.

A number of putative transcriptional consensus elements are detected within the DNA sequence of the RR1 promoter from −217 to +29 (Fig. 2A) (64). These include the octamer/TAATGARAT element flanked by a GA-rich stretch, a sequence common to all HSV-1 α gene promoters. There is a single copy of this VP16 response element in this promoter, similar to that observed for the HSV-2 RR1 promoter, along with single copies of SP1 and AP1 binding sites. Finally, a TATA box element is also indicated, 18 bp upstream of the start of transcription. To define critical *cis*-acting elements within the −217 to +198 region, 5′ nested deletions were generated starting at −217 and progressing toward +1. These promoter deletion constructs still retain the upstream region from −1700 to −218. Cotransfections similar to those described in the legend to Fig. 1 were carried out with each deletion plasmid (Table 1).

Deletion plasmids in which the octamer/TAATGARAT element was intact (pRRΔ−217/−157 and pRRΔ−217/−142) were *trans* activated by VP16 (Table 1). However, deletions that extended to −121 and further downstream, which removed this element, reduced VP16 *trans* activation to background levels. These findings are consistent with the hypothesis that responsiveness of the octamer/TAATGA

TABLE 1. Deletion mutation analysis of the RR1 promoter[a]

RR1 deletion mutant plasmid	Fold increase of CAT activity after cotransfection with:		
	pVP16	pα0	pα4
pRRΔ−217/−157	17.1	23.8	2.2
pRRΔ−217/−142	10.9	22.7	1.7
pRRΔ−217/−121	1.8	25.6	2.7
pRRΔ−217/−106	1.3	20.5	2.5
pRRΔ−217/−81	1.0	29.8	2.0
pRRΔ−217/−72	2.8	18.4	2.5
pRRΔ−217/−62	1.5	27.6	7.5
pRRΔ−217/−51	2.0	23.4	6.5
pRRΔ−217/−39	1.0	13.0	3.1
pRRΔ−217/−20	1.0	3.3	1.1
pRRΔ+17/+198	20.6	17.4	1.1

[a] 5′ nested deletions which start at −217 and progress toward the start site of transcription were constructed. These deletions still retain the upstream sequences from −1700 to −218. The promoter deletion constructs were cotransfected into Vero cells together with pUC18 or ICP0-, VP16-, or ICP4-encoding plasmids (see legend to Fig. 1). pRR1CAT was also included as a control in all cotransfection experiments. The size of each promoter deletion is shown in the first column. CAT activity is again expressed as the fold increase over the activity observed by cotransfection with pUC18. The values are averages of at least three independent transfections, each carried out in duplicate.

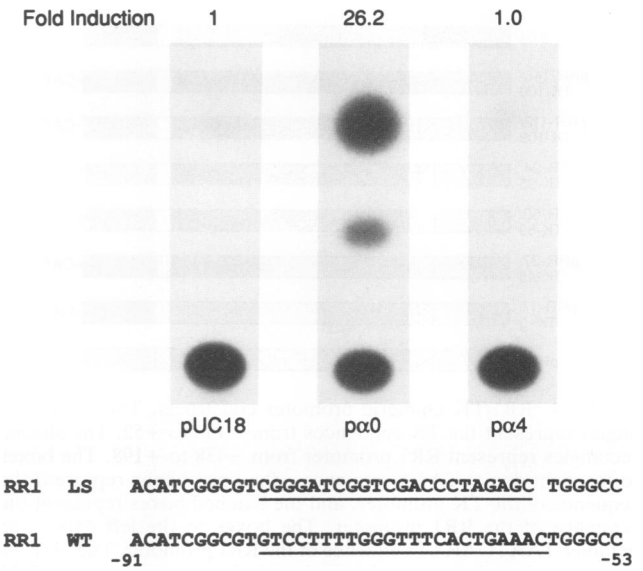

FIG. 3. Linker substitution mutagenesis of the RR1 promoter. Twenty-three nucleotides of the RR1 promoter, from −81 and −59, were replaced with 22 nucleotides of the restriction enzyme linker sequences. CAT activities induced by the substituted plasmid cotransfected with pUC18, pα0, and pα4 are shown.

RAT element to *trans* activation by VP16 probably accounts for the immediate-early expression of the RR1 gene.

trans activation by ICP0 was not affected by deletion of all the putative *cis*-acting elements upstream of the TATA box, as shown by the result of the pRRΔ−217/−39 and pα0 cotransfection (Table 1). Deletion of the TATA box (pRRΔ−217/−20) abolished the induction of CAT gene expression by ICP0. It appears that ICP0 induction of the RR1 promoter may be achieved solely by the presence of the TATA box region and the downstream sequences. To determine the role of the RR1 sequences downstream of the TATA box for ICP0 transactivation, a deletion which spans from +17 to +198 was introduced into pRR1CAT (pRRΔ+17/+198, Table 1). This deletion leaves the entire promoter and transcriptional start site intact but removes most of the untranslated sequence of the RR1 gene. A normal amount of induction resulted in cells cotransfected with the α0 plasmid and this construct. We interpret the data to indicate that the downstream sequences of the RR1 promoter play no apparent role in the induction of the RR1 gene by ICP0.

The RR1 promoter deletion constructs were also cotransfected with the plasmid that encodes ICP4 (Table 1). As expected from our preliminary observations, the deletion plasmids spanning −217 and −72 failed to show CAT activity. Surprisingly, deletion of sequences downstream of −72 resulted in a significant increase of CAT activity over the basal induction. This increase in CAT activity was reproducible and was observed with the deletion constructs that extend from −62 to −39. Two explanations may account for this observation. First, it is possible that the deletions resulted in the shift of upstream viral DNA sequences into closer proximity to the RR1 TATA region, altering the response of this promoter to ICP4. Second, the sequences between −72 and −62 may have an inhibitory effect on activation of RR1 transcription by ICP4. To distinguish between these alternatives, LS mutations were generated in this region. If the sequence between −72 and −62 was involved in negative regulation of the ability of ICP4 to induce the RR1 promoter, then the introduction of a mutant sequence in that region should eliminate the inhibitory effect and result in ICP4 induction of CAT activity. If, on the other hand, the first explanation is correct, then altering the sequence but maintaining the number of base pairs in this region should still prevent the induction of the promoter by ICP4. In the construct designated pRR1-LS-CAT, RR1 sequences from −81 to −59 were replaced with linker sequences (Fig. 3). Although the linker replacement was not precise, with 23 nucleotides replaced with 22, the overall spacing was conserved. CAT gene expression from this promoter construct was induced by ICP0 but not by ICP4 (Fig. 3). Therefore, the observed *trans* activation by ICP4 of the RR1 promoter deleted 3′ of −72 must be caused by upstream sequences being moved closer to the promoter region. Deletion of DNA sequences between −1700 and −218 from pRRΔ−217/−62 eliminated ICP4 *trans* activation (data not shown), supporting the presence of a critical *cis*-acting element in this region. No attempt to find this element has yet been made. We conclude that this element is not an integral part of the RR1 *cis*-acting regulatory sequences.

Transient induction analysis of RR1/TK chimeric promoter constructs. The unique induction of the RR1 gene by ICP0 but not by ICP4 offers the opportunity to identify the *cis*-acting element(s) responsive to ICP4 in an ICP4-inducible promoter. To this end, we have constructed a series of chimeric promoters containing various portions of the regulatory sequences of the RR1 and TK genes. The TK promoter is *trans* activated by both ICP0 and ICP4 in transient transfection assays and primarily by ICP4 during infections. The TK promoter contains a TATA element and a CCAAT box surrounded by SP1 sites (Fig. 2B) (97). By replacing RR1 sequences with TK sequences, it should be possible to determine which TK elements are necessary for the acquisition of ICP4 *trans* activation.

A series of nested 3′ deletions of the RR1 promoter were

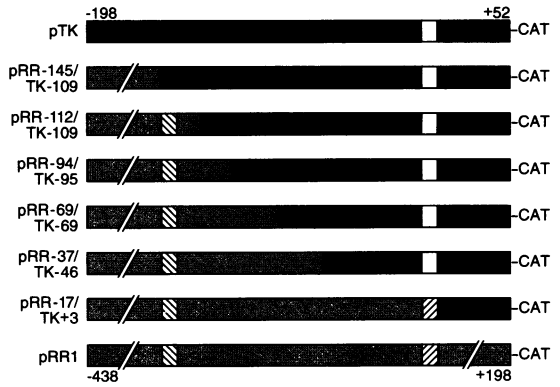

FIG. 4. RR1/TK chimeric promoter constructs. The solid rectangles represent the TK sequences from −198 to +52. The shaded rectangles represent RR1 promoter from −438 to +198. The boxes to the right depict TATA sequences, the open boxes represent the sequence of the TK promoter, and the hatched boxes represent the sequence of the RR1 promoter. The boxes to the left depict the octamer/TAATGARAT sequence of the RR1 promoter. The plasmid names on the left provide the nucleotide boundaries between RR1 and TK sequences, separated by a *Bam*HI restriction enzyme site.

joined to 5′ deletions of the TK promoter derived from a series of TK LS mutations and fused to the CAT gene coding sequence. As shown in Fig. 4, six chimeric promoters were constructed from RR1 sequences spanning from −438 to +198 and TK sequences from −198 to +52. As depicted in Fig. 4, the plasmid with the shortest TK sequence contains RR1 sequences from −438 to −17 joined to TK sequences +3 to +52, separated by a *Bam*HI linker. It has been named pRR−17/TK+3 to reflect the junction point between the RR1 and TK promoter sequences. The five other chimeric promoter plasmids were named in a similar manner, reflecting increasing TK and decreasing RR1 sequences; these are pRR−37/TK−46, pRR−69/TK−69, pRR−94/TK−95, pRR−112/TK−109, and pRR−145/TK−109. It should be noted that in each of these constructs the RR1-TK junctions are separated by a *Bam*HI restriction enzyme site as a consequence of the method of their construction. However, none of the linker sequences disrupts any of the known transcriptional signals of either the RR1 or TK promoter.

These plasmids were tested for induction of CAT activity in cotransfections with pUC18 as a negative control or a plasmid containing either the VP16, α0, or α4 gene. Plasmids containing either the RR1 promoter region from −438 to +198 driving CAT (pRR1CAT) or the TK promoter region from −198 to +52 driving CAT were used as controls. The results of these transfections are summarized in Table 2. CAT activity was induced when the normal TK promoter plasmid was cotransfected with either the α4 or α0 plasmid but not when cotransfected with the VP16 plasmid. The α0 and VP16 cotransfections with the normal RR1 promoter plasmid gave significant CAT induction, in contrast to the absence of CAT activity with the α4 plasmid. All the chimeric promoter constructs were induced by ICP0, demonstrating that the sequences of both promoters were interchangeable with respect to ICP0 transactivation. All chimeric promoters containing the octamer/TAATGARAT element from the RR1 promoter responded to VP16 induction. pRR−145/TK−109, in which the octamer/TAATGARAT element was replaced with TK sequences, was not induced by VP16. These findings confirm the importance of this element for VP16 induction. Five of the six chimeric promoter constructs, pRR−37/TK−46, pRR−69/TK−69, pRR−94/TK−95, pRR−122/TK−109, and pRR−145/TK−109, were induced by ICP4. The sixth plasmid, pRR−17/TK+3, failed to respond to ICP4. Thus, replacing the RR1 bases between −36 and −17 with the TK −46 to +3 sequence was sufficient to impart ICP4 responsiveness, despite the fact that the rest of the RR1 promoter elements in pRR1−37/TK−46 from −438 to −37 remained intact. An examination of the nucleotide sequences of the two promoters (Fig. 2) revealed that pRR−37/TK−46 substitutes the TK TATA region for the RR1 TATA region and that pRR−17/TK+3 retains the RR1 TATA box (Fig. 2A and B). Therefore, the data suggest that responsiveness to *trans* activation by ICP4 resides in the bases around and including the TK TATA sequence of the promoter. The data further suggest that sequences upstream or downstream of the TATA homologies play little or no role in ICP4 inducibility. pRR−37/TK−46 contained all of the *cis*-acting regulatory sequences of the RR1 promoter except the TATA region, yet it was converted to ICP4 responsiveness. pRR−17/TK+3 contained TK 3′ bases from +3 to +52 but did not respond to ICP4 *trans* activation. We have not ruled out the possibility that the TK sequence downstream of the TATA region may have some modulatory effect on ICP4 induction when in *cis* to the TATA region.

ICP0 activates the RR1 promoter during infection. The previous sections of this article have established that the HSV-1 RR1 promoter was *trans* activated in transient transfection assays by ICP0 but not by ICP4. Most other HSV-1 genes are *trans* activated by both ICP0 and ICP4 in such assays. The important question is whether the RR1 promoter in infection is *trans* activated by ICP4 or ICP0 or both. To examine the role of these immediate-early gene products in the control of expression of the RR1 gene, it is necessary to eliminate the activation due to the VP16-octamer/TAATGARAT interaction. To test the hypothesis that the RR1 gene is activated by ICP0 during infection, four mutant viruses were constructed.

The first virus had the wild-type RR1 promoter driving the CAT coding sequence and the wild-type RR2 promoter driving the *lacZ* gene, both at their normal locations in the viral genome. This virus was designated TAATGARAT$^+$.

TABLE 2. Activation of RR1/TK chimeric promoters cotransfected with plasmids carrying the α4, α0, or VP16 gene

Plasmid	Fold induction of CAT activity after cotransfection with[a]:							
	pTK	pRR−145/TK−109	pRR−112/TK−109	pRR−94/TK−95	pRR−69/TK−69	pRR−37/TK−46	pRR−17/TK+3	pRR1
pVP16	1.1	1.0	6.5	7.7	11.6	8.2	16.7	11.0
pα0	13.8	10.1	6.9	11.2	14.9	12.5	16.7	16.5
pα4	17.0	15.4	12.9	8.4	9.3	8.9	1.0	1.0

[a] The values are averages of at least three independent transfections, each carried out in triplicate.

The second virus, designated TAATGARAT$^-$, was identical to the first virus, except that the octamer/TAATGARAT element was mutated at three different positions (see Materials and Methods). These mutations rendered the RR1 promoter unresponsive to VP16 *trans* activation in transfections (data not shown). The purpose in introducing these mutations into the octamer/TAATGARAT *cis*-acting element was to eliminate that portion of the induction of the RR1 gene due to VP16 *trans* activation, allowing the assessment of the roles of ICP0 and ICP4 in RR1 induction independent of that of VP16 during infection. The results of the infections described below suggest that this mutated sequence was, indeed, not responsive to VP16 induction during infection. Confirmation that the unresponsiveness of the octamer/TAATGARAT sequence to VP16 induction came from cycloheximide block-actinomycin D reversal infections (21a). TAATGARAT$^+$ and TAATGARAT$^-$ infections were carried out in the presence of cycloheximide, an inhibitor of protein synthesis, under conditions which allow the accumulation of message not requiring de novo protein synthesis. At 6 h postinfection, cycloheximide was removed and actinomycin D was added. Actinomycin D prevented further message synthesis, while the reversal of the cycloheximide block permitted the translation of message formed during the first 6 h. After an additional 2-h incubation with actinomycin D, infected cell extracts were prepared for CAT assays. The TAATGARAT$^+$ infection gave high levels of CAT activity, but the TAATGARAT$^-$ infection resulted in no CAT activity. It produced background levels similar to zero time point infections, the result expected if the mutations knocked out the responsiveness of the octamer/TAAT GARAT sequence to VP16 induction. The third and fourth viruses both carried the TAATGARAT$^-$ mutant RR1 promoter. The third virus, designated TAATGARAT$^-\Delta\alpha0$, in addition was deleted for most of the α0 gene coding sequence (the Δdlx 3.1 deletion [85]). The fourth virus, designated TAATGARAT$^-\Delta\alpha4$, was deleted for most of the α4 gene coding sequence (the d120 deletion [21]). The four viruses were used to infect Vero cells at a multiplicity of infection of 10 PFU per cell. At 6 h postinfection, cell extracts were assayed for CAT and β-Gal activities and compared with extracts of zero time point infections. Duplicate samples of the infected cells were incubated for 48 h, and then virus titers were determined. The results of the four infections are shown in Table 3.

The TAATGARAT$^+$ and TAATGARAT$^-$ infections induced CAT activity. The TAATGARAT$^-\Delta\alpha4$ infection also gave CAT activity. Since the mutant octamer/TAATGARAT element in this virus was not responsive to VP16 and since the α4 gene deletion should render that gene inactive, this result suggests that the observed CAT activity was the consequence of the *trans* activation of the RR1 promoter by a virus gene product other than ICP4. This suggestion was substantiated by the result of the TAATGARAT$^-\Delta\alpha0$ virus infection. In this infection, the infected cell lysate showed no induction of CAT activity over that of zero time point infections, directly implicating ICP0 in RR1 *trans* activation. This virus carried the normal α4 gene, yet no CAT activity was observed, indicating that in this infection ICP4 does not substitute for ICP0 for the induction of the RR1 promoter. The absence of any CAT activity in the TAATGARAT$^-\Delta\alpha0$ infection suggests that the mutations in the octamer/TAAT GARAT sequence have, indeed, eliminated the responsiveness to VP16 activation. We tentatively conclude that ICP0 *trans* activated the RR1 gene during infection.

The RR2 β-Gal activity results and virus yields provided excellent internal controls for the CAT induction assays and substantiated the conclusions drawn from the CAT induction data. In the first three infected lysates listed in Table 3, both β-Gal activities and virus yields were normal. In contrast, no β-Gal activity or virus production resulted from the TAATGARAT$^-\Delta\alpha4$ infection, the expected consequences of the absence of ICP4 *trans*-inducing activity. Yet in this infection a high level of CAT activity was detected, supporting the conclusion that ICP4 was not able to activate the RR1 promoter. In the TAATGARAT$^-\Delta\alpha0$ infection, ICP4 must be functional because (i) the β gene RR2 promoter was activated to produce β-Gal activity and (ii) a normal virus yield was obtained, yet no CAT activity was observed. The data support the conclusion that ICP4 cannot replace ICP0 in activating the RR1 gene. Taken together, the data permit the conclusion that the RR1 gene was *trans* activated in infection by ICP0 and not by ICP4 under the conditions of these infections.

TABLE 3. *trans*-activation of the RR1 promoter during infection

Infecting virus[a]	Fold induction of[b,c]: RR1-driven CAT activity	Fold induction of[b,c]: RR2-driven β-Gal activity	Virus yield[c]
TAATGARAT$^+$	42	15	1.0×10^9
TAATGARAT$^-$	27	17	4.2×10^8
TAATGARAT$^-\Delta\alpha0$	1.2	15	3.3×10^8
TAATGARAT$^-\Delta\alpha4$	34	0.7	$<10^2$

[a] In all viruses, the RR1 promoter drives CAT and the wild-type RR2 promoter drives *lacZ* reporter gene. TAATGARAT$^+$ has the wild-type RR1 promoter driving CAT. TAATGARAT$^-$ has a 3-bp mutation in the octamer/TAATGARAT element which eliminates its responsiveness to VP16. TAATGARAT$^-\Delta\alpha0$ has both the 3-bp mutation in the RR1 promoter and a deletion in the ICP0 gene. TAATGARAT$^-\Delta\alpha4$ has both the 3-bp mutation in the RR1 promoter and a deletion in the ICP4 gene.

[b] Fold induction, determined at 6 h, was calculated by using zero time point infection lysates as uninduced controls.

[c] Data are the averages of at least three independent infections carried out in triplicate.

DISCUSSION

The findings reported in this article support and expand the work of other laboratories and permit a more complete description of the regulation of the RR1 gene of HSV-1. The regulation of the RR1 gene has features of both α and β genes. The presence of an octamer/TAATGARAT sequence in the 5′ regulatory region of the HSV-1 and HSV-2 genes, which is responsive to *trans* activation by VP16 in transient transfection assays, accounts for the characteristics of α gene expression attributed to the RR1 genes: (i) RR1 message is transcribed within 2 h of the onset of infection (14, 43) in the presence of cycloheximide (98) and (ii) the RR1 protein is synthesized very early in infection (45, 46). As expected of a β gene during infection, the RR1 promoter is activated in the absence of VP16 induction (Table 3). This second induction, in agreement with the transfection data, is due to ICP0 *trans* activation. The RR1 promoter is thus unique among the HSV β and γ genes for its activation by ICP0. Further, the absence of CAT activity in the TAATGARAT$^-\Delta\alpha0$ infection allows the conclusion that none of the other α gene products can substitute for ICP0 in the induction of the RR1 gene in these infections. Thus the RR1 promoter is regulated in a dual manner and is responsive to both VP16 and ICP0 during infection.

Our findings provide an explanation for the production of the RR1 protein during infection with α4 and α0 gene mutants (21, 61, 65, 85). In α4 mutant infections, the *trans* activators of the RR1 gene, VP16 and ICP0, are functional and, therefore, the infection should result in RR1 protein synthesis. Some RR1 protein synthesis should occur in the α0 mutant infections as a consequence of VP16 *trans* induction. The VP16 activation may outweigh that caused by ICP0, or, in the absence of ICP0, activation as a result of an interaction between ICP4 and VP16 might be possible. We are currently attempting to determine the extent of the contribution of VP16 *trans* activation to the overall expression of the RR1 gene. Sze and Herman (93) reported that the RR1 promoter responds weakly to VP16.

The deletion analysis of the RR1 promoter and the studies of the RR1/TK chimeric promoters in transfections suggest a dominant role for the TATA region in the regulation of the RR1 gene. Deletions removing all of the recognizable RR1 regulatory sequences upstream or downstream of the TATA region have little or no effect on the induction of CAT activity by ICP0 in transfections. Deletion of the TATA region eliminates the induction of CAT activity by ICP0. The interchangeability of the *cis*-acting elements of the RR1 and TK promoters shows that they have little or no effect on *trans* activation by ICP0. The data suggest that the TATA region is capable of responding to ICP0 independently of the other elements. The specificity of the induction of the RR1 promoter by ICP0 and not by ICP4 also appears to reside in the TATA region. This conclusion derives from the results of transfections with the pRR−37/TK−46 and pRR−17/TK+3 chimeric plasmids. pRR−37/TK−46 contains the TK TATA region, TK sequences from −46 to +52, and all the regulatory sequences of the RR1 promoter except its TATA region. The acquisition by pRR−37/TK−46 of inducibility by ICP4, which is characteristic of the TK promoter, indicates that the RR1 upstream sequences by themselves play little or no part in the unique regulation of the RR1 promoter. The replacement of the TK TATA region with the RR1 TATA region, as in pRR−17/TK+3, results in the loss of ICP4 induction, suggesting that the TK sequences from +3 to +52 by themselves do not influence ICP4 induction. The data suggest that the acquisition of the response to ICP4 induction resides in the TK TATA region but do not rigorously rule out a modulatory role for the sequences downstream of the TK TATA element.

It must be pointed out that the RR1 TATA has an inherent capacity to respond to ICP4, as determined by transient assays. Deletion of the sequences between −217 and −62 allows the RR1 TATA region to respond to ICP4 induction, presumably as a consequence of bringing an upstream element closer to the TATA region (Fig. 3). Using a cell line stably transformed for the α4 gene, Persson et al. (76) demonstrated the induction of RR1 message after superinfection in the presence of cycloheximide. They interpreted the expression as being due to transactivation of the RR1 gene by ICP4 produced in the transformed cell. This result could also have been due to transcription as a result of the interaction of VP16 and Oct1 with the octamer/TAATGARAT sequence of the RR1 gene. These findings suggest that it may be premature to rule out a role for ICP4 in the induction of the RR1 gene.

The RR1 gene controls the expression of a number of phenotypes and functions. The RR1 protein is multifunctional, coding for protein kinase activity in addition to the large subunit of the reductase. The protein kinase and RR activities reside in nonoverlapping regions on the RR1 gene product, the kinase activity in the amino third of the protein and the reductase activity in the carboxyl two-thirds of the protein (2, 3, 12, 17, 18, 24, 58, 73). The kinases are of the serine/threonine type, and the HSV-2 enzyme is capable of phosphorylating both viral proteins and cellular substrates. The HSV-1 enzyme can autophosphorylate, but it is not clear that it is capable of transphosphorylation. The large and small subunits of RR of both HSV-1 and HSV-2 are themselves phosphorylated. Mutational separation of the kinase from the reductase has been achieved (12, 58). There is evidence that this separation also occurs by proteolysis of the RR1 protein during infection (56). The coding sequence of the HSV-2 RR1 gene also encodes a sequence responsible for the ability of the virus to induce morphological transformation and tumorigenic conversion (41, 51, 52). A 95-bp fragment derived from this sequence of the HSV-2 RR1 gene functions as a silencer when placed adjacent to a heterologous promoter (53). A novel origin of replication capable of using cellular factors to replicate viral DNA has been demonstrated within the coding sequence of the HSV-1 RR1 gene. It is suggested that this origin is active in neurons harboring latent virus (86). Finally, HSV-1 strains which contain mutations in the RR1 gene have been shown to be spectacularly less neurovirulent (8, 48, 95) and to be less able to reactivate from latency (50, 83) than the wild type. Whether neurovirulence or reactivation is correlated with the protein kinase or RR function of the protein or both remains to be determined. However, mutant virus in the RR2 gene is also greatly attenuated, suggesting that the reductase activity is the major factor in neurovirulence. The connection between the unique mode of regulation of the RR1 gene and any of the above-mentioned phenotypes is not clear.

Why is the RR1 gene regulated in this unique manner? The responsiveness to VP16 implies an immediate-early requirement for RR1 function during infection, and the *trans* induction by ICP0 implies a unique β-type regulation. The distinctive regulation of the RR1 gene is unlikely to be associated with the RR function of its gene product. Since enzyme activity requires the dimerization of the two subunits, the RR1 and RR2 genes might be expected to be regulated similarly. However, the RR2 gene is expressed with typical β gene kinetics, lacks VP16 responsiveness, and is *trans* activated by ICP4 and not by ICP0 during infection. It can be speculated that the unique regulation of the RR1 gene is related to the kinase activity of its gene product rather than to its reductase activity. As an early step in infection or reactivation, the protein kinase activity may be necessary to activate critical cellular and/or viral proteins for viral replication. *trans* activation of the RR1 gene by VP16 would account for rapid initial production of kinase activity during infection. Production of RR1 for assembly with the RR2 subunit resulting in active reductase may be the consequence of ICP0 *trans* activation. Recently, ICP0 was found to be associated with purified virions, probably located in the tegument (101). Whether these molecules have a functional role in infection remains to be determined.

Why is ICP0 and not ICP4 the immediate-early activator of the RR1 gene? ICP0 is a nuclear phosphoprotein with a molecular mass of 110 kDa (1, 74). It is a ubiquitous *trans* activator of all classes of HSV-1 and of eukaryotic genes in transfection assays (6, 25–27, 29, 34, 35, 62, 67, 68, 71, 72, 75, 79, 88). The α0 gene is diploid, encoded into the inverted repeat sequences of the unique long segment of the HSV genome. ICP0 is not essential for viral replication (85, 91); replication of mutant virus can be complemented by an

ICP0-like cellular function (7). ICP0 mutants show recognizable phenotypes under conditions in which the cellular ICP0-like function is minimally expressed. (i) The mutants grow poorly during infection at low multiplicities but achieve high titers after high-multiplicity infections (7, 11, 28, 85, 91). The expression of viral proteins is reduced during low-multiplicity infections with α0 mutant virus. (ii) Transfections with ICP0 mutant viral DNAs produce significantly less progeny virus than do transfections with wild-type viral DNA in Vero cells. ICP0 may act to enhance β and γ gene expression during productive infection (7, 11). (iii) Fewer cells transfected with mutant DNA express ICP4 than cells transfected with wild-type DNA. Cotransfection with an ICP0-expressing plasmid reverses this reduction and results in a full yield of progeny virus (7). (iv) ICP0 mutants reactivate poorly from latency (13, 57, 83). Since production of virus is initiated in transfection in the absence of VP16, Cai and Schaffer (7) suggested that transfection mimics reactivation from latency in this respect. They further suggested that ICP0 plays a central role in reactivation of virus from latency, perhaps through the regulation of induction of ICP4. The RR1 gene may also respond early in reactivation to induction by ICP0 to provide protein kinase activity for activation of other essential gene products and to provide RR to synthesize deoxyribonucleotides for viral DNA synthesis in the neuronal bodies. In this connection, it is of particular interest that an RR1 deletion mutant has been shown to be defective for reactivation from latency in mice (50).

We have produced a virus in which the RR1 promoter is replaced with the chimeric promoter present in pRR−37/TK−46. This is a virus in which the RR1 promoter should be *trans* activated by ICP4 during infection. Comparisons between this virus and virus carrying the wild-type RR1 promoter in infection and latency may provide clues to the unique role of ICP0 in the activation of the RR1 gene.

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