Introduction and expression of the bacterial PaeR7 restriction endonuclease gene in mouse cells containing the PaeR7 methylase

T.Jesse Kwoh1,2, Patrice S.Obermiller1, Ann W.McCue2, Deborah Y.Kwoh2, Sue Ann Sullivan1 and Thomas R.Gingeras1,2

1La Jolla Biological Laboratories, PO Box 85350, San Diego, CA 92138 and 2The Salk Institute Biotechnology/Industrial Associates Inc., PO Box 85200, San Diego, CA 92138, USA

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ABSTRACT
To study the factors essential for a functional restriction system, the PaeR7 restriction-modification system has been introduced and expressed in murine cells. Transfer of this system was accomplished in two steps. First, cells containing sufficient PaeR7 methylase to completely methylate the mouse genome were constructed. In the second step, the mouse metallothionein promoter-regulated, endonuclease expression vector linked to the hygromycin B resistance selection marker was used to transfet the high methylase-expressing cells. Sixty percent of the clones isolated contained PaeR7 endonuclease enzymatic activity. Transfected cells expressing both methylase and endonuclease were incapable of blocking infection by DNA viruses, and possible explanations are discussed.

INTRODUCTION
Restriction-modification systems are one of the principal bacterial mechanisms for resisting bacteriophage attack and invasion by foreign DNA (reviewed in 1-3). In classical restriction-modification, incoming bacteriophage DNA is degraded by restriction endonucleases which recognize specific DNA sequences. The host bacterial genome is protected from endonucleolytic cleavage by methylation of the DNA at the endonuclease target sequence. Restriction systems have been observed to reduce bacteriophage plaque-formation efficiency by four to five orders of magnitude if the bacteriophage DNA has not been methylated at the target sequence. However, the efficiency of restriction is dependent upon the level of endonuclease expression and the number of recognition sites within the genome of the infecting bacteriophage. Bacteriophages which escape restriction are able to do so because their DNA has been methylated by the modification system before it can be cleaved. As a consequence, these methylated phages are capable of productively infecting other host cells.
Restriction-modification systems have been categorized into three types, based upon the biochemistry of the enzymes involved (2,3). The simplest of the three system types is the type II system, in which the endonuclease and methylase are separate proteins which act independently at their recognition sequence (2). The two enzymes also have very simple cofactor requirements compared to type I and type III systems (3). In recent years, the genes for a number of type II restriction-modification systems have been cloned (see citations in 4). Transfer and expression of the cloned PaeR7 (5), HhaII (6), PstI (7), DdeI (8), and PvuII (9) type II systems in restriction-deficient Escherichia coli increases the resistance of the recipient bacteria to infection by bacteriophages. However, E. coli expressing the complete TaqI system do not display increased phage restriction (4). Further, the PaeR7 and TaqI endonuclease genes can be expressed in E. coli in the absence of their cognate methylase genes, and such bacteria are unable to restrict phage (4,5). These findings indicate that while endonuclease gene expression is essential for restriction, additional factors must be satisfied as well in order to increase phage resistance in the host bacteria.

We have sought to examine the factors required to obtain a functional restriction system by attempting to reconstitute a type II restriction-modification system in a totally foreign host, the mammalian cell, and, in so doing, to establish mammalian cell lines which are refractile to DNA virus infection. The PaeR7 system, which has been isolated from the Pseudomonas aeruginosa plasmid, pMG7 (4), served as a model system. The PaeR7 system, an isoschizomer of the XhoI system, recognizes the sequence CTCGAG, cleaves between the first cytosine and thymine, and modifies the adenine residue in this sequence (4). The gene organization and nucleotide sequence of the cloned genes have been determined, and the protein products have also been partially characterized (10). To establish the PaeR7 system in mammalian cells, it was first necessary to protect the host cell genome from the PaeR7 endonuclease by the expression of the PaeR7 methylase gene. Cell lines containing the methylase gene have previously been described and were shown to express the gene by: 1) immunoblotting of cell extracts with anti-methylase antiserum; 2)
detection of enzymatic activity in cell extracts; and 3) observation of complete methylation in cotransfected DNA containing XhoI test sequences by Southern blot-hybridization of cellular DNA (11). In addition, murine adenovirus grown on these cell lines became fully methylated at PaeR7/XhoI sites (11). These results suggested that the genomes of transfected mammalian cells and of infecting virus, when replicating in the nucleus of the host cell, are accessible to the bacterial methylase.

In this report we describe the construction of mouse metallothionein promoter-regulated vectors for the expression of the PaeR7 endonuclease in animal cells. These vectors were used to transfec mouse L cells expressing varied levels of the methylase. Clones have been isolated which efficiently express the PaeR7 endonuclease, but these cells do not restrict viral infection. Possible explanations for the lack of resistance are discussed.

MATERIALS AND METHODS

Plasmid DNA Purification

Plasmid pSV2-hyg (12) was obtained from Dr. R. M. Liskay and plasmids pMMT341 and pMTneo302 (13) from Dr. M.-F. Law. Plasmids pCH110 and pMSG were both purchased from Pharmacia. The construction of pPA0R1.9, pPA0M2.7, pPA0694MT-1, and pACYC177-PAOM2.7 has been described previously (5,10,11). Plasmids were introduced by CaCl₂-mediated transformation into and maintained in Escherichia coli strain MM294 (K-12, F⁻, λ⁻, rK⁻, mK⁺, endA, thi) or in strain DH1 (MM294, gyrA, recA) (14). For plasmid DNA isolation from chloramphenicol-treated cultures, cleared cell lysates were made either by a sodium-lauryl-sulfate-NaCl method (14) or a lysozyme-Triton X-100 method (15) and plasmid DNA was purified by centrifugation through CsCl/ethidium bromide gradients (15). "Mini-preparations" of plasmid DNA were obtained by the phenol lysis method (16).

Construction of PaeR7 Expression Plasmids for Mammalian Cells

Endonuclease expression plasmids were constructed using the same approach previously employed for the methylase gene (11). All restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs, Bethesda Research Laborato-
Figure 1. Restriction map of plasmids. The source and the construction of these plasmids are described in the Materials and Methods. Each plasmid is displayed as linear DNA opened at an arbitrary restriction site. Abbreviations: Ba = BamHI; Bg = BglII, Bsa = BstXI, P1 = PvuI, P2 = PvuII, R1 = EcoRI, H3 = HindIII, S = Sall, X = XhoI, E-Pae = PaeR7 endonuclease gene, M-Pae = PaeR7 methylase gene, MMT-P = mouse metallothionein promoter, MTV-LTR = mouse mammary tumor virus long-terminal repeat, SV-A = SV40 early RNA processing sequences, SV-P = SV40 early promoter, amp = β-lactamase, gpt = guanine-xanthine phosphoribosyltransferase gene, hyg = hygromycin resistance gene, neo = Tn5 neomycin resistance gene, and lacZ = E. coli lacZ gene.

Briefly, after Bal31 digestion of BamHI-cleaved plasmid pPAORl.9 (Figure 1A), containing the PaeR7 endonuclease gene, the fragments were ligated to a BglII linker oligonucleotide using T4 DNA ligase and were used to transform E. coli. Deletion mutants
containing a BglII linker fused to the endonuclease ATG start codon were identified by hybridization of lysed colonies fixed to nitrocellulose filters with a 32p-labeled oligonucleotide probe. The position of the BglII linker was confirmed by DNA sequence analysis of M13mp19 clones containing each deletion mutant (data not shown). Plasmid pPAOΔ2317 (Figure 1B) was selected for further work. A second BglII linker was inserted into the unique HindIII site, and the resulting plasmid was designated pPAOΔ2317BB (Figure 1C). The 1.5 kb BglII endonuclease gene fragment from pPAOΔ2317BB was inserted into the unique BglII site of pMMT341 (Figure 1D). Transformations for this latter construction were performed in MM294 containing pACYC177-PAOM2.7 (Figure 1H), which expresses the PaeR7 methylase and thus insures the viability of cells containing an endonuclease-expressing plasmid. Clones in which the endonuclease gene is in the same transcriptional direction as the mouse metallothionein promoter were designated pPAO2317MT-1 (Figure 1E), and clones containing the opposite endonuclease gene orientation were designated pPAO2317MT-2 (Figure 1F).

A set of vectors in which selectable marker cassettes were added to pMMT341, pPAO2317MT-1, and pPAO2317MT-2 was also constructed. Each plasmid was digested with BamHI, treated with calf alkaline phosphatase, and then ligated to BamHI fragments containing either a xanthine-guanine phosphoribosyltransferase (gpt) or hygromycin B resistance (hyg) selectable marker cassette. Both the bacterial gpt (Figure 1M) and hyg (Figure 1I) cassettes were composed of the coding sequences of each marker fused to the SV40 early promoter and to the SV40 early RNA processing sequence. The derivatives of pMMT341 and pPAO2317MT-1 and -2 that contain the hyg selection cassette were named pHYG-MMT341 (Figure 1J), pHYG-PAO2317MT-1 (Figure 1K), and -2 (Figure 1L), respectively; the gpt derivatives are pGPT-MMT341 (Figure 1N), pGPT-PAO2317MT-1 (Figure 1O), and -2 (Figure 1P), respectively.

Growth and Transfection of Mouse L Cells

Mouse Ltk- cells were grown at 37°C in 5% CO2/95% air in DME (Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 100 units penicillin G/ml and 100 μg streptomycin sul-
fate/ml (GIBCO)]. The medium for cell lines A24b1, B6b2, B6C1 and B11b1 was supplemented with 15 μg/ml hypoxanthine, 1 μg/ml aminopterin, and 5 μg/ml thymidine (HAT; all purchased from Sigma) (11). The medium for transfectants containing the neo selection marker was supplemented with 400 μg/ml G418 (GIBCO) (17). Hyg transfectants were maintained in medium containing 400 μg/ml hygromycin B (CalBiochem) (12). For gpt transfectants, the medium was supplemented with 2 μg/ml mycophenolic acid (GIBCO), 300 μg/ml xanthine (Sigma), 2 μg/ml azaserine (Sigma) and 15 μg/ml hypoxanthine when the cells were tk- (18) or with 20 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, 2 μg/ml aminopterin and 10 μg/ml thymidine when the cells were tk+ (19).

Transfection experiments to obtain stable colonies were performed by the calcium phosphate method (20) on 100 mm dishes (NUNC) seeded the day before with 1 x 10^6 cells/dish. Cells were incubated with the calcium phosphate-DNA coprecipitate containing linear plasmid DNA and chromosomal Ltk DNA (total DNA = 20 μg/plate) for 14-16 hours. The cells from each plate were distributed to five new plates prior to addition of selective media. The resulting colonies were either stained with 0.1% crystal violet in 20% ethanol or individual colonies were cloned by sealing a glass cloning cylinder around an isolated colony with vaseline and detaching the cells within the cylinder with trypsin-EDTA (GIBCO). The isolated cells were dispersed in 10 ml medium on a 100 mm diameter plate. For hygromycin B-selected colonies, the drug concentration was reduced to 250 μg/ml until the cell titer reached 10^5-10^6 cells/plate.

Transfections for transient expression of the PaeR7 endonuclease were performed on plates seeded with 2 x 10^6 cells/dish. The calcium phosphate-DNA coprecipitate was made with 10 μg supercoiled, circular plasmid DNA per transfection plate. After 14-16 hours of incubation with the precipitate, the cells were washed and fed with fresh media. Cells were harvested at various times after the transfection by washing the cells with 5 ml phosphate-buffered saline per plate, scraping the cells into 2 ml saline per plate using a rubber scraper and pelleting the cells.
by centrifugation at 3000 rpm for 5 minutes. The drained pellet was frozen at -70°C for later analysis.

**PaeR7 Endonuclease Enzymatic Assays**

The cell pellet from two 100-mm plates was resuspended in 200 µl 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 6 mM 2-mercaptoethanol. Cells were lysed by sonication with Branson Sonifier model 350 attached to a microtip probe (50% duty cycle, output = 5, 10 seconds). The lysate was cleared by centrifugation in an Eppendorf microfuge for 15 minutes at 4°C. A 40 µl reaction mixture containing 100 mM NaCl, 10 mM Tris·HCl, pH 7.4, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin, 1 µg pMSG DNA, and 1 µl EcoRI was pre-incubated at 37°C for one hour and then 2-25 µl cell extract was added. Incubation was continued for 15-30 minutes at 37°C and the reaction was terminated by the addition of Na₂EDTA to 30 mM. The reactions were fractionated by agarose gel electrophoresis, and the DNA was visualized with ethidium bromide and ultraviolet light. Total protein was determined using the BioRad protein assay kit.

**DNA Hybridization and Immunoblotting**

Cellular DNA was prepared by phenol extraction of 1 x 10⁸ cells lysed with Proteinase K and sodium lauryl sulfate, as described previously (21). Analysis of chromosomal DNA by Southern blot-hybridization used 20 µg of DNA per sample and was performed as described previously (11). In all cases, ³²P-labeled probes were prepared either from purified restriction fragments or from M13 clones containing the desired probe sequence in order to avoid hybridization to plasmid sequences in the cells.

Analysis of cellular proteins by immunoblotting has been described previously (11). Briefly, cells were lysed by brief sonication and samples of the crude extract were fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by electrophoretic, and filters were probed with rabbit anti-methylase antiserum (11) and ¹²⁵I-labeled protein A.

**Virus Growth and Plaque Assay**

Herpes simplex virus type 1, strain KOS, obtained from Dr. V. Misra, and Vaccinia virus, obtained from Dr. M. B. A. Oldstone, were maintained on mouse Ltk cells. Stocks were prepared by
infecting cells at a multiplicity of infection of 0.005. Once substantial cytopathic effects were observed, the medium and cells were collected, subjected to one rapid freeze/thaw cycle, and centrifuged at 400 x g for 5 minutes. The supernatant was stored at -70°C until needed. Growth of murine adenovirus was described previously (11). For the plaque assay, the thawed virus was serially diluted, and 0.1 ml of each dilution was incubated on 5 x 10^5 cells for 1 hour at 37°C. The virus suspension was then removed and the infected cells were overlaid with 2 ml of the appropriate medium containing 0.75% methylcellulose and incubated at 37°C for 5-10 days. The plates were then washed with phosphate-buffered saline and stained with 0.1% crystal violet in 20% ethanol.

RESULTS

Construction of Cell Lines with High PaeR7 Methylase Expression

In order to examine the factors necessary for functional expression of a restriction-modification system, we have attempted to reconstitute the type II PaeR7 system in mouse Ltk cells. In bacteria, the efficient transfer of such systems to heterologous host cells often requires the protection of the host genome by the methylase gene prior to transfer and expression of the endonuclease gene (8). Previously, we described the isolation of four mouse cell clones (B6b2, B6c1, B11b1, and A24b1), which express the PaeR7 methylase gene (11). Although all of the cotransfected bacteriophage φ80 DNA, which contains eight PaeR7 sites, in these cell lines was fully modified (11), further examination of endogenous chromosomal PaeR7 sites in the c-fos, epidermal growth factor, and hypoxanthine phosphoribosyltransferase genes indicated that only partial methylation of these sites had occurred (data not shown). Interestingly, the single PaeR7 site located in the mouse mitochondrial DNA was totally unmethylated (data not shown).

To construct cells with complete methylation of chromosomal PaeR7 sites, additional methylase expression was sought in one of the original methylase-expressing clones. B6b2 was chosen based upon the stability of its thymidine kinase selection marker gene (Table I). Each of the clones was removed from HAT selection and
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TABLE I

STABILITY OF SELECTIVE MARKERS IN CLONES

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell Titera</th>
<th>Stabilityb</th>
<th>DME</th>
<th>HAT</th>
<th>HAT+G418</th>
<th>tk</th>
<th>neo</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A24b1</td>
<td>2.9 x 10^6</td>
<td>1.4 x 10^6</td>
<td>NA</td>
<td>ND</td>
<td>0.48</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B6b2</td>
<td>1.6 x 10^6</td>
<td>1.9 x 10^6</td>
<td>NA</td>
<td>ND</td>
<td>1.19</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B6c1</td>
<td>4.9 x 10^5</td>
<td>1.3 x 10^5</td>
<td>NA</td>
<td>ND</td>
<td>0.27</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B1b2</td>
<td>3.0 x 10^5</td>
<td>4.2 x 10^4</td>
<td>NA</td>
<td>ND</td>
<td>0.14</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>E2 pool</td>
<td>6.2 x 10^6</td>
<td>8.0 x 10^6</td>
<td>3.6 x 10^6</td>
<td>ND</td>
<td>1.29</td>
<td>0.45</td>
<td></td>
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<tr>
<td>E2a2</td>
<td>ND</td>
<td>8.3 x 10^6</td>
<td>2.7 x 10^6</td>
<td>ND</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2a3</td>
<td>ND</td>
<td>7.4 x 10^6</td>
<td>2.3 x 10^6</td>
<td>ND</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Cell lines were grown in DME (HAT for E2a2 and E2a3) for 32 days. Serial dilutions of the cells were then plated and grown for 14 days in various media. Colonies were counted after staining with crystal violet. Cell titer = number of cells in 10 cm diameter tissue culture plates. ND = not done. NA = not applicable.

b. Stability of the thymidine kinase (tk) gene = titer in HAT + titer in DME. Stability of the neomycin phosphotransferase (neo) gene = titer in HAT + G418 + titer in HAT.

grown for 32 days in DME. Serial dilutions of the cells were then made, and the colony-forming efficiency of each clone in medium with and without HAT was determined. Greater than 100% of the B6b2 cells were able to grow in HAT-supplemented medium. The greater than 100% survival rate in HAT compared to DME probably results from a growth stimulation by HAT. Only 14-50% of the cells from clones B6c1, B1b2, and A24b1 were able to form colonies after the resumption of HAT selection. These results suggest that the thymidine kinase transgene in B6b2 is stably integrated, whereas the transgenes in the other cell lines were only partially stable.

B6b2 was cotransfected with 15 ng SalI linear pMMTneo302 (Figure 1G) and 1 μg/ml SalI linear pPAO694MT-1 per plate (Figure 1Q) and grown in HAT medium supplemented with G418 for 2 weeks. Ten colonies were isolated at random and grown to mass culture. In addition, approximately 300 colonies from the same plates were pooled together as a mass culture and grown. Crude extracts of the individual clones, designated the E2 clones, were assayed for methylase protein levels by immunoblotting with anti-methylase antiserum (Figure 2). Most of the clones contained higher meth-
Figure 2. Immunoblotting of methylase-expressing cell lines. Extracts of sonicated cells were fractionated by NaDodSO4/polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose filters by electroblotting. After the filters were probed with rabbit anti-PaeR7 methylase antiserum, the bound antibodies were detected using $^{125}$I-labeled protein A. Lanes: A. Ltk; B. B6b2; C. E2b4; D. purified PaeR7 methylase; and E. E2a2.

The expression levels ranged from 20-fold higher (E2a2) down to a level equivalent (E2b4) to that found in B6b2.

To determine whether the increased levels of methylase expression resulted in complete methylation of the mouse cell chromosome, the PaeR7 sites in the c-fos gene of the E2 clones were examined (Figure 3). In the majority of clones, the c-fos BamHI fragment was completely resistant to XhoI cleavage, as exemplified by E2a2. However, clones such as E2b5, which showed only partial protection, similar to that previously observed for B6b2, were also obtained. The stability of the neo transgene in the E2 pool and the two E2 clones containing the highest methylase levels, E2a2 and E2a3, 20- and 15-fold higher, respectively, was determined (Table I). After 32 days of growth in DMEM, 45% of the cells in the E2 pool retained the neo transgene and 30% of the cells from the two clones retained the transgene.

Expression of the Endonuclease Gene in Mouse Ltk Cells

To determine whether the recombinant plasmids containing the PaeR7 endonuclease gene were able to express active endonuclease in mammalian cells, we measured the synthesis of enzymatic activ-
Figure 3. C-fos gene in methylase-expressing cell lines. Twenty µg samples of total cellular DNA from mouse Ltk cells and clones E2b5 and E2a2 were either untreated (U) or digested with BamHI alone (B), XhoI alone (X), or both XhoI and BamHI (X/B). After agarose gel electrophoresis, the digested DNA was transferred to nitrocellulose filters. A DNA fragment containing the cloned v-fos gene was 32p-labeled by nick translation and used as the hybridization probe.

Figure 4. Transient expression of PaeR7 endonuclease in mouse L cells. Mouse Ltk cells were transfected with pPAO2317MT-1 (1-8), pGPT-PAO2317MT-1 (11-12), pGPT-PAO2317MT-2 (13-14), pHYG-PAO2317MT-1 (14-15), and pHYG-PAO2317MT-2 (17-18). Cells were harvested at day 0 (1-2), day 1 (3-4), day 2 (5-6 and 11-18), and day 3 (7-8) after the transfection. Five µl (odd numbers) and 25 µl (even numbers) samples of each cell extract were used for 15-minute digests on 1 µg pMSG linearized by EcoRI. Digests were fractionated on 1% agarose gels along with EcoRI (lanes 9 and 19) and EcoRI and XhoI (lanes 10 and 20) control digests of pMSG. DNA was visualized by ethidium bromide staining and ultraviolet light.
### TABLE II
TRANSFECTION OF CELLS WITH pHYG-PAO2317MT-1 COLONIES

<table>
<thead>
<tr>
<th>Recipient Cells</th>
<th>Endonuclease Vector</th>
<th>Control Vector</th>
<th>Endonuclease + Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTK</td>
<td>8</td>
<td>237</td>
<td>0.03</td>
</tr>
<tr>
<td>B6b2</td>
<td>143</td>
<td>1116</td>
<td>0.13</td>
</tr>
<tr>
<td>E2 pool</td>
<td>306</td>
<td>965</td>
<td>0.32</td>
</tr>
<tr>
<td>E2a2</td>
<td>197</td>
<td>582</td>
<td>0.33</td>
</tr>
</tbody>
</table>

a. Average colony number in plates containing 1/5 of the cells from a transfection plate after two weeks in 400 μg hygromycin B per ml. Cells were transfected with 0.1 pmoles of either pHYG-PAO2317MT-1 (650 ng) or pHYG341 (550 ng) and with 20 μg LTK DNA per plate. Cells were incubated with calcium phosphate-DNA coprecipitate for 16 hours.

b. pHYG-PAO2317MT-1

c. pHYG341

3 (Figure 4). No endonuclease activity was observed in cells that received pMMT341 or pPAO2317MT-2 (data not shown). The amount of product DNA produced that comigrates with the EcoRI + XhoI control DNA is, at best, equivalent to 0.1 μg (day 3). The endonuclease expression levels at 2 days post-transfection by the hyg and gpt derivatives were also examined (Figure 4). The amount of product produced by extracts of pHYG-PAO2317MT-1 and pGPT-PAO2317MT-1 is comparable to that made by pPAO2317MT-1. These amounts of enzyme activity are significant, since only a small fraction (0.1-1%) of the cells received DNA in the transfection (22). In addition, the amount of activity is probably an under-estimation since the mouse cell extracts contain high non-specific deoxyribonuclease levels. Use of large amounts of extract or longer incubation times results in complete non-specific degradation of the linearized pMSG substrate DNA. Even in the brief incubation assays observed in Figure 4, significant non-specific DNase activity is seen, especially in extracts made at 3 days post-transfection, where higher cell numbers are found. In these assays, EcoRI restriction enzyme was present during the incubation of DNA with PaerR7 extracts to linearize the substrate plasmid DNA. Interestingly, the addition of EcoRI was found to partially block the action of the non-specific mouse cell nucle-
Figure 5. Enzymatic activity in clones expressing the PaeR7 endonuclease. Clones from high methylase-expressing cells transfected either with pHYG-PAO2317MT-1 (M1D2, M1F2, P1A1, P1B2, and P1C2) or with pHYG341 (M2A1 and P2A1) were assayed for endonuclease activity. Extracts were prepared by sonication and incubated with 1 μg pMSG DNA and EcoRI at 37°C for 15 minutes. For quantitation of the enzymatic activity, dilutions of PaeR7 purchased from New England Biolabs were each mixed with 5 μl M2A2 extract and added to pMSG DNA. After terminating the reactions by the addition of Na₂EDTA, the digests were subjected to agarose gel electrophoresis. Lanes: 1. clone M2A1, 5 μl extract; 2. P2A1, 5 μl; 3. M1D2, 5 μl; 4. M1F2, 5 μl; 5. P1A1, 5 μl; 6. P1B2, 5 μl; 7. P1B2, 1 μl; 8. P1C2, 5 μl; 9. P1C2, 1 μl; 10. P1C2, 0.5 μl; 11. 4 units PaeR7; 12. 2 units; 13. 1 unit; 14. 0.5 units; 15. 0.25 units; 16. 0.125 units; 17. 0.064 units; 18. 0.032 units; 19. 0.016 units; 20. 0.008 units. The arrow indicates the position of pMSG digested with EcoRI and XhoI.

ases. Other restriction enzymes also have the protective effect. Complete degradation of the circular pMSG DNA substrate was observed in experiments in which the EcoRI was omitted (data not shown).

Transfection of the Endonuclease Gene into Methylase-Expressing Cells

High methylase-expressing cells from the E2 pool and from the clone E2a2 were transfected with pHYG-PAO2317MT-1 which contains
the endonuclease gene linked to the hyg selection cassette. These high methylase-expressing cells have a higher survival to transfection with the endonuclease vector than Ltk or B6b2 (Table II). While the transfection efficiency of E2a2 and E2 pool was 3-fold lower for pHYG-PAO2317MT-1 compared to pHYG341, the reduction for Ltk was 30-fold and for B6b2 was 8-fold. Colonies from each methylase-expressing cell line (E2 pool and E2a2) were isolated from the transfection and grown as clonal cell lines. Extracts from individual clones were assayed for endonuclease enzymatic activity (Figure 5). Five of the eight isolated clones that had received pHYG-PAO2317MT-1 displayed enzymatic activity. Clone P1C2, a derivative of E2a2, had the highest activity. Based upon the comparison to enzyme of known activity, the specific activity in extracts from P1C2 was estimated at 14 units/mg protein. The lowest specific activity was estimated at 0.8 units/mg for clone M1F2. These results suggest that the endonuclease gene can efficiently be introduced and expressed in animal cells containing high levels of the methylase enzyme. The level of methylase protein in these clones, as determined by immunoblotting, was found to be similar to E2a2 (data not shown).

**Viral Challenge of Endonuclease Transfectants**

Cells containing the endonuclease gene were challenged with murine adenovirus, herpes simplex virus and Vaccinia virus. No substantial difference in viral plaque-forming efficiency was observed between cells containing the PaeR7 endonuclease and control cells (data not shown). A 2- to 5-fold lower viral titer was observed in some cells transfected with the endonuclease gene, but this could not be differentiated from a variability of plaque-forming efficiency observed in control cells.

**DISCUSSION**

We have transferred the genes of the PaeR7 restriction-modification system into mouse L cells in order to study the factors necessary for reconstitution of a functional system. While restriction-modification systems are primarily found in bacteria, such systems have been observed in several blue-green algae (23) and even as part of the genomes of viruses that infect Chlorella-like green algae (24). However, no other single- or multi-cellular eukaryotic organisms appear to have retained this form of
viral resistance. We have isolated several mouse L cell transfectants which express the PaerR7 endonuclease gene. Despite the presence of endonuclease activity in cell extracts, these cells did not display increased resistance to infection by murine adenovirus, herpes simplex virus, or Vaccinia virus, compared to transfectants which did not contain the endonuclease gene.

There are several explanations which could account for the lack of viral restriction in the clones which have detectable levels of PaerR7 endonuclease. First, the endonuclease-specific activity observed in the clones is 1000-fold lower than that found in bacteria. In the mouse cell clones, enzymatic specific activity ranged from 0.8 to 14 units/mg total protein. The endonuclease is present at $10^4$ units/mg in E. coli containing the complete PaerR7 system (10). While these bacteria exhibit a $10^3$-fold restriction of bacteriophage 80 infection, a $10^3$-fold reduction of the endonuclease specific activity in these bacteria eliminates the restriction phenotype (5,10).

A second possible explanation is the high level of methylase gene expression in the clones. With high methylase activity in the cells, infecting viral DNA may rapidly become sufficiently methylated to escape the action of the endonuclease. The level of methylase of E2a2, one of the cells used for introduction of the endonuclease gene, is 10-fold higher than other clones of the E2 series, which also displayed complete methylation of the XhoI site in the c-fos gene. This suggests that the transfection host cells used may have much more methylase activity than is required for complete methylation of the cell genome, and this excess methylation activity prevents the clones expressing the endonuclease from restricting infecting virus. Herpes simplex virus grown on E2a2 is completely resistant to XhoI cleavage.

A third explanation is that, unlike prokaryotic cells, eukaryotic cells are compartmentalized. Both adenovirus and herpes simplex virus replicate in the mammalian cell nucleus. The concentration of the PaerR7 endonuclease in the nucleus is likely to be lower than in the cytoplasm, since our vectors were not engineered to direct active transport of the endonuclease to the nucleus. In our cells, the endonuclease does enter the nucleus, because transfection of mouse L-cells which do not
express the \textit{PaeR7} methylase with the endonuclease-expressing plasmid pHYG-PAO2317MT-1 is a lethal event. The mode of entry into the nucleus is probably by passive transport, as is the case for \textit{Saccharomyces cerevisiae} expressing the EcoRI endonuclease (25). However, the lack of restriction of infection by \textit{Vaccinia} virus, which replicates in the cytoplasm, argues against compartmentalization as an explanation. But, it remains a possibility that \textit{Vaccinia} and the endonuclease are separated into different locations within the cytoplasm.

The fourth possible explanation for the lack of restriction by mouse cell transfectants expressing the endonuclease gene is the stability of the transfected genes. Most clones isolated from transfection initially display instability of the introduced genes (21,26). Growth of these clones may yield a mixed cell population in which cells vary in the amount of introduced DNA that is retained. In terms of the transfectants expressing the endonuclease gene, infection of cells containing little or no endonuclease will yield fully methylated virus which can successfully infect cells capable of restriction. Transfectants that have been cultured in the absence of selection for long time periods require reselection and subcloning to obtain clones which will grow as pure cell populations (26).

In constructing mammalian cells that express a restriction endonuclease gene like the \textit{PaeR7}, two factors were found to be important in order to obtain a high gene transfer efficiency. The most important factor is prior establishment of complete methylation by the cognate methylase. In transfections with the endonuclease expression vector on cells whose genome is completely methylated (E2 pool and E2a2), 60% of the clones isolated contained easily detectable endonuclease activity, whereas none of the isolates from transfections on cells possessing partial DNA methylation (B6b2, B6c1, and B1lb1) exhibited endonuclease expression. The second factor important for efficient introduction and expression of the endonuclease gene is linkage of the endonuclease gene with the selection marker gene. A large variability in the copy number of the endonuclease gene was observed in clones from cotransfection experiments in which the selection marker was not linked to the endonuclease vector.
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