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# Multiple Transforming Regions of Human Cytomegalovirus DNA

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*Departments of Microbiology and Pediatrics and Vincent T. Lombardi Cancer Center, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007,[1] and Armand Hammer Cancer Research Center, Linus Pauling Institute of Science and Medicine, Palo Alto, California 94306[2]*

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**The transforming (focus forming) activity of defined cloned DNA fragments from human cytomegalovirus Towne and AD169 was carried out in immortalized rodent cells. The frequency of focus formation in NIH 3T3 cells by Towne *Xba*I fragment E was 80- to 100-fold higher than that observed with Towne *Xba*I fragments AO, O, C, or carrier DNA alone but was similar to that observed with pCM4127, a transforming fragment from HCMV AD169 (J. A. Nelson, B. Fleckenstein, D. A. Galloway, and J. K. McDougall, J. Virol. 43:83–91, 1982; J. A. Nelson, B. Fleckenstein, G. Jahn, D. A. Galloway, and J. K. McDougall, J. Virol. 49:109–115, 1984). Foci were first detected in Towne *Xba*I fragment E-transfected NIH 3T3 cells at 5 to 6 weeks posttransfection, whereas foci were detected at 2 to 3 weeks after transfection with AD169 pCM4127. Digestion of Towne *Xba*I fragment E with *Bam*HI did not significantly reduce its focus-forming activity. When *Bam*HI subclones of Towne *Xba*I fragment E were assayed individually for focus formation in NIH 3T3 and Rat-2 cells, transforming activity was localized within each terminal fragment (EJ and EM). Foci induced by EJ or EM DNA alone were smaller compared with those induced by Towne *Xba*I fragment E. Isolated focal lines exhibited growth in soft agar and were tumorigenic in immunocompetent syngeneic animals. High-molecular-weight DNAs from transformed and tumor-derived lines were analyzed by Southern blot hybridization with intact EM and a 1.5-kilobase subfragment lacking cell-related sequences. Virus-specific EM sequences were detected at less than one copy per cell in Towne *Xba*I fragment E-transformed NIH 3T3 cells and at multiple copies in rat tumor-derived cell lines. In contrast, virus-specific EJ sequences were barely detected in EJ-transformed and tumor-derived lines with intact EJ as probe.**

Human cytomegalovirus (HCMV) is associated with several neoplastic diseases. Evidence for virus infection has been found in cells of prostatic carcinoma (7), adenocarcinoma of the colon (11), cervical carcinoma (18), and Kaposi's sarcoma (3, 8, 9). HCMV DNA, RNA, and antigen have been found in Kaposi's sarcoma tissue (8, 9). In addition, HCMV has been associated with the Kaposi's sarcoma seen in acquired immune deficiency syndrome patients (5). Furthermore, HCMV has been shown to exhibit neoplastic transforming activity for cells in culture (1).

Two distinct regions of HCMV DNA have been shown to induce neoplastic transformation of rodent cells in vitro. Nelson et al. (16, 17) described a region in HCMV AD169 capable of causing focal transformation of primary Wistar rat and established NIH 3T3 cells. Our laboratories (4) identified from HCMV Towne a different region which was capable of causing immortalization of primary-diploid SHE cells and tumorigenic transformation of established NIH 3T3 cells. This Towne *Xba*I fragment E lacked homology to the transforming fragment of AD169 (4) but was homologous to the *Bgl*II-C-transforming fragment of herpes simplex virus type 2 DNA (12).

In the current investigation we compared the transforming activity of Towne *Xba*I fragment E with other HCMV Towne DNA fragments in parallel with the transforming DNA fragment (pCM4127) of HCMV AD169. In addition, we examined the transforming potential of five individual *Bam*HI subclones of Towne *Xba*I fragment E to localize further the area responsible for neoplastic transformation. Our results, obtained in two assay systems (NIH 3T3 and Rat-2), identified neoplastic transforming activity within each of the terminal *Xba*I-*Bam*HI EJ and EM fragments. Furthermore, the analysis of transformed and tumor-derived cell line DNAs by Southern blot hybridization consistently showed the retention of EM sequences whereas EJ sequences were barely detectable.

## MATERIALS AND METHODS

**Cells.** NIH 3T3 cells (provided by G. Vande Woude) were grown in Dulbecco modified Eagle medium containing 10% calf serum. A flat subclone was isolated and used for the transformation studies described here. Rat-2 cells (provided by G. Reyes) were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum.

**Plasmid constructions.** Plasmid pACYC184 derivatives containing individual *Xba*I fragments of HCMV Towne were obtained from M. Stinski, and their construction has been reported (22). The internal *Bam*HI subclones of Towne *Xba*I fragment E were constructed in our laboratory as follows. The HCMV Towne *Xba*I fragment E plasmid was digested to completion with *Bam*HI, ligated to *Bam*HI-cleaved pBR322, and used to transform *Escherichia coli* HB101. Recombinant plasmids were prepared from ampicillin-resistant and tetracycline-sensitive colonies (2), and the presence of Towne *Bam*HI inserts A, D, or T was verified by restriction enzyme analysis (R. L. Lafemina and G. Hayward, personal communication). The terminal *Xba*I-*Bam*HI subclones of Towne *Xba*I fragment E were constructed as follows. The HCMV Towne *Xba*I fragment E plasmid was digested to completion with *Xba*I and *Bam*HI restriction endonucleases, self-

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ligated, and used to transform *E. coli* HB101. Recombinant plasmids from chloramphenicol-resistant and tetracycline-sensitive colonies were isolated, and the presence of Towne *Xba*I-*Bam*HI-EJ and -EM inserts were confirmed by restriction enzyme analysis. Plasmid pCM4127 from HCMV AD169 (17) was obtained from J. Nelson.

**Transformation of NIH 3T3 and Rat-2 cells.** Transfection of NIH 3T3 cells and detection of transformed foci was carried out at Georgetown University as described by Clanton et al. (4). Transfection involved precipitation of 15 μg of HCMV recombinant plasmids with 5 μg of salmon testes DNA by the calcium phosphate technique (10) onto subconfluent monolayers of NIH 3T3 cells. After 24 to 48 h cells were trypsinized and divided at a 1:3 ratio in medium containing 10% calf serum. Medium was changed at 3- to 4-day intervals for 5 to 6 weeks, and foci of refractile morphologically altered cells were monitored with a light microscope.

The focus assay in Rat-2 cells was carried out at the Linus Pauling Institute as described by Jariwalla et al. (14). Briefly, circular pACYC184 derivatives containing HCMV inserts *Xba*I-*Bam*HI-EJ or -EM were individually precipitated (10) with calcium phosphate and applied to subconfluent monolayers of Rat-2 cells ($5 \times 10^5$ cells per 60-mm diameter dish). After 24 to 48 h cells were trypsinized and seeded into three 100-mm-diameter passage 1 (P1) dishes in growth medium containing 10% fetal bovine serum. Two of the P1 dishes were refed growth medium twice a week and maintained for 6 weeks to monitor the development of transformed foci. The third P1 dish was incubated in growth medium for 2 to 3 weeks, was subdivided again into three 100-mm-diameter dishes (P2), and was maintained for 6 weeks as described above. Foci of refractile, morphologically altered cells were scored in P1 and P2 dishes by light microscopy.

**Assay of transformed phenotype.** Individual foci isolated from NIH 3T3 and Rat-2 transfected dishes were assayed for growth in 0.3% agarose (BBL Microbiology Systems) and 0.3% agar (Difco Laboratories), respectively, as described previously (4, 13). Tumorigenicity of transformed Rat-2 foci was assayed in 5-week-old immunocompetent Fisher rats by subcutaneous inoculation of $2 \times 10^6$ cells per animal. Tumors were monitored by palpation once a week. Tumors were excised and trypsinized, and cell lines were established by seeding cells in 100-mm-diameter dishes containing growth medium.

**Southern blot analysis of cellular DNA.** High-molecular-weight cellular DNA was isolated from nuclei of transformed and tumor-derived cell lines as described previously (13). Cellular DNA was digested with restriction enzymes *Bam*HI and *Xba*I, separated on 0.8% agarose gels, and transferred to nitrocellulose paper (19). The filters were prehybridized at 43°C for 2 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% each of Ficoll (Pharmacia Fine Chemicals) and polyvinylpyrrolidone–50 mM sodium phosphate (pH 7.0)–1% glycine–0.1% sodium dodecyl sulfate, containing 250 μg of sonicated and denatured salmon testes DNA per ml. Hybridization was done at 43°C with nick-translated $^{32}$P-labeled probe ($5 \times 10^6$ cpm/ml) for 18 h in the above buffer lacking glycine. Filters were washed once in 2× SSC–0.1% sodium dodecyl sulfate for 10 min at room temperature followed by two washings with the same buffer, each for 15 min at 62°C, and by an additional four washings with 0.1× SSC–0.1% sodium dodecyl sulfate, each for 15 min at 62°C. The filters were exposed to film (X-Omat AR; Eastman Kodak Co.) at −70°C with intensifying screens.

TABLE 1. Transforming activity of HCMV DNA fragments in NIH 3T3 cells

| DNA source | Restriction endonuclease | DNA (μg/dish) | Foci/no. of dishes | Transformation frequency ($10^{-5}$) |
|---|---|---|---|---|
| Towne *Xba*I-AO | *Xba*I | 15.0 | 1/6 | 0.03 |
| | | 15.0 | 5/6 | 0.16 |
| Towne *Xba*I-O | *Xba*I | 15.0 | 1/6 | 0.03 |
| | | 15.0 | 6/6 | 0.20 |
| Towne *Xba*I-C | *Xba*I | 15.0 | 4/6 | 0.13 |
| | | 15.0 | 3/6 | 0.10 |
| Towne *Xba*I-E | *Xba*I | 15.0 | 93/6 | 3.10 |
| | | 15.0 | 72/6 | 2.40 |
| Towne *Xba*I-E | *Xba*I-*Bam*HI | 15.0 | 67/6 | 2.20 |
| | | 15.0 | 73/6 | 2.40 |
| AD169 pCM4127 | *Bam*HI-*Hin*dIII | 15.0 | 112/6 | 3.73 |
| | | 15.0 | 86/6 | 2.87 |
| Salmon testes | | 20.0 | 1/6 | 0.03 |
| | | 20.0 | 1/6 | 0.03 |
| $Ca_3[PO_4]_2$ | | | 1/6 | 0.03 |
| | | | 1/6 | 0.03 |
| No treatment | | | 1/6 | 0.03 |

## RESULTS

**Transforming activity of various HCMV DNA fragments.** Recombinant plasmids containing HCMV Towne *Xba*I fragments E, AO, O, and C and the HCMV AD169 plasmid pCM4127 (17) containing the 558-base-pair transforming fragment were applied onto NIH 3T3 cells. The results in Table 1 show a comparison of their focus-forming activities. Foci of refractile, morphologically altered cells were detected in cultures transfected with Towne *Xba*I fragment E as well as with AD169 pCM4127 but not with Towne *Xba*I fragments AO, C, and O. The transformation frequency of Towne *Xba*I fragment E and AD169 pCM4127 was similar; however, there was a marked difference in the time of appearance of foci. pCM4127-induced foci were detectable by 3 weeks, whereas Towne *Xba*I fragment E-induced foci were first detected at 5 weeks.

We also examined the focus-forming activity of Towne *Xba*I fragment E digested with *Bam*HI which introduces four cuts within the Towne *Xba*I fragment E insert (Fig. 1). The data in Table 1 show that the focus-forming activity of *Bam*HI-digested Towne *Xba*I fragment E was not significantly different from that of undigested DNA.

**Transforming potential of subclones of Towne *Xba*I fragment E.** Since *Bam*HI digestion did not significantly alter the transforming potential of Towne *Xba*I fragment E, the *Bam*HI and *Xba*I-*Bam*HI subclones (EJ and EM) were used to localize the region responsible for transforming activity. Subclones containing *Xba*I-*Bam*HI fragments EJ or EM were both active in the focus assay, with foci first detected at 5 weeks (Table 2). The transformation frequency observed with *Xba*I-*Bam*HI-EM was comparable to that observed for *Xba*I-*Bam*HI-EJ. In contrast to the large foci induced by *Xba*I fragment E, those induced by *Xba*I-*Bam*HI-EM and *Xba*I-*Bam*HI-EJ were predominantly small to medium in size. No foci were detected with either *Bam*HI-A, -D, or -T.

To confirm that both *Xba*I-*Bam*HI-EJ and -EM subclones can individually induce foci in established rodent cells, we tested their transforming potential in the independently derived Rat-2 cell strain. Transfected cells were assayed for focus formation at posttransfection passages P1 and P2. Morphologically transformed foci were first detected at 5 weeks in cultures transfected individually with *Xba*I-*Bam*HI-EJ or -EM (Table 3). At P1 only small foci were

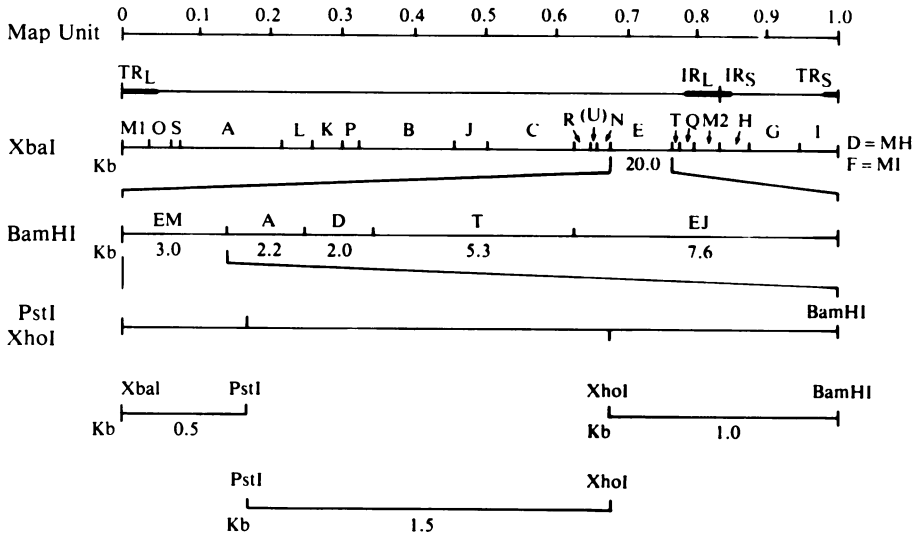


FIG. 1. Physical map of the *Bam*HI restriction sites (Lafemina and Hayward, personal communication) within Towne *Xba*I fragment E and the *Pst*I-*Xho*I sites within the *Xba*I-*Bam*HI-EM fragment (Razzaque et al., unpublished).

detected. Prominent large foci were observed when transfected cells were subcultured at least two times before the focus assay (Table 3, P2).

**Phenotypic properties of the transformants.** Individual foci observed in NIH 3T3 cells were picked and grown into mass culture. Focal lines induced by *Xba*I-*Bam*HI-EJ or -EM fragments were examined for anchorage-independent growth in 0.3% agarose (Table 4). All lines exhibited cloning efficiency ranging from 0.7 to 3.4% compared with the controls. The colonies were predominantly medium sized, with large colonies appearing at low efficiency.

Rat-2 focal lines RBJ1, RBJ3, RBM2, and RBM3 that were transformed independently with *Xba*I-*Bam*HI-EJ and -EM fragments, respectively, also formed predominantly medium-sized colonies in 0.3% agar (Table 3). When $2 \times 10^6$ cells were subcutaneously inoculated into 5-week-old immunocompetent Fisher rats, they formed palpable tumors at the site of inoculation (Table 3). Tumors were first detected at 4 to 5 weeks postinoculation, and they grew to a size of 4 cm with further incubation. Tumors were excised, trypsinized, and established in culture. Cell lines RBM 2T1 and RBM 3T1 derived from EM-induced tumors were examined for the presence of HCMV DNA sequences.

**HCMV DNA sequences in rat tumor-derived cells.** The presence of the Towne terminal *Xba*I-*Bam*HI-EJ or -EM fragments in tumor-derived cell lines was investigated by Southern blot hybridization. To detect the presence of EM sequences, we first probed an EM-induced rat tumor-derived cell line (RBM 2T1) with the 3.0-kilobase (kb) EM fragment. Genomic DNA from RBM 2T1 was first restricted with *Xba*I-*Bam*HI (Fig. 2, 2 cut), the terminal restriction sites of the EM fragment, and another sample of the same digest was further restricted with *Xho*I and *Pst*I (Fig. 2, 4 cut) to liberate the three subfragments (1.5, 1.0, and 0.5 kb) of EM. As a control, genomic DNA from untransformed Rat-2 cells was similarly digested and used. One genome equivalent of authentic EM DNA as well as the 1.5-, 1.0-, and 0.5-kb subfragments of EM were used as markers. A Southern blot was hybridized under stringent conditions by using $^{32}$P-labeled EM DNA as probe. Intense hybridization representing multiple copies was detected in RBM 2T1 (Fig. 2, 2 cut) at a position comigrating with the 3.0-kb EM marker. In RBM 2T1 (4 cut) multiple copies of virus-specific sequences comigrating in positions corresponding to the 1.5-, 1.0-, and 0.5-kb EM subfragment markers were detected. In the 2-cut digest, additional bands were observed in a position different from that of the 3-kb marker. Bands higher than 3 kb may represent integrated EM deleted sequences lacking either the *Xba*I or *Bam*HI site, whereas bands lower than 3 kb may represent extensively deleted EM sequences. Normal Rat-2 DNA exhibited faint multiple bands hybridizing to the EM probe.

To determine whether the bands detected in RBM 2T1 were derived from transfected viral sequences or represented endogenous cellular sequences, we mapped the region of cellular homology within the EM fragment to obtain a viral DNA probe lacking cell-related sequences. Genomic DNAs (10 μg) from untransformed NIH 3T3 and Rat-2 cells were restricted with *Xba*I-*Bam*HI and *Xho*I-*Pst*I, and Southern blots were prepared. Hybridization was first performed with the EM fragment as probe. Hybridization of EM DNA to multiple bands of NIH 3T3 and Rat-2 DNAs is shown in Fig. 3A. To localize the cell-related sequences, individual subfragments of EM (Fig. 1) were used to probe normal NIH 3T3 DNA cleaved with *Xba*I-*Bam*HI and *Xho*I-*Pst*I. Hybridization was detected between the 1.0-kb EM subfragment

TABLE 2. Transforming activity of the *Bam*HI fragments of Towne *Xba*I fragment E in NIH 3T3 cells

| DNA source | Restriction endonuclease | DNA (μg/dish) | Foci/no. of dishes | Transformation frequency ($10^{-5}$) |
|---|---|---|---|---|
| Towne EM | *Xba*I-*Bam*HI | 15.0 | 105/6 | 3.50 |
| | | 15.0 | 140/6 | 4.60 |
| Towne A | *Bam*HI | 15.0 | 1/4 | 0.05 |
| | | 15.0 | 1/6 | 0.03 |
| Towne D | *Bam*HI | 15.0 | 1/1 | 0.20 |
| | | 15.0 | 1/6 | 0.03 |
| Towne T | *Bam*HI | 15.0 | 1/3 | 0.06 |
| | | 15.0 | 1/6 | 0.03 |
| Towne EJ | *Xba*I-*Bam*HI | 15.0 | 201/6 | 6.70 |
| | | 15.0 | 67/6 | 2.20 |
| Salmon testes | | 20.0 | 1/6 | 0.03 |
| | | 20.0 | 1/6 | 0.03 |

TABLE 3. Transforming potential of Towne *Xba*I-*Bam*HI-EJ and *Xba*I-*Bam*HI-EM fragments in Rat-2 cells

| Transfected DNA | Focus formation (size of foci)[a] | | Cloning in agar | | | Tumorigenicity incidence[b] |
|---|---|---|---|---|---|---|
| | P1 | P2 | Rat-2 focal line Isolated | % Colony efficiency[c] | Size[d] | |
| *Xba*I-*Bam*HI-EJ | S | M, L | RBJ1 | 0.15 | M | 3/3 |
| | | | | 0.05 | L | |
| | | | RBJ3 | 0.005 | M | 2/2 |
| | | | | 0.005 | L | |
| *Xba*I-*Bam*HI-EM | S | M, L | RBM2 | 0.08 | M | 6/6 |
| | | | | 0.02 | L | |
| | | | RBM3 | 0.013 | M | 3/3 |
| | | | | 0.004 | L | |
| pACYC184 vector | | | Mass culture | <0.001 | S | 0/5 |

[a] S, <1.0 mm; M, 1.5 to 2.5 mm; L, >2.5 mm.
[b] $2 \times 10^6$ cells were inoculated subcutaneously in 5-week-old immunocompetent syngeneic Fisher rats.
[c] Number of colonies × 100/number of seeded cells.
[d] M, ≤0.1 to 0.2 mm; L, ≥0.25 mm, based on diameter of colony.

and NIH 3T3 DNA (Fig. 3B). This hybridization pattern was similar to that observed in Fig. 3A. The presence of cell-related sequences in the 1-kb subfragment of Towne EM is consistent with the findings of Staprans and Spector (20) who detected similar homology between the colinear 1.0-kb fragment of strain AD169 and mammalian cell DNA. No hybridization was detected with either the 1.5- or 0.5-kb EM subfragment probes.

We next probed normal Rat-2 DNA (2 cut and 4 cut) with the 1.5-kb EM subfragment, and no hybridization was detected (Fig. 3C). We therefore selected the 1.5-kb EM subfragment as our virus-specific probe for further studies. By using the 1.5-kb EM subfragment, we reprobed RBM 2T1 DNA as described in the legend to Fig. 2. A multicopy 3-kb band was present in the 2-cut digest, comigrating with the one-genome-equivalent EM marker (Fig. 4). In the 4-cut digest, we detected a multicopy band comigrating with the 1.5-kb EM subfragment marker (Fig. 4A). An independently derived EM-transformed cell line (RBM 3T1) was also probed with the 1.5-kb EM subfragment DNA. As shown in Fig. 4B, both the 3- and 1.5-kb bands were detected in the 2- and 4-cut RBM 3T1 DNA, respectively. Thus, the use of the 1.5-kb virus-specific subfragment of EM as probe indicates the presence of EM sequences in the rat tumor-derived cell lines. The detection of the 1.5-kb internal EM subfragment confirms the retention of EM DNA in these lines.

Rat tumor-derived cell lines RBJ 3T2, RBJ 1T, and RBJ 3T1 transformed by the *Xba*I-*Bam*HI-EJ fragment were similarly analyzed for the presence of EJ sequences. A band

TABLE 4. Phenotypic properties of NIH 3T3 cells transformed by Towne *Xba*I-*Bam*HI-EJ and *Xba*I-*Bam*HI-EM fragments

| Focal line | Transfected DNA | Cloning in agarose | |
|---|---|---|---|
| | | % Colony efficiency[a] | Size[b] |
| NBJ1 | *Xba*I-*Bam*HI-EJ | 0.7 | M, L |
| NBJ2 | *Xba*I-*Bam*HI-EJ | 0.7 | M |
| NBJ3 | *Xba*I-*Bam*HI-EJ | 1.4 | M, L |
| NBJ4 | *Xba*I-*Bam*HI-EJ | 2.0 | L |
| NBM8 | *Xba*I-*Bam*HI-EM | 2.0 | L |
| NBM11 | *Xba*I-*Bam*HI-EM | 3.4 | M |
| NBM13 | *Xba*I-*Bam*HI-EM | 0.7 | L |
| NBM14 | *Xba*I-*Bam*HI-EM | 1.4 | L |
| NIH 3T3 (P11) | | 0.005 | |

[a] See Table 3, footnote *c*.
[b] M, ≤0.2 mm; L, ≥0.25 mm, based on diameter of colony.

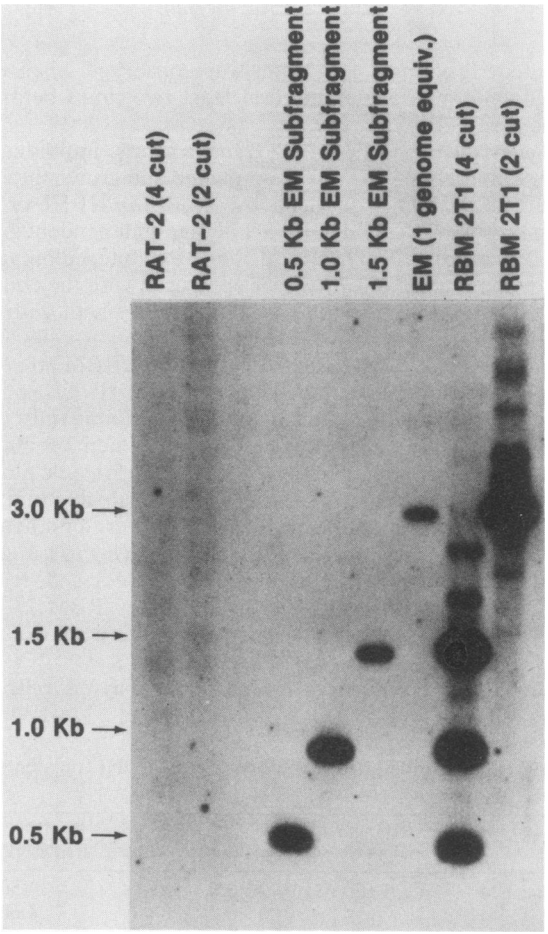


FIG. 2. Southern hybridization of *Xba*I-*Bam*HI-EM-transformed Rat-2 tumor-derived cellular DNAs to $^{32}$P-labeled *Xba*I-*Bam*HI-EM DNA probe. Normal Rat-2 and tumor DNAs (RBM 2T1) (10 μg each) were digested with two enzymes (*Xba*I and *Bam*HI) (2 cut) or four enzymes (*Xba*I, *Bam*HI, *Pst*I, and *Xho*I (4 cut) and hybridized as described in Materials and Methods. EM subfragments of 1.5, 1.0, and 0.5 kb and one genome equivalent of 3.0-kb EM DNA were used as markers.

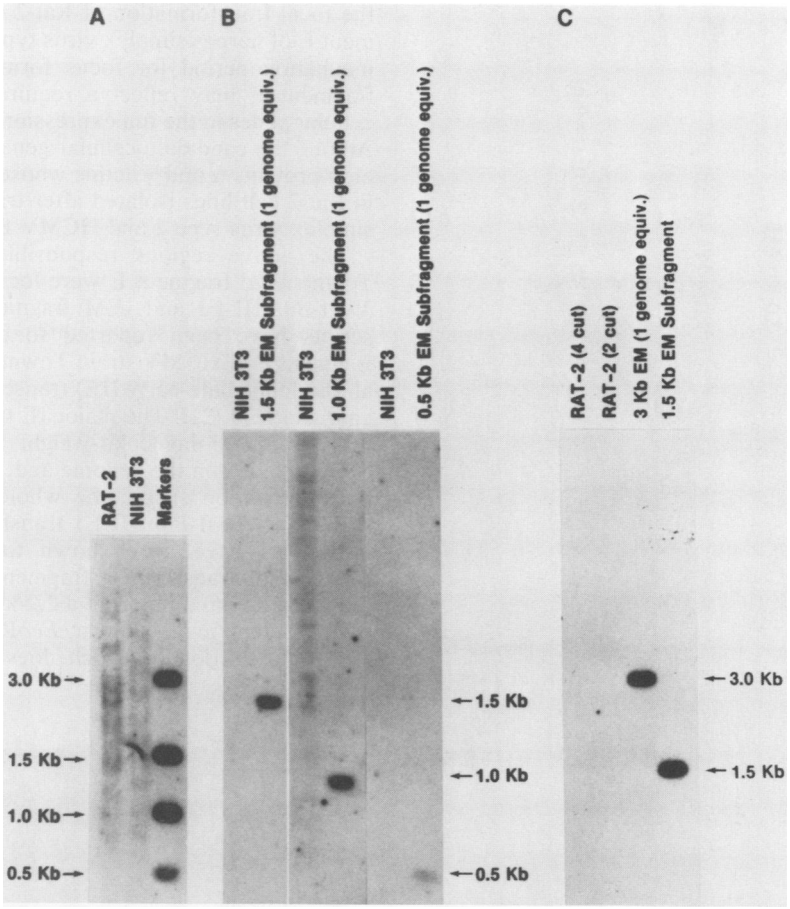


FIG. 3. Southern hybridization of normal Rat-2 and NIH 3T3 celluar DNAs to EM and *Pst*I-*Xho*I EM subfragment probes. (A) Normal Rat-2 and NIH 3T3 cellular DNAs (10 μg each) were digested with four enzymes (*Xba*I, *Bam*HI, *Pst*I, and *Xho*I) and hybridized with $^{32}$P-labeled EM DNA as probe. One genome equivalent each of 3.0-kb EM and EM subfragments (1.5, 1.0, and 0.5 kb) was used as marker. (B) A composite of three blots independently probed with EM subfragments. Each blot contains 10 μg of normal NIH 3T3 DNA digested with four enzymes as above and an EM subfragment as marker. (C) 10 μg of normal Rat-2 DNA was digested with *Xba*I and *Bam*HI (2 cut) or with four enzymes as above (4 cut) and hybridized with the $^{32}$P-labeled *Pst*I-*Xho*I 1.5-kb EM subfragment.

of 7.6 kb (<0.1 copy per cell) was detected in RBJ 1T after prolonged exposure, whereas no hybridization to the EJ probe was seen in normal Rat-2 RBJ 3T1 and RBJ 3T2 DNAs (data not shown).

**HCMV DNA sequences in Towne *Xba*I fragment E-transformed cells.** Towne *Xba*I fragment E transforms NIH 3T3 cells (Table 1). The detection of HCMV-specific sequences in EM fragment-transformed lines led us to investigate the presence of virus-specific sequences in the Towne *Xba*I fragment E focus-derived transformed line (NXE-11). Genomic DNAs from NXE-11 and normal NIH 3T3 cells were initially restricted with *Xba*I-*Bam*HI and further digested with *Xho*I-*Pst*I to liberate subfragments of EM. A Southern blot of this DNA was first probed with $^{32}$P-labeled EM DNA under stringent conditions as described above. The hybridization of EM probe with one genome equivalent of authentic EM DNA as well as with the marker 1.5-, 1.0-, and 0.5-kb EM subfragments of EM is shown in Fig. 5A. Sequences homologous to EM were detected in NXE-11, and they comigrated in position identically to the 1.5- and 1.0-kb EM subfragment markers. In addition, bands differing in size from marker fragments were observed. However, no band was detected in a position that comigrated with the 0.5-kb EM subfragment marker under the hybridization conditions used. Normal NIH 3T3 DNA exhibited faint hybridization with the EM DNA probe. To verify the presence of virus-specific sequences in NXE-11 DNA, we used the 1.5-kb EM subfragment probe which lacks cell-related sequences. Genomic NXE-11 DNA was digested with *Xba*I-*Bam*HI and *Xho*I-*Pst*I and hybridized. A single band that comigrated with the 1.5-kb EM subfragment marker was detected (Fig. 5B). No other bands were observed. The additional bands, which were detected with the intact EM probe (Fig. 5A), may therefore represent cellular sequences related to the 1.0-kb EM subfragment. Similar results have been recently obtained with focus-derived transformed lines NXE-2 and NXE-12 (data not shown).

To determine the presence of EJ sequences, a Southern blot of the *Xba*I-*Bam*HI-digested DNAs from NIH 3T3, NXE-11, and one-genome-equivalent EJ reconstruction was probed with the transforming EJ subfragment of Towne *Xba*I fragment E as probe. A single band (<0.1 genome equivalent) homologous to the EJ probe was detected in NXE-11 at a position comigrating with the 7.6-kb EJ fragment (Fig. 5C). Compared with the one-genome-equivalent EJ reconstruction, the retention of EJ was barely detectable.

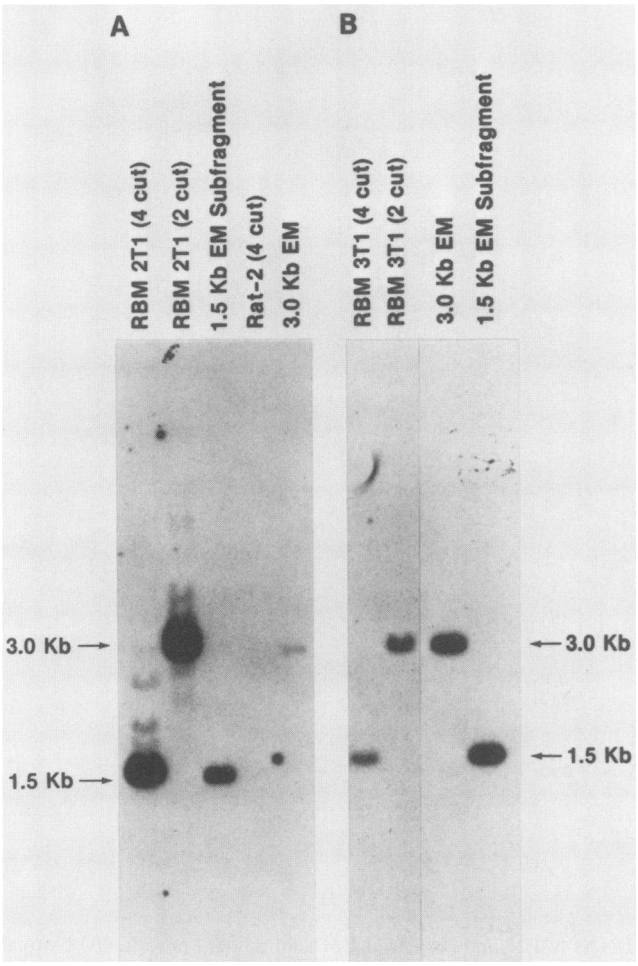


FIG. 4. Southern hybridization of *Xba*I-*Bam*HI-transformed rat tumor-derived cellular DNAs to $^{32}$P-labeled *Pst*I-*Xho*I 1.5-kb EM subfragment. (A) Normal Rat-2 and rat tumor (RBM 2T1) DNAs (10 μg each) were digested with *Xba*I-*Bam*HI (2 cut) or with four enzymes (*Xba*I, *Bam*HI, *Pst*I, and *Xho*I) (4 cut) and hybridized with $^{32}$P-labeled 1.5-kb EM subfragment as probe. Markers used were a 3-kb EM and 1.5 kb EM subfragment. (B) DNA (10 μg) from another independent rat tumor-derived cell line (RBM 3T1) was digested with two (2 cut) or four enzymes (4 cut) as above and hybridized with $^{32}$P-labeled *Pst*I-*Xho*I 1.5-kb EM subfragment as probe. The 1.5-kb EM subfragment and one genome equivalent of 3.0-kb EM were used as markers.

## DISCUSSION

The use of various cloned HCMV DNA fragments has further demonstrated that the Towne *Xba*I fragment E (4) and its terminal fragments *Xba*I-*Bam*HI-EJ or -EM can individually cause the tumorigenic conversion of immortalized rodent cells. DNA sequences contained within Towne *Xba*I DNA fragments AO, C, and O did not induce the transformation of immortalized cells. Both Towne *Xba*I fragment E and the 558-base-pair subclone of (pCM4127) of AD169 induced morphologically transformed foci on transfected NIH 3T3 cells at a comparable frequency. However, Towne *Xba*I fragment E-induced foci were detected on an average of 2½ weeks later than those induced by AD169 pCM4127. The delayed appearance of transformed foci after transfection with HCMV Towne *Xba*I fragment E resembles the focal transformation of Rat-2 cells by the *Bam*HI fragment E of herpes simplex virus type 2 DNA (14). The longer incubation period for focus formation with Towne *Xba*I fragment E may reflect a requirement for modulation of cellular genes in the full expression of the transformed state. Among the candidate cellular genes are the major cytoskeleton proteins α and γ actins whose expression is modulated in Rat-2 cell lines isolated after transformation with herpes simplex virus type 2 and HCMV DNA fragments (15).

The active regions responsible for transformation by Towne *Xba*I fragment E were localized within the terminal *Xba*I-*Bam*HI-EJ and -EM fragments. A number of transcripts have been reported to originate from the *Xba*I fragment E of HCMV strain Towne (21). This area codes for all the immediate-early (IE) transcripts and has been highly characterized (21). The major IE transcript (1.95 kb) coding for the 72,000-molecular-weight IE polypeptide maps to 0.739 to 0.751 on the genome and, with the exception of the polyadenylation signal, the whole of this gene is located within the *Xba*I-*Bam*HI-EJ transforming fragment. No IE transcripts have been shown to map within the *Xba*I-*Bam*HI-EM transforming fragment, however Staprans and Spector (20) have reported a 2.2-kb transcript coding for an early protein in a colinear *Eco*RI-R fragment of HCMV AD169, a portion of which does overlap with the 1.0-kb

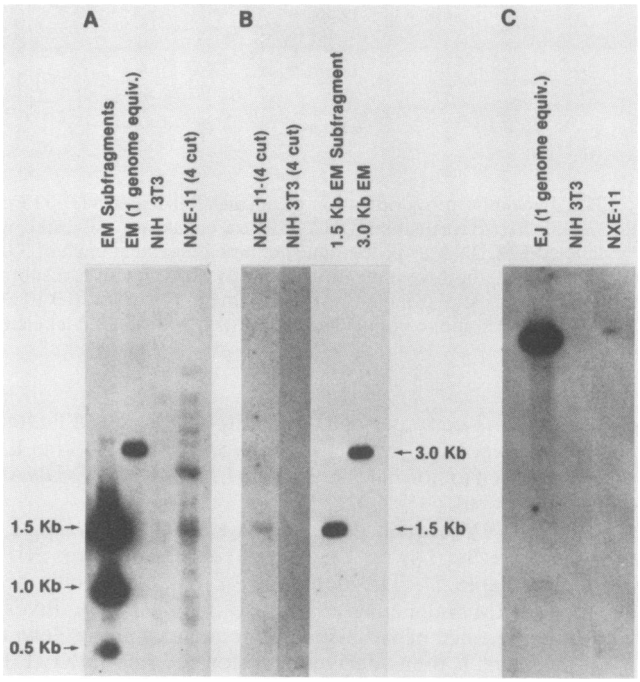


FIG. 5. Southern hybridization of Towne *Xba*I fragment E-transformed NIH 3T3 cellular DNA (NXE-11) with EM (panel A), 1.5-kb EM subfragment (panel B), and EJ (panel C) as probes. (A) Normal NIH 3T3 and NXE-11 DNAs (10 μg each) were digested with four enzymes as described in the legend to Fig. 4 (4 cut) and hybridized with $^{32}$P-labeled EM as probe. EM subfragments (1.5, 1.0, and 0.5 kb) and one genome equivalent of 3-kb EM were used as markers. (B) NXE-11 and normal NIH 3T3 DNAs (10 μg each) were digested with four enzymes (4 cut) and hybridized with the $^{32}$P-labeled 1.5-kb EM subfragment. One genome equivalent of EM and the 1.5-kb EM subfragment were used as markers. (C) Normal NIH 3T3 and NXE-11 cellular DNAs (10 μg each) were digested with *Xba*I-*Bam*HI and hybridized with $^{32}$P-labeled EJ DNA. One genome equivalent of EJ DNA was used as marker.

*Xho*I-*Bam*HI subfragment of EM as determined by nucleotide sequence analysis (A. Razzaque et al., unpublished data). In addition, sequence analysis also indicates a small 240-nucleotide potential open reading frame within the 1.5-kb *Pst*I-*Xho*I subfragment of EM (A. Razzaque et al., unpublished).

Each of the terminal fragments EJ and EM was capable of inducing focus formation in transfected NIH 3T3 and Rat-2 cells. Focal lines isolated after transfection with *Xba*I-*Bam*HI-EJ and -EM fragments formed medium-to-large (0.1 to 0.25 mm) colonies in soft agar and were tumorigenic in syngeneic Fisher rats. Tumors were detected in each case after a latent period of 4 to 6 weeks. No apparent difference in cellular properties was detected between *Xba*I-*Bam*HI-EJ- and -EM-transformed cells. However, we recently observed that cotransfection of Rat-2 cells with a combination of *Xba*I-*Bam*HI-EJ and -EM fragments results in foci that form very large, macroscopic (≥0.5 mm) colonies in agar and highly tumorigenic cells capable of forming tumors in Fisher rats within 1 week (R. Jariwalla et al., manuscript in preparation). This may suggest that *Xba*I-*Bam*HI-EJ and -EM fragments provide distinct functions that cooperate in the multistep process of tumor progression.

A Southern blot analysis of Towne *Xba*I fragment E-transformed cell DNA and *Xba*I-*Bam*HI-EJ or -EM tumor-derived DNA was next carried out with the transforming EJ and EM fragments as DNA probes. With the EM probe (Fig. 2 and 5) we readily detected EM sequences in all three lines examined (NXE-11, RBM 2T1, and RBM 3T1), whereas faint hybridization was detected in normal NIH 3T3 and Rat-2 DNAs. Similarly, two other Towne *Xba*I-E focus-derived transformed cell lines (NXE-2 and NXE-12) also showed the retention of EM-specific sequences (data not shown). Since the EM sequence showed faint hybridization with normal cellular DNAs, it was necessary to determine whether the bands that we detected in transformed and tumor-derived cell lines were derived from endogenous cellular sequences. To address this possibility we examined by Southern hybridization the homology of the Towne EM subfragments to normal NIH 3T3 and Rat-2 DNAs. Cell-virus homology was localized (Fig. 3B) to the 1-kb *Xho*I-*Bam*HI-EM subfragment, which is colinear to a sequence within the *Eco*RI R fragment of HCMV AD169 that was recently shown (20) to hybridize to uninfected human, murine, or sea urchin DNAs. Since the 1.5-kb *Pst*I-*Xho*I-EM subfragment did not exhibit any homology by hybridization to normal rodent DNAs, it was selected as probe to distinguish between virus-specific and cellular sequences in the transformed and tumor-derived lines. When RBM 2T1 (Fig. 4A) was reprobed with the 1.5-kb EM subfragment DNA, multiple copies of EM sequence were detected. Furthermore, upon digestion of genomic DNA with *Pst*I-*Xho*I, the internal 1.5-kb EM subfragment was detected. In another independent tumor-derived rat line (RBM 3T1) approximately one copy of EM and the 1.5-kb internal EM subfragment was detected (Fig. 4B). Thus, the identification of the 1.5-kb internal EM subfragment confirmed the retention of the virus-specific sequences. Recently we analyzed other *Xba*I fragment E-induced NIH 3T3 focal lines as well as EM-induced Rat-2 focus-derived transformed lines with the 1.5-kb EM subfragment as virus-specific probe. Sequences homologous to EM DNA were detected at less than one copy per cell in *Xba*I fragment E-transformed NIH 3T3 lines and were also barely detected in EM-transformed Rat-2 lines (data not shown). Several other EM-induced tumor-derived lines are currently being analyzed. The Towne *Xba*I fragment E-tranformed NIH3T3 lines were probed with the *Bam*HI-A, -D, and -T subfragments of *Xba*I fragment E. The *Bam*HI T subfragment did not exhibit any hybridization to transformed cell DNAs, whereas *Bam*HI-A and -D subfragments exhibited faint hybridization to both normal and transformed cellular DNA, indicating the presence of cell-related sequences in these subfragments.

A possible explanation for the presence of a low amount of EM sequence in the transformed cells is that not all of the cells retain viral sequences. Whether those cells that do not contain viral sequences remain fully transformed by a hit-and-run mechanism is not known. The observation that both tumor-derived lines consistently retained single or multicopy virus-specific bands suggests the possibility that EM-containing cells have a selective growth advantage for tumor formation in vivo. These data shed little information as to the state of the viral DNA sequences in the transformed cells. Experiments to elucidate the state of EM DNA in tumor-derived cell lines are in progress. In contrast to EM DNA, EJ sequences were rarely detected in focus-derived as well as tumor-derived lines, suggesting their instability in transformed cells. Since the transformed phenotype of EJ lines was not reversed, we favor the possibility that EJ sequences are not required for the maintenance of a transformed phenotype but may be required for the initiation of transformation. In this respect, EJ sequences resemble pCM4127 which transforms rodent cells but is not subsequently retained in transformed cells (6). Studies are currently underway to determine the minimal size of EJ required for transformation and to determine whether the 72,000-molecular-weight IE viral gene product may play a role in mediating transformation. On the other hand, the retention of EM sequences indicates that they may have a role in the maintenance of transformation. It has still not been determined whether a product of the HCMV-transforming sequences retained in the transformed or tumor-derived cell lines is required for the induction or maintenance of the neoplastic state, or both. Therefore, the expression of EM sequences in transformed and tumor-derived cell lines is currently under investigation.

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