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The Human Cytomegalovirus mtrII Colinear Region in Strain Tanaka Is Transformation Defective

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The morphological transforming region II (mtrII) of human cytomegalovirus (HCMV) strain Towne has been localized to a 980-base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids (aa). In addition, noncoding DNA sequence elements which have the potential to form stem-loop structures were also observed within mtrII. To determine what elements within HCMV Towne mtrII are important in transformation, colinear regions in other HCMV strains (AD169 and Tanaka) were isolated and a comparison of transforming potential was performed. The results indicated that the 2.2-kilobase colinear region in strain AD169 was transforming, whereas the colinear mtrII region in strain Tanaka showed significantly reduced transforming potential. Analysis of the nucleotide sequence data of these colinear regions revealed the presence of the 79-aa ORF in strains Towne and AD169 and its absence in strain Tanaka. In addition, *Bgl*II-digested Towne mtrII, which was cleaved within the 79-aa ORF, was shown to display significantly reduced transforming potential. Since the 83- and 34-aa coding sequences were interrupted in both the transforming AD169 colinear region and the nontransforming Tanaka strains, these ORFs were thought not to be important in transformation. Analysis of the stem-loop structures within each of the mtrII colinear regions did not reveal significant changes among the transforming and nontransforming colinear fragments. Thus, the comparative data indicate an important role for the 79-aa ORF in transformation.

Human cytomegalovirus (HCMV) infection has been associated with several neoplastic diseases, including prostatic carcinoma (6), adenocarcinoma of the colon (9), cervical carcinoma (15), and Kaposi's sarcoma (7, 8). Furthermore, inactivated HCMV was shown to transform hamster embryo fibroblasts in culture (1). In subsequent studies, three distinct HCMV-transforming fragments were located in the long unique region of the viral genome (Fig. 1) (2, 3, 13, 14). A minimal region of 558 base pairs (bp) (pCM4127) was mapped in the *Xba*I-*Hin*dIII fragment of HCMV strain AD169 and has been designated morphological transforming region I (mtrI) (13, 14). This sequence was noncoding and contained a stem-loop structure (5) similar to an insertion-like element. A 20-kilobase (kb) *Xba*I E fragment (map units 0.680 to 0.770) in HCMV strain Towne which immortalized and transformed primary diploid SHE cells was identified (2), and further investigation revealed two distinct morphological transforming regions within the *Xba*I E fragment (3). A left-handed EM fragment, designated mtrII, and a right-handed EJ fragment, designated mtrIII, were both identified and independently shown to transform established rodent cells (3). The EM sequence was retained in both EM-transformed rodent cells as well as in EM-induced tumor-derived cell lines, whereas the EJ sequence was not retained in either EJ-transformed cells or EJ-induced tumor-derived cell lines. There is no information on whether mtrII or mtrIII has the ability to immortalize primary diploid cells. However, we have recently reported greater tumorigenicity when cells were transformed by mtrII plus mtrIII than when cells were transformed by either alone (11).

The EM transforming domain has been localized to a 980-bp *Ban*II-*Xho*I fragment (mtrII) containing three open reading frames (ORFs) of 79, 83, and 34 amino acids (aa) in addition to DNA elements capable of forming stem-loop structures (16). Analysis of cells lytically infected with HCMV by S1 nuclease analysis revealed several distinct early RNA species, two of which were large enough to contain the coding sequences for the 79-, 83-, and 34-aa proteins, indicating the expression of mtrII sequences in infected cells. In the current investigation, we isolated the colinear mtrII regions from HCMV strains AD169 (13, 14) and Tanaka (19) and compared the transforming abilities of these colinear regions on immortalized NIH 3T3 cells. The colinear Tanaka mtrII region was generated from a *Hin*dIII C fragment of strain Tanaka (supplied by S. Ihara) (Fig. 1). The colinear 2.2-kb *Xba*I-*Bam*HI fragment of strain AD169 was prepared from JN201 (supplied by J. Nelson). The Tanaka 980-bp *Ban*II-*Xho*I fragment was cloned into pBR327 and the 2.2-kb *Xba*I-*Bam*HI fragment was cloned into pACYC184 as previously described for the isolation of the Towne mtrII fragment (16).

A comparison of transforming activities of the minimal *Ban*II-*Xho*I (mtrII) regions of strains Towne and Tanaka as well as the colinear 2.2-kb *Xba*I-*Bam*HI fragment of AD169 on immortalized NIH 3T3 cells is shown in Table 1. Compared with Towne mtrII, the AD169 colinear region showed the same transforming activity. The numbers of large foci, previously shown to correlate with tumorigenic potential (3, 10), as well as the colony efficiencies in 0.3% agarose were similar for the Towne mtrII and the AD169 2.2-kb fragment. In contrast, the Tanaka mtrII region showed 25% of the overall transforming activity and only 4.3 to 5.5% of the activity for large-focus formation. Evidence of the importance of the unique *Bgl*II restriction site within Town mtrII is also provided in Table 1. The restriction enzyme *Bgl*II cuts once within Towne mtrII specifically at nucleotide 436 within the 79-aa ORF (Fig. 1). In this case, dishes transfected with *Bgl*II-digested Towne mtrII showed a 56% reduction in transforming potential compared with intact Towne mtrII.

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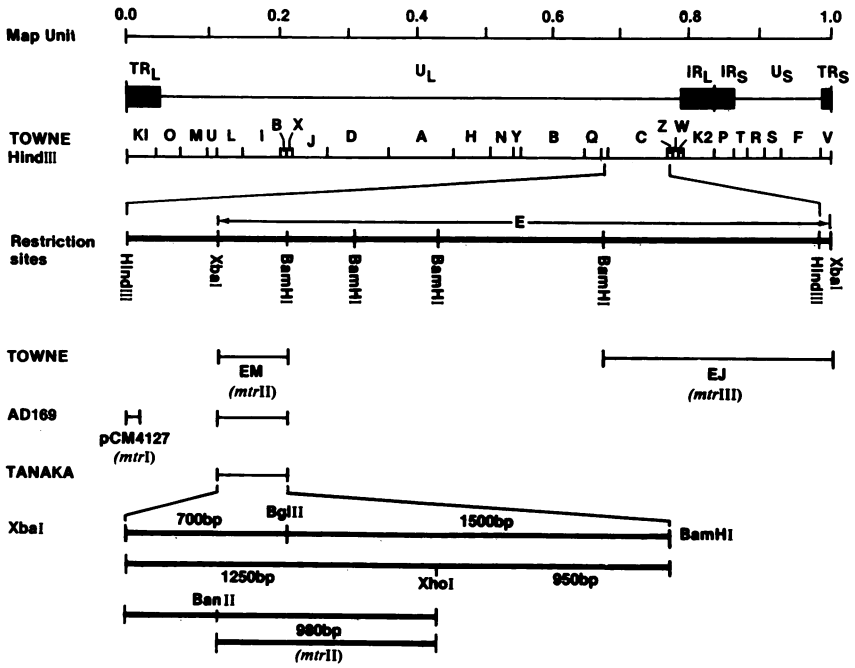

FIG. 1. Restriction map of HCMV DNA indicating the map locations for the *Hin*dIII C and *Xba*I E fragments. The map locations for mtrI, mtrII, and mtrIII are shown as well as the mtrII colinear regions in strains Towne, AD169, and Tanaka. TR_L and TR_S, Long and short terminal repeats; U_L and U_S, unique long and short regions; IR_L and IR_S, inverted long and short repeats.

The individual foci were next picked and grown into mass culture, and focal lines induced by Towne mtrII or AD169 2.2-kb fragment were examined for anchorage-independent growth in 0.3% agarose (Table 1). All lines induced by either Towne mtrII or by the AD169 colinear 2.2-kb fragment exhibited cloning efficiencies ranging from 0.48 to 3.5%, and the colonies which grew were macro in size. In contrast, focal lines induced by Tanaka mtrII exhibited negligible cloning efficiency ranging from 0.005 to 0.01%, which was similar to that of the controls. Moreover, colonies arising from the transfection with Tanaka DNA which grew in agarose were predominantly micro in size. These results indicate that the mtrII colinear region in strain Tanaka did not exhibit significant transforming activity compared with Towne mtrII and the colinear 2.2-kb region in strain AD169. These experiments provide no information concerning immortalization, since primary cells were not used.

TABLE 1. Transforming potential of HCMV colinear mtrII regions in established NIH 3T3 cells

Transfected DNA	Focus formation[a]	Cloning in agarose[b]	
		Colony efficiency	Colony size
Towne mtrII (980 bp)	43 (23 L, 11 M, 9 S)	1.4–3.5	Macro
Tanaka mtrII (980 bp)	11 (1 L, 6 M, 4 S)	0.005–0.01	Micro
Towne mtrII (*Bgl*II digested)	19 (10 L, 5 M, 5 S)	NT	
AD169 colinear 2.2-kb region	43 (18 L, 14 M, 11 S)	0.48–1.09	Macro
Salmon testes	2 (1 M, 1 S)	0.003	Micro
Control	2 (1 M, 1 S)	0.003	Micro

[a] Average number of foci per 100-mm-diameter dish from two independent experiments. NIH 3T3 cells were transfected as previously described (2, 3) with 15 μg of each respective HCMV DNA together with 5 μg of salmon testes DNA used as a carrier. After 48 h, cells were trypsinized and divided at a 1:3 ratio, and morphologically transformed foci were scored 5 to 6 weeks later. Focus diameter size: L, >2.5 mm; M, 1.5 to 2.5 mm; S, >1.0 mm.

[b] Cells were seeded in 0.3% agarose at 10^5 cells per 60-mm-diameter dish, and 3 to 4 weeks later, colonies were scored. Colony efficiency is defined as (number of colonies × 100)/(number of seeded cells). Data shown are the range for colony efficiency for all focal lines tested. Colony diameter size: micro, 0.1 to 0.25 mm; macro, >0.5 mm. NT, Not tested.

To ascertain what may be responsible for the differences in transforming potential, the sequence analysis of the Tanaka mtrII was carried out and compared with the published sequences of Towne mtrII (16) and the colinear region in strain AD169 (12). The nucleotide sequence of Tanaka mtrII was determined by the Sanger dideoxynucleotide chain termination method (17) with single-stranded recombinant bacteriophage DNA as templates (18). Alignment and analysis of the Tanaka mtrII sequence were performed by using IBI sequence analysis software on an IBM PC XT computer as previously reported for Towne mtrII (16). Comparisons of the AD169 and Tanaka sequences with the complete Towne sequence are presented in Fig. 2. The sequence analysis of Towne mtrII and the designations for the ORFs are presented in Fig. 3A, and a comparison of the effects of these base changes on the ORFs in strains Towne, AD169, and Tanaka is shown in Fig. 3B. The 79-aa ORF in strain AD169 is identical to that in strain Towne, while the 83-aa ORF in AD169 is truncated and the 34-aa ORF is changed by frameshift, producing a somewhat larger ORF (Fig. 3B). The nucleotide sequence of strain Tanaka revealed that the 83- and 34-aa ORFs were similar to those in AD169. Interestingly, the sequence analysis indicated a major change in the 79-aa ORF in strain Tanaka compared with the ORFs in strains Towne and AD169. Since the colinear fragment in strain AD169 containing the 79-aa ORF was found to be transforming while the colinear fragment in strain Tanaka

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AGCCCACCAC CTTTTTTTTA AGAGAGGAGG AATTTCGTCT TGATCTCCAG CCGGAGATAA ( 60)
CGGCGGTGGT GGTGGTGGCG GGAGAGACTT CAAGGCAATG AAAAAAAAAA ATTTCGTTTT ( 120)
GCCATCAAGT GGTGACGATA ACCCGTCAGA TTGATAATTG GTTCCTACAG AAACTATTCT ( 180)
AACCGCGGAA GAAAGAAATT GAAAAAAAAA AATTGACAAA AAACATCATA ACATAAAGGA ( 240)
CCACCTACCT GGGACGCGCA GTTGGGCGGC GGACTGGGCG GCATGCTGCG GTGATGCTGT ( 300)
CGGTGATGGT CTCTTCCTCT CTGGTCCTGA TCGTCTTTTT TCTAGGCGCT TCCGAGGAGG ( 360)
CGAAGCCGGC GACGACGACG ACGATAAAGA ATACAAAGCC GCAGTGTCGT CCAGAGGATT ( 420)
ACGCGACCAG ATTGCAAGAT CTCCGCGTCA CCTTTCATCG AGTAAAACCT ACGTTGGTAG ( 480)
GTCACGTAGG TACGGTTTAT TGTGACGGTC TTTCTTTTCC GCGTGTCGGG TGACGTAGTT ( 540)
TTCCTCTTGT AGCAACGTGA GGACGACTAC TCCGTGTGGC TCGACGGTAC GGTGGTCAAA ( 600)
GGCTGTTGGG GATGCAGCGT CATGGACTGG TTGTTGAGGC GGTATCTGGA GATCGTGTTC ( 660)
CCCGCAGGCG ACCACGTCTT AATCCCGGAC TCAAGACGGA ATTGCATAGT ATGCGCTCGA ( 720)
CGCTAGAATC CATCTACAAA GACATGCGGC AATGCGTAAG TTGGTTCCTC TGTGGCGGCG ( 780)
CTGTCCCGAG AGGTAACAAC GTGTTCATAG CACGCTGTTT TACTTTTGTC GGGCTCCCAG ( 840)
CCTCTGTTAG GTTGCGGAGA TAAGTCCGTG ATTAGTCGGC TGTCTCAGGA GGCGGAAAGG ( 900)
AAATCGGATA ACGGCACGCG GAAAGGTCTC AGCGAGTTGG ACACGTTGTT TAGCCGTCTC ( 960)
GAAGAGTATC TGCACTCGAG                                             (1020)
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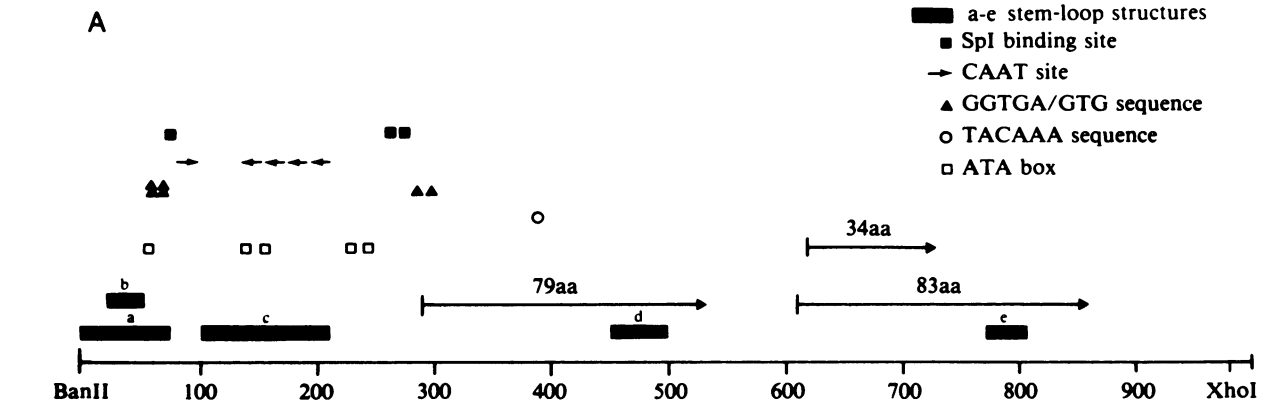
FIG. 2. Complete nucleotide sequence of Towne mtrII from the *Ban*II site to the *Xho*I site. Base changes in uppercase letters are for AD169, while changes in lowercase letters are for the Tanaka sequence. Symbols: ○ nucleotide deletion in AD169; ● deletion in the Tanaka sequence; →, insertion of sequence.

lacking the 79-residue ORF showed reduced transforming activity, the data implicate a role for the 79-aa ORF in transformation. This conclusion is further substantiated by the reduced transforming potential seen in *Bgl*II-digested Towne mtrII transfections. In addition, since the sequence differences that disrupt the 83- and 34-residue ORFs were observed in both the colinear regions of the transforming AD169 and the nontransforming Tanaka colinear regions, the data suggest that these ORFs may be less important in transformation.

Other data also implicate the role of the 79-aa ORF in transformation (16). We have previously carried out transfection assays using a *Bgl*II-*Bam*HI subclone of Towne EM. When a clone extending from the *Bgl*II cut to the *Bam*HI cut was tested, a 62 to 66% reduction in focus formation was detected and no tumors were produced in animals inoculated with these focal lines (16). These data provide further evidence that *Bgl*II cuts within the transforming mtrII region, specifically within the 79-aa ORF which may play a major role for the full transforming activity of mtrII. Alternatively, the *Bgl*II site might separate the upstream transcriptional regulatory sequences from the downstream 83- or 34-residue protein-coding sequences.

Transforming domains of herpesviruses have been mapped to small DNA fragments (2, 3, 13, 14, 16). Sequence analyses have revealed these transforming domains to be noncoding, which brings into question the role of virally encoded polypeptides in transformation. Recently, however, we reported (16) on the localization and DNA sequence analysis of HCMV Towne mtrII. In contrast to other herpesvirus transforming domains, mtrII was retained in transformed and tumor-derived cell lines and was found to contain ORFs of 79, 83, and 34 aa. Furthermore, S1 analysis identified RNA transcripts in cells lytically infected with HCMV which were large enough to code for the ORFs.



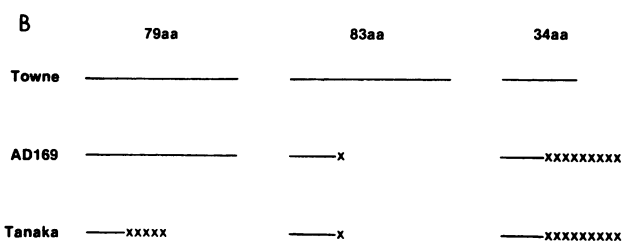


FIG. 3. (A) Map positions of three putative ORFs of 79, 83, and 34 aa and the upstream regulatory sequences. Also shown are the map positions for the stem-loop structures located within mtrII. (B) Comparison of the mtrII ORFs from HCMV strains Towne, AD169, and Tanaka. Data taken from sequence analysis shown in Fig. 2. Sequence analysis was carried out on the Towne and Tanaka mtrII regions in our laboratory and compared with the sequence of AD169 as published by Kouzarides et al. (12). Pictured are the amino acid schematics for the translated sequences of the 79-, 83-, and 34-aa ORFs obtained in the transforming Towne mtrII compared with the colinear regions in the transforming AD169 mtrII and the nontransforming colinear region in Tanaka. Solid line indicates homology; X indicates changes in amino acid sequence.

In the current study, we provide further insight into the significance of these ORFs by comparing transforming activities of mtrII colinear regions in strains AD169 and Tanaka. In contrast to Towne mtrII and AD169 colinear transforming fragments, the Tanaka mtrII had substantially reduced transforming activity and produced significantly reduced numbers of focal lines. Nucleotide sequence comparisons of the ORFs in strains Towne, AD169, and Tanaka are consistent with the hypothesis that the 79-aa ORF may play a role in transformation.

Stem-loop structures have been proposed to be involved in herpesvirus transformation (5). Along these lines, we have previously identified three stem-loop structures in the first 300 nucleotides of the Towne mtrII sequence (Fig. 3A) (16). These three stem-loop structures were virtually unaltered in their sequence in the Tanaka mtrII, the transforming AD169 2.2-kb, and the Towne mtrII colinear regions. Thus, the importance of these stem-loop structures in transformation is not consistent with the data presented. Two other stem-loop structures were also located within Towne mtrII. One of these is found at nucleotides 457 to 499 within the 79-aa ORF. The other is found at nucleotides 757 to 803 within the 83-aa ORF. In the case of the stem-loop structure located within the 79-aa ORF, its sequence is the same in the HCMV Towne, AD169, and Tanaka colinear regions. Thus, the substantial reduction in transforming activity of Tanaka mtrII is consistent with the truncation of the 79-aa ORF. We cannot at this time rule out the possibility that the residual focal transforming activity of Tanaka mtrII is due in part to this stem-loop structure or to some partial activity of the truncated ORF seen in Tanaka mtrII. Finally, a fifth stem-loop structure is found in the 83-aa ORF. However, it is dramatically altered in both AD169 and Tanaka colinear regions, leading us to believe that it also may not be important in transformation. At present, an extensive deletion analysis of mtrII is being carried out to identify the element(s) responsible for transformation. Moreover, we are trying to determine whether expression of the 79-aa ORF can be correlated with transforming activity. Alternatively, the expression of the 79-aa ORF may alter transcription of cellular genes (4, 11). Both studies may yield further insight into the mechanism of transformation by HCMV mtrII.

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