Key words: cytomegalovirus, human/Enhancer/immediate early genes

The Role of a Repetitive Palindromic Sequence Element in the Human Cytomegalovirus Major Immediate Early Enhancer

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SUMMARY

The major enhancer, extending from nucleotides −530 to −120 upstream of the transcription initiation site of immediate early (IE) genes 1 and 2 in human cytomegalovirus (HCMV), contains four groups of repeated sequence motifs that consist of 17, 18, 19 or 21 bp, respectively. One of these elements, the 19 bp repeat, is a symmetrical palindrome that is also part of IE regulatory sequences of other cytomegalovirus-type herpesviruses, but not of unrelated members of the herpesvirus group. Synthetic oligonucleotides representing the 19 bp repeat unit strongly reduced the activity of the IE1/2 enhancer/promoter in cotransfection assays after transient expression. The HCMV enhancer can substitute for the 72 bp repeats of simian virus 40 (SV40). Replication-competent deletion mutants of SV40/HCMV enhancer recombinants were constructed that contained a single palindromic 19 bp repeat with a central cleavage site for AhaII. If deletions were introduced into the single remaining 19 bp repeat most of the mutant viruses were still replication-competent in CV-1 monkey kidney cells. Insertion of two nucleotides into the single AhaII site did not significantly alter transient SV40 T antigen expression. Deletion of four nucleotides or more from the single 19 bp palindrome reduced the stimulation of T antigen synthesis by the HCMV enhancer/SV40 promoter unit down to about 50%. More extended deletions (28 to 80 bp) did not further reduce T antigen expression. All mutants without an intact 19 bp repeat contained the 18 bp and/or the 21 bp sequence motif. DNase I footprinting and gel retardation assays indicated sequence-specific protein binding by the 19 bp palindrome. Altered palindromes, correlating with reduced enhancer activity, lost most of their protein-binding properties. Thus, the 19 bp repeat element is one of several protein-binding sites that contribute to enhancer strength. However, the 19 bp sequence motif can be deleted entirely to leave reduced activity. The HCMV IE1/2 upstream sequence appears to be the perfect model of an enhancer as a complex of multiple binding sites for trans-activating proteins in a modular fashion.

INTRODUCTION

Cis-acting transcription-enhancing DNA sequences were first identified upstream of the early promoters in small DNA viruses, namely in group C human adenoviruses (Hearing & Shenk, 1983; Hen et al., 1983) and in papovaviruses, such as simian virus 40 (SV40) (Banerji et al., 1981; Benoist & Chambon, 1981; Gruss et al., 1981), mouse polyoma virus (De Villiers & Schaffner, 1981), human BK virus (Rosenthal et al., 1983), simian B-lymphotropic papovavirus (Mosthaf et al., 1985) and various papillomaviruses (Spalholz et al., 1985; Haugen et al., 1987; Seeberger et al., 1987). Enhancer elements were also localized in a long translational reading

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frame of hepatitis B virus DNA upstream of the cap site for core antigen mRNA (Shaul et al., 1985), and within the long terminal repeats of proviral DNA from retroviruses, e.g. Moloney murine sarcoma virus (Laimins et al., 1984). As various viral enhancer sequences were shown to substitute in general for the major functional enhancer domains of SV40 (De Villiers et al., 1982; Levinson et al., 1982; Tognoni et al., 1985; Swift et al., 1987), a ‘trap assay’ was developed for the detection of enhancer sequences in the complex genomes of large viruses such as herpesviruses (Weber et al., 1984). Linearized SV40 genomes with the two major functional enhancer domains deleted (Davidson et al., 1986) were cotransfected into permissive cells with randomly fragmented DNA of two betaherpesviruses, human cytomegalovirus (HCMV) (Boshart et al., 1985) and mouse cytomegalovirus (Dorsch-Häsl er et al., 1985), and with DNA from the gamma-2-herpesvirus herpesvirus saimiri (Schirm et al., 1985). Intracellular ligation then resulted in replication-competent SV40 recombinant viruses with herpesvirus sequences replacing the deleted SV40 enhancer sequences. The respective regulatory DNA sequence from HCMV was identified as a very strong cis-acting element that fulfilled all essential criteria of a eukaryotic transcription enhancer. The HCMV enhancer has been shown to stimulate transcription of fused genes in transient expression assays (Boshart et al., 1985) and after stable integration (Foecking & Hofstetter, 1986). It is active in a broad spectrum of cell types, including frog kidney cells (Boshart et al., 1985). It is also remarkably effective in in vitro assays for RNA polymerase II enhancement using HeLa cell extracts (Thomsen et al., 1984; Ghazal et al., 1987, 1988b).

A block of four genes from the HCMV genome (strains AD169 and Towne), immediate early (IE) genes, is constitutively expressed in the initial phase of virus replication (Stinski et al., 1983; Jahn et al., 1984; Wilkinson et al., 1984). The HCMV enhancer sequence was found to extend between nucleotides (nt) −530 and −120 upstream of the transcription initiation site for the major IE gene (IE1) (Boshart et al., 1985) which encodes a nuclear phosphoprotein of 64K, as derived from the size of the single larger open reading frame in the threefold spliced mRNA (Stinski et al., 1983; Stenberg et al., 1984, 1985; Akrigg et al., 1985). Additional regulatory domains have been identified in the far upstream region of IE1. This region, termed the ‘modulator’, is implicated in suppression of IE1 transcription in undifferentiated teratocarcinoma cells (Nelson & Groudine, 1986; Nelson et al., 1987). The major IE enhancer/promoter unit of HCMV strain AD169 is strictly switched off during later phases of virus replication (Jahn et al., 1984). The sequence motifs that mediate silencing of the IE regulatory unit through virus-encoded polypeptides have not been identified so far.

Nucleotide sequencing of the HCMV enhancer revealed an array of four groups of direct repeats, consisting of 17, 18, 19 or 21 bp (Thomsen et al., 1984; Akrigg et al., 1985; Boshart et al., 1985; Stinski & Roehr, 1985). Each sequence element is present three to five times; some repeats are imperfect and separated by irregular stretches of non-repetitive DNA. The enhancer is preceded by four binding sites for nuclear factor 1 (NF1) (Hennighausen & Fleckenstein, 1986; Jeang et al., 1987). Enhancers of similar structure and strength have also been identified within the IE genes of simian and murine cytomegaloviruses. The simian cytomegalovirus enhancer (Jeang et al., 1984, 1987; Mosca et al., 1987) has at least 20 NF1-binding sequences in the upstream region. The murine cytomegalovirus IE enhancer, however, contains longer (94 bp) abutting sequence elements that are sixfold repeated (Dorsch-Häsl er et al., 1985; Koszinowski et al., 1986). More recently, Weston (1988) reported a new IE enhancer from HCMV, located in the short unique Us region. The HCMV Us IE enhancer reiterates two sequence motifs, one of which is the known 18 bp sequence from the major HCMV IE enhancer. Footprinting analyses have shown that each of the HCMV repeat elements correlates with a specific sequence site of DNA–protein interaction (Ghazal et al., 1987, 1988a).

The 19 bp repeat of the major HCMV IE enhancer is remarkable in structure, as 18 nt of it form a symmetric palindrome. Deletion analyses by Boshart et al. (1985) and by Stinski & Roehr (1985) suggested that the 19 bp palindromic repeat is a functionally important component of the HCMV enhancer. Further studies by Ghazal et al. (1988b) showed that synthetic oligonucleotides representing 19 bp repeats competed for transcription factors in an in vitro assay using HeLa cell nuclear extracts. The work described in the present paper was aimed at
Palindromic repeat in HCMV enhancer

a functional definition of the 19 bp palindrome by competition in vivo with synthetic oligonucleotides, by extensive deletions, by replacement of the SV40 enhancer with short HCMV sequence modules and by correlating protein-binding properties with enhancer function.

METHODS

Virus and cell culture. CV-1 cercopithecus kidney cells were obtained from W. Schaffner (Zürich, Switzerland), and cultured as described by Weber et al. (1984). An SV40 wild-type virus stock was also a gift from W. Schaffner.

Plasmid cloning. The vector clone psIEMBL was a gift from H. Lehrach, London, U.K. The unique AhaII site was destroyed by a filling-in reaction with DNA polymerase I large fragment (Klenow), resulting in psIEMBL'. This served as a cloning vector for deletion clones 5, 7, 9 and 10 (Boshart et al., 1985). Cloning reaction conditions followed the suppliers' manuals. Bal 31 shortening reactions were done according to Silhavy et al. (1984), and filling-in reactions with Klenow fragment followed a protocol described by Maniatis et al. (1982). Plasmid DNA was isolated from bacterial cells by disintegration with N-lauroyl sarcosinate and shearing of chromosomal DNA (Rüger et al., 1984) or by disintegration with Triton X-100 (Messing, 1983). Quick lysates of bacteria containing plasmids were prepared according to the protocols of Holmes & Quigley (1981) or Zagursky et al. (1985). Viral DNA from cell cultures was obtained by Hirt extraction (Hirt, 1967) or by a total cellular lysis protocol (Rüger et al., 1984).

Nucleic acid hybridization, oligonucleotide synthesis and DNA sequencing. 'Bluescribe' (Vector Cloning Systems, San Diego, Ca., U.S.A.) and pUC18 subclones were sequenced from double-stranded DNA as described by Zagursky et al. (1985). Oligonucleotides were generated by the phosphoramidite procedure using a Cyclone DNA synthesizer (Biosearch, San Rafael, Ca., U.S.A.) and purified by PAGE and ion-exchange chromatography through DEAE-Sephadex G-25 columns (Pharmacia). Southern blots were done according to standard protocols (Rüger et al., 1984).

Transfection assays. Transfection experiments were performed by the calcium phosphate precipitation method (Graham & Van der Eb, 1973) on approximately 80% subconfluent monolayers of CV-1 or HeLa cells. The indirect immunofluorescence essay assays were done as described by Banerji et al. (1981), as modified in the manual of Oncogene Science (Mineola, N.Y., U.S.A.) who supplied a monoclonal antibody against the SV40 large T antigen (AB-1). A goat fluorescein isothiocyanate-labelled antibody directed against mouse immunoglobulin was purchased from EY Laboratories (San Matteo, Ca., U.S.A.) and AhaII site of pUC18. The resulting expression clone pRR55 (10 μg) was cloned into the CAT plasmid (10 μg) with increasing amounts (up to 15-fold molar excess) of plasmid pRR63 as a functional definition of the repeated 19 bp palindrome. Enhancer/promoter activity was tested in transient expression assays by competition with synthetic oligonucleotides. A DNA fragment containing the HCMV IE1/2 enhancer and promoter between a BalI cleavage site at nt −671 and a HpaII site at nt +52 (Fig. 1) was cloned in front of the CAT gene using the pUC18 vector. The CAT gene fragment contained 701 nt of prokaryotic DNA, the small SV40 T antigen intron and an SV40 polyadenylation signal (Gorman et al., 1982). Transfection of the resulting expression clone pRR55 (10 μg) into 3 x 10^6 subconfluent HeLa cells gave high CAT enzyme activity upon transient expression, as determined by conversion of 14C-labelled chloramphenicol to acetylated forms in standard thin-layer chromatography. A synthetic double-stranded oligonucleotide representing a 19 bp repeat sequence (Fig. 1; nt −469 to nt −451) was cloned into the SmaI site of pUC18. The resulting clone (pRR63) contained five tandemly inserted copies of the 19 bp repeat. Cotransfection of the CAT plasmid (10 μg) with increasing amounts (up to 15-fold molar excess) of plasmid pRR63 as

RESULTS

Competition in vivo by synthetic oligonucleotides

As a first approach to defining the functional relevance of the repeated 19 bp palindrome, enhancer/promoter activity was tested in transient expression assays by competition with synthetic oligonucleotides. A DNA fragment containing the HCMV IE1/2 enhancer and promoter between a BalI cleavage site at nt −671 and a HpaII site at nt +52 (Fig. 1) was cloned in front of the CAT gene using the pUC18 vector. The CAT gene fragment contained 701 nt of prokaryotic DNA, the small SV40 T antigen intron and an SV40 polyadenylation signal (Gorman et al., 1982). Transfection of the resulting expression clone pRR55 (10 μg) into 3 x 10^6 subconfluent HeLa cells gave high CAT enzyme activity upon transient expression, as determined by conversion of 14C-labelled chloramphenicol to acetylated forms in standard thin-layer chromatography. A synthetic double-stranded oligonucleotide representing a 19 bp repeat sequence (Fig. 1; nt −469 to nt −451) was cloned into the SmaI site of pUC18. The resulting clone (pRR63) contained five tandemly inserted copies of the 19 bp repeat. Cotransfection of the CAT plasmid (10 μg) with increasing amounts (up to 15-fold molar excess) of plasmid pRR63 as
The palindromic 19 bp repeats of the HCMV enhancer contain a recognition site for the restriction endonucleases \textit{AhaII} and \textit{AatII}, but no other reiterations or non-repetitive sequences in the HCMV enhancer and SV40 DNA are cleaved by these enzymes. In order to construct a series of deletion mutants from the HCMV-SV40 recombinants, the recombinants C2 and C4 (Fig. 1) were cleaved to completion with \textit{AhalI}, religated, and cloned into vector pUC8 by using the single \textit{BamHI} site, resulting in deletion mutants 5, 7, 9 and 10 as described by Boshart et al. (1985). Each deletion variant containing a single 19 bp repeat expressed SV40 T antigen when transfected into CV-1 monkey kidney cell cultures. Releasing the HCMV-SV40 recombinant DNA from the pUC vector by \textit{BamHI} digestion and transfecting it into permissive cells resulted in replication-competent viruses that had a reduced growth rate. In subsequent experiments, the HCMV-SV40 hybrids were cloned as \textit{BamHI} fragments into the vector pslEMBL in which the single \textit{AhaII} cleavage site had been destroyed. The map positions of HCMV and SV40 sequences in the resulting clones pRR27, pRR36, C66.7 and D10.7 relative to the original viruses are schematically summarized in Fig. 4. Each of these clones was linearized by cleavage

Fig. 1. Nucleotide sequence of the HCMV IE1 enhancer/promoter unit. The extension of the HCMV-specific sequences within the SV40-HCMV recombinants C2 and C4 are indicated with brackets. The four groups of repeated motifs are underlined: \(\ldots\) 21 bp; \(\ldots\) 19 bp; \(\ldots\) 18 bp; \(\ldots\) 17 bp. The repeat units homologous to the synthetic oligonucleotides used in competition assays are underlined prominently. The \textit{AhaII} restriction sites within the palindromic 19 bp repeats are indicated by arrows as well as the \textit{Ball-HpaII} fragment fused to the CAT gene in the plasmid pRR55. CAAT and TATAA consensus sequences, the initiation site of transcription and a splice donor motif are boxed.

competitor resulted in significant reduction of CAT expression (Fig. 2). For all \textit{in vivo} competition experiments, plasmid pUC18 was added to reach a final amount of 210 \(\mu\text{g}\) DNA in each transfection assay. Parallel experiments with the 18 bp repeats (Fig. 1; nt \(-428\) to nt \(-411\)), cloned in the plasmid pRR64 as a dimer in tandem orientation, showed strong reduction in CAT activity (down to 1.5 \(\%\) at 1:14 molar excess; Fig. 3, Table 1). Competition with the two types of repeats at intermediate concentration (molar ratio 1:7 for both cloned repeats) did not reach the same degree of CAT reduction (Fig. 3), indicating that the two oligonucleotide sequences are recognized by different transcription factors, independently.

These data could be taken as evidence that the 19 bp palindrome, like the 18 bp repeat, acts as a protein-binding site that functionally contributes to the overall transcription-stimulating activity of the HCMV major IE enhancer.

\textit{Construction of deletion mutants}

The palindromic 19 bp repeats of the HCMV enhancer contain a recognition site for the restriction endonucleases \textit{AhaII} and \textit{AatII}, but no other reiterations or non-repetitive sequences in the HCMV enhancer and SV40 DNA are cleaved by these enzymes. In order to construct a series of deletion mutants from the HCMV-SV40 recombinants, the recombinants C2 and C4 (Fig. 1) were cleaved to completion with \textit{AhaII}, religated, and cloned into vector pUC8 by using the single \textit{BamHI} site, resulting in deletion mutants 5, 7, 9 and 10 as described by Boshart et al. (1983). Each deletion variant containing a single 19 bp repeat expressed SV40 T antigen when transfected into CV-1 monkey kidney cell cultures. Releasing the HCMV-SV40 recombinant DNA from the pUC vector by \textit{BamHI} digestion and transfecting it into permissive cells resulted in replication-competent viruses that had a reduced growth rate. In subsequent experiments, the HCMV-SV40 hybrids were cloned as \textit{BamHI} fragments into the vector pslEMBL in which the single \textit{AhaII} cleavage site had been destroyed. The map positions of HCMV and SV40 sequences in the resulting clones pRR27, pRR36, C66.7 and D10.7 relative to the original viruses are schematically summarized in Fig. 4. Each of these clones was linearized by cleavage.
Palindromic repeat in HCMV enhancer

Fig. 2

Palindromic repeat in HCMV enhancer

Fig. 3

Table 1. HCMV enhancer activity under competition with synthetic oligonucleotides

<table>
<thead>
<tr>
<th>Transfected and cotransfected plasmids</th>
<th>DNA (µg)</th>
<th>Molar ratio of repeated elements</th>
<th>pRR55 activity (%)</th>
<th>CAT conversion (% acetylation)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRR55 + pUC18</td>
<td>200</td>
<td>1:14</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>pRR55 + pRR63</td>
<td>80</td>
<td>-</td>
<td>8</td>
<td>6.6</td>
</tr>
<tr>
<td>pRR55 + pRR64</td>
<td>120</td>
<td>1:14</td>
<td>1:5</td>
<td>1:2</td>
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<tr>
<td>pRR55 + pRR63 + pUC18</td>
<td>200</td>
<td>1:14</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>pRR55 + pUC18</td>
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<td>1:14</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>pRR55 + pUC18 + pRR63</td>
<td>100</td>
<td>1:14</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>pRR55 + pUC18 + pRR64</td>
<td>60</td>
<td>1:7</td>
<td>36</td>
<td>30</td>
</tr>
</tbody>
</table>

* Acetylation rate calculated in relation to the basal activity of expression plasmid pRR55.
Fig. 4. Schematic maps of SV40-HCMV recombinants. The first two lines show the position of the 72 bp and 21/22 bp repeats in the SV40 genome and the enhancer trap molecule. The vertical lines in between show identical map positions of cleavage sites, the origin of replication, and the borders for inserted HCMV DNA. The locations of the HCMV sequences from the recombinants C2 and C4 are given for the third line. Plasmids pRR27, pRR36, C66.7, and D10.7 contain deletion mutations of C2 or C4 with a single 19 bp motif, which is reconstituted from two half repeat motifs after AhaI digestion. The DraI and HpaII restriction sites used for cloning of the various deletion mutants into 'Bluescribe' vectors are indicated. Map locations of the four groups of repeated elements in the HCMV enhancer sequence are symbolized by arrows at the bottom.

A series of deletion mutants derived from the HCMV enhancer-SV40 recombinants C2 and C4 was tested for the ability to give rise to replicating SV40-like viruses. The clones described in
Palindromic repeat in HCMV enhancer

(a) Schematic map of the deletion mutants generated from the plasmids pRR27, pRR36, C66.7 and D10.7 after Bal 31 and Klenow polymerase treatment. The nucleotide positions of the *Ahal* sites in the middle of the 19 bp repeats and of the borders of C2 and C4 are indicated on top. The four types of dashed columns represent the relative location of the repetitive elements within the HCMV IE1/2 enhancer (C4, 17 bp; C2, 18 bp; C1, 19 bp; C1T, 21 bp). The deletion mutants are arranged in groups, each preceded by the respective clone that resulted from *Ahal* digestion and religation (pRR27, pRR36, C66.7, D10.7). The extents of the deletions are shown for the nucleotide sequence and in comparison with the localizations of the repeated elements and the *Ahal* sites. For the sequence data the first and the last remaining nucleotides after Bal 31 digestion are given in nt numbers (Fig. 1). +1 or +2 indicates an insertion after a filling-in reaction with the Klenow fragment. (b) Mutations within the palindromic 19 bp repeat. The 19 bp motif is shown on top as a dsDNA sequence; the central *Ahal* site is marked. Below, the sequences of particular mutated clones are given for the 19 bp motif only. The two vertical lines stand for the *Ahal* site; insertions are written in between; • indicates the deletion of one nucleotide pair. The length of the mutation is indicated by + for insertions and - for deletions.
Table 2. Replication competence of recombinant deletion mutants

<table>
<thead>
<tr>
<th>Virus deletion</th>
<th>Time from transfection to c.p.e. (&gt;80% lysis) in days*</th>
<th>Virus growth verified by hybridization</th>
<th>Time from infection (2nd passage) in days†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRR27</td>
<td>22 ± 10</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>2701</td>
<td>24 ± 5</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>27151</td>
<td>38 ± 14</td>
<td>NT‡</td>
<td>8</td>
</tr>
<tr>
<td>pRR27.1</td>
<td>30</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>27301</td>
<td>46 ± 8</td>
<td>NT</td>
<td>8</td>
</tr>
<tr>
<td>pRR27.2</td>
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<td>NT</td>
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<td>7</td>
</tr>
<tr>
<td>pRR27.4§</td>
<td>&gt;80</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>pRR27.5§</td>
<td>&gt;80</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>pRR27.15</td>
<td>29</td>
<td>NT</td>
<td>7</td>
</tr>
<tr>
<td>3604</td>
<td>58</td>
<td>NT</td>
<td>9</td>
</tr>
<tr>
<td>36154</td>
<td>48 ± 6</td>
<td>NT</td>
<td>9</td>
</tr>
<tr>
<td>36301</td>
<td>49 ± 10</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>C66.7</td>
<td>27</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>D10.7</td>
<td>57</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>C4</td>
<td>16 ± 8</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>SV40 wild-type</td>
<td>8 ± 2</td>
<td>+</td>
<td>7</td>
</tr>
</tbody>
</table>

* If two to five experiments were performed, standard deviation is given for each mutant.
† Second passages from cultures with c.p.e. were performed at high m.o.i. (>10).
‡ NT, Not tested.
§ Plasmids pRR27.4 and pRR27.5 did not yield infectious virus after very long periods of cultivation (>80 days) in repeated experiments. Southern blots of such cultures always remained negative.

Estimation of enhancer strength by mutagenesis in the 19 bp palindrome

All deletion variants of HCMV–SV40 recombinants, cloned in the vector pslEMBL' by the unique BamHI site of the SV40 late region, were transfected by the calcium phosphate precipitation procedure (1.5 × 10⁶ cells; 6 μg DNA). SV40 T antigen was monitored by indirect immunofluorescence after 48 h. Immune staining with AB-1 showed a typical nuclear...
fluorescence with clearly defined nucleoli that was absent in mock-transfected cells. About 3600 cells were counted in each culture per 20 cm² Petri dish. All clones were tested at least three times. There was no significant difference in the staining intensity of positive cells after transfection with the different deletion clones. The numbers of T antigen-expressing cells are shown in Fig. 6. All deletion clones (pRR27, pRR36, C66.7, D10.7) with a single 19 bp repeat induced significantly less T antigen than the recombinant C4 which contains a long (262 nt) genuine stretch of HCMV enhancer sequence (Fig. 6). Insertion of one or two additional nucleotide pairs into the centre of the palindromic 19 bp repeats did not significantly alter T antigen expression. If, however, four to 13 nucleotides were excised from the 19 bp repeat, the activity was significantly lower, resulting in reduction of T antigen-expressing cells to about 50%. Removal of longer stretches (25 to 80 nucleotides) did not further reduce enhancer activity of the HCMV sequences in recombinants (Fig. 6). A single remaining sequence motif (21 bp repeat) (clone 36301) still yielded an equivalent percentage of T antigen-positive cells, with the same bright immune staining as the wild-type. The clones pRR27.4 and pRR27.5, both devoid of HCMV enhancer sequences, did not induce any T antigen expression.

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### Table: Palindromic repeat in HCMV enhancer

<table>
<thead>
<tr>
<th>Clone</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
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<tbody>
<tr>
<td>pRR27</td>
<td>0</td>
<td>11315</td>
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</tr>
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<td>11658</td>
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</tr>
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<td>6.96</td>
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</tr>
<tr>
<td>C4</td>
<td>-</td>
<td>11509</td>
<td>1.274</td>
</tr>
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</table>

Fig. 6. T antigen expression in immunofluorescence tests after transient expression of the HCMV-SV40 deletion mutants in CV-1 cells. (a) Designation of the plasmids containing mutated HCMV-SV40 recombinants; (b) number of nucleotides deleted (−) or added (+) in each mutant; (c) total number of cells counted for each mutant in immunofluorescence tests; (d) average percentage of T antigen-positive cells in immunofluorescence and standard deviations from nine independent samples for each mutant.
The 19 bp palindrome as a protein-binding site

To study protein-binding properties further, a series of oligonucleotides was synthesized containing the perfect palindrome, half of the palindrome, a transcription factor AP1 consensus sequence that resulted from omitting a single nucleotide pair, and three mutant sequences that had been used for functional assay in HCMV-SV40 recombinants (Fig. 7).

Fig. 8 shows the gel retardation of the 32P-labelled oligonucleotides by HeLa cell nuclear protein extracts. While the perfect palindrome was almost entirely bound by protein (lane 5), the AP1 consensus oligonucleotide (lane 8) and the insertion mutant sequence derived from clone 2701 (lane 10) were shifted to the same positions, although with slightly reduced efficiency. In contrast the half of the palindrome (lane 9) and the oligonucleotides from the non-functional deletion variants (clone 27151, lane 11; clone 66151, lane 12) showed weaker nuclear protein binding. Apparently, all sequence motifs were bound by the same protein(s), as was evident from their identical positions in the gels. Complexing of the half site oligonucleotide and the AP1 consensus sequence could be competed with by the canonical palindrome (Fig. 9). When an unrelated sequence, the 18 bp repeat, was used for gel shift competition experiments in high molar excess, complexing of the 19 bp palindrome with protein was not affected (data not shown). This indicated that the activity of the 19 bp repeat as an enhancer element correlated with specific protein-binding affinities.

Sequence comparisons

Computer searches in gene libraries indicated that a 12 nt core sequence of the 19 bp repeat is conserved in the IE upstream sequence of cytomegalovirus-type herpesviruses (betaherpesviruses) but not within the entire sequence of varicella-zoster virus (alphaherpesvirus) (Davison & Scott, 1986) and Epstein-Barr virus (gammaherpesvirus) (Baer et al., 1984) or in any of the known herpes simplex virus IE genes. The palindromic 19 bp repeat unit is largely conserved in the mouse cytomegalovirus enhancer (Dorsch-Häslar et al., 1985) and in the simian cytomegalovirus IE upstream regulatory sequence where it occurs 11 times between nt − 50 and − 500 (Jeang et al., 1987) (Table 3). A remarkable similarity exists with a sequence upstream of the tml promoter of the T plasmid in Agrobacterium tumefaciens; this sequence is a functional eukaryotic promoter in plant cells (Gielen et al., 1984). The inner palindromic core of the HCMV 19 bp repeat also corresponds to a transcription regulatory element of the human
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Chorionic gonadotropin alpha subunit promoter which is a cAMP response element (CRE) for transcription enhancement (Silver et al., 1987). Other CREs with core sequences identical or very similar to the 8 bp core palindrome of the HCMV 19 bp repeat (TGACGTCA) have been found in the upstream gene sequences of the hormones somatostatin (Montminy et al., 1986) and vasoactive intestinal polypeptide (Tsukada et al., 1985), of proenkephalin (Terao et al., 1983; Comb et al., 1987), of the proto-oncogene c-fos (Van Beveren et al., 1983) and of the enzyme phosphoenolpyruvate carboxykinase (Wynshaw-Boris et al., 1986).

DISCUSSION

The enhancer located about 520 to 120 nt upstream of the cap site in the IE1 gene of HCMV strongly activates transcription in a broad spectrum of vertebrate cells. It also efficiently enhances transcription from the cognate promoter, if tested in vitro using HeLa cell extracts. The HCMV enhancer sequence is characterized by a complex array with four groups of non-abutting direct repeats. Here we show by extensive deletion analysis and competition in vivo that the 19 bp sequence is a protein-binding site that contributes, as one of several modular elements, to the constitutive activity of the enhancer.
Table 3. Sequence homologies to the 19 bp palindrome

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Localization (nt)</th>
<th>Gene*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>gCCC ATTGAC GTCAtT a a t</td>
<td>-469/-451</td>
<td>HCMV major</td>
<td>Boshart et al. (1985)</td>
</tr>
<tr>
<td>tCCt ATTGAC GTCAtT GGG</td>
<td>-416/-398</td>
<td>IE enhancer</td>
<td></td>
</tr>
<tr>
<td>CCCt ATTGAC GTCAtT GGG</td>
<td>-333/-315</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCt ATTGAC GTCAtT GGG</td>
<td>-147/-129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCt ATTGAC GTCAtT GGG</td>
<td>-73/-55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCt ATTGAC GTCAtT GG t</td>
<td>-488/-470</td>
<td>SCMV IE enhancer</td>
<td>Jeang et al. (1987)</td>
</tr>
<tr>
<td>tCCt ATTGAC GTCA t a t GG</td>
<td>-453/-435</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCCt ATTGAC GTaAT AT GG c</td>
<td>-430/-412</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCt ATTGAC GTCA A t c</td>
<td>-406/-388</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gCCC ATTGAC GTCA a GG</td>
<td>-363/-345</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cacc ATTGAC GTCA GGG</td>
<td>-338/-320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCC ATTGAC GTCA GGG</td>
<td>-284/-266</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCCC ATTGAC GTCA GGG</td>
<td>-167/-149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCC ATTGAC GTCA GGG</td>
<td>-113/-95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gggC AaTGAC GeA AT GGG</td>
<td>-91/-73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t tCC ATTGAC GTCAtT GG c</td>
<td>-70/-52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCC ATTGAC GTCA GGG</td>
<td>-236/-218</td>
<td>MCMV IE enhancer</td>
<td>Dorsch-Häsler et al. (1985)</td>
</tr>
<tr>
<td>gCCC ATTGAC GTCA tT GG t</td>
<td>11752/11770</td>
<td>pTi plasmid of A. tumefaciens</td>
<td>Gielen et al. (1984)</td>
</tr>
<tr>
<td>a a ATTGAC GTCA t g G t a</td>
<td>-128/-111</td>
<td>Human chorionic gonadotropin alpha subunit</td>
<td>Silver et al. (1987)</td>
</tr>
</tbody>
</table>

* Abbreviations: SCMV, simian cytomegalovirus, MCMV, murine cytomegalovirus.

To determine enhancer strength, recombinant SV40 viruses containing HCMV enhancer fragments instead of the 72 bp repeats were tested for replication competence in permissive cells and for T antigen expression by indirect immunofluorescence. In this test system, the number of positive cells generally correlates with transcription rate (Weber et al., 1984; Boshart et al., 1985; Schirm et al., 1985); it has certain advantages over other common methods such as RNA or CAT analyses, as it utilizes autonomous genetic units instead of plasmid constructions. Weintraub (1988) showed that the presence of enhancer sequences increases the probability of formation of stable transcription complexes. As there are only a few templates per cell, enhancer activity was proven to be represented by a dramatic increase in the number of cells expressing the reporter gene. High sensitivity is the result of examining individual cells and not missing low levels of activity since positive cells yield bright fluorescence. All recombinant SV40-type viruses containing a single intact 19 bp palindrome strongly expressed T antigen and were replication-competent. Our deletion analyses on the HCMV enhancer motifs are compatible with an earlier study on the IEI promoter-regulatory sequence by Stinski & Roehr (1985) who determined activity of various fusion genes in transient and stable expression; the previous study, however, emphasizing the role of 18 and 19 nt repeats, did not allow conclusions on the individual repeat sequence elements, as most deletion variants contained several copies of each.

There was generally a good correlation between the protein-binding activity of the 19 bp sequence and its function as an enhancer component. If one or two nucleotides were inserted into the centre of the palindromic 19 bp repeat in the deletion mutants with a single copy of this motif, enhancing activity appeared not to be significantly changed, and the respective oligonucleotide was complexed by protein. Removing four nucleotides from the centre of the palindrome diminished transcription as much as deleting the entire repeat, and protein binding was weak. The residual binding, however, was to the same protein(s), as it could be competed for by oligonucleotides with the entire palindrome. In view of the known similarities between the target sequences for transcription factor AP1 (Angel et al., 1987; Lee et al., 1987), for the c-jun oncoprotein (Struhl, 1987) and for the CRE-binding proteins (Montminy & Bilezikjian, 1987), we studied common binding characteristics. As the related synthetic AP1 consensus sequence...
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was shifted to the identical position and could be competed for by the symmetrical palindrome, the 19 bp repeat appears to be complexed with a protein with AP1-like binding properties. The 19 bp motif seems to be a symmetrical protein-binding site which interacts with two identical molecules of a cellular trans-acting factor as short insertions did not affect the function. Removal of a few nucleotides, however, strongly diminished the protein-binding capability and transcription-enhancing activity of the unit. This is consistent with the 'enhanson model' proposed by Ondek et al. (1988) emphasizing the precise spacing of small enhancer subunits.

DNA sequence motifs with dyad symmetry related to transcription control were identified in prokaryotic and, in few cases, in eukaryotic genes. They may be imperfect palindromes that are symmetrical around a central nucleotide, such as the binding sites for cro and repressor proteins of bacteriophage lambda (Takeda et al., 1983) or the GCN-4 protein in yeast (Hill et al., 1986; Struhl, 1987). Alternatively, the two strands could form an ideal palindrome, similar to the ocs element in the plant octopine synthase enhancer (Ellis et al., 1987), a 14 bp consensus from drosophila heat-shock genes (Parker & Topol, 1984), and regulatory response elements in the human glycoprotein hormone (Delegeane et al., 1987; Silver et al., 1987) and in the rat somatostatin gene (Montminy et al., 1986), and a short imperfect palindrome of 10 bp in the U5 IE enhancer of HCMV (Weston, 1988). As far as has been investigated in yeast and in the prokaryotic system, each half site of the dyad element was found to be a binding site for a monomer of the respective dimeric proteins (Takeda et al., 1983; Ptashne, 1986; Hope & Struhl, 1987).

The sequence comparisons had indicated that the inner palindromic core of the 19 bp repeat of HCMV is identical in nucleotide sequence with the centre of numerous CREs. We found evidence that the HCMV enhancer/promoter (plasmid pRR55) is stimulated by the cAMP analogue 8-BrcAMP; this, however, does not prove unambiguously that the 19 bp palindrome is a CRE, since it has been shown that the AP2-binding site can mediate cAMP activation through protein kinase A and phorbol ester stimulation through protein kinase C via the phosphatidylinositol pathway (Imagawa et al., 1987; Mitchell et al., 1987). AP2-binding sites have consensus sequences that are typically GC-rich (70 to 100\%, Imagawa et al., 1987). Similar sequence motifs were seen in the HCMV IE1/2 enhancer. Thus, more experiments are required to determine whether the 19 bp palindrome is a cAMP response element. It may be reminiscent of human adenoviruses, where a transcription control region shares structural and functional properties with CREs (Hardy & Shenk, 1988; Leza & Hearing, 1988).

The peculiar array of repeat elements and protein-binding sites provides a plausible model for the modular organization of eukaryotic enhancers where multiple sequence elements are often redundant (Schaffner, 1985; Serfling et al., 1985). Regulated and constitutive elements of different tissue specificity are combined in a single functional unit (Schirm et al., 1987; Ondek et al., 1987; Schaffner et al., 1988). All cytomegalovirus-type major IE enhancers had the 19 bp repeat (Boshart et al., 1985; Dorsch-Häслer et al., 1985; Jeang et al., 1987). Other classes of repeats can be missing, still leaving reduced but sufficient activity. The architecture of betaherpesvirus IE enhancers also suggests that some defined elements are not required for function; the 17 bp repeat is lacking in mouse cytomegalovirus, though present in the simian cytomegalovirus IE regulatory region. Conversely, the 18 bp repeat is part of the mouse cytomegalovirus enhancer, but is not found in simian cytomegalovirus (Dorsch-Häsler et al., 1985; Keil et al., 1987; Jeang et al., 1987). The total absence of these sequence elements from herpesviruses of other subgroups (alpha- and gammaherpesviruses) contributes to the general view that regulatory elements of herpesviruses diverge more than protein-coding sequences. Strong constitutive enhancers of defined functions have been found only in cytomegalovirus-type betaherpesviruses up to now. The adaptation of herpesviruses to various patterns of pathogenicity or host and cell tropism may have been determined more by evolutionary modification at the level of gene regulation than by mutations of protein-coding sequences.

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