Localization and DNA sequence analysis of the transforming domain (*mtrII*) of human cytomegalovirus

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ABSTRACT To define the morphological transforming region II (*mtrII*) of human cytomegalovirus (HCMV), a series of subclones of the *Xba* I/*Bam*HI fragment EM was constructed *in vitro* and tested for focus-forming activity and tumorigenicity. A 980-base-pair subclone of fragment EM was identified, and its nucleotide sequence revealed three small open reading frames (ORFs), encoding 79, 83, and 34 amino acid residues. S1 nuclease analysis of HCMV-infected cells identified several distinct early RNA species within *mtrII*, two of which (P1 and P2) were of particular interest, since the length of the protected DNA fragments would position the 5' end of the RNAs upstream of the open reading frames. In addition, the 980-base-pair transforming sequence revealed DNA elements capable of forming stem-loop structures. Thus the transforming *mtrII* domain of HCMV strain Towne contains both small open reading frames that are expressed in lytically infected cells and sequences resembling insertion-like structures that may be involved in transformation.

DNA of human cytomegalovirus (HCMV) contains three transforming fragments, which have been mapped in the long unique region of the viral genome (see Fig. 1 *Left*). A minimal region of 558 base pairs (bp) (pCM4127) was localized in the *Xba* I/*Hind*III fragment of HCMV strain AD169 (map unit 0.123–0.140) and designated morphological transforming region I (*mtrI*). pCM4127 DNA was reported to cause one-step focal transformation of primary Wistar rat embryo cells and NIH 3T3 mouse cells (1, 2). This sequence was noncoding and contained a stem-loop structure (3) analogous to an insertion-like element.

Two distinct transforming regions were mapped in the 20-kilobase (kb) *Xba* I fragment E (*Xba*I-E) of HCMV Towne DNA (map unit 0.680–0.770). Cloned *Xba*I-E will immortalize diploid Syrian hamster embryo cells and induce the neoplastic transformation of established rodent cell lines (4). The left-hand 3-kb *Xba* I/*Bam*HI EM segment of *Xba*I-E, designated *mtrII*, and a right-hand 7.6-kb *Xba* I/*Bam*HI EJ segment, designated *mtrIII*, were independently capable of inducing tumorigenic transformation of established rodent cells (5). Cell lines transformed individually by EJ or EM and their tumor derivatives retained the EM but not the EJ sequence (5). Similarly, only EM sequences were retained in the transformed and tumor-derived cells induced by cotransfection of cloned EJ and EM plasmids (unpublished data). These data suggested that EM sequences were required both to initiate and to maintain the transformed phenotype. In contrast, EJ sequences were required only for the initiation of transformation, perhaps by a hit-and-run mechanism analogous to the *Bgl*II-N fragment of herpes simplex virus 2 or pCM4127 of HCMV AD169 (3).

In the present study we localized a minimal 980-bp transforming *Ban* II-*Xho* I region within the HCMV Towne *Xba* I/*Bam*HI EM fragment and determined its nucleotide sequence.[¶] Nucleotide sequence analysis identified three potential open reading frames (ORFs) within the 980-bp fragment, and S1 nuclease protection assay revealed the presence of "early" RNA transcripts in infected cells. In addition, noncoding DNA sequence elements were observed with the potential to form stem-loop structures.

MATERIALS AND METHODS

Construction of Recombinant Subclones. The recombinant EM plasmid (5) was digested with *Pst* I and *Xho* I to generate three subfragments; 0.5-kb *Xba* I/*Pst* I, 1.5-kb *Pst* I/*Xho* I, and 1.0-kb *Xho* I/*Bam*HI (Fig. 1 *Left*). The 0.5-kb *Xba* I/*Pst* I subfragment was ligated to *Xba* I/*Pst* I-digested pUC18 DNA. For cloning of the 1.5- and 1.0-kb subfragments, the *Xho* I site of EM was converted to the *Xba* I site by the addition of synthetic *Xba* I linkers. The ligated mixture was then digested with *Pst* I or *Bam*HI to generate 1.5-kb *Pst* I/*Xba* I and 1.0-kb *Xba* I/*Bam*HI subfragments, respectively. These fragments were gel purified and cloned in pUC18. Furthermore, a 980-bp *Ban* II/*Xho* I fragment was subcloned in pBR327 by using the recombinant pUC18 clone containing the 1.5-kb *Pst* I/*Xho* I (*Xba* I) EM subfragment. Briefly, the unique *Ban* II site in the viral DNA was converted to an *Eco*RI site by standard procedures. The recombinant plasmid was then digested with *Eco*RI and *Xba* I to release the 980-bp *Ban* II/*Xho* I fragment, which was subsequently cloned in pBR327 at the *Eco*RI/*Bam*HI sites (*Bam*HI site being converted to *Xba* I site in pBR327). The plasmid clones were propagated in *Escherichia coli* and screened by the alkaline lysis procedure (6).

For construction of deletion clones, EM DNA was cloned in M13mp19 at the *Eco*RI site. A sequential series of overlapping subclones was generated in both orientations by T4 DNA polymerase digestion of linearized DNA using the IBI Cyclone 1 Biosystem (7). Additionally, EM subfragments *Bgl* II-*Bam*HI, *Bgl* II-*Xba* I, and *Xho* I-*Xba* I were cloned in M13mp10. The recombinant M13 clones were used to transform competent *E. coli* JM101, and the phage DNA with deletions was harvested as described (8).

DNA Sequence Analysis. The nucleotide sequence of the *Xba* I-*Bam*HI EM fragment was determined by the Sanger di-deoxynucleotide chain termination method (9), using single-stranded recombinant phage DNA as templates (8). Alignment and analysis of the DNA sequences were performed using IBI sequence analysis software on an IBM PC XT computer.

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Abbreviations: HCMV, human cytomegalovirus; *mtr*, morphological transforming region; m.p., map position; ORF, open reading frame.
[¶]The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03822).

FIG. 1. (Left) Restriction map of HCMV DNA and the features contained within the Towne *XbaI*-E fragment and the *XbaI*/BamHI EM subfragment. TR, terminal repeats; U, unique region; IR, inverted terminal repeats; subscripts L and S, long and short, respectively. (Right) Sequencing strategy for the *PstI*/*XhoI* fragment containing the 980-bp transforming region.

S1 Nuclease Assay. Human fibroblasts were infected with HCMV at a multiplicity of infection of 1–5 plaque-forming units per cell. For isolation of immediate-early (IE) RNA, the cells were infected and maintained in the presence of 100 μ g of cycloheximide per ml (10) and were harvested 14 hr after infection. For isolation of early RNA, cells were harvested 24 hr after infection (11) and RNA was extracted by the hot acid/phenol method (12). Single-stranded DNA probes labeled to high specific activity with [32 P]dCTP were generated (13). RNA was analyzed by the S1 nuclease assay as described by Loeken *et al.* (14).

Transformation and Tumorigenicity Assays. Culture conditions for NIH 3T3 mouse cells and Rat-2 rat cells, DNA transfection, and transformation assays of focus-formation and anchorage-independence were performed as previously described (4, 5). Tumorigenicity of rat cell lines was assayed in 90-day-old immunocompetent Fisher rats. Each animal was inoculated subcutaneously with a suspension of 1×10^6 cells in 1 ml of Dulbecco's phosphate-buffered saline. Tumor appearance was monitored by palpation once a week for 8 weeks. Latent period (i.e., time until detection of first tumor) was recorded and tumor diameters were measured with a caliper.

RESULTS

The physical map of the three nonhomologous morphological transforming regions (*mtrI*, *mtrII*, and *mtrIII*) of HCMV is

depicted in Fig. 1 Left. To define precisely the minimal transforming region of Towne *mtrII*, recombinant subclones of EM were generated and assayed for transforming activity in both NIH 3T3 and Rat-2 cells. Initially, the EM clone in pACYC184 was cleaved with *PstI* and *XhoI* and the unfractionated DNA digests were used in transformation assays. No difference in transforming activity was detected among these digests and the intact EM (data not shown). The three subfragments of *PstI*/*XhoI*-digested EM (Fig. 1 Left) were subcloned in pUC18 and were individually assayed for transforming activity. Results shown in Table 1 demonstrated that only the 1.5-kb *PstI*-*XhoI* subfragment of EM showed significant transforming activity. No activity was detected with the left-hand *XbaI*-*PstI* or right-hand (*XhoI*-*BamHI*) subfragments of EM.

To delineate further the transforming region within the *PstI*-*XhoI* subfragment, a 980-bp subclone extending from the *BanII* to the *XhoI* site was constructed. The *BanII* site is located 610 bp to the right of the *PstI* site. When tested for transforming activity, the *BanII*-*XhoI* clone induced foci at a transforming efficiency similar to that of EM (Table 1). The relative proportion of large foci was higher in *BanII*-*XhoI*-transfected cells than in EM-induced cultures. The restriction enzyme *BglII* cuts once within the *BanII*-*XhoI* fragment. When a clone extending from the *BglII* cut to the *BamHI* cut was tested, a 62–66% reduction in focus forma-

Table 1. Transforming activities of the HCMV *mtrII* subclones in NIH 3T3 and Rat-2 cells

DNA	Foci per dish*		Tumorigenicity of Rat-2 foci†	
	NIH 3T3	Rat-2	Incidence	Latency, days
<i>XbaI</i> EM <i>BamHI</i>	8L, 12M, 14S	2M, 22S	9/10	28–35
<i>XbaI</i> <i>PstI</i>	0	1M, 5S	—	—
<i>PstI</i> <i>XhoI</i>	11L, 23M, S	5M, 45S	ND	—
<i>BanII</i> <i>XhoI</i>	21L, 9M, 4S	17L	20/20	20–22
<i>BglII</i> <i>BamHI</i>	4L, 9M, S	2M, 6S	0/5	—
<i>XhoI</i> <i>BamHI</i>	0	1M, 2S	—	—
Vector pUC18	1	3S	—	—
Vector pBR327	1	ND	—	—
Salmon testes	0	ND	—	—

Tumorigenicity of Rat-2-derived focal lines was assayed in immunocompetent syngeneic rats as described (5). ND, not done.

*Focus-formation data are averages of two experiments (focus sizes: L, large; M, medium; S, small).

†Tumor incidence of *BanII*/*XhoI*-transformed cells is the cumulative value for four independent foci.

tion was detected and no tumors were produced in animals inoculated with these focal lines. These data suggested that *Bgl* II cleaved within the transforming *Ban* II-*Xho* I region (Table 1). Control plasmid or replicative form M13 vector DNAs showed rare small foci in both of the cell lines tested.

To determine the phenotypic properties of the foci induced by the 1.5-kb *Pst* I-*Xho* I subfragment, its 980-bp *Ban* II-*Xho* I subclone, and the *Bgl* II-*Bam* HI fragment, isolated foci were tested. Foci induced by the 1.5-kb *Pst* I-*Xho* I and the 980-bp *Ban* II-*Xho* I fragments formed large colonies in agar, while those induced by the *Bgl* II-*Bam* HI fragment formed small colonies (data not shown). Focal lines derived from the Rat-2 transfected cells were assayed for tumorigenicity in immunocompetent syngeneic animals (Table 1). Focal lines obtained from the *Ban* II-*Xho* I fragment induced palpable tumors (0.8–1.0 cm in size) within 2½ weeks in all animals, whereas the *Bgl* II-*Bam* HI-induced lines failed to produce tumors even after 8 weeks. Rare focal lines derived from control plasmid or M13 vector DNAs did not form large colonies in soft agar or produce tumors in animals by 8 weeks. These results place the *mtl* transforming domain within the 980-bp *Ban* II-*Xho* I fragment, approximately 1 kb to the left of the *Bam* HI site at map position (m.p.) 0.68 on the Towne HCMV genome (Fig. 1 Left).

DNA Sequence Analysis of the Transforming Region. The complete nucleotide sequence of the *Pst* I-*Xho* I fragment was determined by the strategy shown in Fig. 1 Right. The nucleotide sequence of the transforming *Ban* II-*Xho* I fragment containing 980 bp is shown in Fig. 2. Sequence analysis of this region revealed several unique features. First, three small ORFs of 79, 83, or 34 amino acid residues are located between m.p.s 294 to 530, 612 to 860, and 622 to 723, respectively. The 79-residue ORF contains the initiation

sequence CGGTGATGC and the 34-residue ORF contains the initiation sequence GCGTCATGG. These initiation motifs have 55% or 78% identity with the Kozak consensus translational initiation sequence of CC^ACCATGG (15) respectively. Second, a polyadenylation signal, AATAAA, located approximately 500 bp downstream of the first termination codon TGA at m.p. 530, is present in the *Xho* I-*Bam* HI fragment. Moreover, sequences at the 5' terminus of the ORFs contain several transcriptional regulatory sequences. For example, CAAT sequences have been identified at m.p.s 96, 153, 160, 201, and 215 (16). In addition, three potential binding sites for transcription factor Sp1 are located at m.p.s 75–82, 264–269, and 276–281 (17). The sequence TACAAA, similar to that shown by Huang *et al.* (18) to function as a weak transcriptional initiation signal in the adenovirus *E1a* late promoter is located at m.p. 391. In addition, six copies of the heptanucleotide sequence GGTG^AGTG are present in the upstream region at m.p. 62, 65, 68, 71, 290, and 302. These repeats have approximately 75% identity with the simian virus enhancer core consensus sequence (19). No consensus splice donor or acceptor signals are present in the sequence.

Third, the 980-bp *Ban* II-*Xho* I fragment contains numerous repetitive elements that have the potential to form stem-loop structures. Those presented in Fig. 3 were chosen on the basis of the thermodynamic stability of the stem, size of the loop, and the presence of repetitive sequences in the flanking region of the stem structure. Three of these are located in the presumed promoter region (sequences a–c in Fig. 3). The first (a) has no viral flanking sequence on one side because the viral *Ban* II site is at the first nucleotide position. The second (b) has no loop and resembles a palindromic sequence. The

FIG. 2. Map positions of three putative ORFs of 79, 34, and 83 amino acids (aa) and the upstream regulatory sequences are shown, together with the complete nucleotide sequence of *mtl*. P1 and P2 indicate the RNA transcripts detected in infected cells by the S1 nuclease analysis. The m.p. for the *Bgl* II site is at nucleotide 436.

FIG. 3. Stem-loop structures within the *Ban* II-*Xho* I transforming region. A small circle indicates a base mismatch in the stem. Nucleotide positions are indicated in parentheses. The arrows indicate direct repeats in flanking 10 nucleotides of the stem-loop structure. The size of the loop (in nucleotides) is indicated by the number in the loop.

third (c) is interesting because it contains a perfect direct repeat (10/10 bases match) in the flanking sequence, which is contained in an A + T-rich repeat. The last two structures (d and e) contain small loops and stable stems and are located within the 79- and 83-residue ORFs, respectively. The stem-loop structure in d contained with nucleotides 457-499 might be important since it is within the 79-residue ORF in which *Bgl* II cleaves and reduces transforming activity.

There are several ORFs in the opposite DNA strand. However, the translational initiation sequence of each of these peptides is weaker than those discussed above. In addition, there are no potential regulatory signals that would serve as a transcriptional promoter element.

Analysis of the *mtlII* Transcripts. To determine if *mtlII* transcripts were expressed in HCMV lytically infected cells, the S1 nuclease analysis was carried out (Fig. 4). When the *Ban* II/*Xho* I probe, representing the entire 980-bp DNA fragment was used, the S1 nuclease analysis revealed several distinct RNA species in the 24-hr HCMV-infected cultures

but not in the 14-hr RNA samples (Fig. 4C). These have been designated P1 through P6. The transcripts P1 and P2 were of particular interest because the length of the protected DNA fragments would position the 5' end of the RNA upstream of the putative ORFs. The P1 transcript was determined to be approximately 720 nucleotides in length and, depending upon the exact position of the 5' end, may contain the coding sequences for the 79-, 83-, and 34-residue proteins. The major RNA transcript, P2, was determined to be 410 nucleotides in length. Identically sized protected DNA fragments were detected with both the *Ban* II/*Xho* I and *Bgl* II/*Xho* I probes, demonstrating the validity of the S1 mapping technique. This would position the 5' end of the RNA at approximately 570 ± 10 , 30-40 nucleotides upstream of the AUG initiation codons for the 83- and 34-residue polypeptides, respectively.

DISCUSSION

Transforming domains among the herpesviruses have been localized in small DNA fragments (2, 3, 21). To date, no

FIG. 4. S1 nuclease analysis of *mtlII* RNA. The *Ban* II/*Xho* I *mtlII* DNA fragment was inserted into M13mp10 for preparation of DNA probes. The synthesis and purification of the [³²P]DNA probes and the S1 nuclease analysis were performed as described previously (20). Two probes were utilized in this study: the 980-base *Ban* II/*Xho* I probe covered the entire *mtlII* sequence, and the *Bgl* II/*Xho* I probe covered the 3' 540 bases. (A) Analysis of 980-base *Ban* II/*Xho* I and 540-base *Bgl* II/*Xho* I probes. (B) S1 nuclease analysis of RNAs in uninfected human fibroblasts (HF), or human fibroblasts infected with HCMV for 14 or 24 hr (HF CMV 14 hr and HF CMV 24 hr), using the *Bgl* II/*Xho* I probe; b, bases. (C) S1 nuclease analysis of RNAs as in B, using the *Ban* II/*Xho* I probe. (D) Longer exposure of C. Molecular weight markers: *Hinf*I restriction digest of simian virus 40 DNA.

transforming polypeptide has been identified in HCMV-induced transformation. As a result, viral DNA elements have been proposed to be responsible for transformation through modulation of cellular genes by transcriptional activation (22), DNA amplification (23), or genetic recombination and rearrangement (22–24). In this study, we have identified the *mtrII* of HCMV Towne to be a 980-bp fragment. Our analysis of HCMV lytically infected cells has identified two *mtrII*-specific RNA transcripts large enough to code for the 70-, 83-, and 34-residue polypeptides. The *mtrII* domain also contains secondary structural elements—i.e., stem-loops—similar to those found in other herpesvirus transforming domains (3, 21).

The sequence analysis of the *mtrII* domain revealed sequence elements upstream of the ORFs that are characteristic of a promoter. These include CAAT boxes, Sp1-binding sites, TACAAA (18) and ATA (25) transcriptional initiation signals, and six copies of the heptanucleotide sequence GGTG^AGTC, which has similarity to the enhancer core consensus sequence of simian virus 40 (19). All of these were found in the first 300 nucleotides upstream of the ORFs (Fig. 2). Furthermore, the 79-residue ORF and the 34-residue ORF contain motifs that have 55–78% identity with the Kozak consensus translational initiation sequence of CC^AGCCATGG (15).

The S1 analysis identified several *mtrII* transcripts in lytically infected cells at early times after infection (24 hr) and indicates that these transcripts may have been transcribed from the putative ORFs. It is unlikely that the RNA species represent splice acceptor junctions and not authentic 5' ends. We have analyzed the *mtrII* DNA sequence for homologies to the consensus splice donor sequence AGGTAAGT, and the splice acceptor sequence, Y₁₀CAG (Y = T or C), and no homologies were detected. The next step is to analyze *mtrII*-induced tumors and transformed cells for the expression of *mtrII* transcripts to determine their involvement in transformation.

A 2.2-kb early RNA transcript was shown to originate from a region of HCMV AD169 that is colinear to the *Xho* I/*Bam*HI subfragment of HCMV Towne EM (Fig. 1 *Left*) (26). This RNA transcript maps outside of the 980-bp transforming domain described in our study.

In our tumorigenicity assay (Table 1), untransformed Rat-2 cells do not clone in agar but can form tumors in Fisher rats after a long latency (10–12 weeks) (27, 28). In contrast, Rat-2-derived foci induced by *mtrII* formed large (>0.25 mm) colonies in agar and induced tumors in Fisher rats within 2½ weeks. Previous studies showed that large agar colonies correlated with tumorigenic potential (4, 5). Taken together, these data indicate that *mtrII* affects a critical step in tumor progression associated with the induction of anchorage-independent growth and enhanced tumorigenicity. The inability of rat-derived-*Bgl* II-*Bam*HI foci to form large colonies in agar or tumors *in vivo* (up to 8 weeks) is consistent with the interpretation that *Bgl* II cleaves within a region required for the full transforming activity of *mtrII*. In fact, since *Bgl* II cuts into the 79-residue ORF this might indicate that this ORF is required for transformation. Alternatively, the *Bgl* II restriction site might simply separate the upstream transcriptional regulatory sequences from the downstream 83- or 34-residue protein coding sequences.

We have compared the Towne *mtrII* colinear sequence in strain AD169 published by Kouzarides *et al.* (29) and in strain Tanaka determined in our laboratory (unpublished data). The results indicated the presence of the 79-residue ORF in AD169 and its absence from strain Tanaka. This finding becomes interesting since the colinear fragment in AD169 containing the 79-residue ORF was found to be transforming

whereas the colinear fragment in strain Tanaka lacking the 79-residue ORF was not (unpublished data). Other sequence differences that disrupt the coding sequence for the Towne 83- and 34-residue ORFs were observed in both the colinear regions of the transforming AD169 and the nontransforming Tanaka colinear regions, leading us to believe that these ORFs may be less important in transformation.

In view of the presence of transcriptional control signals and stem-loop structures in HCMV *mtrII*, it is possible that *mtrII* transforms cells by increasing the transcription of critical cellular genes (22). Alternatively, *mtrII* may induce transformation by altering the structure of host DNA through fusion and gene rearrangement (23, 24). Cloning *mtrII* upstream of a basal eukaryotic promoter should enable us to determine if the transforming domain can act as a transcriptional enhancer or activator. Furthermore, the analysis of the HCMV *mtrII* integration sites in transformed cells will be necessary to determine if *mtrII* causes structural alteration of cellular genes.

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