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Herpes Simplex Virus Type 2 Mutagenesis: Characterization of Mutants Induced at the *hprt* Locus of Nonpermissive XC Cells

LOUISE PILON,* YVES LANGELIER, AND ANDRÉ ROYAL

Institut du Cancer de Montréal, Centre Hospitalier Notre-Dame, Montreal, Quebec, Canada H2L 4M1

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In a previous report, herpes simplex virus type 2 (HSV-2) was shown to increase the frequency of mutation at the hypoxanthine phosphoribosyltransferase (*hprt*) locus of nonpermissive rat XC cells (L. Pilon, A. Royal, and Y. Langelier, J. Gen. Virol. 66:259–265, 1985). A series of 17 independent mutants were isolated after viral infection together with 12 spontaneous noninfected mutants to characterize the nature of the mutations induced by the virus at the molecular level. The DNA of the mutants isolated after viral infection was probed with cloned HSV-2 fragments representing the entire genome. In these mutants, no authentic HSV-2 hybridization could be detected. This was indicative of a mechanism of mutagenesis which did not require the permanent integration of viral sequences in the host genome. The structure of the *hprt* gene was determined by the method of Southern (J. Mol. Biol. 98:503–517, 1975), and the level of *hprt* mRNA was analyzed by Northern blots. Except for the identification of one deletion mutant in each of the two groups, the HPRT$^-$ clones showed no evidence of alteration in their *hprt* gene. A total of 7 of 12 spontaneous mutants and 11 of 15 mutants isolated from the infected population transcribed an *hprt* mRNA of the same size and abundance as did the wild-type cells. Thus, the majority of the mutants seemed to have a point mutation in their *hprt* structural gene. Interestingly, the proportion of the different types of mutations was similar in the two groups of mutants. This analysis revealed that HSV-2 infection did not increase the frequency of rearrangements but rather that it probably induced a general increase of the level of mutations in the cells. This type of response is thought to be compatible with the biology of the virus, and the possible mechanisms by which HSV-2 induces somatic mutations in mammalian cells are discussed.

Defined regions of the herpes simplex virus type 2 (HSV-2) genome can transform rodent cells in vitro (12, 20), but the existence of a viral oncogene responsible for the maintenance of the transformed state has been questioned because of the lack of evidence that a specific viral sequence was retained in these cells (13). In addition, transformation by HSV is less efficient (13, 20, 21) than what is generally observed with viral oncogenes (3, 29). These considerations suggested that the role of the virus was transient and led to the hypothesis that HSV acts as a mutagen (13, 17, 42, 61). The first evidence that HSV could cause mutations came from observations that infection often results in fragmentation and pulverization of cellular chromosomes (4, 16, 45, 57). It was subsequently shown by Schlehofer and zur Hausen (41) that infection of human permissive rhabdomyosarcoma cells by inactivated HSV-1 could result in an increased mutation frequency at the hypoxanthine phosphoribosyltransferase (*hprt*) locus. In a previous report, we made use of the XC cell line, which is not permissive for HSV-2, to demonstrate that intact viral particles could increase the frequency of HPRT$^-$ mutants by factors ranging from 2.4 to 10.3 (38). Other DNA viruses such as simian virus 40 and adenovirus type 2 can cause mutations at several loci in nonpermissive infections (14, 27, 28, 50, 52, 60). However, the nature of the virally induced mutations has not been systematically studied at the molecular level, and the mutational mechanism of these viruses is not known.

Several reports suggest that simian virus 40 mostly induces point mutations (47, 51, 56). In these studies, the nature of the mutations was determined by analyzing the HPRT enzyme activity and electrophoretic mobility and by screening for the presence of cross-reacting material. In a study of the mutagenic potential of adenovirus type 2, where revertants of an HPRT$^-$ Chinese hamster cell line were obtained after infection, electrophoresis analysis of the enzymes suggested that most reversion events could be ascribed to the occurrence of a second intragenic mutation (39). However, except for the demonstration that the reversion did not require the stable integration of viral sequences, the nature of the genetic change could not be determined. Mutations induced by herpesviruses have not been characterized, but several mechanisms of mutagenesis can be envisaged. HSV is a complex virus that codes for several enzymes involved in its DNA metabolism (44). Hence, HSV could induce mutations by interfering with the process of cellular DNA replication. Another general mechanism which is envisioned is the insertion of viral sequences. Finally, it is possible that infection could result in the induction of the host cell repair system.

To gain better understanding of the mutational mechanism of HSV-2, we attempted to determine the molecular basis of mutations induced in the infected population by analyzing the HPRT$^-$ mutants isolated previously (38). The *hprt* gene was chosen for these studies because the selection assay is well characterized (6). More importantly, the HPRT$^-$ phenotype can result from a variety of mutations such as insertions, deletions, and point mutations (46, 49), thus allowing several mechanisms of mutagenesis to be detectable. By using an *hprt* cDNA probe (23), we analyzed the restriction pattern of this gene and the status of the *hprt* mRNA in our HPRT$^-$ mutants. We also screened our

* Corresponding author.

mutants to determine whether HSV-2 DNA sequences were integrated in the host genome.

MATERIALS AND METHODS

Cells and virus. The XC cell line, which was derived from a Rous sarcoma virus-induced rat sarcoma, was obtained from the American Type Culture Collection (CCL 165). These cells were grown in Eagle reinforced medium containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 2 mM glutamine, streptomycin, and penicillin G. The cells were infected with HSV-2 (HG-52) obtained from J. H. Subak-Sharpe. Independent colonies resistant to 6-thioguanine (6 μg/ml) were selected from control uninfected or HSV-2-infected XC cell populations as previously described (38). The $HPRT^-$ phenotype of the resistant colonies was confirmed by the low level of HPRT activity (EC 2.4.2.8) as assayed by the rate of formation of [^{3}H]IMP from [G-^{3}H]hypoxanthine in the presence of 5-phosphoribosyl-1-PP_i (38).

DNA analysis. High-molecular-weight cellular DNA was extracted from confluent cultures by the method of Bellard et al. (2) and dialyzed against 1 mM Tris hydrochloride (pH 8.0) and 0.1 mM EDTA. DNAs were digested to completion with *Eco*RI or *Hin*dIII (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), fractionated on 0.7% agarose gels, and transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) as described previously (43). The *hprt* gene restriction pattern was studied by using the mouse *hprt* cDNA probe pHPT5, kindly provided by C. T. Caskey (23). Cell lines were probed for the presence of HSV-2 sequences by using a series of plasmids containing *Hpa*I or *Hin*dIII viral fragments representing the entire genome, kindly provided by A. Kessous. In most cases, inserts were purified by electroelution before nick translation (30).

Prehybridization of Southern blots was performed at 42°C in 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0]), 1× Denhardt solution (9), 50 mM Tris hydrochloride (pH 7.6), and 250 μg of sonicated salmon sperm DNA per ml. For hybridization, [α-^{32}P]dCTP nick-translated probes were added to a solution of four parts of prehybridization buffer and one part of 50% dextran sulfate, and incubated at 42°C for 18 to 36 h. Membranes were washed with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature and then sequentially washed at 65°C with 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS. Autoradiography was performed at −70°C by using Kodak XAR or presensitized PR-X-Omat film (Eastman Kodak Co., Rochester, N.Y.) and amplifying screens.

RNA analysis. RNA was extracted from confluent cultures as described by Murphy et al. (34). Briefly, the cells were lysed in the presence of SDS and vanadyl ribonucleoside complex (VRC), treated with proteinase K, and phenol extracted. After ethanol precipitation, the RNAs were treated with DNase I (RNase free) in the presence of VRC, phenol extracted, and precipitated with ethanol. To remove the VRC, the RNA was dissolved in H_2O, and EDTA (pH 8.0) was added to a final concentration of 50 mM. The solution was extracted twice with phenol-chloroform-isoamyl alcohol (50:49:1) and precipitated with 2 volumes of ethanol after the addition of sodium acetate (pH 6.0) to a final concentration of 0.3 M. The RNA pellet was washed twice with 70% (vol/vol) ethanol, dissolved in H_2O, and stored at −70°C. The concentration of the RNA was evaluated by spectrophotometry.

A 20-μg amount of RNA was denatured with glyoxal as previously described (31), fractionated on a 1% agarose gel, and transferred to a nylon membrane (Pall Biodyne) (53). Prehybridization of Northern blots was carried out in 50% deionized formamide–5× SSPE (1× SSPE is 0.18 M NaCl plus 10 mM sodium phosphate buffer [pH 8.3] plus 1 mM EDTA)–5× Denhardt solution–0.3% SDS–250 μg of sonicated salmon sperm DNA per ml–50 μg of bakers' yeast tRNA per ml at 42°C. After hybridization, performed as described above for Southern blots, membranes were washed with 2× SSC–0.1% SDS at room temperature and then several times with 0.1× SSC–0.1% SDS at 50°C. Filters were sequentially hybridized with pHPT5 for the detection of *hprt* mRNA and with a β-actin cDNA clone (10) for the detection of actin mRNA. Where indicated, autoradiographs were scanned on a densitometer (Hoefer Scientific Instruments), and areas under peaks were determined by using a Carl Zeiss MOP-3 integrator.

RESULTS

In our study of the mutagenic properties of HSV-2, we generated a number of $HPRT^-$ mutants, all with very low enzymatic activities (38). The mutants analyzed in this report were isolated during the course of experiments where the frequency of mutation at the *hprt* locus for the infected cultures was 4.5, 4.4, and 2.5 times higher than for the corresponding noninfected population (Table 1).

Analysis of mutant DNAs for retention of viral sequences. An obvious possible mechanism by which HSV could induce mutation is by insertion of viral sequences in the cell genome, more specifically in the *hprt* gene. To examine this question, DNAs isolated from mutant cell lines were probed with cloned HSV-2 DNA fragments *Hpa*I-a, *Hpa*I-dh, *Hin*dIII-ao, and *Hin*dIII-h, which represent the unique sequences of the long and short arms of the genome, and fragments *Hin*dIII-jm and *Hin*dIII-ki, which contain the repeated sequences (Fig. 1A). First, in a reconstruction experiment designed to determine the level of sensitivity of the method, a mixture of restriction fragments equivalent to 0.1 or 1 copy per haploid cell genome was added to 20 μg of XC cell DNA digested with *Eco*RI and transferred to a nitrocellulose membrane. Hybridization was then carried out with a pBR322 plasmid containing the HSV-2 fragment *Hin*dIII-ao (39.7 kilobase pairs) which constituted a complex probe. A fragment of 475 base pairs (bp) present at the level of 1.0 copy per haploid genome on the filter and complementary to approximately 1% of the probe, could be detected (Fig. 1B). Also, a 600-bp fragment present at the level of 0.1 copy per haploid genome and corresponding to 1.5% of the probe was visualized. Fragments of 368 and 315 bp were also present but could not be detected in this experiment. By using the same conditions, a total of 16 independent $HPRT^-$ clones isolated after HSV-2 infection (designated h) were examined. In each case, a *Hin*dIII digest and an *Eco*RI digest were independently probed with each of the fragments described above (Fig. 1A), and no viral sequences could be detected with any of these probes. Representative results are shown in Fig. 1C, in which the DNAs from mutant lines h22, h23, and h44 to h48 were probed with the HSV-2 fragment *Hin*dIII-a. When HSV-2 fragments *Hin*dIII-ki and *Hin*dIII-jm were used as probes, homologies were detected with all cellular DNAs including the parental XC cell DNA as previously described for mouse and human DNA (37). However, hybridization to authentic HSV-2 DNA sequences was undetectable with these probes. Therefore, integration

of viral sequences did not seem to contribute to the mutagenic effect of HSV-2.

Analysis of the *hprt* gene in mutants. Because HSV is known to induce chromosomal damage (4, 16), our next step was to determine, by Southern blot analysis of the *hprt* gene structure, whether the phenotype of the HSV-induced mutants could be associated with major rearrangements such as deletions or translocations. In XC cells, the restriction pattern of the *hprt* gene probed with the plasmid pHPT5 revealed seven *Hin*dIII fragments of 10.5, 7.9, 4.2, 2.15, 1.15, 0.9, and 0.55 kilobases (kb) (Fig. 2A) and four *Eco*RI fragments of ~23.5, 9.5, 6.8, and 1.85 kb (Fig. 2B). This indicated that the gene extended over 30 kb, which is consistent with the results obtained with hamster, mouse, and human DNA (11, 32, 36).

A total of 17 mutants isolated after HSV infection and 12 spontaneous mutants were examined. Of these, one spontaneous and one HSV-induced mutant showed evidence of rearrangements by analysis of *Hin*dIII and *Eco*RI digests. In clone s41, the 2.15-kb *Hin*dIII band (Fig. 2A, lane 5) and the 6.8-kb *Eco*RI band (Fig. 2B, lane 3) were missing. In clone h15, the two larger *Hin*dIII bands were replaced by a 9-kb band and the 0.55-kb band was missing (Fig. 2A, lane 2), and the 13.5-kb *Eco*RI band was lost without any evidence of new hybridizing fragments (Fig. 2B, lane 2). Therefore, these two mutants seemed to have a deletion in their *hprt* gene. Also, the complete loss of hybridizing bands indicated that the *hprt* gene is hemizygote in XC cells. For the other clones examined, no modification of the restriction pattern could be observed. These results indicate that the frequency of rearrangement detectable by our approach is low in the two groups of mutants.

Analysis of the *hprt* mRNA transcribed in the HPRT$^-$ mutants. To further characterize the mutations, the status of the *hprt* mRNA was determined by Northern hybridization. This analysis revealed that most clones transcribed an *hprt* mRNA of the expected size (1.55 kb) and with the same abundance as the control XC cell *hprt* mRNA (Fig. 3). This conclusion was substantiated by densitometric analysis of the autoradiograms where the amount of *hprt* mRNA was compared to the amount of actin mRNA (results not shown). A total of 7 of 12 spontaneous mutants and 11 of 15 mutants isolated after infection were of this type. The other mutants had undetectable levels of *hprt* mRNA. These included mutants s41 and h15, which suffered a deletion event, and mutants which did not seem to have any genetic rearrangements as determined by DNA blot hybridization.

The results of the analysis of each mutant are summarized in Table 1. Three different classes could be distinguished, based on the characterization of gene structure, presence of mRNA, and enzyme activity. First, mutants having approximately normal amounts of mRNA, with no evidence of genomic rearrangements, were probably the result of point mutations. They represented more than half of all the mutants isolated and had a very low level of HPRT activity, ranging from undetectable to 6.1% of that of wild type. A second class of mutants was identified which had no genomic rearrangement and no detectable mRNA. Within this class some mutants had detectable enzyme activity. Finally, mutants s41 and h15, which had lost hybridizing DNA, did not seem to transcribe an *hprt* mRNA and did not have any enzyme activity.

TABLE 1. Summary of analysis of spontaneous or HSV-2-induced HPRT$^-$ mutants

Increase factor[a] and mutant type	*hprt* gene restriction pattern[b]	Status of *hprt* mRNA[c]	HPRT activity (% wild type)[d]
4.4			
s10	NA	−	<1.5
s11	NA	−	<1.5
h8	NA	+	6.1
h9	NA	+	5.1
h12	NA	+	3.1
h13	NA	−	4.7
h14	NA	ND	6.9
h15	A	−	<1.5
4.5			
s16	NA	−	5.5
h17	NA	ND	4.3
h18	NA	−	3.6
h19	NA	−	4.4
h21	NA	+	3.6
h22	NA	+	ND
h23	NA	+	ND
2.5			
s40	NA	+	2.2
s41	A	−	<1.5
s42	NA	+	ND
s43	NA	+	2.5
s49	NA	+	1.8
s50	NA	+	3.2
s51	NA	−	<1.5
s52	NA	+	2.7
s53	NA	+	1.7
h44	NA	+	2.1
h45	NA	+	<1.5
h46	NA	+	2.1
h47	NA	+	1.7
h48	NA	+	2.3

[a] Factor of increase of the mutation frequency of the infected cells compared with the spontaneous frequency for three independent experiments.
[b] Absence (NA) or presence (A) of gene alteration.
[c] Absence (−) or presence (+) of gene *hprt* mRNA. ND, Not determined.
[d] Average of two assays. ND, Not determined.

DISCUSSION

This is the first study attempting to define the type of mutations present in cells after HSV-2 infection. Our data suggest that the predominant type of mutation at the *hprt* locus occurring spontaneously or after viral infection is point mutation. The most striking observation we have made is that there does not seem to be any difference in the distribution of these two groups of mutants in the different classes we have defined.

The majority of the mutants were identified as having a point mutation in their *hprt* gene since they had very little detectable HPRT activity, an intact gene, and expressed an mRNA of the expected size and abundance. These mutants may have had HPRT enzymes with reduced affinity for their substrate, which could have accounted for the low levels of activity we measured. In this analysis, small variations in the size of the bands observed by Southern blot or in the size of the mRNA transcribed by these mutants cannot be ruled out. However, the frequency at which we observed point mutations was similar to the frequencies reported in other studies at this locus for spontaneous mutants or for mutants induced by several mutagens (for a review, see reference 49).

Several mutants were characterized by the absence of detectable mRNA, although no evidence of rearrangements in the gene could be obtained. The nature of these mutations is not known, but they have been described by others, who

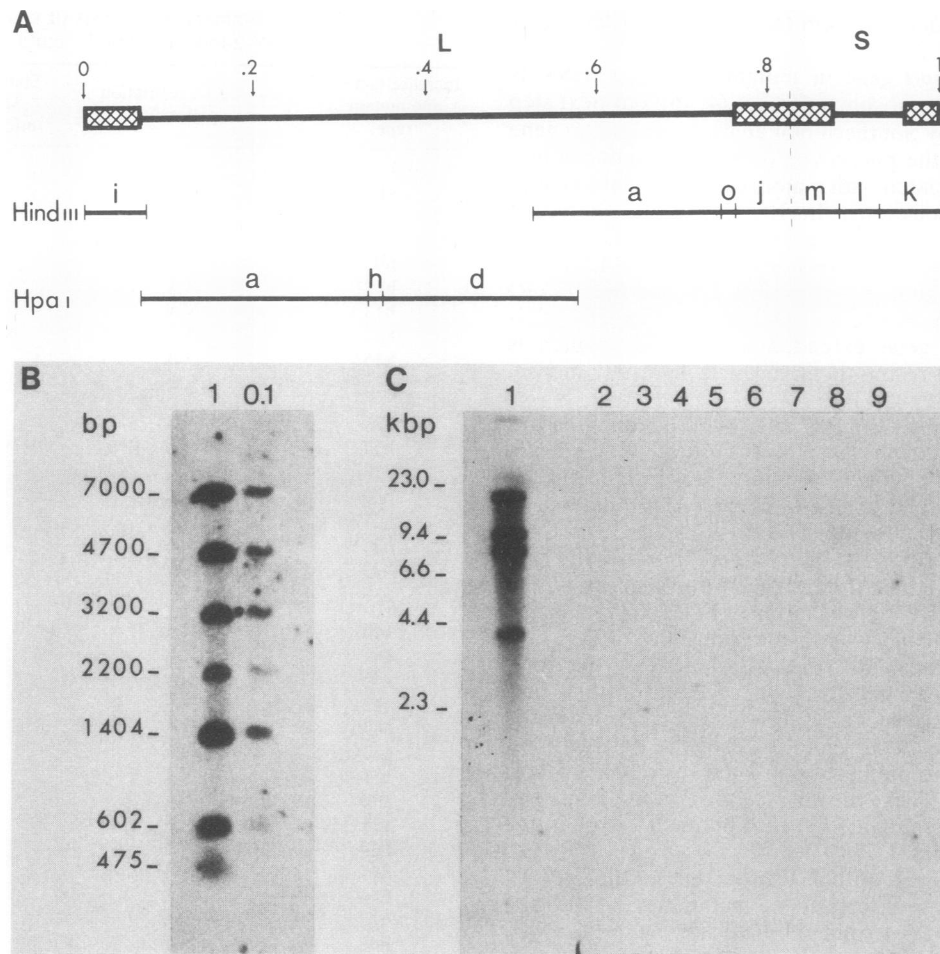

FIG. 1. DNA of HPRT⁻ mutants isolated after viral infection screened for the presence of integrated viral sequences. (A) Location of the HSV-2 (HG-52) fragments used as probes. Fragments *Hin*dIII-a, *Hin*dIII-l, *Hpa*I-d, and *Hpa*I-a were purified, whereas fragments *Hin*dIII-ao, *Hin*dIII-jm, *Hin*dIII-ki, and *Hpa*I-dh were used without being separated from their bacterial sequences. (B) Reconstruction experiment in which 1.0 and 0.1 copy per haploid genome of defined plasmid fragments ranging from 315 to 7,000 bp were hybridized with a plasmid containing the HSV-2 fragment *Hin*dIII-ao. The filter was exposed for 24 h. (C) Hybridization of an *Eco*RI digest of wild-type DNA (lane 2) and mutant DNAs (lanes 3 through 9) with HSV-2 fragment *Hin*dIII-a. Lane 1, 1.0 copy per haploid genome of HSV-2 *Hin*dIII-a cut by *Bgl*II. This is a 3-day exposure of the filter. Exposure up to 2 weeks did not reveal any HSV-2-specific hybridization.

found them at similar frequencies (22, 35). In some instances, the phenotype may be reminiscent of spontaneous events, where gene expression is thought to be shut off over large chromosomal regions (5, 54). Other genetic events can also be envisaged, such as point mutations resulting in the instability of the mRNA, incorrect splicing, or inhibition of mRNA transport. Within this class, some mutants have residual HPRT enzyme activity which may result from mutations outside the gene which could drastically reduce the level of transcription. Clearly, further studies are needed to determine the exact nature of the lesions in the mutants; again, we must consider that small genetic rearrangements may have escaped our analysis.

Two mutants which have undergone distinct deletion events were observed. One mutant occurred spontaneously, whereas the other was isolated from the infected population. This low frequency is consistent with other reports for this locus (11, 22), but the fact that we observed only one deletion event within our mutants isolated after infection is quite surprising, since HSV infection often results in chromosome breaks and rearrangements (45, 57). It may be that those types of damage caused by the virus lead to cell lethality and do not contribute to the mutagenic effect of HSV-2. We have in fact previously observed cytopathic effects during HSV-2 infection of nonpermissive XC cells (38). We may conclude from our results that HSV-2 infection does not increase the frequency of deletion mutations at the *hprt* locus in XC cells.

Viral DNA sequences were not detected in the mutant isolated after infection. However, we must consider that this type of analysis is complicated by the fact that HSV shows homology with mammalian DNA, especially in the inverted repeats (37). Thus, we cannot exclude the possibility that small dispersed viral sequences were integrated in our HPRT⁻ mutants. Nonetheless, integration of viral sequences appears to be an infrequent event in this system. Similar results were reported for mutants induced after nonpermissive infection by adenovirus type 2 (39), in which, as mentioned above, no integration of viral sequence was found. These observations point to a mechanism of mutagenesis by DNA viruses seemingly independent of the integration of viral sequences. This is different from what is observed with retroviruses, which are known to cause insertional mutagenesis in mammalian cells (55, 59).

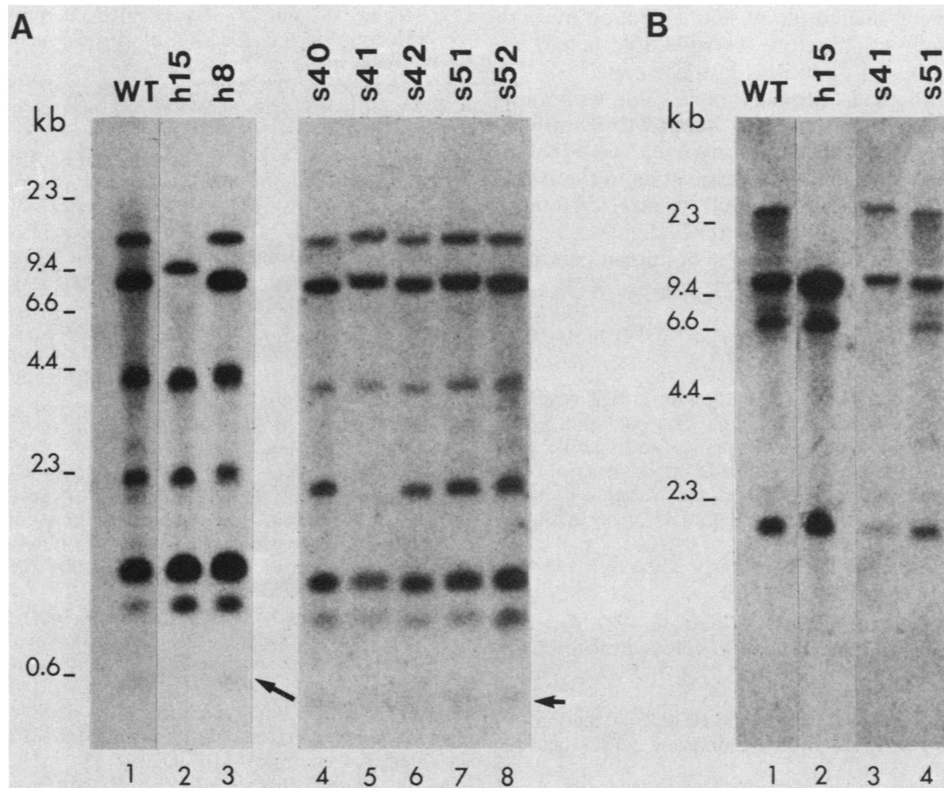

FIG. 2. Southern blot analysis of HPRT⁻ mutants. (A) *Hin*dIII digest of DNA from wild-type (WT), HPRT⁻ mutants isolated after viral infection (h15 and h8), and spontaneous mutants (s40, s41, s42, s51, and s52). The 550-bp fragment (arrow) was clearly visible upon longer exposure. (B) *Eco*RI digest of DNA from wild-type cells (WT), DNA from an HSV-2-infected mutant (h15), and spontaneous mutants (s41 and s51). The DNAs were hybridized with the purified nick-translated pHPT5 insert.

Two general hypotheses remain consistent with the fact that we observed a general increase of the level of mutation in XC cells. First, infection could disturb the host cell DNA repair system, leading to an increase in the stabilization of spontaneous mutation events, as suggested previously (61). This would also explain why viral mutagenesis is a universal phenomenon. Alternatively, expression of viral enzymes involved in DNA metabolism could account for the observed increase in mutation frequency (13). Lending support to this hypothesis is the fact that HSV has a ribonucleotide reductase (8, 25) and a thymidine kinase (48) which have allosteric properties that are different from those of the corresponding cellular enzymes (8, 19). The expression of these enzymes has been implicated in the generation of nucleotide pool imbalances (1, 18, 40), and it is known that deregulation of intracellular levels of dNTPs can be mutagenic (24, 33, 58). In addition, the viral DNA polymerase (7) is known to be error prone during the replication of its own DNA (15). Thus, expression of this viral enzyme may confer a mutator phenotype to the cell, since alterations in the cellular polymerase have such a capacity (26). Viral thymidine kinase, ribonucleotide reductase, and DNA polymerase are expressed in XC cells (L. Beaudet, A. Royal, and Y. Langelier, manuscript in preparation) and could induce

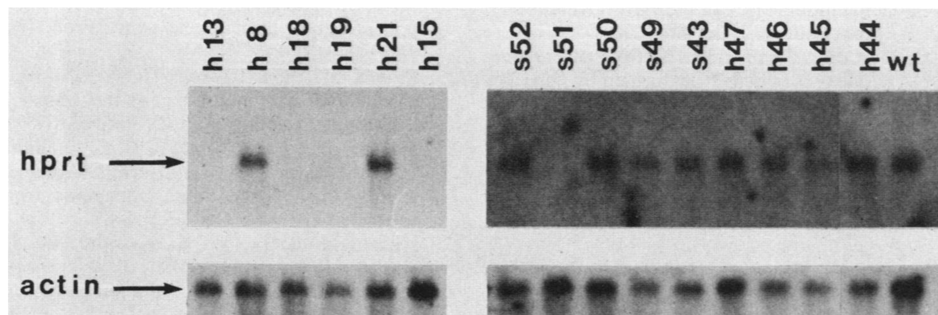

FIG. 3. Northern blot analysis of mRNA from wild-type XC cells (wt) and from several spontaneous HPRT⁻ mutants (s52, s51, s50, s49, and s43) and mutants isolated after HSV-2 infection (h13, h8, h18, h19, h21, h15, h47, h46, h45, and h44). A total of 20 μg of RNA was denatured, electrophoresed in a 1.0% agarose gel, and hybridized with nick-translated pHPT5 to identify the *hprt* mRNA. The same filters were dehybridized and rehybridized with a nick-translated β-actin probe.

mutations by increasing nucleotide misincorporation during cellular DNA replication. Therefore, considerable potential exists for the generation of mutations in these cells.

These two hypotheses are equally consistent with our results, and further experimentation is needed to discriminate between them. Once these alternatives have been defined, significant information will be gained as to the type of responses occurring in mammalian cells after HSV infection. This could ultimately lead to a better understanding of the contribution of HSV-2 to the etiology of human cervical carcinoma (13, 61).

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