Tumor Progression Mediated by Two Cooperating DNA Segments of Human Cytomegalovirus

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The terminal fragments (EJ and EM) of the *Xba*I-E transforming segment of human cytomegalovirus can independently induce the tumorigenic conversion of immortalized cells. To study their interaction, Rat-2 cells were transfected singly or with a combination of cloned EJ and EM DNAs. Large transformed foci were induced at a 10-fold higher frequency by EJ plus EM than by either DNA fragment alone. Focus-derived lines transformed by EJ plus EM produced tumors in syngeneic rats at a much faster rate (5 to 7 days) than did cell lines transformed by EJ or EM alone (25 to 35 days). Southern hybridizations showed that EM-homologous DNA was retained, exhibiting a complex pattern of multiple and amplified bands in EJ-plus-EM lines compared to a simple pattern in EM-induced lines. EJ DNA was not detected in the single or double transformants. The levels of p29, a 29-kilodalton transformation-sensitive marker in Rat-2 cells, were decreased 10- to 100-fold in cell lines transformed by EJ or EM fragment alone. Synthesis of p29 was shut off in EJ- plus-EM transformants. These data demonstrate that two unlinked transforming regions of human cytomegalovirus can cooperate to produce an aggressive tumorigenic phenotype.

Human cytomegalovirus (HCMV), a member of the herpesvirus group, is a ubiquitous human pathogen, implicated as an etiologic agent in congenital birth defects of newborn infants as well as in severe opportunistic disease of immunosuppressed individuals, including acquired immunodeficiency syndrome (AIDS) patients (6, 22). Epidemiologic and molecular studies (4, 9) have implicated HCMV as a possible cofactor in Kaposi's sarcoma. However, its role in the pathogenesis of neoplastic disease remains obscure.

Oncogenic potential of HCMV has been demonstrated for human and rodent cells in vitro (1, 8). Three transforming domains (5, 15) have been mapped in HCMV DNA (Fig. 1). A 558-base-pair fragment (pCM4127) of HCMV AD169 (mtrI; Fig. 1) was shown to be capable of inducing the morphologic transformation of both primary and established cells (15, 16). The *Xba*I fragment E (*Xba*I-E) of HCMV Towne caused the immortalization of primary diploid cells and tumorigenic transformation of established cell lines (5). The Towne *Xba*I DNA fragment is noncolinear to mtrI of AD169. The two terminal segments of *Xba*I-E, termed EM and EJ (mtrII and mtrIII, respectively; Fig. 1), were shown to independently transform immortalized lines (7). The low potential of anchorage-independent growth conferred by individual EJ or EM DNA (7) relative to whole *Xba*I-E (5) led us to investigate the combined effect of both DNA fragments on the in vitro phenotypic properties and oncogenic potential of transformed cells. In this article, we report that EJ and EM fragments cooperate in immortalized preneoplastic cells to produce enhanced tumorigenic transformation.

NIH 3T3 and Rat-2 cells were previously shown to undergo neoplastic transformation following transfection with either cloned *Xba*I-E or its terminal EJ or EM subfragments from HCMV Towne (7). In this investigation, Rat-2 cells were chosen for study because of the availability of a syngeneic animal host for evaluation of tumorigenic potential. For cotransformation experiments, we employed the

terminal *Xba*I-BamHI EJ and EM subclones constructed in plasmid pACYC184. Subconfluent Rat-2 monolayers were transfected with either EJ or EM alone or in combination, and the appearance of transformed foci was monitored after one or two passages in vitro (Table 1). Cells transfected with EJ or EM alone developed medium to large foci after 6 to 9 weeks. On the other hand, cells cotransfected with EJ-plus-EM DNA displayed prominent large foci, first detected at 3 weeks. By 6 to 9 weeks, the frequency of large foci in cotransfected cells was increased 10-fold higher than in cultures transfected with EJ or EM alone.

To investigate potential phenotypic differences between single-fragment and double-fragment transformants, foci were picked, expanded in mass culture, and tested for colony formation in 0.3% agar and for tumorigenic potential in Fisher rats. Focal lines transformed by EJ or EM formed predominantly microscopic (<0.25-mm) colonies in soft agar. Upon subcutaneous injection into animals, these lines produced tumors after a latent period of 25 to 35 days. In contrast, lines transformed by EJ plus EM formed macroscopic (>0.5-mm) colonies in soft agar and gave rise to tumors within 5 to 7 days (Table 1).

Tumors induced in rats were excised, trypsinized, and established into cell lines. To investigate the presence of transfected viral sequences, genomic DNAs from focus- and tumor-derived EM-plus-EJ lines were digested sequentially with *Xba*I-BamHI and *Pst*I-XhoI to release the three subfragments (1.5, 1.0 and 0.5 kilobases [kb]) of EM (7). As a control, genomic DNAs from untransformed Rat-2 cells as well as from the EM-induced tumor line (RBM3T1) were similarly digested and analyzed (Fig. 2A). Previous hybridization analysis of EM-induced tumors, using the 1.5-kb *Pst*I-XhoI subfragment (Fig. 1) as a probe, demonstrated the retention of EM-specific sequences (7). This probe was originally selected because it lacked homology to normal cell DNA. However, upon prolonged exposure a single faint band of ~4 kb was observed in untransformed Rat-2 DNA (Fig. 2A, lane 2). A comigrating band was also detected in the EM-derived RBM3T1 tumor line (Fig. 2A, lane 1).

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FIG. 1. Map locations of the EM and EJ transforming regions in HCMV Towne DNA.

Consistent with previous data (7), RBM3T1 exhibited a simple hybridization profile and retained the 1.5-kb EM-specific band. In contrast, all EJ-plus-EM focus- and tumor-derived lines displayed a complex pattern of multiple hybridizing bands with the retention of the 1.5-kb virus-specific band in most lines (Fig. 2B). The 1.5-kb EM-specific band is amplified in one line (J+M F4). Furthermore, the 4-kb cellular band was observed in most cell lines and amplified significantly in J+M 4T1. Multiple bands higher than 4 kb were seen in EJ-plus-EM lines and could represent sequence rearrangements caused by the interaction of EM fragment with cellular DNA. To determine the presence of EJ sequences, a replicate of the Southern blot shown in Fig. 2B was probed with the 7.6-kb EJ fragment under identical conditions. No hybridization to the EJ probe was detected in untransformed Rat-2 cells or in any of the EJ-plus-EM cell lines (data not shown).

To look for modulation of host cell proteins as a result of transformation, untransformed Rat-2 cells and tumor-derived lines transformed by EJ alone, EM alone, and EJ plus EM were analyzed. Cells were labeled with [³⁵S]methionine, and the lysates were electrophoresed on two-dimensional polyacrylamide gels (2, 3) to generate protein profiles (Fig.

3). Computerized microdensitometric analysis was employed to match and quantitate 50 individual spots across the protein profiles of Rat-2 and transformed cells (S. Lawson, D. Goldstein, G. Latter, E. Zuckerkandl, and R. J. Jariwalla, *Carcinogenesis*, in press). This analysis revealed no significant alterations in protein levels except for major regulatory changes in α -actin (13) and in a polypeptide of 29 kilodaltons (p29) with an isoelectric point (pI) of about 7.5. Polypeptide p29 was abundantly expressed in untransformed Rat-2 cells (Fig. 3). Synthesis of p29 was diminished about 10- to 100-fold in cell lines transformed individually by EM and EJ fragments (data not shown). Expression of p29 was not detectable in EJ-plus-EM tumor derivatives (Fig. 3), indicating shutoff of this gene.

The effect of the HCMV EJ and EM segments, introduced singly or together into established Rat-2 cells, may provide important clues to understanding how these fragments transform cells. While EJ or EM individually transformed these cells, the frequency of formation of large transformed foci and soft-agar colonies, an *in vitro* correlate of tumorigenicity (7, 11), was relatively low. When both EJ and EM segments were cotransfected (Table 1), the frequency of focus formation was 10-fold greater, and these cell lines formed tumors

TABLE 1. Transforming potential of HCMV Towne DNA fragments in established Rat-2 cells

Transfected DNA	Focus formation ^a	Cloning in agar ^b		Tumorigenicity ^c	
		% Colony efficiency	Size	Incidence	Latency (days)
EJ	15M, 4L	0.005–0.15	Micro	15/15	25–35
EM	18M, 3L	0.004–0.08	Micro	18/20	28–35
EJ plus EM	>100M, 45L	0.3–0.8	Macro	8/8	5–7

^a Average number of foci per 100-mm-diameter dish. Rat-2 cells were transfected (10) singly or with a mixture of 5 μ g of each recombinant plasmid containing the respective HCMV DNA fragment. Vector pACYC184 DNA was used as a carrier to bring the concentration in each transfection mix to 10 μ g per dish. At 48 h posttransfection, cells were trypsinized and plated at a 1:3 split ratio. After 2 weeks, cells were replated at a 1:3 ratio, and morphologically transformed foci were scored 6 to 7 weeks later. Focus size: M, medium; L, large (7). Cells transfected with control vector DNA gave only small rare foci per dish.

^b Cells were seeded in 0.3% agar at 10⁵ cells per 60-mm-diameter dish, and 3 to 4 weeks later, colonies were scored. Colony efficiency is defined as number of colonies $\times$ 100/number of seeded cells. Colony size is defined by diameter: Micro, 0.1 to 0.25 mm; Macro, >0.5 mm. Data shown are the range for percent colony efficiency and size of two focus-derived lines. Colony efficiency of vector DNA control was <0.001%.

^c Syngeneic Fisher rats were inoculated subcutaneously with 2 $\times$ 10⁶ cells per animal and palpated twice weekly for tumor development. Incidence is defined as number of positive animals/total number inoculated. Latency is time until appearance of first tumor. Data shown are values for three independent focal lines transformed individually by EJ or EM and two lines transformed by EJ plus EM.

FIG. 2. Southern blot analysis of high-molecular-weight cellular DNA from Rat-2 cells (A), EM-induced tumor derivative (A), and EJ-plus-EM-transformed and tumor-derived lines (B). DNA samples (10 μ g) were digested with *Xba*I, *Bam*HI, *Xho*I, and *Pst*I, electrophoresed, transferred to nitrocellulose filters (18), and hybridized with a nick-translated, 32 P-labeled 1.5-kb *Pst*I-*Xho*I internal subfragment of EM. Hybridization and washing conditions were as previously described (7). The 1.5-kb *Pst*I-*Xho*I DNA and one genome equivalent of 3-kb EM DNA were used as reconstruction markers.

at a more rapid rate than did lines transformed by EJ or EM alone.

A previous study (7) localized a region of homology between Towne EM and normal rodent DNA within the 1.0-kb *Xho*I-*Bam*HI EM subfragment derived from the right-hand one-third of EM. This region of Towne EM is colinear

to a sequence within the *Eco*RI R fragment of HCMV AD169 that was shown to hybridize to uninfected human, murine, or sea urchin DNA (19). The detection of a 4.0-kb hybridizing fragment in untransformed Rat-2 cells with the 1.5-kb *Pst*I-*Xho*I EM subfragment as a probe (Fig. 2A) identifies a second putative region of cell-virus homology within EM. The 4.0-kb Rat-2 fragment may represent a cellular homolog of the oncogenic region of EM, since the minimal transforming region (mtrII) in EM has been mapped to a 980-base-pair segment within the *Pst*I-*Xho*I subfragment (17). In this respect, amplification of the 4.0-kb hybridizing fragment in the J+M 4T1 cell line resembles the genetic alterations in the cellular homolog of the herpes simplex virus type 2 *Bam*HI E fragment associated with tumorigenic transformation (12).

While the transformation of primary cells by herpes simplex virus type (11) requires at least two functions, we do not know how EJ and EM cooperate to produce enhanced transformation. Although we have previously shown that the 20-kb *Xba*I E fragment of HCMV Towne (containing both EJ and EM) can immortalize and transform primary Syrian hamster embryo cells (5), these segments were linked and it was not possible to determine whether they mediated separate phenotypic events. EJ or EM alone induced a similar transformed phenotype in established Rat-2 cells. Whether tumorigenic cooperation involves an immortalization function of EJ or EM must await further studies examining the effects of EJ, EM, and EJ plus EM in primary cells.

EJ encodes a major immediate-early 72-kilodalton protein (IE1) implicated in transactivation and autoregulation (20, 21). The 980-base-pair minimal transforming region of EM contains three small open reading frames with transcripts expressed early in lytically infected cells (17). Thus we propose three possible mechanisms for the tumorigenic cooperation we observed: (i) EJ may regulate EM-encoded open reading frames; (ii) EJ may promote the insertion or amplification of EM DNA and its cellular homolog; and (iii) EJ and EM may exert independent effects on cellular func-

FIG. 3. Autoradiograms of total cellular proteins in Rat-2 cells and in a tumor-derived line transformed by EJ plus EM, as resolved by two-dimensional gel electrophoresis (2, 3). Polypeptide p29 is indicated by an arrow.

tion(s) in established cells. The first mechanism would involve the transactivation by EJ sequences. However, since EJ DNA is not maintained in transformed and tumor-derived cell lines, its interaction with EM must be transient. In the second mechanism, EJ may induce chromosomal aberrations resulting in promotion of the frequency of EM integration into host DNA. The detection of multiple bands of EM homologous sequences in EJ-plus-EM-transformed cells (Fig. 2) is consistent with this possibility. Chromosomal aberrations may also lead to alteration of EM-related cell sequences and could be responsible for amplification of the 4.0-kb hybridizing band in the J+M 4T1 cell line. In support of this mechanism, HCMV has been shown to damage metaphase chromosomes following virus infection (14). Third, EJ and EM may independently affect the expression of cellular genes that cooperate to augment tumorigenesis. Consistent with this possibility is the down-regulation of p29, a transformation-sensitive marker associated with tumor progression (Lawson et al., in press). Recently, the IE1 protein encoded by EJ was shown to associate with metaphase chromosomes in mitotic cells (R. LaFemina and G. S. Hayward, personal communication), suggesting that EJ may also act to stimulate host genes involved in cell proliferation. Further studies defining the transforming, chromatin-associated, and regulatory domains of EJ will provide greater understanding of the relationship between these functions and EJ-plus-EM-induced tumorigenic cooperation.

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