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Genetics

# A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2

(complementing cell line/*gpt* selection/mycophenolic acid)

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ABSTRACT **Cell lines that produce viral gene products and that can support the growth of viral mutants lacking those products have been valuable in the genetic analysis of the transforming regions of several animal viruses. To extend the advantages of such complementing cell systems to regions of the adenoviral genome not directly involved in transformation, we have constructed a cell line that will support the growth of a defective adenoviral deletion mutant, H2*dl*808, that lacks most of early region 4 (E4). The right-hand terminal adenovirus 5 *Eco*RI restriction fragment, which contains all of E4, was first inserted into the vector pSV2*gpt*, and the recombinant plasmid was introduced into Vero cells by calcium phosphate precipitation. Clones containing the hybrid plasmid were selected by their resistance to mycophenolic acid. Five mycophenolic acid-resistant clones were then tested for the ability to support the growth of H2*dl*808. One of the five lines, W162, permits plaque formation by H2*dl*808 at an efficiency that is $>10^6$-fold higher than that of the parental Vero cells and allows the production of high-titer, helper-free H2*dl*808 stocks. Thus, W162 cells are permissive for at least one defective E4 mutant. The line carries, as expected, an intact E4, detected by hybridization. Using an H2*dl*808 lysate produced on W162 cells, we have accurately mapped the 808 deletion. It extends from between *Bcl* I and *Sma* I sites at positions 91.4 and 92.0, respectively, to just beyond a *Hin*dIII site at position 97.2 and, therefore, falls entirely within E4. H2*dl*808 and W162 should be of value in determining the physiological role of E4 in adenoviral infection.**

Studies that probe the functions of viral genes are frequently dependent upon the availability of viral mutants. Most of the mutants that have proved useful in studying viral gene function are conditionally defective; in animal virus systems, the majority of these are temperature sensitive (1, 2). An alternative approach to the isolation of conditionally lethal mutations exploits the fact that some virally transformed cells will support the growth of mutants with defects in transforming regions. For example, polyoma virus-transformed mouse cells support the growth of *hr-t* mutants which carry lesions in the polyoma early region (3); COS cells, a line of simian virus 40 (SV40)-transformed monkey cells, support the growth of SV40 early region mutants (4); and 293 cells, a line of human cells transformed by sheared adenoviral DNA (5), support the growth of mutants of adenoviral early region 1 (6–8). In each of these cases, mutant viruses can be propagated efficiently on the transformed cell line, and their phenotypes subsequently can be analyzed in normal, nonpermissive cell types. The value of a complementing cell line in the isolation and propagation of viral mutants is probably best illustrated by the last example; the examination of a wide variety of mutants of adenoviral early region 1 (E1) has provided a detailed picture of the functions of that region (6–13).

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In the examples cited above, the expression of integrated viral DNA is presumably responsible both for the cells' transformed phenotype and for their ability to complement the defective mutants. However, many segments of viral DNA do not transform cells, and there is no direct selection for cells that contain such DNA and that might support the growth of mutants in those regions of a viral genome. In an effort to extend the complementing cell approach to segments of viral DNA that do not transform cells and to make possible the analysis of mutants of adenoviral early region 4 (E4), we have used the *Escherichia coli gpt*-based selective system of Mulligan and Berg (14, 15) to introduce E4 DNA into cells that are permissive for human adenoviruses. E4 lies at the right end of the adenoviral genome, and although it is required for viral growth (see below), its role in the viral life cycle is not known. E4 is genetically ill-characterized, and so a cell line that would complement E4 mutants and simplify their isolation and analysis would be useful. One of the lines that we have obtained supports the growth of a defective adenoviral mutant, H2*dl*808 (16), which lacks most of E4. This paper describes the isolation of this line, its partial characterization, and the accurate mapping of the 808 deletion.

## MATERIALS AND METHODS

**Cells and Viruses.** Vero cells were obtained from A. M. Lewis and 293 cells from F. Graham. Vero cells were grown in monolayers in Eagle's minimal essential medium containing 10% calf serum (medium A), and 293 cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (medium B). Mycophenolic acid-resistant Vero cell derivatives were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and selective drugs as described below.

Wild-type adenovirus type 2 (Ad2) was originally from A. Lewis. H2*dl*808 is an Ad2 deletion mutant lacking the viral DNA between about positions 92 and 97.1 on the standard map; its isolation was described by Challberg and Ketner (16).

**Transformation and Mycophenolic Acid Selection.** Derivatives of the pSV2*gpt* plasmid of Mulligan and Berg (14, 15) containing adenoviral DNA (see *Results*) were introduced into Vero cells by the calcium phosphate precipitation technique (17, 18). About $5 \times 10^5$ cells were plated in a 9-cm Petri dish on the day before they were to be transformed; 15–24 hr later, the medium was removed from the dishes, and 0.5 ml of a suspension of precipitated DNA (12.5 μg of plasmid DNA per 0.5-ml aliquot) was added to each dish. After 20 min at room temperature, the plates were filled with 9 ml of medium A and transferred to a 37°C incubator. Four hours later this medium was replaced with selective medium: Dulbecco's modified Eagle's medium containing calf serum (10%), mycophenolic acid (25 μg/ml), xanthine (250 μg/ml), hypoxanthine (15 μg/ml),

Abbreviations: Ad2, adenovirus type 2; Ad5, adenovirus type 5; E1 and E2, early regions 1 and 2 of adenoviral genome; SV40, simian virus 40.

amethopterin (2 μg/ml), and thymidine (10 μg/ml) (16). Mycophenolic acid was the generous gift of the Eli Lilly Research Laboratories. Mycophenolic acid-resistant colonies first became visible 7–9 days later and were picked after 14–20 days by using cloning cylinders cut from plastic Eppendorf centrifuge tubes. The transformants were grown up and are maintained in the selective medium. It is at present unclear whether continued selection is necessary if W162 cells are to retain their biological activity over long periods, although a brief period without selection does not affect complementing ability (see below).

**Preparation of W162 Monolayers for Plaque Assays.** To conserve mycophenolic acid, plaque assays on W162 monolayers were performed in the absence of selective drugs. When suddenly withdrawn from selective medium, however, W162 cells grew very poorly. This difficulty could be avoided by a single passage through Eagle's minimal essential medium containing hypoxanthine, xanthine, and thymidine without amethopterin and MPA (medium C). Therefore, W162 cells to be used for plaque assays were transferred first into medium C and, after 2 to 3 days, into 5-cm dishes containing Eagle's minimal essential medium. The resulting monolayers could be used without further special treatment.

**Southern Transfers and Hybridization.** Cellular DNAs, digested with the restriction endonucleases *Eco*RI or *Hin*dIII, were fractionated on 3-mm thick vertical slab gels and transferred to nitrocellulose filter sheets by the Southern procedure (19). Restriction fragments containing adenovirus type 5 (Ad5) sequences were detected by hybridization (20, 21) to adenoviral DNA labeled with $^{32}P$ by nick-translation (22) and subsequent autoradiography.

## RESULTS

**Construction of Cell Lines.** The initial goal of these experiments was to introduce adenoviral early region 4 (E4) into a cell line permissive for human adenoviruses. To do so, we used the *gpt*-based selective system developed by Mulligan and Berg (14, 15). This system permits the selection of cells that take up one of a series of plasmid vectors containing the *E. coli gpt* gene linked to SV40 sequences that allow its expression in animal cells. The basis of the selection is the novel ability of such cells to utilize exogenous xanthine as a source of GMP, when *de novo* GMP synthesis is blocked by the drug mycophenolic acid: *gpt*-containing cells are resistant to mycophenolic acid in the presence of xanthine, whereas normal cells are not. Mycophenolic acid resistance is dominant, and the recipient cells need not possess any special properties. We constructed two derivatives of one of the *gpt* vectors, pSV2*gpt*. These plasmids (pE4*gpt*6 and pE4*gpt*16) both contain the Ad5 *Eco*RI B fragment inserted at the vector's single *Eco*RI cleavage site but differ in the orientation of the viral DNA segment. Ad5 *Eco*RI B covers the region 84–100 on the viral genome and contains all of E4, the fiber gene, and part of early region 3. The Ad5 *Eco*RI B fragment that we used has been modified by the addition to its right end of a synthetic *Eco*RI site and was kindly provided by K. Berkner. pE4*gpt*16 is diagrammed in Fig. 1.

The two E4-bearing plasmids were introduced into Vero cells by calcium phosphate precipitation, and mycophenolic acid-resistant clones were selected. On the average, two or three transformants were obtained from each plate exposed to plasmid DNA. A total of 13 clones were picked, 5 made with pE4*gpt*6 and 8 made with pE4*gpt*16. Four clones produced with pE4*gpt*16 (W162 through W165) and one clone produced with pE4*gpt*6 (W6B) were selected for further examination.

**Assay for Complementing Activity.** To determine whether any of the selected mycophenolic acid-resistant cell lines were capable of complementing an E4 defect, we tested each one for the ability to support growth of the defective E4 deletion mutant H2*dl*808 (16). The 808 deletion covers sequences from about 92 to about 97 map units on the Ad2 genome (see below) and, thus, is entirely within E4 (23, 24). A mixed stock containing both H2*dl*808 and its Ad5 *ts* helper, enriched for the deletion mutant by four cycles of CsCl equilibrium density gradient centrifugation, was titrated on each of the five mycophenolic acid-resistant lines listed above. The apparent titer of the stock on the five lines ranged from about $2 \times 10^5$ plaque-forming units/ml (W162, W163, W165, and W6B), to about $3 \times 10^4$ plaque-forming units/ml (W164). Five plaques produced on each of the lines W162–W165 and 10 plaques produced on W6B were picked and used to produce small lysates in cells of the same line. These lysates were then used to prepare small amounts of $^{32}P$-labeled viral DNA, which were digested either with *Eco*RI or *Xba* I and analyzed by agarose gel electrophoresis. Judged by the restriction fragments produced, all of the plaques formed on four of the lines (W163–W165 and W6B) contained H2*dl*808 and its Ad5 helper, the helper alone, or Ad2/Ad5 recombinants lacking the 808 deletion. Therefore, none of these lines seems to support the growth of pure H2*dl*808. In contrast, three of the five plaques picked from lawns of W162 contained only H2*dl*808. No restriction fragments characteristic of the Ad5 helper were observed in digests of DNA from these plaques, and the Ad2 fragments affected by the 808 deletion (*Eco*RI C and *Xba* I C), were entirely replaced by the expected novel fragments. The remaining plaques contained both H2*dl*808 and Ad5. An *Eco*RI digest of DNA from descendants of one of the mutant plaques, subsequently replaqued and grown up on W162, is presented in Fig. 2.

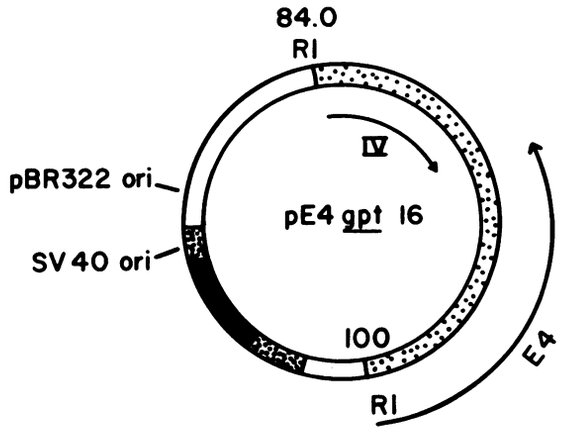


FIG. 1. Structure of pE4*gpt*16. pE4*gpt*16 consists of the Ad5 *Eco*RI B fragment (positions 84–100) inserted at the single *Eco*RI site of pSV2*gpt* (14). On the map, adenoviral sequences are indicated by light stippling, SV40 sequences by heavy stippling, and *E. coli gpt* sequences by the solid bar. The coordinates indicated are from the adenovirus physical map. Arrows indicate the direction of transcription and extent of adenoviral E4 and of the fiber gene (IV).

**Viral Growth on W162.** To confirm that H2*dl*808 is defective on normally permissive cell lines and that it forms plaques efficiently on W162, we titrated Ad2 and H2*dl*808 stocks on W162 and on the parental Vero strain. The H2*dl*808 stock used was produced in W162 cells from virus purified by three successive rounds of plaque formation on W162 monolayers. As shown in Table 1, H2*dl*808 formed plaques more than $10^6$-fold more efficiently on W162 cells than on Vero monolayers. We conclude that W162 complements a defect in H2*dl*808 that renders the mutant defective. That lesion is presumably the 808 deletion; thus, it seems certain that W162 will complement at least some defective E4 mutants.

**Viral DNA in W162.** Because we expected the complementing activity of W162 to be dependent upon the presence

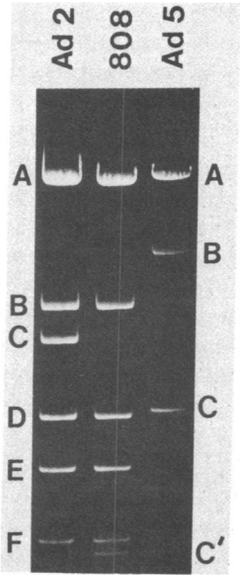


FIG. 2. *Eco*RI digest of H2*dl*808 DNA. DNAs obtained from purified H2*dl*808, Ad2, and Ad5 virions were digested with the *Eco*RI restriction endonuclease. The resulting fragments were fractionated by electrophoresis on a 1.4% agarose gel, stained, and photographed. In the digest of mutant DNA, the Ad2 C fragment is replaced by a shortened derivative (C'), which is slightly smaller than Ad2 *Eco*RI F.

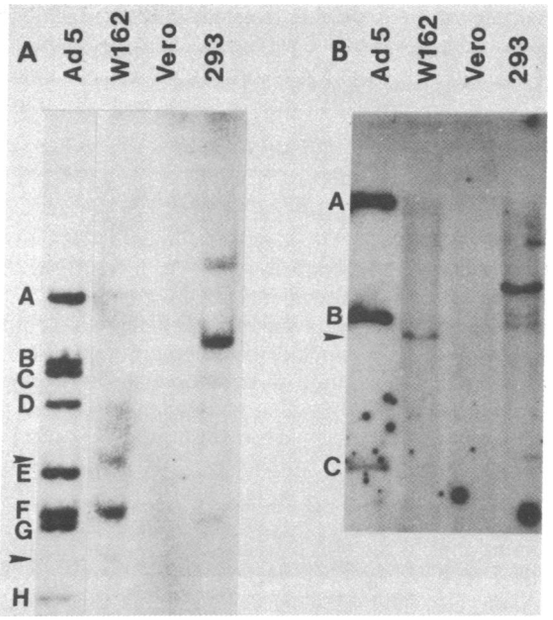


FIG. 3. Adenoviral DNA sequences in W162. W162, Vero, and 293 DNAs (10 μg each) were digested with *Hin*dIII or with *Eco*RI, transferred to nitrocellulose by the Southern procedure (19), and hybridized to $^{32}$P-labeled Ad2 DNA. (*A*) *Hin*dIII digests. The eight largest Ad5 *Hin*dIII fragments are indicated by letters. The arrowheads mark the positions of two of the bands that contain viral sequences in the lane containing W162 DNA; the third band lies next to *Hin*dIII F. (*B*) *Eco*RI digests. The three Ad5 *Eco*RI bands are labeled, and an arrowhead marks the position of the band containing viral sequences in the digest of W162 DNA. The Ad5 standard contains viral DNA equivalent to about 10 copies per genome.

of viral E4 DNA, we assayed W162 for viral DNA sequences by the Southern transfer procedure (19). W162, Vero, and 293 DNAs (10 μg each) were digested with either the *Hin*dIII or *Eco*RI restriction endonucleases, fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized to $^{32}$P-labeled Ad2 DNA. The *Hin*dIII digest of W162 DNA contained three bands that hybridized to viral DNA (Fig. 3). One of these comigrated with Ad5 *Hin*dIII F (89.5–98.2 map units), which contains most of E4. *Eco*RI digestion of W162 DNA produced one fragment containing viral sequences that had a slightly greater mobility than had Ad5 *Eco*RI B. Vero DNA contains no viral sequences, whereas 293 DNA digested with either enzyme yielded three fragments that hybridized with viral DNA. Because the fragment observed in the *Eco*RI digest of W162 DNA did not comigrate precisely with the Ad5 *Eco*RI B marker, the pE4*gpt*16 DNA present in these cells must have suffered some rearrangement during its incorporation. However, both the phenotype of the line and the presence of the *Hin*dIII F fragment suggest that W162 carries an intact, functional E4.

**The 808 Deletion.** The deletion in H2*dl*808 had previously been mapped by electron microscopy to coordinates 92.0–97.1 (14). To refine these measurements, we assayed DNA obtained from plaque-purified H2*dl*808 virions for the presence of several restriction sites near the ends of the deletion. The results of these mapping experiments (summarized also in Fig. 4) indicate that *Sma* I and *Hin*dIII cleavage sites at positions 92.0 and 97.2 (25), respectively, are missing from H2*dl*808 DNA, while *Bcl* I and *Sma* I sites at positions 91.4 and 98.4 (25), respectively, are present. Therefore, the left end point of the deletion falls in the roughly 200-base region between positions 91.4 and 92.0, and the right end point falls between positions 97.2 and 98.4. The size of the 808 deletion, measured by electron microscopy and estimated from the mobility of the novel restriction fragments produced in H2*dl*808 DNA by the deletion (Fig. 2) is just over 5%. It is likely, therefore, that the right end point lies quite close to the *Hin*dIII site at position 97.2 as shown in Fig. 4.

The 808 deletion, which does not cover the *Bcl* I site at position 91.4, cannot be any closer to the presumed polyadenylylation site for fiber mRNA (position 91.1; refs. 25 and 26) than about 100 bases. It is likely, therefore, that fiber mRNA is not directly affected by the 808 deletion. This is of particular interest in light of the observation that, even in W162 cells, H2*dl*808 substantially underproduced fiber protein (data not shown). Therefore, the 808 deletion may define a downstream site, outside of the sequences incorporated into stable mRNA,

Table 1. Titration of Ad2 and H2*dl*808 on W162 and Vero cells

| | Ad2 | H2*dl*808 |
|---|---|---|
| W162 | $4.0 \times 10^7$ | $7 \times 10^7$ |
| Vero | $1.5 \times 10^7$ | $<1 \times 10^1$* |

Ad2 and H2*dl*808 lysates were titrated on W162 and Vero cells. The titers of the two stocks on these cell lines appear above, expressed as plaque-forming units per ml.

* No plaques appeared on either of two dishes inoculated with 1.0 ml of the H2*dl*808 stock diluted 1:10.

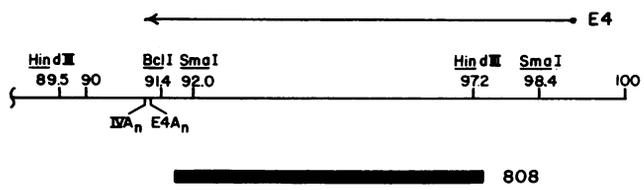


FIG. 4. Map of the H2*dl*808 deletion. The extent of the H2*dl*808 deletion, deduced from restriction digests and electron microscopy, is diagrammed. The end points of the deletion (black bar) fall between a *Bcl* I site at position 91.4 and a *Sma* I site at position 92.0 on the left and between a *Hin*dIII site at position 97.2 and a *Sma* I site at position 98.4 on the right. The positions of the presumed polyadenylylation signals for fiber (IVA$_n$) and E4 (E4 A$_n$) RNAs and the approximate extent of E4 transcription (arrow) are indicated (23, 24).

that is necessary for efficient expression of the fiber gene. More thorough analysis of H2*dl*808 will presumably shed light on this possibility and on the nature of the mutant's biochemical defect.

## DISCUSSION

The genetic analysis of the transforming regions of several animal viruses has been facilitated by the fact that some transformed cell lines support the growth of otherwise defective viral mutants with lesions in those regions. Such transformed cell lines contain and express segments of viral DNA (3–5) and presumably are capable of supplying the essential products of those DNA segments to viral mutants that cannot produce them. The experiments described here were undertaken to extend the benefits of such complementing cell systems to a viral DNA segment not directly involved in transformation, early region 4 of human adenoviruses. In these experiments, a segment of adenoviral DNA containing early region 4 sequences derived from Ad5 was introduced into Vero cells as part of a plasmid containing the Ad5 *Eco*RI B fragment and the dominant, selectable *E. coli* gene *gpt* (14). Several of the resulting cell lines were then screened for biological activity and one, W162, was found to support the growth of the defective E4 deletion mutant H2*dl*808 (16). Thus, it is possible to construct complementing cell lines for at least some segments of viral DNA that do not transform cells, and a line that should be useful in the analysis of E4 was obtained. Recently, Shiroki *et al.* (27) and Babiss *et al.* (28) reported the use of the *gpt* selective system to construct KB cell derivatives containing adenoviral E1 sequences. Like 293 cells, some of these lines support the growth of E1 mutants.

Vero cells, which are of monkey origin but permissive for human adenoviruses, were chosen for these experiments rather than a human cell line partly for technical reasons: Vero cells grow well, form durable monolayers, and were easily transformed to mycophenolic acid resistance. Further, in Vero derivatives, resident E4 sequences should remain silent because Vero cells do not contain the adenoviral E1 sequences required for efficient E4 expression (9, 10). Thus, even if E4 expression is lethal, there ought to be no selection against E4-containing Vero transformants, as there might be against similar derivatives of, for example, 293 cells. We presumed that in E4-bearing Vero derivatives, infecting adenovirus would activate the resident E4 DNA by providing E1 products.

Five mycophenolic acid-resistant lines, all of which presumably carry the *gpt* gene and attached E4 DNA, were originally chosen for close examination. Of these, only one seems to complement the E4 mutant that we have used to test biological activity. The reason for the inactivity of the other four lines is not known. All of the lines examined, including W162, grow well and form long-lived monolayers.

E4 is one of the few segments of the adenoviral genome for which no function in the viral life cycle is known. This is due in part to the lack of E4 mutants: no conditionally defective mutants are available, and H2*dl*808, which until now has been propagated in the presence of a helper virus, carries a deletion too small to make its physical purification practical. One deletion mutant lacking E4 sequences (H2*dl*807; ref. 14) has been characterized, but the interpretation of its phenotype is complicated by the fact that it is missing a substantial amount of DNA outside of E4. The difficulties encountered in the genetic analysis of E4 should be considerably reduced by W162, which will make the analysis of H2*dl*808 possible immediately and should permit the eventual isolation of new E4 mutants.

Using lysates produced on W162 cells, we have begun the characterization of H2*dl*808, which lacks viral sequences from between positions 91.4 and 92.0 to just beyond position 97.2. This deletion falls entirely within E4 (ref. 24; see Fig. 4) and would disrupt all but the most promoter-proximal of the hypothetical protein-coding regions in E4 proposed on the basis of sequence data (25, 26). We are not yet certain of the level at which the growth of H2*dl*808 is blocked in nonpermissive cells. It is of interest that even in W162 cells, H2*dl*808 synthesizes little fiber protein. Because fiber mRNA ought not to be directly affected by the deletion, the missing DNA may contain a novel genetic element required for efficient expression of the fiber gene.

The W162 cell line should soon shed light on the functions of adenoviral early region 4. The method used in the construction of the line also should be of general utility in producing similar complementing cell lines for other regions of interest in animal virus genomes.

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