SEQUENCE SPECIFICITY IN TRANSCRIPTION AND TRANSLATION

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Contents

III. TERMINATOR SELECTION

IV. TRANSCRIPTIONAL REPRESSORS AND ACTIVATORS

Sequence Specificity in Transcription and Translation, pages 511-520

THE STRONG ENHANCER ELEMENT IN THE IMMEDIATE EARLY REGION OF THE HUMAN CYTOMEGALOVIRUS GENOME[1]

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ABSTRACT The human cytomegalovirus (HCMV), a member of the herpesvirus group, was found to possess a strong transcription enhancer in the immediate early gene region. Co-transfection of enhancerless SV40 DNA with randomly fragmented HCMV DNA yielded two SV40-like recombinant viruses, each containing HCMV DNA fragments that were substituting for the missing SV40 enhancer. The two inserts, 341 and 262 bp in length, are overlapping segments of genuine viral DNA representing part of the 5'flanking region of the major immediate early gene in HCMV. Studies with deletion mutants showed that different non-overlapping subsets of the HCMV enhancer region can substitute for the 72 bp repeats of SV40. Transient expression assays indicated that the HCMV enhancer is significantly stronger than the SV40 element, activating cis-linked heterologous promoters in a wide spectrum of cultured cells. It appears that the HCMV enhancer is positively regulated by viral immediate early genes.

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INTRODUCTION

Human cytomegalovirus (HCMV), one of the four herpesviruses of man, is a pathogenic agent of widespread occurrence. The virus has clinical importance in prenatal infections, and it causes severe pneumonitis and septicemia in immunocompromised patients. The genome of HCMV is a linear double-stranded DNA molecule of about 235 kbp. The virion DNA consists of a long (U_L) and a short (U_S) unique region, each flanked by a pair of inverted repeats (1, 2). Both regions, U_S and U_L, may be oriented in either direction, resulting in four isomeric forms of the viral genome (3). The viral genes are transcribed in three phases. The proteins of the immediate early (IE) phase seem to have regulatory functions and to be necessary for initiation of the second (early) phase of transcription. This phase is followed by DNA replication and synthesis of late gene products. Four IE genes are localized in the HindIII E-fragment of the HCMV strain AD169 (4). A polyadenylated 1.9 kb-transcript enconding a 72 Kd phophoprotein represents the most abundant IE RNA of HCMV (5). Transcription of this gene is blocked during the later phases of virus replication. The stregth of the major IE gene promoter seemed us to point to a possible role of an enhancer-like element in the control of this transcription unit.

A number of heterologous enhancers can substitute for the enhancer of SV40 or polyomavirus, resulting in viral DNA replication and T antigen expression (6, 7, 8, 9, 10). This allowed to detect enhancer elements in large genomes by an "enhancer trap" assay, if short randomly fragmented heterologous DNA was cotransfected with linearized SV40 DNA lacking its own enhancer. Incorporation of foreign DNA with enhancer function led to lytically growing SV40-type recombinants (11). Applying this method, we identified an enhancer sequence in the IE region of HCMV which is unusual in stregth and functional organisation.

RESULTS

1. Identification of HCMV sequences with enhancer activity

The enhancerless linear SV40 genome lacking the entire 72 bp repeat region was excised from the vector sequence of plasmid pET-1 (11).

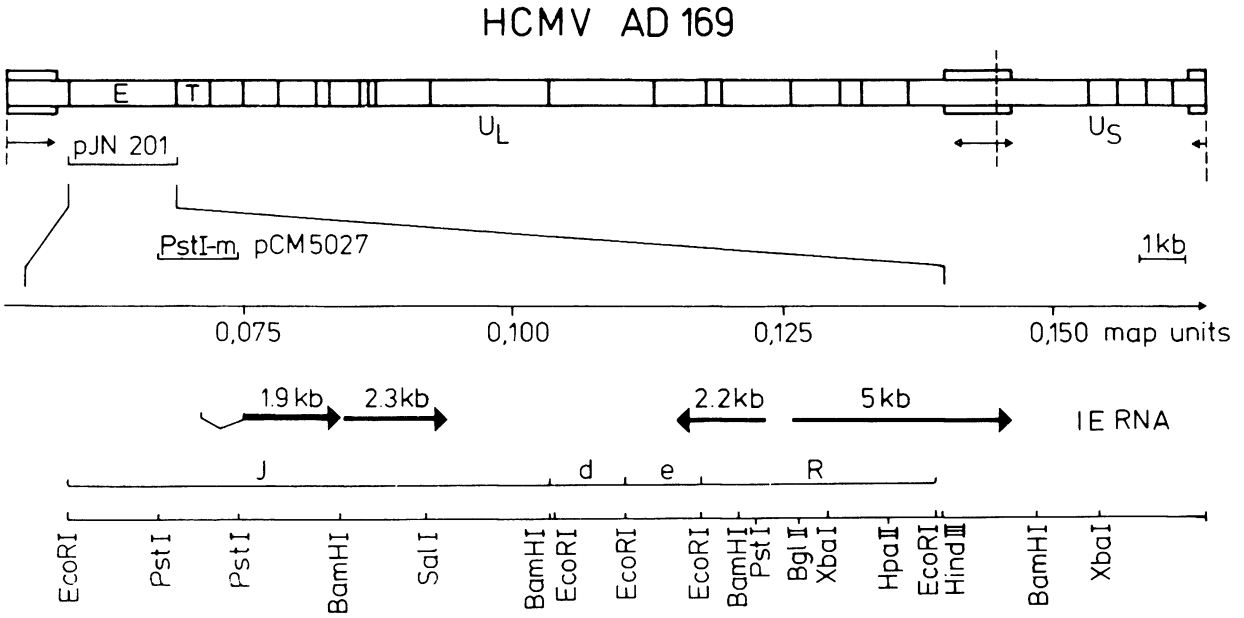

FIGURE 1. The 235 kbp genome of human cytomegalovirus strain AD169. The inverted repeats flanking the long (U_L) and the short (U_S) unique region of the genome are boxed. HindIII cleavage sites are indicated by vertical lines. The HindIII E-fragment spanning most of the immediate early (IE) region is magnified to demonstrate the map position of the PstI m-fragment, and the EcoRI sites within the IE region are indicated. The orientations of the major IE transcripts are indicated by arrows. The first intron in the 1.9 kb-gene is indicated.

Cotransfections of this enhancer trap with randomly sheared (~ 300 bp) DNA fragments of HCMV strain AD169 showed, that sequences from the promoter region of the dominant 1.9 kb-IE mRNA can restore SV40 growth (Fig. 1). Two replication competent SV40-HCMV recombinants, designated C2 and C4, were isolated and analyzed by DNA sequencing. The sizes of the integrated HCMV segments are 341 and 262 bp in recombinant C2 and C4, respectively. These sequences overlap by 196 bp. Both regions are identical with the genomic HCMV virion sequence. Concluding from structural similarities between HCMV strain AD169 and Towne (12), the inserted sequence of C2 was found to be located between -118 and -458 upstream of the cap site for the dominant 1.9 kb IE mRNA, and the segment contained in recombinant C4 represented nucleotides -263 to -524. Four repeated elements of 17 bp, 18 bp, 19 bp, and 21 bp length are found within the HCMV-enhancer region. The repeats are imperfect in part and are separated from each other by non-repetitive DNA (Fig. 2). The 18 bp sequence is repeated four times and shows an 11 bp homology with a core consensus sequence in the 72 bp repeats of SV40 (13). The 19 bp-sequence (repeated five times) froms perfect palindroms. The 21 bp repeats contain the hexanucleotide CCGCCC or the related motif CCCGCC reminiscent of the 21 bp repeats in the early SV40 promoter and of herpes simplex virus immediate early and early upstream regions (14, 15, 16). The recombinant viruses grew about as well as wild type SV40 on CV1 cells. T-antigen expression after transfection into various cells such as Hela, human fibroblasts, human hepatoma cells, mouse fibroblasts and frog kidney cells implicated a broad host range at least as wide as it is characteristic for the SV40 enhancer.

2. Stimulation of transcription of a cis-linked heterologous gene

HCMV enhancer sequences were excised from both recombinant viruses and were inserted in either orientation downstream of a cloned rabbit ß-globin gene. In parallel the PstI m-fragment of HCMV genomic DNA (Fig. 1) was cloned downstream of the ß-globin-DNA. Enhancement of ß-globin transcription was quantitated by S1 nuclease analysis of cytoplasmic RNA after transient expression in Hela cells. The HCMV sequences from the recombinents C2 and C4 enhanced the synthesis of correctly initiated ß-

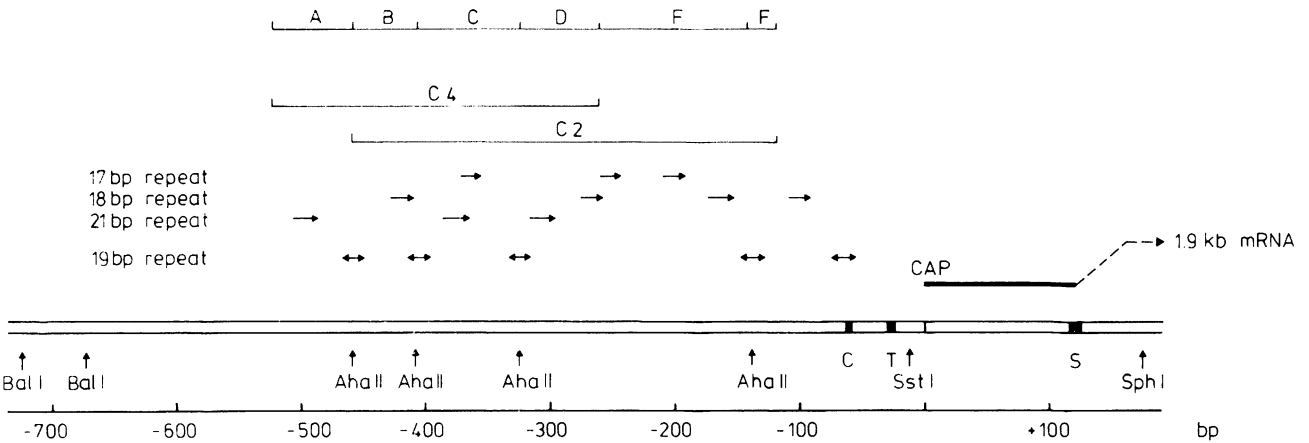

FIGURE 2. Schematic presentation of the enhancer region extending between nucleotides -118 and -524. Brackets indicate the enhancer region as defined by the recombinant viruses C2 and C4. The CAAT-(C) and TATA-(T) consensus sequences, first splice donor signal (S) and location of the capping site (CAP) for the 1.9 kb IE-mRNA are indicated. The elements of the four classes of imperfect direct repeats are represented by arrows. The domains of the enhancer region, defined by AhaII sites within the palindromic 19 bp repeat, are designated A, B, C, D, E, and F (table 1).

globin transcripts by at least two orders of magnitude irrespective of orientation. While the C2 sequence was about as active as the SV40 enhancer, the enhancer in recombinant C4 was three to five times stronger. Insertion of the HCMV PstI m-fragment, containing the complete enhancer promoter/region and the transcriptional start site of the 1.9 kb IE-mRNA, showed strong polar effects. In a construct, where the directions of transcription of the 1.9 kb IE-mRNA and the ß-globin mRNA are opposite to each other and the enhancer is separated from the ß-globin gene by plasmid DNA on one side and by the HCMV IE promotor on the other side almost no enhancing activity was detected. Transfection into Hela cells of a construct where the direction of RNA synthesis from the IE promoter of HCMV and from the ß-globin gene are identical, revealed synthesis of correctly initiated ß-globin transcripts in similar amounts as stimulated by the SV40 enhancer. The S1 nuclease analysis revealed an addtitional strong band of a shortened protected fragment (306 nucleotides); this is probably formed by aberrant splicing to an acceptor sequence at position +48 of the rabbit ß-globin gene (17). Transcription seems to be initiated from the strong IE promoter of HCMV, and the IE-RNA leader is spliced to the ß-globin sequences. Co-transfection of intact HCMV IE-genes (HindIII-E and EcoRIJ, see Fig. 1) with ß-globin/HCMV enhancer fusion plasmids resulted in significantly increased amount of correctly initiated ß-globin transcripts. It indicates that the HCMV enhancer is stimulated by gene product(s) that is (are) encoded in the 1.9 and 2.3 kb IE-mRNA.

3. Deletion mutagenesis in the enhancer sequence

The restriction enzyme Aha II cuts within the palindromic 19 bp repeat sequences, defining six structural domains in the HCMV-enhancer sequence (Fig. 2; Table 1). Deletion mutants of the SV40-HCMV recombinants were constructed with partial AhaII digestion products of recombinants C2 and C4. Transfection into CV1 cells revealed that smaller deletions had little or no effect on viral replication and T-antigen expression. Large deletions reduced T-antigen expression and resulted in slow virus growth. However, remarkably, each single deletion mutant was viable, thus retaining at least the minimum enhancer activity allowing replication of viral DNA.

TABLE 1
COMPOSITION OF SV40-HCMV RECOMBINANTS WITH DELETIONS IN THE HCMV-ENHANCER REGION

Domains of the enhancer region	A	B	C	D	E	F	
	64	53	83	62	124	21	(bp)
Recombinant virus C2	-	+	+	+	+	+	
" " C4	+	+	+	+	-	-	
Deletion mutant #1	-	+	+	+	-	-	
" " 2	+	-	+	+	-	-	
" " 3	+	+	-	+	-	-	
" " 4	+	+	+	-	-	+	
" " 5	+	-	-	+	-	-	
" " 6	+	+	-	-	-	+	
" " 7	-	+	-	+	-	-	
" " 8	-	+	+	-	-	+	
" " 9	+	-	-	-	-	+	
" " 10	-	+	-	-	-	+	

DISCUSSION

The SV40 enhancer trap system allowed to identify a functional enhancer region upstream of the major immediate early gene of HCMV strain AD169. The region extends between nucleotides -118 and -524 upstream of the capping site. The enhancer stimulates correctly initiated transcription, if it is inserted downstream of a cloned rabbit ß-globin gene in either orientation. The HCMV-enhancer region has a host range at least as wide as the SV40 enhancer. Deletion mutants containing non-overlapping subsets of the enhancer can still substitute for the SV40 72 bp repeats.

The herpesvirus group is highly heterogenous in the functional genome organisation. Some herpesviruses such as Herpesvirus saimiri or pseudorabies virus have a single immediate early region (18, 19). Other herpesviruses have several IE-transcription units, for example herpes simplex virus (HSV), which contains five IE-genes. These IE-genes of HSV are stimulated in trans by a structural component of the virus particle. The regulation is mediated by far upstream regions, and it is suggested that these regions

share a consensus motif TAATGARATNC which plays an important role for trans-activation (15, 20, 21, 22, 23, 24). The sequence mediating the trans-activation for the HSV IE-mRNA 3 has weak enhancer-like effects, if fused with the HSV thymidine kinase gene (25, 26). Sequence comparison of the upstream regions of the five HSV IE genes with the HCMV-enhancer region did not reveal homologies $\geq$ 70% to any of the repeated motifs in the HCMV-enhancer, and the TAATGARATNC motif was not found in the HCMV sequence. The sequence dissimilarities appear to reflect different mechanisms in the regulation of IE-transcription in HSV and HCMV, correlating with the known fundamental differences in the biology of those herpesviruses.

It has been shown that duplications can increase the activity of enhancer elements (11, 27). The HCMV enhancer region contains four groups of directly repeated sequences. The observation that different subsets of the HCMV enhancer substitute for the SV40 72 bp repeats may suggest that the strong enhancer sequence of HCMV has arisen through amplification and diversification of an original element. This may explain why the HCMV enhancer sequence is relatively long and one of the strongest of these elements known so far.

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