

A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus

(transcription enhancer/transcription factor binding site)

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ABSTRACT Using the simian virus 40 “enhancer trap” approach, we have identified a transcription enhancer located just upstream of the major immediate early gene of murine cytomegalovirus. This enhancer has several striking properties. (i) Together with the enhancer of human cytomegalovirus, it is the strongest transcription enhancer found to date. (ii) It is an extremely long enhancer, spanning >700 base pairs. (iii) It consists of a rather complex pattern of sequence repeats, the longest of which is 181 base pairs. Also, several types of short sequence motifs are scattered throughout the enhancer in monomeric, heterodimeric, or homodimeric (palindromic) form. These motifs have been identified to be components of other enhancers and promoters, and they are presumably binding sites for specific nuclear factors. Our analysis suggests that enhancers are composed of a modular arrangement of short conserved sequence motifs and that enhancer strength is correlated with the redundancy of these motifs.

Murine cytomegalovirus (MCMV) is a member of the highly diverse group of herpesviruses. Even among cytomegaloviruses of different host species there can be wide variation. For example, MCMV differs considerably from the human cytomegalovirus (HCMV) with respect to biological properties, immediate early (*IE*) gene organization, and overall nucleotide sequence. The 235-kilobase-pair (kbp) genome of MCMV also lacks large internal and terminal repeats characteristic of HCMV. Accordingly, no isomeric forms of the MCMV genome exist (1, 2).

As in other members of the herpes group, MCMV gene expression is temporally regulated. *IE* genes are expressed in the absence of protein synthesis, early gene products depend on the synthesis of at least one *IE* gene product, and late RNAs are synthesized after the onset of viral DNA synthesis.

MCMV has one major *IE* region (3, 4). This region codes for one major 2.75-kb and five minor polyadenylated *IE* RNAs (unpublished data). Low levels of *IE* transcripts have also been detected from both termini of the genome (3, 4). In an attempt to answer the question of how transcription is regulated, we decided to look for a transcription enhancer that might control the *IE* promoter.

Transcription enhancers are *cis*-acting DNA elements that are able to activate RNA polymerase II-transcribed genes in either orientation from a distance of up to several kbp from the promoter site (5, 6), even when located downstream of the transcribed sequences (ref. 5; for reviews, see refs. 7–9). Originally detected in papovaviruses, their presence has since been demonstrated in a number of animal viruses, including herpesviruses such as herpes simplex (10), HCMV

(11), and *Herpesvirus saimiri* (12). Some enhancers—e.g., the enhancers from simian virus 40 (SV40) and HCMV—can function in a number of different cell types, while others show a distinct host-cell preference. Enhancers that are associated with cellular genes—e.g., immunoglobulin genes (13–15), rat insulin (16), and a murine class II major histocompatibility antigen gene (17)—are often strictly cell-type specific.

The fact that in SV40 the enhancer can be substituted by heterologous enhancers has led to the development of the SV40 “enhancer trap,” whereby enhancerless SV40 DNA regains infectivity by incorporating an exogenous enhancer (18). The enhancer trap allows the selection of a transcription enhancer from a large excess of DNA. By cotransfecting enhancer trap DNA and the entire MCMV genome or DNA from its major *IE* region, we have been able to recover a very strong transcription enhancer upstream of the major *IE* region of MCMV. The MCMV enhancer has been sequenced and its location with respect to the *IE* region has been determined. Sequence analysis revealed a complex arrangement of repeats and also short DNA motifs found in other enhancers, which presumably are binding sites for nuclear proteins.

MATERIALS AND METHODS

Recombinant DNAs. *Xba* I- and *Kpn* I-digested enhancer trap DNA (1 μ g) (clone pET-1; ref. 18) was mixed with 10 μ g of sonicated MCMV-specific DNA and used for transfection of monkey kidney CV-1 cells by the calcium phosphate technique. Viral DNA was extracted from CV-1 cells, cloned into the *Bam*HI site of pBR327, and analyzed as described (18). The molecular cloning of MCMV DNA has been described (1, 4).

Cell and Virus Culture and Transfections. HeLa and CV-1 cells were cultured as described (18). Propagation of MCMV (strain Smith, ATCC VR-194) in BALB/c mouse embryo fibroblasts, purification of MCMV virus particles, viral DNA, and viral RNA have been described (1, 4).

Transfections using either the calcium phosphate or the DEAE-dextran technique were performed as described (18).

For transient expression assays, cytoplasmic RNA was isolated from HeLa cells 42–48 hr after transfection. S1 nuclease analysis has been described (19, 20).

Abbreviations: bp, base pair(s); kbp, kilobase pair(s); MCMV, murine cytomegalovirus; HCMV, human cytomegalovirus; SV40, simian virus 40; *IE*, immediate early.

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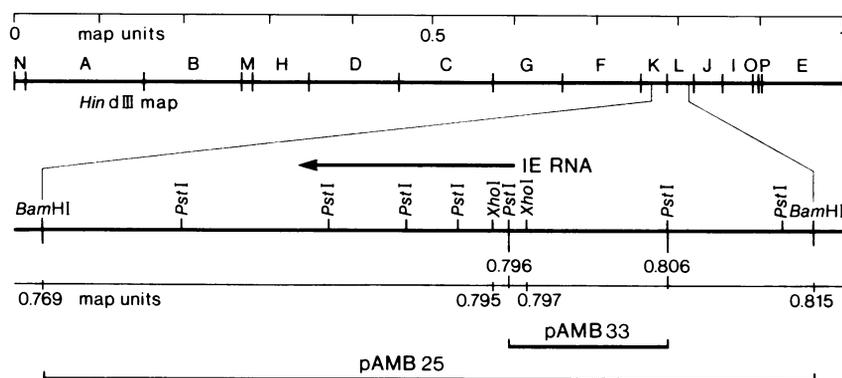


FIG. 1. *Hind*III restriction map of the MCMV genome. The region encoding the 2.75-kbp major IE transcript is expanded. The 10.8-kbp *Bam*HI fragment (clone pAMB25) and the 2.27-kbp *Pst* I fragment (clone pAMB33) were used together with enhancer trap DNA for cotransfection into monkey CV-1 cells. The *Xho* I fragment spanning map units 0.795–0.797 was used to determine the site of IE RNA initiation by S1 nuclease analysis. The direction of transcription of the 2.75-kb IE RNA is indicated by an arrow.

RESULTS

A Strong Transcription Enhancer in the MCMV Genome.

Although viral transcription enhancers usually are associated with *IE* genes (11, 21), we decided to screen the entire genome of MCMV, in addition to the region coding for IE mRNA, for the presence of transcription enhancers. Cloned enhancerless SV40 DNA (clone pET-1; ref. 18) was cotransfected into monkey kidney CV-1 cells with the following MCMV-specific DNAs: (i) total MCMV DNA isolated from purified virions; (ii) DNA from clone pAMB25, spanning map units 0.769–0.815 of the MCMV genome and coding for the major IE transcript; (iii) DNA from clone pAMB33, spanning map units 0.796–0.806 (Fig. 1) and containing the promoter region of the major IE transcript. In all cases, the CV-1 cells began to lyse 2–3 weeks after transfection, indicating that a viable SV40-like virus had been generated.

Recombinant SV40–MCMV viral DNAs were isolated and cloned in the bacterial plasmid pBR327. Individual recombi-

nant virus clones were reclaimed from the plasmid and tested for viability by transfection into monkey CV-1 cells using the DEAE-dextran method. Fifteen of 16 clones originating from transfection with the entire MCMV genome, 8 of 8 clones originating from the pAMB25 transfection, and 6 of 8 clones originating from the pAMB33 transfection were viable and lysed CV-1 cells 12–16 days after transfection. This is about the same time it takes to lyse the cells when transfection is performed with SV40 wild-type DNA or SV40–HCMV recombinant DNA (11).

By Southern blot hybridization, it was shown that all the analyzed clones hybridized exclusively to the pAMB33 fragment spanning map units 0.796–0.806 on the MCMV genome (data not shown), indicating that there is a strong enhancer located in the major *IE* region. The expectation that this enhancer activates transcription from the major IE promoter was confirmed by deletion analysis of the MCMV enhancer/promoter region (unpublished data). Additional enhancers, if they exist in the MCMV genome, must be weak,

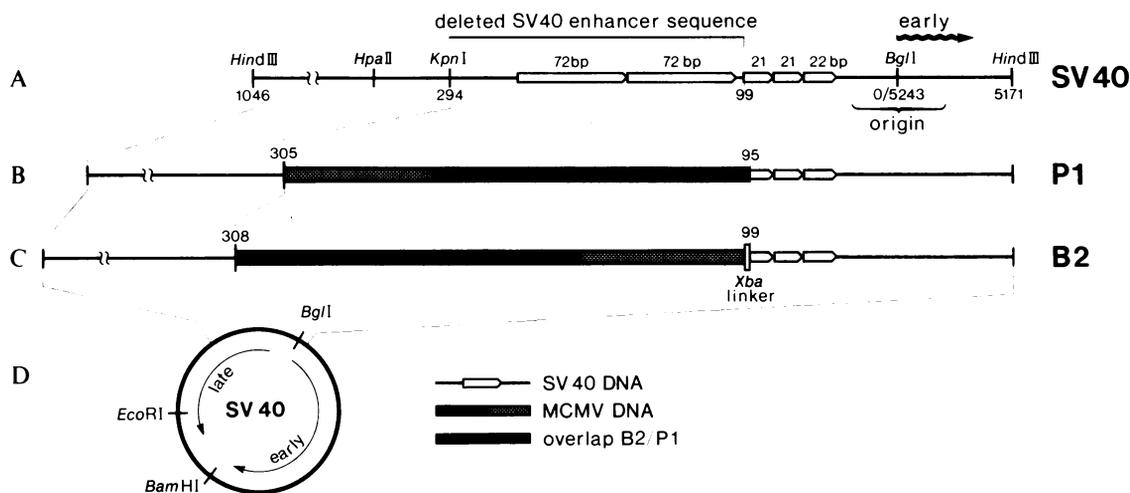


FIG. 2. The SV40 enhancer trap experiment. (A) *Hind*III C fragment of wild-type SV40 (nucleotide positions 5171–1046 of the SV40 map; see ref. 22). This fragment encompasses the origin of DNA replication, the major initiation site of early RNA, and the enhancer. The 21- to 22-bp repeats and the 72-bp repeats are shown by bold arrows. The SV40 enhancer trap clone pET-1 is an enhancer deletion mutant (nucleotides 99–294 are deleted) and has an *Xba* I restriction site inserted at position 99 (18). (B) *Hind*III C fragment from the recombinant SV40–MCMV P1 clone containing the MCMV enhancer. It originated from a cotransfection of enhancer trap DNA and the MCMV-specific 2.27-kbp pAMB33 fragment into CV-1 cells. The inserted MCMV sequence is 311 bp long. At the *Xba* I site, the *Xba* I linker and four nucleotides of SV40 DNA are deleted; at the *Kpn* I site, 11 nucleotides of SV40 DNA are deleted. (C) *Hind*III C fragment from the recombinant SV40–MCMV B2 clone. It was obtained by cotransfection of enhancer trap molecules with the MCMV-specific 10.8-kbp pAMB25 fragment into CV-1 cells. The MCMV insertion is 342 bp long and is inserted between the *Xba* I linker and the *Kpn* I site, where 14 nucleotides of SV40 DNA are deleted. The sequence overlapping in the MCMV enhancer segments present in the two recombinant clones is shown by black bars. (D) Map of the SV40 genome indicating the direction of early and late transcription units.

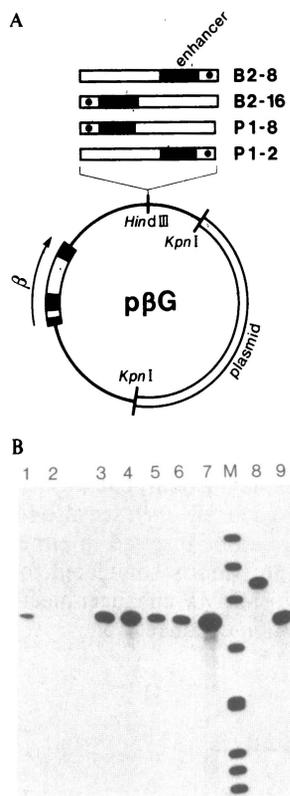


FIG. 4. Functional analysis of the MCMV enhancer. (A) To test the strength of the MCMV enhancer, the *Hind*III C fragments from the recombinants B2 and P1 (Fig. 2) containing the MCMV enhancer were inserted downstream of the rabbit β -globin gene in the *Hind*III site of clone p β G (26) in either orientation. Closed circle indicates the position of the SV40 origin of replication. (B) S1 nuclease assay. A β -globin gene clone lacking the first intervening sequence (27) was end-labeled at the *Bam*HI site. The single-stranded probe was hybridized to 20 μ g of cytoplasmic RNA that had been isolated from HeLa cells transfected with the enhancer-containing p β G clones. The hybrids were digested with S1 nuclease, denatured, analyzed by gel electrophoresis, and autoradiographed. Lanes: 1, p β G-WT3 containing the *Hind*III C fragment of SV40 wild-type DNA inserted downstream of the rabbit β -globin gene; 2, p β G-HC Δ E containing the *Hind*III C fragment from reclosed viral enhancer trap DNA lacking the enhancer sequence; 3, p β G-MCMV B2-8; 4, p β G-MCMV B2-16; 5, p β G-MCMV P1-8; 6, p β G-MCMV P1-2; 7, HCMV enhancer C4 located downstream of the rabbit β -globin gene (11); 8, full-length probe (453 nucleotides); 9, p β G-MCMV B2-8, the same clone as in lane 3, but a different plasmid preparation was used for transfection. The sequences protected from S1 nuclease digestion are 354 nucleotides long. Lane M, DNA marker (pBR322 cleaved with *Hpa* II).

Initiation Site of the 2.75-kb Major IE RNA. To determine the position of the MCMV enhancer relative to IE transcription, the initiation site of the major IE RNA was determined. S1 nuclease analysis was performed by using a single-stranded end-labeled *Xho* I fragment spanning the putative promoter region (Fig. 1, map units 0.795–0.797) and hybridizing it to RNA isolated from MCMV-infected cells 4 hr after infection. After S1 nuclease digestion, a single band of 280 nucleotides was detected (Fig. 5, lane 1). This band was not seen when RNA from uninfected mouse 3T6 cells was used or when the opposite strand of the *Xho* I fragment was used for hybridization (lanes 2 and 3). These data localize the initiation site of the major IE RNA (Fig. 3 A and C). The same result has been found by an alternative mapping technique, where 32 P-labeled antisense cRNA from the IE gene of MCMV was hybridized to IE RNA and the hybrids were analyzed by gel electrophoresis after digestion with RNases A and T1 (unpublished data). Analysis of the sequence

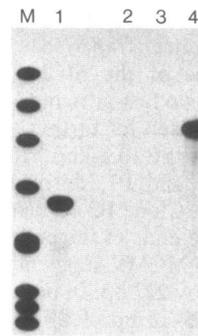


FIG. 5. Initiation site of the major IE RNA. Cytoplasmic RNA was isolated 4 hr after infection from mouse embryo fibroblasts infected with MCMV in the presence of cycloheximide (4). RNA (10 μ g) was hybridized to the end-labeled single-stranded MCMV *Xho* I fragment spanning map units 0.795–0.797 (see Fig. 1). The hybrids were digested with S1 nuclease, denatured, fractionated by gel electrophoresis, and subjected to autoradiography. Lanes: 1, hybridization of the single-stranded *Xho* I fragment to IE RNA; 2, the same probe hybridized to RNA from uninfected mouse 3T6 cells; 3, the opposite strand of the end-labeled *Xho* I fragment hybridized to MCMV IE RNA; 4, full-length probe; M, DNA marker (see Fig. 4).

upstream of the cap site reveals a TATA box located 26 bp upstream of the initiation site determined by S1 nuclease mapping (Fig. 3). The promoter-proximal end of the B2 enhancer segment is located 101 bp upstream of the initiation site. This is in agreement with all the other enhancer trap experiments where the most proximal promoter components of heterologous enhancer/promoters, notably the TATA box, seem to be excluded by the enhancer trap (refs. 11, 18, 28; unpublished data).

DISCUSSION

By using the SV40 enhancer trap, we have been able to locate a very strong enhancer within the MCMV genome. The enhancer sequence incorporated by the recombinant virus is very long (342 bp), as are enhancers from other members of the herpesvirus group (HCMV, *H. saimiri*; see refs. 11 and 12). Despite the fact that the genome organization of MCMV and HCMV is rather different and that no homology has been found by standard hybridization techniques (unpublished data), the enhancers from the two viruses share some features: the MCMV enhancer recovered by the SV40 enhancer trap is a very strong enhancer—apart from the HCMV enhancer, the strongest one found to date. In addition, the MCMV enhancer shows no obvious cell preference; it can be expressed in a number of different cell types—e.g., mouse 3T6 fibroblasts, human epithelial cells (HeLa), monkey kidney CV-1 cells, and frog kidney B cells (unpublished data).

A computer analysis of 885 bp of upstream sequence containing the enhancer indeed revealed a rather complex system of long repeated sequences, mostly located in the far upstream enhancer region and upstream of the region recovered by the enhancer trap (Fig. 3). This far upstream region also displays strong enhancer activity: in a functional test, a fragment of 840 bp (position about –1330 to –488; see Fig. 3) was found to contain at least as much enhancer activity as the MCMV enhancer sequence present in the SV40–MCMV recombinants (position –487 to –147; Fig. 3) (data not shown). These expression data and the structure of this DNA region with several repeats harboring conserved enhancer motifs (see below and Fig. 3) indicate that the MCMV enhancer is an extremely long enhancer, spanning over 700 bp. Its full potential is not revealed by enhancer trap experiments; because of the packaging constraint of the SV40 genome, only subsets of this long and highly redundant sequence can be incorporated.

Interestingly, the MCMV enhancer also harbors several short sequence motifs that have been identified functionally and/or structurally to be important components of other enhancers. The sequence CACCATTGACGTCAATGGG is organized as an inverted repeat (palindrome). It is almost identical to a 19-bp palindrome present in 5 and 12 copies in HCMV (11) and monkey cytomegalovirus (K.-T. Jeang and G. Hayward, personal communication), respectively. This palindrome, which can be viewed as a homodimer of two adjacent sequence motifs, occurs only once in the MCMV enhancer. Its right-hand monomer, however, is present another two times (Fig. 3B). An additional conserved sequence (TCAATAGGGACTTTCCA_A^T) exists within the

MCMV enhancer, which is homologous to an 18-bp sequence in the HCMV enhancer. In both enhancers, this sequence is present in several copies. Similar to the homodimeric palindrome, the 18-bp sequence seems to be composed of two adjacent sequence motifs (Fig. 3B), which are, however, not identical. Again, one motif (the left-hand half) of this heterodimer can be found an additional six times in the MCMV enhancer. The other (right-hand) motif constitutes an enhancer "core" that has been identified in a variety of enhancers (29) and is usually read in the complementary

strand as the GTGG^{AAA}_{TTT}G consensus sequence. The MCMV enhancer also harbors one copy of the adenovirus enhancer consensus sequence (21), one CCGCCC motif (24, 25, 30), and several CAAT boxes, often found inverted relative to the orientation generally described (23, 31). All these motifs have been determined to be important components of other enhancer/promoter regions (for reviews, see refs. 8 and 9).

The structure of the MCMV enhancer is compatible with our current view of enhancers having a modular structure with highly redundant information in which conserved motifs of several kinds are separated by less-conserved DNA sequences (8, 9). The conserved motifs apparently are binding sites for specific nuclear proteins. To date the only protein that has been characterized is the positively acting transcription factor Sp1, which binds to CCGCCC motifs (30). Each of the larger conserved sequences (the 19-bp palindrome and the 18-bp sequence) most likely represents two adjacent protein binding sites. Accordingly, we anticipate the palindrome to bind two identical factors and the 18-bp sequence to bind two nonidentical factors. A similar interpretation for binding of two identical factors can, for example, be drawn for the inverted repeat sequence regulating the heavy metal response of the mouse metallothionein I gene (32, 33). Thus, multiple copies of a variety of sequence motifs apparently contribute to the activity of the MCMV enhancer, and it seems that information redundancy ("the longer the better") is nature's way of obtaining particularly strong enhancers, such as the one of MCMV.

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