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Properties of Cells Carrying the Herpes Simplex Virus Type 2 Thymidine Kinase Gene: Mechanisms of Reversion to a Thymidine Kinase-Negative Phenotype

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We have isolated cells with a thymidine kinase-negative (tk^-) phenotype from cells which carry the herpes simplex virus type 2 tk gene by selection in 5-bromodeoxyuridine or 9-(2-hydroxyethoxymethyl)guanine. Both selection routines generated revertants with a frequency of 10^{-3} to 10^{-4}, and resistance to either compound conferred simultaneous resistance to the other. tk^- revertants fell into three classes: (i) cells that arose by deletion of all virus sequences, (ii) cells that had lost the virus tk gene but retained a nonselected virus-specific function and arose by deletion of part of the virus-specific sequence, and (iii) cells that retained the potential to express all of the virus-specific functions of the parental cells and retained all of the virus-specific DNA sequences.

The herpes simplex virus (HSV) thymidine kinase (tk) gene can be introduced as a stable genetic character into mammalian cells by treatment of cells either with inactivated virus (31), with sheared or restricted virus DNA (1, 25, 47), or with DNA from cells which carry the resident virus gene (29). Selection for the HSV tk gene may result in simultaneous acquisition of other nonselected virus specific functions, which can be detected by the ability of these "biochemically transformed" cells to support the growth of temperature-sensitive mutants at the nonpermissive temperature (27, 29).

The resident virus tk gene is not under the same regulatory control as the cell-specific enzyme (23), and expression of the virus gene may, in some instances, be regulated by superinfecting virus, since infection of transformed cells with a tk^- HSV mutant may result in elevated levels of enzyme activity (16, 21). The stability of the tk gene in transformed cells varies from one cell line to another (1, 6), and reversion to a kinase-negative phenotype results from at least two mechanisms: some reversion events are irreversible and have, in some instances, been shown to result from loss of virus DNA sequences (2, 4, 19, 28), whereas others are reversible and apparently result in the maintenance of the kinase gene in a suppressed state (6, 15, 39).

The stability and control of the HSV tk gene in transformed cells is of some interest since this gene is now being used as a vector system to introduce other genes into mammalian cells (20, 26, 48), but the information currently available is difficult to interpret because it refers to different revertant cells which have been derived from different parental transformed lines obtained by diverse procedures. The rationale behind the work described here was to isolate a large number of revertants from two well-characterized transformed cell lines and to attempt to identify the mechanisms by which those revertants arise. The transformed cells used, $D2_1$ and $D2_5$, carry the HSV type 2 (HSV-2) tk gene together with other nonselected virus-specific markers, and these characters are stable to extensive passage in selective or nonselective medium (27, 29). Both cell lines contain a low copy number (one to two copies per cell) of a virus-specific DNA sequence which lies within map coordinates 0.2 to 0.4 of the HSV-2 genome (28). Thymidine kinase-negative revertants of $D2_1$ and $D2_5$ cells have been derived by selection in 5-bromodeoxyuridine (BUdR) or 9-(2-hydroxyethoxymethyl)guanine (acyclovir), and the following questions have been asked: (i) Can the revertants be induced to synthesize thymidine kinase by infection with a tk^- HSV mutant? (ii) Is the loss of the tk^+ phenotype accompanied by loss of the nonselected virus-specific characters found in the parental tk^+ cells? (iii) Can the revertant cells be distinguished from the parental cells by their virus DNA content or by the integration site of virus DNA? (iv) Can the DNA from revertant cells be used as a donor of the tk gene in transfection experiments?

MATERIALS AND METHODS

Cells and viruses. Cell lines $D2_1$, $D2_3$, $D2_5$, $D2_6$, and $D2_9$ are tk^+ cell lines derived from LMTK$^-$ cells by transformation with sheared HSV-2 DNA. The derivation, maintenance, and characterization of these cells have been described elsewhere (27-29). Cells were grown in Glasgow-modified Eagle medium supple-

mented with 10% newborn calf serum. Selection for the tk^+ phenotype was achieved by supplementing the medium with thymidine, adenosine, guanosine, glycine, and methotrexate (31). Selection for the tk^- phenotype was achieved by supplementing with BUdR (50 μg/ml) or with acyclovir (15 μg/ml; obtained from the Wellcome Foundation).

HSV-2 strain 27766 and HSV-1 strain HFEM were the wild-type viruses used throughout. HSV-1 strain B2006 is a tk^- mutant which makes no recognizable thymidine kinase polypeptide (40) or thymidine kinase-related antigen (3). tsN102 is an HSV-1 mutant isolated by A. Buchan. It falls in complementation group 1-1 (36), and mutants of this group lie within physical map units 0.339 to 0.464 on the HSV-1 genome (34). ts208 is an HSV-2 mutant isolated by D. Purifoy. Its complementation group is unknown, but it efficiently complements tsN102.

Growth inhibition assays. A total of 10^5 cells were plated in 30-mm tissue culture dishes, and after 24 h the medium was supplemented with various concentrations of acyclovir. After a further 72 h, the cells were harvested and counted.

Thymidine kinase induction assays. Monolayers of 5×10^6 cells were mock infected or infected with HSV-1 strain B2006 in duplicate at a multiplicity of 3 PFU/cell. The virus was allowed to absorb for 30 min at 37°C, and the inoculum was then replaced with 3 ml of warm medium. After 8 h the cells were harvested, counted, and washed with phosphate-buffered saline, and the cell pellets were stored at −70°C. The pellets were resuspended in 0.01 M Tris-chloride, pH 7.5, and disrupted by ultrasonic vibration, and the extracts were assayed for thymidine kinase activity (18).

Blot hybridization. DNA was extracted from tissue culture cells (43), and restriction digests were electrophoresed in 1% agarose horizontal slab gels. The products were transferred to nitrocellulose filter sheets (38), and the filters were washed at 70°C for 6 h in sealed plastic bags containing 6× SSC (SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured calf thymus DNA per ml, 0.02% Ficoll, 0.02% polyvinylpyrolidone, and 0.02% bovine serum albumin. The filters were then hybridized with ^{125}I-labeled HSV-2 DNA (28) or with ^{32}P-labeled DNA prepared by nick translation (35) using [α-^{32}P]dCTP (specific activity, >2,000 Ci/mmol; Amersham Corp.). Both types of hybridization probe were used at specific activities of 5×10^7 to 2×10^8 dpm/μg. The hybridization conditions were the same as the washing conditions described above except that SSC was used 3× concentrated and 10% dextran sulfate was included. The hybridization mixture was shaken vigorously throughout. Probe molecules were used at a final concentration of ~2×10^8 copies/ml, and under these conditions of hybridization 50% annealing of the probe was achieved in ~10 h. Hybridization was allowed to proceed for 20 h. Filters were washed with three changes of the prehybridization washing buffer, three changes of 1× SSC–0.5% SDS, and two changes of 0.2× SSC–0.5% SDS. All washes were for 20 min at 70°C in a shaking water bath. Where ^{125}I-labeled probes were used, all hybridization and washing buffers contained 2 mM KI. The washed, dried filters were autoradiographed at −70°C, using intensifying screens.

RESULTS

Induction of thymidine kinase in transformed cell lines. Several transformed cell lines were tested for their ability to exhibit increased thymidine kinase activity after infection with the tk^- mutant B2006. Cells were infected at various multiplicities and assayed for activity after 8 h. The results (Fig. 1) indicate that there were qualitative differences between cell lines; $D2_1$ and $D2_5$ cells were inducible, whereas $D2_3$, $D2_6$, and $D2_9$ cells were relatively insensitive to superinfection. The phenomenon was multiplicity dependent, maximum enzyme levels being achieved when all cells were infected. Since B2006 infection produces no polypeptide corresponding to the HSV thymidine kinase (40) nor any antigen which reacts with antisera prepared against thymidine kinase (3), the increased enzyme levels in $D2_1$ and $D2_5$ cells presumably result from increased expression of the resident

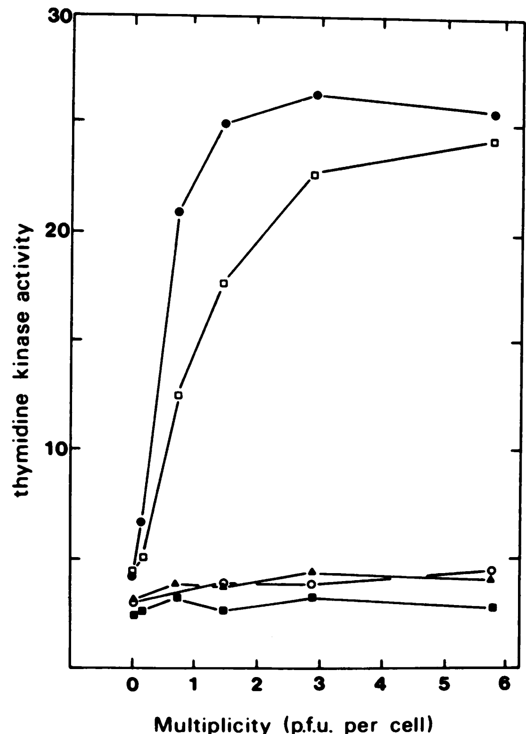

FIG. 1. *Induction of thymidine kinase in transformed cells by infection with tk⁻ virus. Cells were infected with HSV-1 strain B2006, and the thymidine kinase activity was determined after 8 h. Activities are given as picomoles of thymidine phosphorylated by extracts of 10^4 cells in 20 min at 37°C. Symbols:* ●, $D2_1$; □, $D2_5$; ▲, $D2_3$; ○, $D2_6$; ■, $D2_9$.

tk gene. Results similar to these have been reported by Leiden et al. (21) and by Kit and Dubbs (16). The HSV thymidine kinase has been shown to be a member of the β class of HSV polypeptides (10) and hence to require α-polypeptides for its synthesis in infected cells (12). Positive control of β polypeptide synthesis by α polypeptides appears to be at the level of transcription, since transcription in cells infected with mutants defective in α function or in the presence of cycloheximide results in a limited set of transcripts corresponding to the mRNA's for α polypeptides (5, 13, 44, 45). The simplest interpretation of the induction of tk in biochemically transformed cells by infection with tk^- virus is that the infecting virus provides a source of α polypeptides which allow elevated transcription of the resident tk gene from a virus-specific promoter. This view is supported by the results of Kit et al. (17), who found that a mutant in complementation group 1-2 failed to induce high enzyme levels in transformed cells. Mutants in this complementation group are defective in early and late transcription and synthesize only immediate-early transcripts at the nonpermissive temperature (44, 45). Our results are consistent with this interpretation since we know that $D2_1$ and $D2_5$ cells do not contain those sequences which code for α polypeptides (28, 30, 33, 46). The dependence of tk gene transcription on α polypeptides implies that the transcription of the resident tk gene in uninfected $D2_1$ and $D2_5$ cells occurs from a host-specific promoter.

Isolation of tk^- revertant cells. Cells with a tk^- phenotype were derived from $D2_1$ and $D2_5$ cell populations by selection in either BUdR (revertants denoted by a suffix R) or acyclovir (revertants denoted by a suffix A). A total of 10^4 cells were plated in 5-cm dishes, and after 2 weeks single colonies from each dish were picked and established as cell lines. Acyclovir is specifically phosphorylated by the HSV thymidine kinase (9), and the drug is therefore cytotoxic for cells which express this enzyme (32). This is confirmed by the data in Fig. 2, which shows that the drug was more cytotoxic for $D2_5$, $D2_1$, and $D2_6$ cells than for the parental $LMTK^-$ cells from which they were derived. Selection for tk^- cells by using either BUdR or acyclovir gave reversion frequencies for both cell lines of 10^{-3} to 10^{-4}, and resistance to one drug conferred simultaneous resistance to the other. It is worth noting, however, that although revertants selected in BUdR (e.g., $D2_1R5$ and $D2_5R12$) were indistinguishable from $LMTK^-$ cells in their response to acyclovir, cells selected in acyclovir (e.g., $D2_1A1$ and $D2_5A3$) exhibited higher levels of resistance to this drug, and it is likely, therefore, that growth in acyclovir exerts selection pressures in addition to those directed against the thymidine kinase.

Some cell lines selected in BUdR were found to contain reduced but significant levels of thymidine kinase, an observation also reported by Littlefield (24). These cell types were eliminated from this study, and all revertant cell lines described here contained tk levels indistinguishable from that found in $LMTK^-$ cells.

Properties of revertant cells. (i) Detection of virus-specific nonselected functions. The transformed cell lines $D2_1$ and $D2_5$ carry, in addition to the HSV tk gene, other virus-specific markers which can be detected by the ability of these cells to support the growth of temperature-sensitive mutants at the nonpermissive temperature (27, 29). $D2_1$ cells compensate for the defects in tsN102 and ts208, whereas $D2_5$ cells compensate for the defect in ts208 only. We have previously reported that three tk^- revertants of $D2_1$ cells ($D2_1R1$, $D2_1R2$, and $D2_1R3$) have lost these nonselected virus-specific markers (27), and experiments were done to determine whether this would hold true for the larger series of revertant cells isolated in the present study. $LMTK^-$ cells, transformed cells, and revertants were infected in parallel with wild-type and mutant viruses, and the virus yields were determined after 18 h at the nonpermissive tempera-

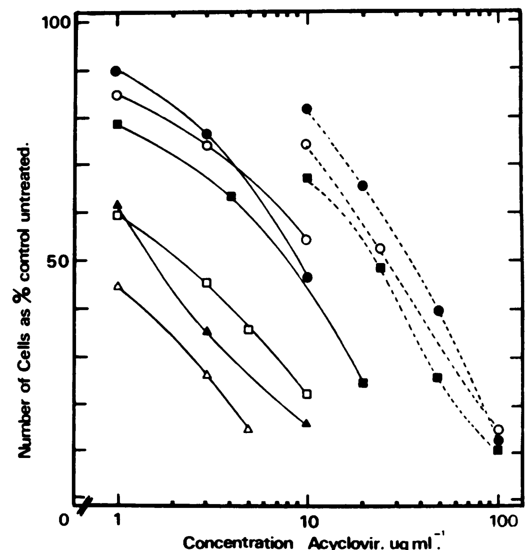

FIG. 2. *Sensitivity of LTK^- cells, transformed cells, and revertant cells to acyclovir. A total of 10^5 cells were seeded in 3-cm dishes, and acyclovir was added after 24 h. Replicate cultures were harvested and counted after a further 72 h, and yields are expressed as a percentage of the yields of untreated parallel cultures. Symbols: ●——●, LTK^-; △, $D2_1$; □, $D2_5$; ▲, $D2_6$, ○——○, $D2_5R12$; ■——■, $D2_1R5$; ●-----●, $D2_1A1$; ○-----○, $D2_5A3$; ■-----■, $D2_1A2$.*

ture (38.5°C). There was no significant difference in the ability of different cell lines to support the growth of wild-type viruses. Yields of mutant viruses are given in Table 1 and are expressed as a percentage of wild-type yield. It is clear that several revertants of both $D2_1$ and $D2_5$ cells retained the nonselected markers present in the parental cells from which they were derived.

(ii) Induction of thymidine kinase by infection of revertant cells. Infection of $D2_1$ or $D2_5$ cells with tk^- virus results in elevated expression of the resident virus gene, presumably as a result of transcription from a virus-specific promoter. To find whether tk^- revertants of these cells contained a quiescent HSV tk gene, cells were infected with HSV-1 B2006 and the tk activity was assayed after 8 h. Many of the revertant cell lines contained an inducible tk gene, and this character correlated well with the presence of the nonselected virus-specific markers in revertant cells: all revertant cell lines which contained an inducible tk retained the nonselected markers found in the parental cells (Table 1).

TABLE 1. *Phenotypic characterization of revertants*

Cell line	Mutant yield[a]		Increase in thymidine kinase activity after infection with B2006[b]
	tsN102	ts208	
LTK^-	0.4	0.6	<1
$D2_1$	54	95	57
$D2_5$	1.2	65	35
$D2_1R1$	0.1	2.1	<1
$D2_1R2$	0.2	2.5	<1
$D2_1R3$	0.1	1.3	<1
$D2_1R6$	4.0	0.2	<1
$D2_5A2$	ND	0.8	<1
$D2_1A1$	112	4.4	<1
$D2_1A2$	16	2.7	<1
$D2_1R4$	37	146	38
$D2_1R5$	107	87	16
$D2_5A1$	ND	126	33
$D2_5A3$	ND	134	14
$D2_5A4$	ND	166	19
$D2_5R4$	ND	68	12
$D2_5R11$	ND	75	23
$D2_5R12$	ND	88	11

[a] 10^5 cells were infected with either tsN102, ts208, HSV-1 wild type, or HSV-2 wild type in parallel. After 18 h at 38.5°C, the cells were harvested and virus yield was assayed at 33°C. Yields of HSV-1 wild type were $2.6 \pm 0.5 \times 10^6$; yields of HSV-2 were $1.2 \pm 0.3 \times 10^5$. Mutant yields are expressed as a percentage of wild-type yield in the same cell line. ND, Not done.

[b] Cells were mock infected or infected with HSV-1 strain B2006. Cells were harvested and activity was assayed after 8 h. Results are presented as the activity in infected cells above that found in mock-infected cells and are expressed as picomoles of thymidine phosphorylated by extracts of 10^4 cells in 20 min at 37°C.

The revertants could be placed into three classes based on their biological behavior (Table 1). Class 1 revertants lacked the potential to express any of the virus-specific functions found in the parental cells; class 2 revertants could not express the virus tk (the selected marker), were unable to support the growth of ts208, but retained the ability to support the growth of tsN102; class 3 revertants retained the potential to express all the virus-specific functions of the parental cells.

We attempted to further characterize these classes of revertants by identifying the virus-specific DNA sequences present in revertant cells and comparing these with the sequences found in the parental tk^+ cells.

Virus DNA sequences in transformed cells $D2_1$ and $D2_5$. We have previoulsy shown, by kinetic hybridization, that these cell lines contain HSV-2-specific sequences lying within the limits of 0.2 to 0.4 map units of the virus genome (28). More precise information was obtained by hybridizing ^{125}I-labeled HSV-2 DNA to nitrocellulose filter blots of *Bam*HI-restricted DNA from transformed cells. The results are shown in Fig. 3, together with the simplest interpretation of these results. This interpretation is not unambiguous, but where a fragment from a transformed cell co-ran with a mixture of virus fragments, we chose that fragment which lay in the 0.2-to-0.4 region of the HSV-2 physical map. Thus, digestion of $D2_1$ or $D2_5$ cell DNA yielded a fragment which co-ran with virus fragments h, i, and j, but of these only i lay in the 0.2-to-0.4 region.

Analysis of $D2_5$ showed one fragment which did not co-run with any virus fragment (marked with an arrow in Fig. 3) and presumably represents a virus sequence linked to cellular DNA. A second "nonvirus" fragment has not been found and may contain insufficient virus-specific sequences to be detected in these experiments. Digestion of $D2_1$ DNA yielded fragments that all co-ran with virus fragments. However, the fragment which co-ran with virus fragment l cannot be fragment l, since this fragment maps in the Us region of the virus genome (N. Wilkie and A. Davison, personal communication), and this region is not represented in these cells (28). The interpretation shown in Fig. 3 has been verified in part by hybridizing similar blots with the HSV-2 *Sal*I fragment g, which maps from 0.281 to 0.319 on the virus genome and contains the functional virus tk gene (G. Reyes and G. Hayward, personal communication). This fragment overlapped *Bam*HI fragments n, r, and s

(see Fig. 3) and detected only these fragments when hybridized to a total *Bam*HI digest of HSV-2 DNA (Fig. 4). Hybridization of this *Sal*I fragment to *Bam*HI digests of $D2_1$ and $D2_5$ DNA (Fig. 5) showed that $D2_1$ cells contained *Bam*HI fragments n, r, and s, whereas $D2_5$ cells contained r and s but not n. The additional band

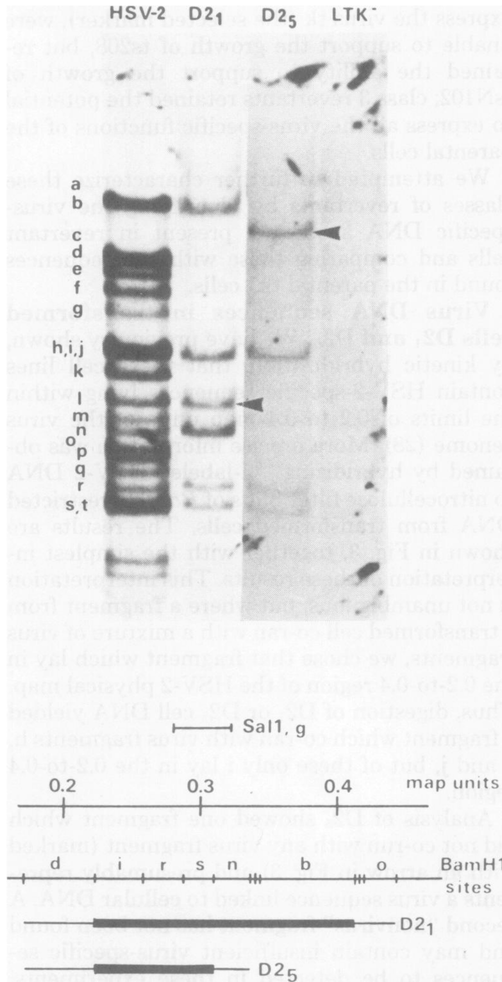

FIG. 3. *Virus sequences in $D2_1$ and $D2_5$ cells. Thirty micrograms of DNA from $D2_1$, $D2_5$, or LTK⁻ cells was digested with BamHI restriction endonuclease, and Southern blots were prepared after electrophoresis in 1% agarose gels. Blots were hybridized with ^{125}I-labeled HSV-2 DNA. The track marked "HSV-2" contained a digest of 30 μg of LTK⁻ cell DNA plus 2 ng of HSV-2 DNA (equivalent to about four genome copies of HSV-2 DNA per cell). Arrows indicate fragments which are not composed solely of virus sequences. The BamHI restriction map shown and the interpretation of the HSV-2 DNA restriction pattern are the unpublished data of N. Wilkie and A. Davidson. The heavy bars represent HSV-2 sequences present in cell lines $D2_1$ and $D2_5$. The lines which flank the bars represent regions of uncertainty.*

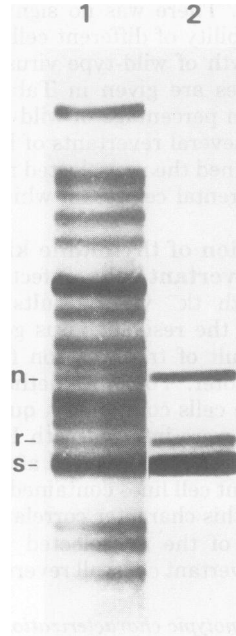

FIG. 4. *Hybridization of SalI fragment g to BamHI fragments of HSV-2 DNA. Southern blots were prepared of BamHI-restricted HSV-2 DNA. Track 1 was hybridized with ^{125}I-labeled HSV-2 DNA; track 2 was hybridized with ^{32}P-labeled SalI fragment g from HSV-2 DNA.*

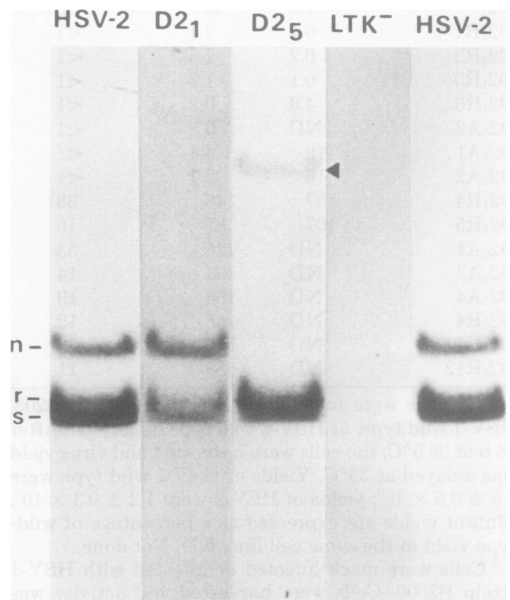

FIG. 5. *Hybridization of SalI fragment g to sequences in $D2_1$ and $D2_5$ cell DNA. The experimental procedures were as described in the legend to Fig. 3 except that the blot was hybridized with ^{32}P-labeled SalI fragment g of HSV-2 DNA.*

visible in the $D2_5$ track represents part of the *Bam*HI n fragment linked to nonviral sequences and corresponds to the band marked with an arrow in Fig. 3. Finally, the interpretation shown in Fig. 3 is consistent with a map position of approximately 0.3 for the HSV-2 tk gene (21, 28, 34a).

The reconstruction experiments shown in Fig. 3 and 4 represent four HSV genome copies per cell. Reconstructions using one copy per cell showed that this was close to the limit of sensitivity of the methods using ^{125}I-labeled HSV-2 DNA as probe. Nevertheless, all *Bam* fragments from a to s were detected in such reconstructions. The sensitivity of the method using the *Sal* g fragment as probe was much higher (the signal was the same, but backgrounds were much lower since the total isotope used was 20-fold less), and sequences of <500 bases have been easily detected in one HSV-2 copy per cell reconstructions using double-enzyme digests. It is worth noting that we have previously reported (28), as a result of kinetic hybridization experiments, that cell line $D2_5$ contains a short sequence within HSV-2 *Eco*RI fragment l (0.63 to 0.7 map units). This sequence has not been detected in experiments reported here, using ^{125}I-labeled HSV-2 DNA as probe. This sequence could not, of course, be detected with the *Sal* g fragment as probe.

Virus DNA sequences in revertant cell lines. *Bam*HI digests of DNA from revertant cells were compared with digests of DNA from parental cells by filter hybridization to ^{125}I-labeled HSV-2 DNA (Fig. 6). Class 1 revertants (represented by $D2_1$R1 and $D2_5$A2) contained no detectable virus-specific DNA and arose by deletion of virus sequences. This is consistent with a previous report that three revertants which are phenotypically of this class ($D2_1$R1, $D2_1$R2, and $D2_1$R3) contain no virus DNA (28). Class 3 revertants ($D2_1$R4 and $D2_5$A1) contained all the virus-specific sequences present in the parental cells. Furthermore, the positions of those fragments which contained both virus and host sequences were unchanged, implying that the integration site is unaltered. Despite the ability of class 2 revertants ($D2_1$A1 and $D2_1$A2) to compensate for the defect in tsN102, we have been unable to detect virus sequences in these cells. The majority of the virus sequence has therefore been deleted, and we presume that a short sequence remains which we cannot detect. The same results were obtained with the *Sal* g fragment as a probe. Note that since $D2_1$ cells, but not $D2_5$ cells, complement tsN102, we predict from Fig. 3 that the gene which complements tsN102 lies outside the *Sal* g fragment.

Transformation of cells to a tk⁺ phenotype, using cell DNA as a gene donor. Nu-

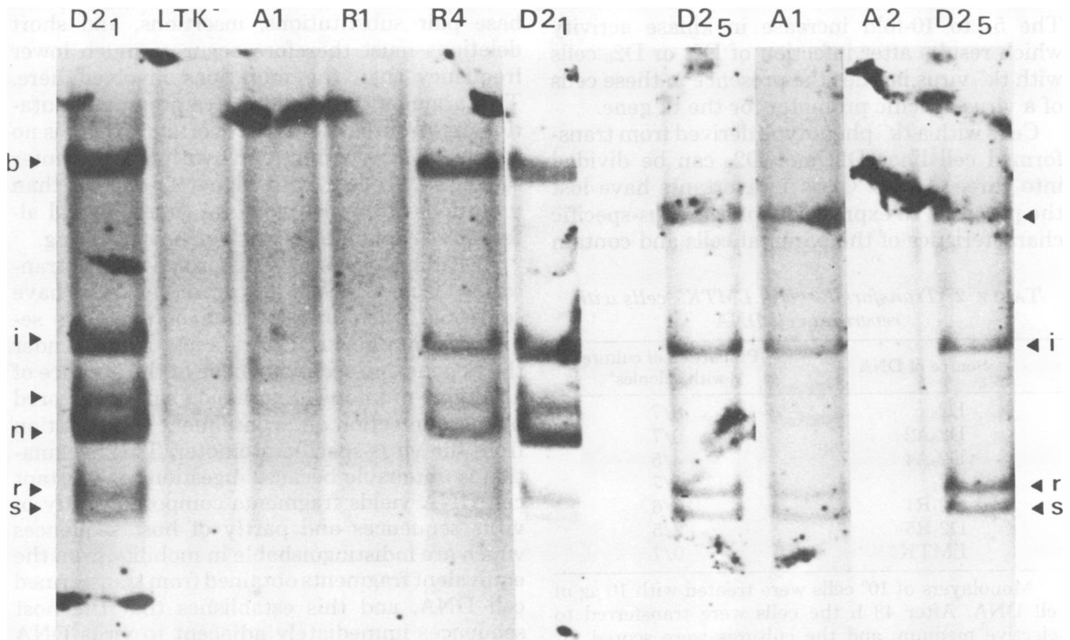

FIG. 6. *Comparison of HSV-2 DNA sequences in transformed cells and tk⁻ revertant cells. Southern blots were prepared of BamHI-restricted DNA from LTK⁻ cells, $D2_1$ cells, $D2_5$ cells, and tk⁻ revertants derived from $D2_1$ and $D2_5$. Blots were hybridized with ^{125}I-labeled HSV-2 DNA.*

cleic acid hybridizations studies have shown that class 3 revertants retain the virus sequences present in parental cells, and induction of enzyme activity by infection with tk$^-$ virus demonstrates the ability of the resident gene to specify a functional polypeptide. We have previously demonstrated that transformed cell DNA can be used as an efficient donor of the tk gene in transformation experiments (29). To obtain further evidence for the presence of a functional tk gene in class 3 revertant cells, DNA from revertant cells was used to transform LMTK$^-$ cells to a tk$^+$ phenotype (Table 2). Although the numbers are very small, it is clear that class 3 revertants (represented by $D2_1R5$ and $D2_5A4$) contain DNA which is a tk vector of comparable efficiency to the parental cell DNA. As expected, DNA from class 1 revertants did not transform.

DISCUSSION

The HSV tk$^+$ cell lines $D2_1$ and $D2_5$ each contain a continuous virus-specific sequence which contains, in addition to the tk gene, nonselected HSV-specific genes which can compensate for the defects in virus ts mutants. It is worth noting that, although these cells produce "constitutive" levels of thymidine kinase, we do not know whether the nonselected functions are constitutive or whether they are only expressed after virus infection, since it is only under these conditions that these functions can be measured. The 5- to 10-fold increase in kinase activity which results after infection of $D2_1$ or $D2_5$ cells with tk$^-$ virus implies the presence in these cells of a virus-specific promoter for the tk gene.

Cells with a tk$^-$ phenotype derived from transformed cell lines $D2_1$ and $D2_5$ can be divided into three classes. Class 1 revertants have lost the potential to express any of the virus-specific characteristics of the parental cells and contain no detectable virus DNA sequences. Class 2 revertants cannot be induced to express thymidine kinase, but retain one of the nonselected markers found in the parental cells. We have been unable to detect virus DNA in these revertants. Most of the virus-specific sequences have therefore been lost, but we presume that a small sequence remains, corresponding to the gene for which tsN102 is defective. Class 3 revertants retain the potential to express all the virus-specific functions of the parent cells and contain all the virus DNA sequences found in the parent cells. T-antigen-negative revertants of simian virus 40-transformed cells have been described which fall into similar classes (42).

Class 1 revertants may arise by chromosome loss or by deletion of virus sequences, presumably by illegitimate recombination. Class 2 revertants are deletions of part of the virus sequence and may have arisen by the same mechanism as class 1 revertants. Class 3 revertants are more interesting and resemble phenotypically the tk$^-$ cells isolated by Davidson et al. (6), which are capable of back-mutation to a tk$^+$ phenotype. These cells contain all the virus-specific sequences found in the parental cells, and since they can be induced to synthesize thymidine kinase by infection with a tk$^-$ virus, they cannot have arisen by a mutation within the thymidine kinase structural gene. Indeed, of the 15 revertants described in this study, none has arisen by mutation in the structural gene, and base pair substitutions, insertions, and short deletions must therefore occur at much lower frequency than the mutations involved here. This argument also eliminates promoter mutations as a source of class 3 revertants; there is no reason to expect mutations within a promoter sequence to occur with higher frequency than mutations within a structural gene. Several alternative explanations are worth considering.

(i) If the tk gene in transformed cells is transcribed from a cell-specific promoter, as we have suggested, then translocation of the virus sequences to a new integration site might render the tk gene quiescent by virtue of the absence of a suitable promoter. Expression can be restored by virus infection and consequent transcription from the virus-specific promoter. This explanation is untenable because digestion of revertant cell DNA yields fragments composed partly of virus sequences and partly of host sequences which are indistinguishable in mobility from the equivalent fragments obtained from transformed cell DNA, and this establishes that the host sequences immediately adjacent to virus DNA sequences are unchanged in revertant cells. However we cannot exclude the possibility that

TABLE 2. *Transformation of LMTK$^-$ cells with revertant cell DNA*

Source of DNA	Proportion of cultures with colonies[a]
$D2_5$	6/7
$D2_5A2$	0/7
$D2_5A4$	4/5
$D2_1$	4/7
$D2_1R1$	0/6
$D2_1R5$	2/5
LMTK$^-$	0/7

[a] Monolayers of 10^6 cells were treated with 10 μg of cell DNA. After 48 h the cells were transferred to selective medium, and the cultures were scored for colonies after 20 days. Methods have been described in detail elsewhere (29).

large DNA fragments (which include host sequences) are translocated to new sites in the cell genome and, as a result, are subject to altered regulation.

(ii) An alternative explanation involves a regulatory mutation at a locus of host origin resulting in loss of expression of the tk gene, which is itself unaltered in sequence or integration site. A dominant regulatory mutation has been described which reduces hypoxanthine guanosine phosphoribosyltransferase activity in heteroploid L cells (14, 37). Such regulatory mutants arise at a high spontaneous frequency of about 10^{-6}, and in the presence of mutagens this is increased to 10^{-4}. However, most mutants of this type synthesize >10% of the enzyme activity in the parent cells, and so are not directly comparable with the tk^- revertants described here, which have enzyme levels which are indistinguishable from those found in $LMTK^-$ cells.

(iii) A further possibility is that the virus sequences in revertant cells are chemically modified such that they are no longer transcribed. Two reports have appeared which suggest that methylation of virus sequences in transformed cells is associated with decreased expression of virus genes (8, 41). However, if virus sequences in revertant cells are modified in this way, the modification must be readily reversible, since virus infection results in expression, and the tk gene from revertant cells transforms $LMTK^-$ cells to a tk^+ phenotype with similar frequency to the gene from parental cells. Nevertheless, we cannot eliminate this possibility.

The isolation of these three classes of revertants in a group of 15 suggests that these classes arise at comparable frequency, and it is therefore tempting to invoke a common mechanism for the generation of all three classes. We can arrive at a possible mechanism by supposing that excised sequences are reintegrated with high frequency. Then class 1 revertants arise by excision and loss of large sequences, including all the virus sequences. Class 2 revertants arise by excision of smaller sequences, including only part of the virus sequences. Class 3 revertants arise by excision of large sequences, but the sequence is reintegrated at a new site which is transcriptionally silent. This model is highly speculative, but at least is consistent with the available data. Phenotypic modification by rearrangement of genetic elements has been established in yeast, where mating type is determined by transposition of sequences from "silent" sites to the mating type locus (11), though the sequences involved are smaller than the virus sequences in $D2_1$ or $D2_5$ cells ($D2_1$ cells contain a virus sequence of ~25 kilobases). However, microscopic studies have shown that rearrangement of very large genetic elements can occur in tissue culture cells (7).

In DNA transfection experiments, selection for the HSV tk gene selects simultaneously for other linked genes (as illustrated by $D2_1$ and $D2_5$ cells). This has been exploited by several groups who have ligated unrelated genes to the HSV tk gene and introduced such genes into mammalian genotypes by selecting for the tk^+ phenotype (20, 26). This general approach of introducing genes by the use of covalently linked markers of selective value has considerable potential. In this context, it is worth noting that in this study revertants have been obtained which lack the tk gene, but retain a nonselected marker (class 2 revertants). It is therefore possible to eliminate the vector gene from transformed cells while retaining sequences which were introduced with it.

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