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Transduction of the Chinese Hamster Ovary *aprt* Gene by Herpes Simplex Virus

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The Chinese hamster ovary adenine phosphoribosyl transferase gene (*aprt*) was reengineered to be flanked by sequences from the thymidine kinase (*tk*) gene of herpes simplex virus. This construct was cotransfected with DNA from herpes simplex virus type 1, and after 3 days, virus was harvested and Tk$^-$ plaques were selected after the virus was plated on Tk$^-$ cells in the presence of bromodeoxycytosine. Recombinant viruses were identified by dot-blot hybridization, and the arrangement of *aprt* and *tk* sequences were determined by Southern blot hybridization. Analysis of the recombinants revealed that acquisition of *aprt* sequences resulted from insertional inactivation of the *tk* locus as a consequence of homology-based recombination. Recombination was precise, as evidenced by the failure to detect plasmid sequences or the synthetic restriction endonuclease sites that bounded the mutant *tk* gene in the *aprt-tk* construct. Infection of Aprt$^-$ mouse or Chinese hamster ovary cells with UV-irradiated virus and selection in medium containing azaserine and adenine resulted in the survival of numerous colonies that stably express the *aprt* gene. Transformed cells synthesized an *aprt* mRNA that is identical to wild-type mRNA as determined by Northern blot and S1 nuclease analyses. Cells lytically infected with the recombinant virus do not appear to transcribe the *aprt* gene. Thus, infected cells differentiate between virus and foreign promoters even when a cellular gene is *cis* to the virus chromosome.

The ability to isolate and manipulate eucaryotic genes has necessitated the development of strategies to reintroduce these altered sequences into the cell from which they were derived. By these means, it will become possible to correlate the effects of specific mutations with the biological activity of a defined genetic element. The observation by Zinder and Lederberg (50) that bacteriophages could successfully transduce bacterial genes has served as a precedent for similar studies with animal viruses as vectors and eucaryotic cells as hosts. Exploitation of eucaryotic viruses as vectors requires that they be easy to manipulate, contain dispensable domains that are suitable targets for deletion and replacement with foreign sequences, and not be subject to rigid packaging constraints. The use of simian virus 40 as a vector for the transient expression of isolated genes (11, 23) has demonstrated both the feasibility of using viruses as vectors and the problems associated with a vector that can only package a limited amount of genetic information. More recently adenoviruses (38, 44), vaccinia virus (19, 30), and retroviruses (34, 43, 47) have been employed as vectors for the introduction and expression of foreign genes into animal cells. We have chosen to explore the feasibility of using herpes simplex virus (HSV) as a transducing vehicle for eucaryotic cells. Our choice of HSV was dictated by several considerations; the virus has a wide host range allowing it to infect virtually any cell of mammalian origin, virus inactivated by irradiation with UV light efficiently transforms thymidine kinase-deficient (Tk$^-$) cells to the thymidine kinase wild-type (Tk$^+$) phenotype (24), the virus genome has the capacity to expand (15), and insertions of foreign DNA at the *tk* locus are readily selected. In this study, we describe the construction of a recombinant HSV type 1 (HSV-1) that contains the genomic sequences that code for the adenine phosphoribosyl transferase gene (*aprt*) from Chinese hamster ovary (CHO) cells. This virus will stably transform adenine phosphoribosyl transferase (Aprt$^-$) cells of both mouse and hamster origin to the adenine phosphoribosyl transferase wild-type (Aprt$^+$) phenotype. These transformed cells transcribe an mRNA that is indistinguishable from that synthesized by Aprt$^+$ CHO cells. Parenthetically, we note that when the *aprt* gene is placed *cis* to the virus chromosome, it is not expressed at any time during the infectious cycle of the virus.

MATERIALS AND METHODS

Cells and viruses. Murine Ltk$^-$ Aprt$^-$ cells were maintained in Dulbecco modified Eagle medium supplemented with 10% inactivated calf serum, 30 μg of bromodeoxyuridine per ml, and 50 μg of 2,6-diaminopurine per ml. A29 Chinese hamster cells were maintained in F-12 medium containing 10% fetal calf serum plus twice-concentrated nonessential amino acids (48). Aprt$^-$ CHO cells were maintained in the same medium supplemented with 50 μg of 2,6-diaminopurine per ml. Medium for the selection of the Aprt$^+$ phenotype contained azaserine and adenine at 4 and 15 μg/ml, respectively, as previously described (48). HSV-1 strain F obtained from B. Roizman was grown and titers were determined on Vero cells as previously described (26). F lacks a *Kpn*I site in the sequences that code for *tk* from HSV type 106 (35).

Transfection. Procedures for the preparation of the calcium phosphate precipitates and transfection of eucaryotic cells were as previously described (48). Transformation of hamster cells was performed in medium containing a 1:1 mixture of Dulbecco modified Eagle medium and F-12 medium. Transformants were identified on the basis of ability to grow in selective medium containing azaserine and adenine, and colonies were scored after growing for 15 to 21 days and were isolated with the aid of cloning cylinders.

Plasmids and M13 phage clones. Plasmids that were used in this study and their sources are noted below; pTK5 (29), pHAPRT (17), pBiM6, and pBiM10 were a gift from Steve McKnight (20), and pPVUII is the 1.8-kilobase (kb) *Pvu*II fragment isolated from pHAPRT and cloned into the unique *Pvu*II site of plasmid pBR328. To generate a probe specific

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TABLE 1. Functional analysis of the *aprt* gene by restriction endonuclease cleavage of cloned DNA[a]

Restriction endonuclease	DNA source	Aprt$^+$ colonies[b]	Tk$^+$ colonies[b]
*Hin*dIII	pHAPRT	110	0
*Bam*HI	pHAPRT	80	ND
*Bgl*II	pHAPRT	106	ND
*Eco*RI	pHAPRT	0	ND
*Pvu*II	pHAPRT	0	ND
*Kpn*I	pHAPRT	67	ND
*Hin*dIII	pCT26	140	0
*Bam*HI	pCT26	95	0
*Bgl*II + *Kpn*I	pCT26	82	0
*Bam*HI + *Hin*dIII	pBiM6	ND	202

[a] pHAPRT or pCT26 DNAs were digested to completion with 5 U of the indicated restriction endonuclease per μg. Digested DNA (20 ng) was transfected onto Aprt$^-$ mouse L cells, and the frequency of transformation was determined. ND, Not determined.

[b] Colonies per plate is the average number of Aprt$^+$ or Tk$^+$ transformants that appeared after 15 to 20 days in selective medium. In each instance, 10 plates were transfected with DNA and selected in medium containing azaserine and adenine or HAT.

for the 5′ end of the *aprt* mRNA, we isolated the *Bam*HI-to-*Pvu*II fragment after subcloning it in pBR328 and inserted it into the replicative form DNA from mp9 at the *Bam*HI and *Sma*I sites. The resulting ligation mixture was transformed into *Escherichia coli* JM103 cells (21). Clear plaques were picked and screened for the presence of the insert by digestion with restriction endonucleases and DNA sequencing. A recombinant designated mp9A-54 was used in subsequent studies.

Isolation of nucleic acids. High-molecular-weight DNA was isolated from mouse or hamster cells as previously described (31). Total cell RNA was isolated from cells lysed with guanidine thiocyanate as described by Chirgwin et al. (8). Polyadenylic acid [poly(A)]-containing RNA was isolated by affinity chromatography on oligodeoxythymidilate-cellulose columns (1). HSV-1 DNA was isolated from purified virions. The purified virus was suspended in 20 mM Tris (pH 8.0)–150 mM NaCl–5 mM EDTA, and after incubation in the presence of 50 μg of boiled pancreatic RNase per ml for 1 h at 37°C, addition of sodium dodecyl sulfate to 0.5%, and digestion with 500 μg of heat-treated pronase per ml for 1 h, the lysed virus suspension was sequentially extracted with phenol and chloroform-isoamyl alcohol (24:1). DNA was precipitated after the addition of an equal volume of isopropanol and suspended in sterile water. Recombinant DNAs were isolated from chloramphenicol-amplified cultures as described by Bolivar et al. (3).

Filter hybridization. DNA from transformed cells or from viruses or plasmids was digested with the appropriate restriction endonucleases under conditions described by the supplier, electrophoresed through agarose gels, and transferred to nitrocellulose paper (39) after depurination by exposure to 200 mM HCl (45) to facilitate quantitative transfer of high-molecular-weight DNA. The filters were baked, blocked, and hybridized to ^{32}P-labeled probes as previously described (49). RNAs were analyzed by blot hybridization after electrophoresis in gels containing 2.2 M formaldehyde as previously described (29). To determine whether the putative Tk$^-$ recombinants contained the CHO *aprt* gene, we used dot-blot hybridization of whole, infected cells as described by Brandsma and Miller (4).

Transduction. Recombinant HSV-*aprt* virions were labeled with ^{3}H by being grown in the presence of [^{3}H]hypoxanthine and were purified by centrifugation through gradients of dextran T10 in 1 mM $NaPO_4$ buffer (pH 7.0) as described by Spear and Roizman (40). The particle-to-PFU ratio of purified virus was determined by measuring the specific activity, the virus was irradiated with UV light, and at various intervals, samples of the irradiated virus were removed and titrated to determine the reduction in plaque-forming ability of the exposed virus. Samples containing virus whose infectivity was decreased by 5 orders of magnitude were used to infect Aprt$^-$ mouse L or CHO cells. The cells were overlaid with medium containing 0.1% purified pooled human gamma globulin to neutralize any infectious virus. At 24 h postinfection, Aprt$^+$ cells were selected by exposure to medium containing azaserine, adenine, and 0.1% pooled gamma globulin, the medium was changed after 4 days, and colonies were scored after 14 to 21 days in culture. Aprt$^+$ transformants were isolated with the aid of cloning cylinders and maintained in the presence of selective medium.

S1 nuclease analysis. The 5′ ends of the *aprt* transcripts were mapped by the procedure of Berk and Sharp (2) with strand-separated, end-labeled probes as described by Weaver and Weissmann (46). The probe was prepared by subcloning the *aprt* sequences from the small *Bam*HI-to-*Pvu*II fragment from pHAPRT to pBR328. The recombinant was cleaved at the unique *Pvu*II site, digested with calf intestine alkaline phosphatase, and end-labeled with [^{32}P]ATP by using polynucleotide kinase from T4-infected cells. The labeled DNA was heated to 65°C for 15 min and then diluted and digested with *Bam*HI. The DNA was then extracted with phenol, precipitated by the addition of 2 volumes of ethanol, and strand-separated by electrophoresis through a 5% acrylamide gel (acrylamide-bisacrylamide, 60:1) after denaturation in formamide. DNA was eluted from the gel by diffusion in 500 mM $NH_4C_2H_3O_2$–10 mM EDTA overnight at 65°C and filtered through a cellulose-acetate membrane to remove gel bits and then concentrated by ethanol precipitation. The probe was precipitated in the presence of the RNA to be analyzed and suspended in 10 μl of Casey-Davidson (7) salts containing 80% formamide. The hybridization mix was heated to 80°C for 10 min and then transferred to a bath maintained at 52°C for 18 h. The samples were diluted in 300 μl of S1 digestion buffer containing 40 mM $NaC_2H_3O_2$ (pH 4.5), 250 mM NaCl, 1 mM $ZnSO_4$, and 2,000 U of nuclease S1 (Boehringer-Mannheim Corp.) and digested at 37°C for 1 h. The reaction was terminated by the addition of EDTA and yeast RNA to 10 mM and 10 μg, respectively, and the product was precipitated with ethanol and analyzed by electrophoresis through a 6% acrylamide gel (acrylamide-bisacrylamide, 20:1) containing 8.3 M urea in 135 mM Tris–45 mM H_3BO_3–2.5 mM EDTA (pH 8.9). Alternatively, a uniformly ^{32}P-labeled, single-stranded probe complementary to *aprt* mRNA was prepared by primer extension on mp9A-54 and used for S1 analysis.

RESULTS

Experimental rationale. Previous studies by Knipe et al. have demonstrated that the HSV genome can be expanded by as much as 7.5 kb (15). Subsequent studies by this group and by Smiley et al. have demonstrated the feasibility of inserting or deleting sequences at the viral *tk* locus (22, 33, 36, 37). By imbedding the sequence of interest within a virus gene, it was possible to transfect this reconstructed sequence along with naked virus DNA into animal cells. The flanking homology served to site direct the recombination events that may result in insertion or deletion of virus

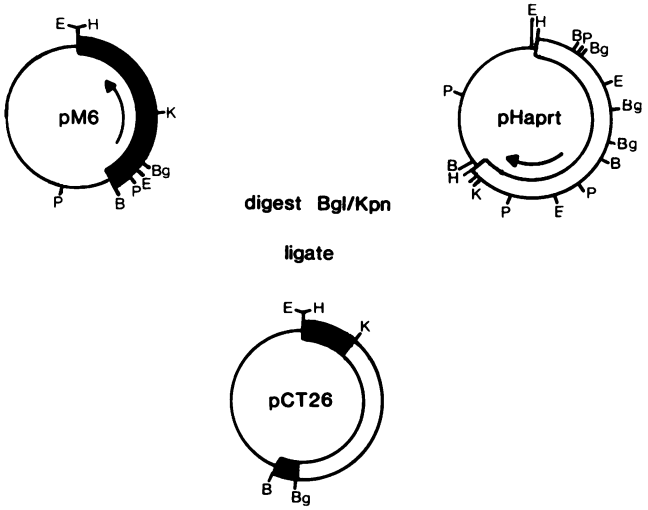

FIG. 1. Construction of an *aprt-tk* hybrid plasmid. pCT26 was constructed from the plasmids pBiM6 and pHAPRT. pBiM6 is a 5.9-kb TK^+ plasmid that contains a synthetic *Bam*HI linker at the 5′ end of the *tk* gene and a *Hin*dIII linker which marks the 3′ end of this gene; pHAPRT is a 7.8-kb plasmid that contains the *aprt* gene cloned from CHO cells. The origin and construction of these plasmids has been described in detail (17, 20). We digested these plasmids with *Bgl*II and *Kpn*I and isolated the *tk* sequences that remained with the pBR322 vector and the unique 3.8-kb, *aprt*-containing sequence. These DNAs were ligated, and ampicillin-resistant recombinants were screened for the presence of both sequences by the colony hybridization technique of Grunstein and Hogness (10). Positive colonies were analyzed for the presence of the appropriate restriction endonuclease sites and the ability to transfer the Aprt but not Tk phenotype to appropriate recipient cells. The direction of transcription and approximate size of each transcript is marked by an arrow in the parental plasmids. The abbreviations used to denote restriction endonuclease cleavage sites are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II. Symbols: →, mRNA; ■, HSV *tk*; □, hamster *aprt*; ——, pBR322.

sequences. The rare recombinants are recognized on the basis of their ability to form plaques in selective medium on the appropriate cell line (6).

On the basis of these studies and the early work of Munyon et al. (24), who showed that the virus *tk* gene was readily transferred to Tk^- cells, we sought to mobilize a heterologous mammalian gene into the body of the HSV chromosome to ask two questions: whether the virus could be used to transduce eucaryotic genes and whether a cellular gene with its own promoter would be expressed when placed *cis* to the virus chromosome. For these purposes, we have employed the *aprt* gene isolated from CHO cells (17). The gene may be selected in both directions by growth in the appropriate medium, and thus it provides an ideal marker for acquisition or loss of the Aprt phenotype. The gene resides on a 7.8-kb *Hin*dIII fragment which contains both murine and hamster sequences (17). We digested the plasmid pHAPRT with a battery of restriction endonucleases to identify the minimal amount of the gene that retained transforming activity (Table 1). These studies demonstrated that the minimal *aprt*-transforming region resided on a fragment bounded by a *Bam*HI site at the 5′ end of the gene and a *Kpn*I site at the 3′ end (Fig. 1).

Generation of a chimeric herpesvirus. Preliminary analysis of the transcript coding for *aprt* has revealed that it initiates at a site between *Bam*HI and *Pvu*II, proceeds through three intervening sequences, and is processed to yield an mRNA that is 900 nucleotides long (I. Lowy and R. Axel, personal communication). To facilitate characterization of a herpesvirus carrying the *aprt* gene, we isolated the 3.8-kb *Bgl*II-*Kpn*I fragment from pHAPRT (Fig. 1) and cloned it into the body of pBiM6, a defined 5′ deletion mutant of the HSV *tk* gene, between the *Bgl*II and *Kpn*I sites (Fig. 1). The construct, pCT26, has a functional *aprt* gene imbedded between flanking *tk* sequences and juxtaposes the *tk* and *aprt* promoters without altering the polarity of transcription. It is bounded on the 5′ end by a synthetic *Bam*HI linker and has unique sites for the enzymes *Bgl*II and *Kpn*I that define the boundaries of the *aprt* sequences; the 3′ end of this construct is marked by a synthetic *Hin*dIII linker. This 9.2-kb plasmid has deleted 379 base pairs (bp) of the *tk*-coding sequence and is therefore incapable of transforming Tk^- cells to the Tk^+ phenotype. We examined the biological activity of this construct by assaying its ability to transform $Aprt^-$ cells. This experiment (Table 1) demonstrated that pCT26 was as proficient as pHAPRT at transforming the *aprt* gene and that it no longer possessed the capacity to convert Tk^- cells to the Tk^+ phenotype.

The *aprt* sequences flanked on both sides by sequences from the *tk* gene were assimilated into the body of the HSV chromosome at the *tk* locus by homologous recombination by the strategy described by Mocarski et al. (22). Briefly, plasmid DNA and purified virus DNA from HSV-1 F were mixed at a 10:1 molar ratio and transfected onto Vero cells in the form of a calcium phosphate precipitate. After 3 days in culture, the virus yield was harvested by freezing and thawing the infected cell monolayers in their growth medium. The liberated virus was titrated on Tk^- 143 cells in the presence and absence of 30 μg of bromodeoxycytosine (BdC) per ml under a 1.5% methylcellulose overlay. This analysis revealed that only 1 in 10^4 progeny were Tk^-.

Tk^- plaques could result from mutation to the Tk^- phenotype or from recombination and insertional inactiva-

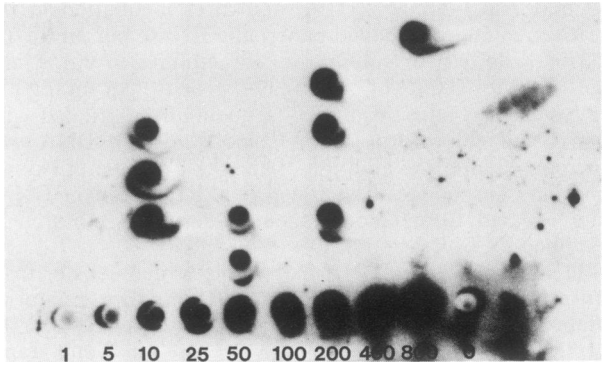

FIG. 2. Identification of HSV-*aprt* recombinants. Individual Tk^- plaques were picked, and a virus stock was prepared by growth in Tk^- 143 cells in the presence of BdC. The virus stocks were then used to infect cells in individual wells of a 24-well cluster dish. When full cytopathic effect had developed, the cells were removed from the wells and gently filtered onto nitrocellulose paper by using a dot-blot apparatus. Virus DNA was denatured in situ and hybridized to a probe specific for CHO *aprt* sequences. After processing the filter, $Aprt^+$ candidates were identified on the basis of the intensity of their hybridization signal. The bottom row is a reconstruction experiment that contains *aprt* DNA mixed with 143 cell DNA at ratios designed to simulate 1, 5, 10, 25, 50, 100, 200, 400, 800, or 0 copies of *aprt* per cell.

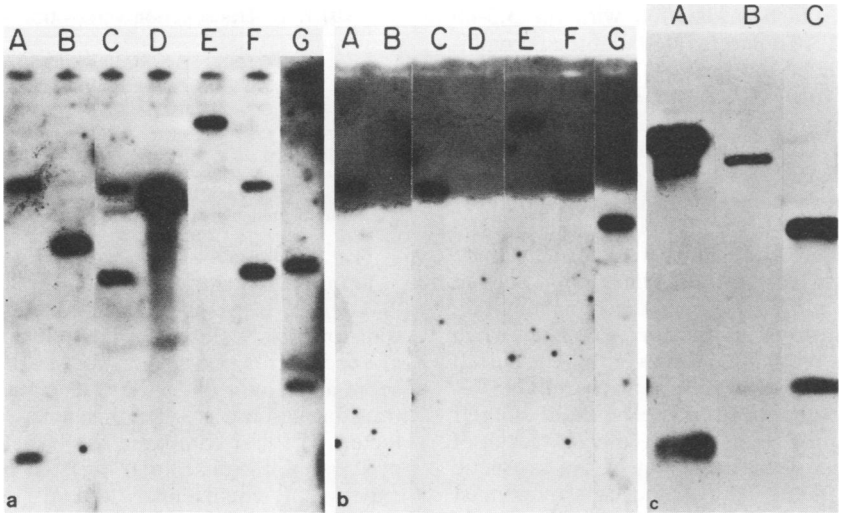

FIG. 3. Characterization of *aprt* and *tk* sequences present in HSV-*aprt*. DNAs from cells infected with HSV-*aprt* or HSV F were digested with restriction endonucleases, electrophoresed on agarose gels, and analyzed by Southern blot hybridization for the presence of sequences homologous to ^{32}P-labeled HSV *tk* DNA (a and c) or CHO *aprt* (b) probes. (a) and (b) HSV-*aprt* digested with *Bam*HI (lane A), HSV F digested with *Bam*HI (lane B), HSV-*aprt* digested with *Kpn*I (lane C), HSV F digested with *Kpn*I (lane D), HSV-*aprt* digested with *Hin*dIII (lane E), HSV-*aprt* digested with *Kpn*I and *Hin*dIII (lane F), and HSV-*aprt* digested with *Bgl*II and *Kpn*I (lane G). (c) DNAs from pCT26 (lane A), HSV-*aprt* (lane B), and HSV F (lane C) were digested with *Bam*HI and *Bgl*II and probed with the 3.5-kb *Bam*HI fragment from pTK5.

tion of the *tk* locus. To differentiate between these possibilities, Tk$^-$ plaques were picked and propagated on 143 cells in the presence of BdC to generate stocks. These stocks were then used to infect 143 cells, and the infected monolayers were screened for the presence of hamster *aprt* sequences by dot-blot hybridization. A representative dot blot is shown in Fig. 2. Of the 120 plaques that were analyzed, 12 hybridized with the *aprt* probe. The apparently high reversion rate to the Tk$^-$ phenotype is not unusual for certain strains of HSV (41). Virus stocks containing *aprt* sequences were prepared after two rounds of plaque purification under conditions designed to eliminate any contaminating Tk$^+$ virus that might have survived the initial selection protocol. A single Tk$^-$ Aprt$^+$ recombinant was chosen for further study; this virus, HSV-1-*aprt*, plaqued with equal efficiency in the presence or absence of BdC and grew with the same kinetics and final yield as its wild-type parent HSV-1 F.

Structure of the HSV-*aprt* chimera. The structure and arrangement of the *aprt* sequences in the chimeric virus were determined by Southern blot hybridization with *tk*- and *aprt*-specific probes to facilitate our analyses. Introduction of the entire *aprt* coding sequence requires that two reciprocal crossovers have occurred within the *tk* locus of the virus. This event would result in interruption of the 3.5-kb *Bam*HI and 5.0-kb *Kpn*I *tk*-containing DNA fragments that are present in parental DNA and the appearance of new fragments in the chimera. Therefore, DNA from cells infected with the prototype and with HSV-*aprt* was digested with *Bam*HI or *Kpn*I and analyzed by Southern blot hybridization. Cleavage with *Bam*HI and hybridization with *tk*-specific probe revealed the presence of a single, 3.5-kb species in DNA from the prototype and two species that migrated at 1.3 and 5.5 kb from the DNA of the recombinant virus (Fig. 3a). When an *aprt*-specific probe was used, only DNA from cells infected with HSV-*aprt* displayed a hybridization signal (Fig. 3b). No hybridization was detected when pBR322 plasmid DNA was used to probe the filters, suggesting that very little, if any, plasmid DNA had been assimilated into the virus chromosome. Cleavage with the enzyme *Kpn*I and hybridization with a *tk* probe revealed the presence of a single, 5.0-kb hybridizing species in DNA derived from cells infected with F and two novel bands that are 5.5 and 2.9 kb in DNA from cells infected with HSV-*aprt* (Fig. 3a). When a duplicate filter was hybridized with an *aprt*-specific probe, a single band of 5.5 kb was detected in DNA extracted from HSV-*aprt* (Fig. 3b). A fragment of this size that hybridizes to both probes is consistent with the introduction of the unique *Kpn*I site that results from fusion of the 3′ end of the *aprt* gene to the 3′ end of the *tk* gene. The 3.5-kb *Bam*HI fragment lies entirely within the 5.0-kb *Kpn*I fragment in F DNA (35); therefore, these fragments are predicted when the recombination events interrupt the 3.5-kb *Bam*HI fragment in F DNA to introduce novel *Bam*HI and *Kpn*I sites in the chimeric virus.

When DNA from the recombinant virus was digested with *Bgl*II and *Kpn*I and hybridized with a *tk*-specific probe, we detected two bands that migrated as 2.9- and 1.6-kb species (Fig. 3a). When this DNA was analyzed with an *aprt*-specific probe, we detected the presence of a unique 3.8-kb band that was not found in F DNA (Fig. 3b). These results demonstrate that the entire 3.8-kb *Bgl*II-*Kpn*I *aprt* fragment was present in the recombinant and that the sequences corresponding to the *Bgl*II-*Kpn*I *tk* fragment from F have been deleted. We next probed the boundaries of recombination by digesting DNA from HSV-*aprt*-infected cells with either *Bam*HI and *Bgl*II or *Hin*dIII and compared the blot hybridization profiles of the cleavage products with those of either wild-type virus or the donor plasmid pCT26. The results of these analyses revealed that *Hin*dIII-cut DNA from the recombinant virus migrated as a unique high-molecular-weight species (Fig. 3a). Analysis of DNA digested with *Kpn*I and *Hin*dIII revealed a pattern that was identical to DNA digested with *Kpn*I alone. Thus, the synthetic *Hin*dIII linker which demarcates the 3′ boundary of the *tk* sequences in pCT26 has not been assimilated into the virus chromosome. A similar analysis of these sequences after digestion

with *Bam*HI and *Bgl*II and hybridization with the 3.5-kb *Bam*HI fragment from pTK5 demonstrated that the recombinant virus genome contained the same 800-bp *Bam*HI-*Bgl*II fragment that was found in DNA isolated from the prototype (Fig. 3c). Therefore the synthetic *Bam*HI linker that identified the 5′ boundary of the chimeric gene in pCT26 was absent from the recombinant (Fig. 3c). By additional experiments with enzymes that recognize multiple sites within the 3.5-kb *Bam*HI-*tk* fragment, we have demonstrated that the sequences that lie between *Bgl*II and where the *Kpn*I site would be in F DNA (if it were present) have been deleted from HSV-*aprt* (Fig. 3a and b). A schematic representation of the events that led to insertion of the *aprt* gene is shown in Fig. 4. One homologous recombination event has occurred between the donor plasmid pCT26 and the tk^+ parent HSV-1 F within the 400-bp region bounded by the synthetic *Bam*HI linker in the plasmid and the *Bgl*II site at +56. The result of this reciprocal crossover is the exclusion of the synthetic linker from the recombinant. The second crossover must have occurred within the region bounded by the *Kpn*I site in the donor plasmid and the synthetic *Hin*dIII linker that serves to identify the endpoint of the *tk* sequences in pCT26. As a result of assimilation of the *aprt* sequences and resolution of the heteroduplex that must be formed during recombination, the *tk* coding sequences from +56 to +435 have been deleted from the recombinant. Thus, the structure of HSV-*aprt* proceeding in a 5′ to 3′ direction is identical to that found in pCT26, except that the synthetic *Bam*HI and *Hin*dIII linkers that mark the boundaries of the *tk* sequences in the chimeric plasmid are not present in HSV-*aprt*.

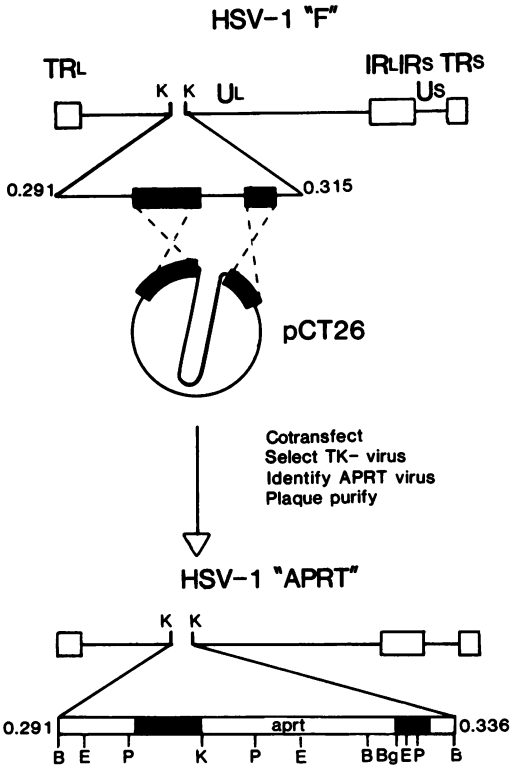

FIG. 4. Generation of a recombinant virus. A schematic representation of the technique used to generate the HSV-*aprt* chimera and the homologous recombination events that were required to assimilate the *aprt* gene into the *tk* locus of the virus are shown. Details are in the text.

TABLE 2. Transduction versus transfection of the *aprt* gene

Source of gene	Complexity	% of molecule that is *aprt*	Efficiency of conversion[a]
pCT26	9.2	41	25
HSV-*aprt* DNA	160	2.4	1
HSV-*aprt* virus	160	2.4	150

[a] Efficiency of conversion to the $Aprt^+$ phenotype was arrived at by measuring the number of CFU per gene equivalent of *aprt*.

Transduction of mutant cells. The studies of Munyon et al. (24) demonstrated that UV-irradiated HSV could be used to transform Tk^- cells to the Tk^+ phenotype. In this next series of experiments, we asked whether the chimeric virus would serve as a donor of the *aprt* gene and what the efficiency of transformation was when this gene was transduced or transfected. We first compared the transformation frequency of pCT26 DNA with *Hin*dIII-digested HSV-*aprt* DNA. In this experiment, equal molar amounts of *aprt* sequences were present in each transformation. When the data was normalized for DNA complexity, the results of this analysis (Table 2) demonstrated that the DNA from pCT26 was about 25-fold more active in transferring the Aprt phenotype than was DNA from the recombinant virus. Because HSV DNA contains nicks and gaps, this result may reflect a difference in the physical state of the donor DNA molecules (13).

To analyze the capacity of the recombinant virus to donate the *aprt* sequence, we exposed ^{3}H-labeled, purified virus to UV light until the plaque-forming capacity was reduced 10^5-fold. This irradiated virus was used to transduce the *aprt* gene into mouse and hamster cells by infecting subconfluent monolayers, and then the cells were overlaid with medium containing 0.1% pooled human gamma globulin to prevent reinfection with any virus that might have been released as a result of completing an infectious cycle. Colonies were scored after 20 days in selective medium. The efficiency of transformation of mouse L cells by transduction was 100-fold and 4-fold greater than that seen by transfection with *Hin*dIII-cleaved virus or pCT26 DNA, respectively (Table 2). Infection of $Aprt^-$ CHO cells with irradiated virus resulted in 90% fewer colonies than infection of mouse cells (data not shown); nevertheless, this still results in an efficiency of transformation that is about 100-fold higher than we can obtain by transfecting CHO cells.

Transformation results from introduction and expression of *aprt* DNA. To demonstrate that transformation resulted from the introduction and expression of the CHO *aprt* gene, we analyzed the DNA and RNA extracted from mouse or hamster cells transduced by the HSV-*aprt* chimera by Southern and northern blot hybridizations and S1 nuclease protection experiments. To demonstrate that $Aprt^-$ CHO cells had acquired virus sequences, DNA from a transductant was cleaved with *Bam*HI and analyzed with a probe specific for HSV *tk* sequences. The results of this analysis show that bands of 5.5 and 1.3 kb, homologous to *tk* DNA, are present in this cell line (Fig. 5a). This result demonstrates that herpesvirus sequences have been stably introduced into these cells by transduction. There are two bands homologous to the *tk* probe because insertion of the *aprt* sequences splits the gene and introduces a new *Bam*HI site between the naturally occurring sites that flank the *tk* gene.

To analyze $Aprt^+$ mouse cells for the presence of CHO sequences, we isolated high-molecular-weight DNA from a transductant, digested it with *Bam*HI, *Hin*dIII, or *Eco*RI, and hybridized blots of these digests with a probe specific for *aprt* sequences. This analysis revealed the presence of two

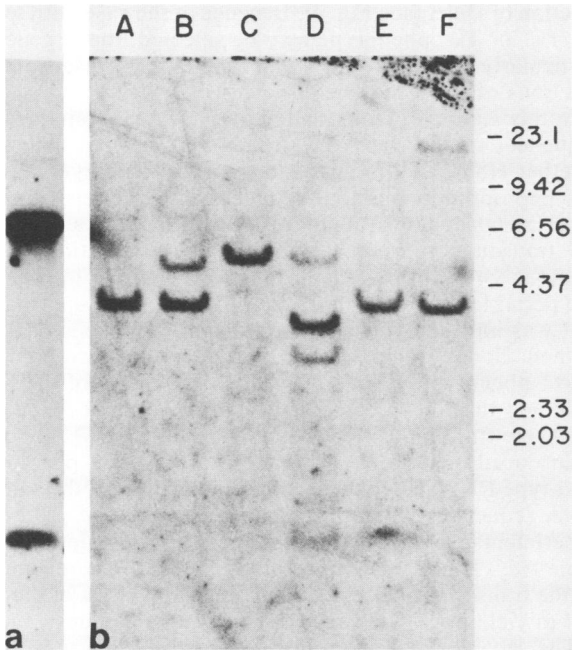

FIG. 5. Analysis of DNA from transduced cell lines. DNA was isolated from Aprt$^-$ mouse and CHO cells and Aprt$^+$ transformants isolated after infection with UV-irradiated HSV-*aprt* and analyzed by Southern blot hybridization for the presence of donor sequences. (a) DNA from Aprt$^-$ CHO cells digested with *Bam*HI and hybridized to the *tk*-specific *Bam*HI fragment from pTK5. (b) DNA from Aprt$^+$ mouse cells digested with *Bam*HI (lane A), *Eco*RI (lane C), or *Hin*dIII (lane E). DNA from L Aprt$^+$ UV-1-digested with *Bam*HI (lane B), *Eco*RI (lane D), or *Hin*dIII (lane F). All lanes were probed with the *aprt*-specific *Pvu*II fragment.

prominent *Bam*HI fragments that were 4.6 and 4.3 kb (Fig. 5b). The 4.6-kb species is unique to the transformant; the other band can be detected in untransformed mouse DNA and represents endogenous murine *aprt* sequences that cross-hybridize with the CHO probe (17). A similar analysis of the hybridization pattern of DNA digested with *Eco*RI revealed the presence of two unique species of DNA that were 3.8 and 3.1 kb and two cross-hybridizing species that could not be distinguished from what was detected in untransformed mouse cells (the lower band of 1.2 kb only appeared after longer exposures of the gel [Fig. 5b]). After digestion with *Hin*dIII, an enzyme that does not cleave within *tk* or *aprt* sequences, a unique 20-kb, hybridizing species was detected in the transduced murine cell line (Fig. 5b). This finding suggests that only a single copy of the transduced *aprt* sequence is present.

Poly(A)-containing RNA was isolated from transductants and analyzed by Northern blot hybridization to demonstrate that transformation resulted from expression of the CHO *aprt* gene. Thus, RNAs from an Aprt$^+$ CHO cell line (A29) and mouse and CHO transductants were probed for the presence of *aprt* mRNA. In these experiments, we used a 3.8-kb *Bam*HI fragment isolated from pHAPRT as probe because the 1.8-kb *Pvu*II fragment that was used in the analyses of virus DNA spans two intervening sequences and therefore lacks sequences that are complementary to a large region of mature *aprt* mRNA. The results of these analyses (Fig. 6a) demonstrated that the transformants accumulated a 0.9-kb poly(A)-containing RNA which was the same size as that synthesized by wild-type CHO cells. This probe did not hybridize to mRNA from untransformed mouse or Aprt$^-$ CHO cells (data not shown).

The 5′ terminus of the *aprt* mRNAs from the transformants was determined by S1 nuclease protection studies. In these experiments, RNA from transformants or A29 cells was hybridized to a single-stranded *Bam*HI-*Pvu*II probe that protected the 5′ end of *aprt* mRNA (see above) and digested with S1, and the size of the protected probe was determined by acrylamide gel electrophoresis under denaturing conditions. This analysis shows that the transductants accumulated a poly(A)-containing RNA whose 5′ end was between 190 and 201 bases upstream from the *Pvu*II site. These RNAs protect the same sequences as RNA from A29 cells does (Fig. 6b). Thus, Aprt$^+$ transformants derived after infection with UV-irradiated HSV-*aprt* result from introduction and expression of the CHO *aprt* gene.

Analysis of *aprt* transcripts synthesized by HSV-*aprt*. Previous studies from this and other laboratories have demonstrated that shortly after infection with HSV, host macromolecular synthesis is disrupted (9, 27, 42). These changes, which include disruption of host polyribosomes and degradation of cellular mRNA, result from the introduction of a virion-associated protein and expression of virus-specified immediate-early gene products (14, 25, 28). To determine whether the *aprt* gene would be recognized as self or not-self, we determined whether it was expressed during the course of lytic infection when it was *cis* to the virus chromosome. Two types of analyses were performed on RNA extracted from cells infected with HSV-*aprt* in the presence (12) or absence of an inhibitor of protein synthesis. First, RNA extracted at different times postinfection was analyzed for the presence of immediate early and *aprt* transcripts. Figure 7a shows the result of probing RNA extracted at different times postinfection for the presence of sequences homologous to the *aprt* probe. We note that at 2, 4, and 6 h postinfection there were no RNAs that hybridized

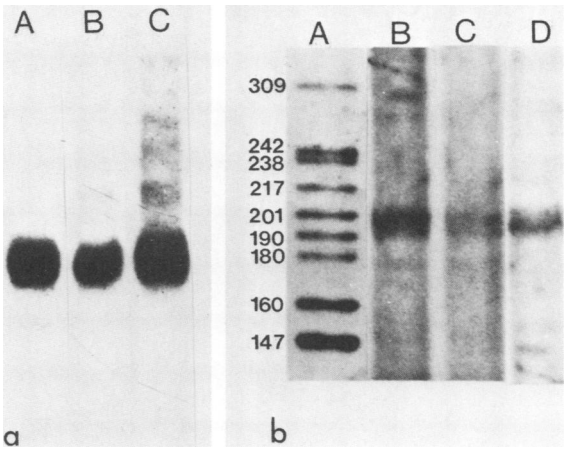

FIG. 6. Analysis of RNA. (a) Poly(A)-containing RNA was isolated from Aprt$^+$ wild-type CHO cells (lane A), transductant CHO-18 (lane B), and transductants L Aprt$^+$ UV-1 (lane C), electrophoresed through an agarose gel, transferred by nitrocellulose, and analyzed for the presence of *aprt* sequences by using a ^{32}P-labeled 3.8-kb *Bam*HI fragment from pHAPRT as the probe. (b) The same RNA samples were hybridized to a strand-separated *Bam*HI-*Pvu*II fragment from the *aprt* gene uniquely labeled at the *Pvu*II site with T4 polynucleotide kinase. S1-resistant hybrids were denatured and analyzed by electrophoresis by using *Hpa*II-cut pBR322 DNA as a size standard (lane A). RNA from A29 cells (lane B), RNA from CHO-18 cells (lane C), and RNA from L UV-1 cells (lane D).

with the probe. Even when cells were maintained in the presence of cycloheximide for 8 h (lane F) we were unable to detect any *aprt* transcripts. The bands in lane D (8-h sample) might represent transcription through the *aprt* sequence which uses a virus promotor that is downstream from the insert. Nevertheless, these RNA transcripts are not of the correct size, and we do not detect transcripts from the appropriate strand that initiate correctly by S1 nuclease hybridization analysis. In a companion study, we probed RNA isolated at different times postinfection to demonstrate that HSV-*aprt* expressed its immediate early genes at the appropriate times and that these transcripts accumulated in the presence of cycloheximide. The results of this analysis are shown in Fig. 7b. In an effort to increase sensitivity, these same samples were subjected to S1 nuclease protection analysis. The results of these studies reveal that the *aprt* sequences are not transcribed during the lytic cycle of the virus either in the presence or in the absence of de novo protein synthesis (data not shown). We conclude that the *aprt* promoter is recognized as foreign and is not activated in infected cells.

DISCUSSION

The chromosome of HSV is large (160 kb) and littered with numerous sites for all of the known restriction endonucleases that have been commonly used to facilitate insertion into other DNA virus vectors such as simian virus 40 and adenovirus. Nevertheless, because the virus recombines at a high rate (5), it is possible to introduce or delete sequences from the chromosome without great difficulty. Previous studies have shown that virus genes can be readily positioned in the HSV chromosome, provided they are flanked by sequences that are normally found to be contiguous in the genome (22, 32, 33, 37). Thus, after transfection of animal cells, homologous recombination between the sequences in the virus chromosome can result in the introduction or deletion of DNA (see Fig. 4). Because of the ease with which the Tk^+ or Tk^- phenotype may be selected, the *tk* gene is a particularly useful site into which mutations, insertions, or deletions of DNA may be directed (16, 36).

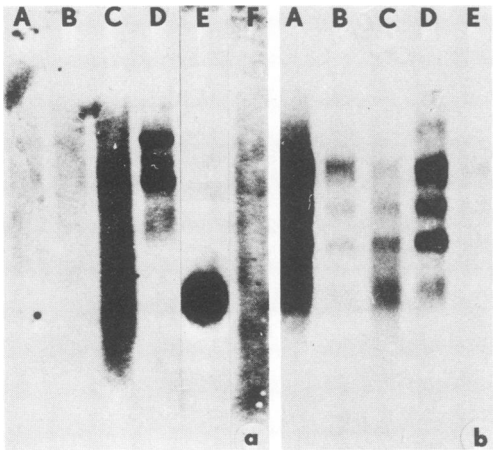

FIG. 7. Analysis of RNA from cells infected with HSV-*aprt*. (a) RNA (25 μg) extracted from cells infected with HSV-*aprt* for 2, 4, 6, and 8 h (lanes A to D, respectively); control A29 cells (lane E); cells infected with HSV-*aprt* in the presence of cycloheximide and maintained in the presence of drug for 8 h (lane F). The probe was the 3.8-kb *Bam*HI fragment described in Fig. 6. (b) Blot hybridization of RNA extracted from cells infected with HSV-*aprt* and maintained in the presence of cycloheximide (lane A). Lanes B to E show the hybridization profile of RNA extracted at 8, 4, 2, and 1 h postinfection, respectively. The blots were probed with ^{32}P-labeled *Eco*JK fragment which encompasses the genes that encode ICP 0, 27, and 4.

In this study, we constructed an HSV that contained the sequences of the *aprt* gene from CHO cells to determine whether HSV could be used to transduce eucaryotic genes into appropriately marked recipient cells. The results of our experiments demonstrate that HSV can serve as a vector for the transduction of eucaryotic genes. Irradiation of the chimeric virus with UV light reduced its ability to replicate and permitted the virus to transfer the selectable *aprt* marker that was introduced in the chromosome. Analysis of transformed clones demonstrated that they had acquired the $Aprt^+$ phenotype as a result of the acquisition of the CHO gene and that they accumulated an mRNA that is indistinguishable from *aprt* mRNA synthesized by CHO A29 cells. Transduced cell mRNAs migrated to the same position as wild-type RNA in denaturing gels and protected the same 5′ DNA sequences when analyzed by S1 nuclease protection experiments, suggesting that they initiate transcription at the same site. Thus, genes transduced by HSV are recognized by the transcriptional and translational apparatus of the host cell to yield bona fide transcripts and polypeptides.

The second reason for constructing this virus was to ask whether the promoter for the *aprt* gene would be recognized and transcribed during the course of the virus replicative cycle or if the virus was able to distinguish self from not-self and repress its transcription. In previous studies, we showed that, during the course of virus infection, cell macromolecular synthesis is shut off. Shutoff occurs in two stages and results from introduction of a virion-associated, heat-labile function that disrupts host protein synthesis (25) and also expression of a virus gene(s) that degrades host mRNA (28). In addition to these early functions, the virion brings in a factor that serves to activate the promoters that direct transcription of five immediate early (alpha) genes (18, 33). We monitored the expression of the *aprt* gene during the course of the virus replicative cycle by a sensitive S1 nuclease protection assay but were unable to detect the accumulation of stable transcripts at any time. In a companion experiment, we were able to demonstrate that the immediate early promoters are activated at the correct stage in the virus life cycle and that the virus replicated as efficiently as its wild-type parent. When we asked whether this gene would be transcribed in the absence of de novo protein synthesis, as the immediate early genes are (12), we found that it was not (Fig. 7). Therefore, we surmise that a virion-associated function acts to inhibit expression of this foreign DNA, even when it is placed *cis* to the virus chromosome. Thus, the virus has evolved a mechanism to differentiate between self and not-self and repress transcription of genes of nonviral origin. The proteins responsible for this function and their mechanism of action are neither known nor understood at this juncture.

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