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# Assessment of the Base Sequence Homology Between the Two Subtypes of Equine Herpesvirus 1†

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The magnitude of the genetic relatedness of the two antigenic subtypes of equine herpesvirus 1 (EHV-1) was determined by DNA-DNA reassociation kinetics. Denatured, labeled viral DNA from one EHV-1 subtype was allowed to reassociate in the presence or absence of the unlabeled heterologous viral DNA. The initial rate of reassociation of either labeled viral DNA was increased by the presence of the heterologous viral DNA to an extent indicating 10 to 20% homology between the two EHV-1 genomes. Similar estimates of the amount of homology between the genomes of the two EHV-1 subtypes were obtained by determining the maximum fraction of labeled viral DNA that could be made resistant to S1 nuclease by hybridization with a large molar excess of the unlabeled, heterologous viral DNA. Analysis of the thermal stability of the subtype 1-subtype 2 heteroduplex DNA indicated approximately 30% base pair mismatching within the hybrid DNA molecules. Cross-hybridization of $^{32}$P-labeled virion DNA to nitrocellulose blots of restriction endonuclease cleavage fragments of each EHV-1 subtype DNA indicated that the observed homology between the two viruses was nonuniformly distributed within the viral genome. No homology could be detected between the DNA of either EHV-1 subtype and that of a strain of equine cytomegalovirus (EHV-2). The data suggest that the two biotypes of EHV-1 have arisen by divergent evolution from a common progenitor herpesvirus.

Equine herpesvirus 1 (EHV-1) is the etiological agent of both upper respiratory infections (rhinopneumonitis) and contagious virus abortion of horses (2). Two antigenic subtypes of EHV-1 have been recognized since 1959, when Shimizu and co-workers (12) demonstrated that EHV-1 isolates could be grouped into either of two distinct but cross-neutralizing serotypes. Subsequent studies have revealed other major differences in the growth rate, in vitro host range, adaptability to hamsters, and abortigenicity of the two EHV-1 subtypes (3, 4, 14; R. Burrows, Abstr. Annu. Cong. Br. Equine Vet. Assoc. 20th, Cambridge, England, p. 18, 1981). Moreover, members representative of the two virus subtypes exhibit differences in the electrophoretic mobility of at least seven of their structural glycoproteins when analyzed in sodium dodecyl sulfate-polyacrylamide gels (16). Investigations by Burrows and Goodridge (3) first suggested that, whereas the majority of respiratory isolates of EHV-1 fall into one antigenic subtype, viruses isolated from aborted equine fetuses belong to the other EHV-1 subtype. Recent comparisons of the restriction endonuclease cleavage patterns of the two viral genomes suggested a greater degree of genetic dissimilarity than was originally suspected, since the two virus DNAs have very few restriction enzyme cleavage sites in common (10, 15, 16).

The purpose of the studies described in this paper was to investigate the degree of base sequence homology between the genomes of the two subtypes of EHV-1, using the techniques of blot hybridization, DNA-DNA reassociation kinetics, and S1 nuclease analysis. Our results showed that the two equine herpesviruses share approximately 17% of their genome sequences.

## MATERIALS AND METHODS

**Viruses and cell culture.** The viruses used in this study were the Army 183 (A-183; subtype 1) and Kentucky T2 (Ky-T2; subtype 2) strains of EHV-1 and the Ky-T162 strain of equine cytomegalo-virus (EHV-2). The origin and propagation of these viruses and their purification from the culture medium of infected equine dermal fibroblasts (KyED cells) have been described previously (16).

**Isolation and labeling of DNA.** The procedures for isolation of DNA from purified EHV virions or from uninfected KyED cells by sodium dodecyl sulfate-pronase digestion and phenol extraction have been

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described elsewhere (1). EHV-1 DNAs were labeled to specific radioactivities of $3 \times 10^6$ to $4 \times 10^6$ cpm/μg by infecting KyED cells in the presence of 800 μCi of [$^3$H]thymidine (77 Ci/mmol) per ml of culture medium. Viral DNAs for blot hybridization were labeled in vitro by nick translation (9) with [$^{32}$P]dCTP (7,000 Ci/mmol) to a specific activity of $10^8$ cpm/μg. All DNAs for hybridization were degraded to fragments 400 to 500 nucleotides in length by boiling in 0.3 N NaOH for 20 min (11). After fragmentation, the DNA solutions were neutralized and dialyzed against TNE buffer (0.1 M Tris-hydrochloride [pH 8.1]–0.015 M EDTA–0.15 M NaCl).

**Hybridization to nitrocellulose blots of restriction fragments.** Unlabeled EHV DNAs were digested to completion with the restriction endonucleases *Bam*HI and *Eco*RI. The resulting cleavage fragments were separated by electrophoresis in horizontal slab gels of 0.6% agarose and then blot-transferred to nitrocellulose for hybridization (13). The conditions for nitrocellulose filter hybridization and subsequent washing were those described by Jeffreys and Flavell (6). Autoradiography of the filter sheets after hybridization was performed at −80°C with Kodak XAR-5 film and Lighting-Plus intensifying screens (Du Pont Co., Wilmington, Del.).

**Kinetic hybridization.** The strategy and design of the DNA-DNA reassociation kinetics experiments for quantitation of the extent of base sequence homology between two viral genomes has been previously described in considerable detail (1, 5). Hybridizations were performed at 65°C in TNE buffer. Reaction mixtures consisted of 100 ng of labeled viral probe DNA per ml, 0 to 2 μg of unlabeled test DNA per ml, and enough calf thymus DNA to give a total DNA concentration of 2 μg/ml. The mixtures were sealed in 100-μl microcapillary pipettes, denatured for 7 min at 110°C in an ethylene glycol bath, and allowed to reanneal at 65°C. Samples were taken after various periods of incubation, quickly chilled in ice, and frozen until assayed by chromatography on hydroxyapatite columns.

Reassociation of labeled viral DNA was monitored by hydroxyapatite (BioGel HTP; Bio-Rad Laboratories, Richmond, Calif.) chromatography at 60°C, as described in detail by Sharp et al. (11). The DNA eluting from the column at 0.14 M phosphate (single-stranded DNA) or 0.4 M phosphate buffer, pH 6.8 (double-stranded DNA) was precipitated with trichloroacetic acid, collected on nitrocellulose membrane filters (type BA85; Schleicher & Schuell Co., Keene, N.H.), and counted by liquid scintillation spectroscopy.

The hybridization results were plotted by the linear transformation method of Wetmur and Davidson (17), with the time interval of hybridization as the independent variable and the reciprocal of the fraction of DNA remaining single-stranded as the dependent variable; the method of least-squares regression was used to determine the slopes of the plotted data.

**Thermal elution chromatography of DNA-DNA duplexes.** The extent of base pair mismatching present within the EHV-1 subtype 1-subtype 2 heteroduplex DNAs was estimated by comparison of their thermal stability with that of the homoduplex DNA of each virus subtype. Reannealed viral DNA in 0.14 M sodium phosphate (pH 6.8)–0.4% sodium dodecyl sulfate (PB) was poured into a jacketed column containing 1 g of hydroxyapatite at 40°C. Single-stranded DNA was washed through the column with PB, and the column temperature was then raised in 2°C increments to 90°C. After each temperature equilibration, 2 ml of a solution of PB containing 8 M urea (7) was allowed to flow through the column, mixed with RIA-Fluor (New England Nuclear Corp., Boston, Mass.) and assayed for radioactivity.

## RESULTS

**Blot hybridization.** Restriction endonuclease fragments of EHV-1, strain A-183 (subtype 1); EHV-1, strain Ky-T2 (subtype 2); and EHV-2, strain Ky-T162 were transferred from agarose gels to nitrocellulose sheets and then hybridized with each of the three viral DNAs that had been radiolabeled by nick translation with [$^{32}$P]dCTP, as described in Materials and Methods. Photographs of the ethidium bromide-stained gels before transfer to nitrocellulose and autoradiograms of the blots after DNA transfer and hybridization are shown in Fig. 1 and reveal several notable features.

No hybridization could be detected, even after prolonged exposure of the film, between EHV-2 DNA and that of either of the two EHV-1 subtypes. Hybridization between the DNAs from the two subtypes of EHV-1 was readily demonstrated but was considerably less efficient than that between each virus genome and its homologous DNA restriction fragments. Autoradiographic exposure times for hybridization between heterologous viral DNAs (i.e., EHV-1 subtype 1 × EHV-1 subtype 2) were approximately five times longer than those required for the same degree of film darkening for homologous hybridizations.

Homology between the DNAs of the two EHV-1 subtypes was not restricted to any selected portion of the genome but was distributed over the entire DNA molecule. However, some restriction fragments appeared to exhibit a greater degree of cross-hybridization than others, indicating that some regions of the genome may have been more highly conserved than others during divergent evolution of the two virus subtypes.

**Reassociation kinetics and computation of homology.** For analysis of the kinetics of reassociation of mixtures of DNAs from both subtypes of EHV-1, labeled DNA from one EHV-1 subtype was denatured and allowed to reanneal in the presence of a 4-, 10-, or 20-fold excess of unlabeled DNA from the heterologous virus subtype (Fig. 2). In each instance, the initial rate of reassociation of the labeled viral DNA was increased by the presence of the heterologous DNA in the hybridization mixture, indicating sequences shared by the two herpesvirus genomes. However, the addition of the unlabeled

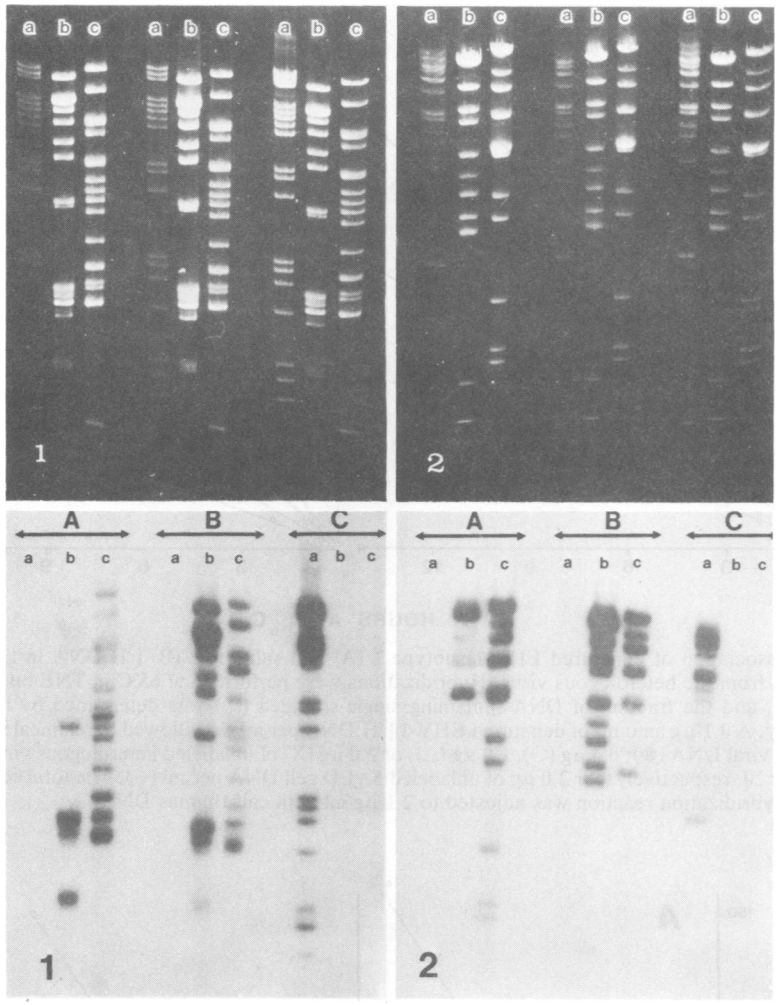


FIG. 1. Hybridization between $^{32}$P-labeled EHV DNAs and fragments of the viral DNAs generated by restriction endonucleases. EHV-2, EHV-1 subtype 2, and EHV-1 subtype 1 DNAs (lanes a, b, and c, respectively) were cleaved with either *Bam*HI (1) or *Eco*RI (2). The resulting cleavage fragments were electrophoresed on 0.6% agarose slab gels, stained with ethidium bromide (top frames), and then blot-transferred to nitrocellulose for hybridization (bottom frames) with either EHV-1 subtype 1 (A), EHV-1 subtype 2 (B), or EHV-2 (C) [$^{32}$P]DNA.

second viral DNA to the hybridization reaction resulted in a biphasic reassociation curve (Fig. 2). These results indicate that the DNA of each EHV-1 subtype contained sequences homologous to only a fraction of the heterologous EHV-1 genome (5).

Fujinaga et al. (5) have derived a formula for quantitating the amount of base sequence homology between two viral genomes utilizing the following two parameters from the DNA reassociation kinetics test: (i) the degree of increase of probe reassociation rate caused by the presence of the second unlabeled viral DNA and (ii) the molar ratio of test to probe DNA. The basis of the Fujinaga equation is to replot the experimental data by a method that emphasizes the initial reassociation reaction of the labeled viral genome in the presence of the unlabeled second viral genome. Such plots are shown in Fig. 3 for the reassociation of labeled subtype 1 viral DNA fragments in the presence of three different concentrations of unlabeled subtype 2 DNA fragments and vice versa. The initial rate of probe reassociation, represented by the slopes of the lines, was, in each case, dependent upon the concentration of added test DNA. The slopes of the plots of single-stranded DNA/double-stranded DNA ratios ($C_{ss}/C_{ds}$) versus $1/t$ during the initial period of reassociation of the probe DNA in the presence (A) and in the

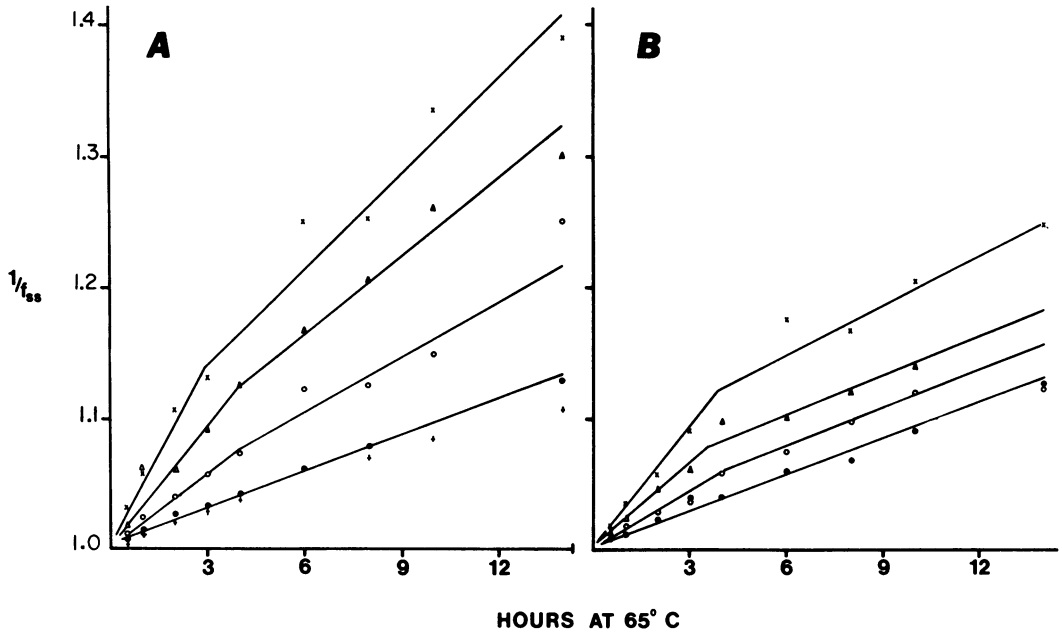


FIG. 2. Reassociation of denatured EHV-1 subtype 1 (A) and subtype 2 (B) [$^3$H]DNA in the presence of unlabeled DNA from the heterologous virus. Hybridizations were performed at 65°C in TNE buffer for various lengths of time, and the fraction of DNA remaining single-stranded ($f_{ss}$) was determined by hydroxyapatite chromatography. A 0.1-μg amount of denatured EHV-1 [$^3$H]DNA per ml was allowed to reanneal in the presence of no unlabeled viral DNA (●); 0.4 μg (○), 1.0 μg (△), or 2.0 μg (X) of unlabeled heterologous viral DNA per ml ($n$ = 0, 4, 10, or 20, respectively); or 2.0 μg of unlabeled KyED cell DNA per ml (+). The total concentration of DNA in each hybridization reaction was adjusted to 2.1 μg/ml with calf thymus DNA.

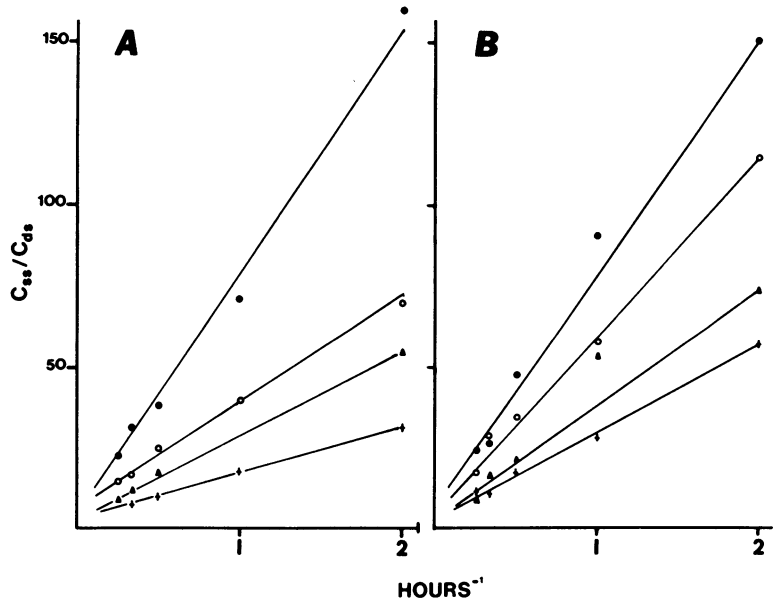


FIG. 3. Reassociation of 0.1 μg of EHV-1 subtype 1 (A) or subtype 2 (B) [$^3$H]DNA per ml in the presence of different concentrations of the unlabeled heterologous subtype viral DNA to give unlabeled-to-labeled DNA ratios ($n$ values) of 0 (●), 4 (○), 10 (△), or 20 (+). Hybridizations were performed at 65°C in TNE buffer for various lengths of time, and the ratio of single- to double-stranded DNA ($C_{ss}/C_{ds}$) was determined by hydroxyapatite chromatography.

absence ($A_0$) of the unlabeled DNA, together with the ratio of test to probe DNA ($n$), can then be used to calculate the fraction of sequences shared by the two viral genomes ($x/f$) from the following relationship (5): $x/f = [1 - (A/A_0)]/[n(A/A_0)]$. Using this equation, the fraction of nucleotide sequences shared between the DNAs of the two EHV-1 subtype viruses was computed (Table 1). It can be seen that the two herpesviruses share only about 17% of their genome sequences.

**Analysis of homology by S1 nuclease analysis.** Heteroduplexes of subtype 1-subtype 2 DNAs were prepared by annealing low concentrations of each labeled viral DNA with a 100-fold excess of unlabeled heterologous viral DNA; hybridization was allowed to continue at 65°C for 12 h. Under these conditions, homoduplexes accounted for less than 25% of the labeled hybrids, whereas heteroduplex formation went virtually to completion. Samples taken at frequent intervals during the hybridization reactions were monitored for reassociation of denatured DNA by S1 nuclease analysis. The values for heteroduplex formation were corrected for self-reannealing of labeled DNA molecules.

The results of this type of analysis of homology indicated that, in the presence of a large excess of heterologous viral DNA, approximately 10 to 12% of the EHV-1 genome could be driven into S1-resistant, double-stranded molecules (data not shown).

**Thermal stability of EHV-1 heteroduplex DNAs.** Because the degree of nucleotide complementarity in nucleic acid hybrids is reflected in their thermal stability, thermal chromatography of subtype 1-subtype 2 heteroduplexes on hydroxyapatite was used to determine the extent of mismatched base-pairing present within the hybrid viral DNAs.

Tritium-labeled heteroduplexes were adsorbed to hydroxyapatite at 40°C along with $^{14}C$-labeled homoduplex DNA. By increasing the temperature of the hydroxyapatite column, melting of the DNA-DNA duplexes was monitored by the appearance of labeled single-stranded DNA in the column effluent (Fig. 4). Homo-

TABLE 1. Fraction of genome sequences shared by the two subtypes of EHV-1

| Unlabeled test DNA/labeled probe DNA ($n$) | Ratio of slopes ($A/A_0$)[a] | Shared fraction of genome ($x/f$)[b] |
|---|---|---|
| EHV-1 subtype 1[c] | | |
| 4 | 0.50 | 0.25 |
| 10 | 0.42 | 0.14 |
| 20 | 0.23 | 0.17 |
| EHV-1 subtype 2[d] | | |
| 4 | 0.58 | 0.18 |
| 10 | 0.40 | 0.15 |
| 20 | 0.29 | 0.12 |

[a] Ratio of slopes of curves for $C_{ss}/D_{ds}$ versus $1/t$ during the intial periods of reassociation of labeled EHV probe DNA in the presence (A) and absence ($A_0$) of unlabeled heterologous test DNA.

[b] Calculated by the equation $(x/f) = [1 - (A/A_0)]/[n(A/A_0)]$.

[c] EHV-1 subtype 1 [$^3$H]DNA was reassociated in the presence of unlabeled subtype 2 DNA.

[d] EHV-1 subtype 2 [$^3$H]DNA was reassociated in the presence of unlabeled subtype 1 DNA.

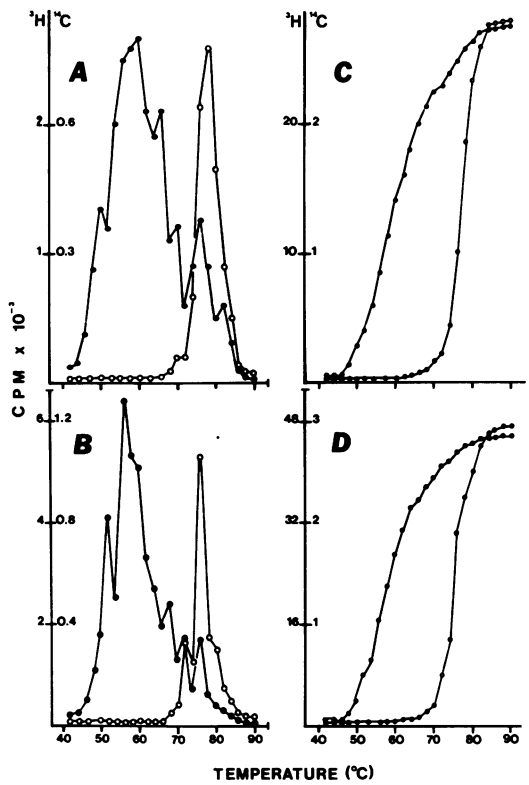


FIG. 4. Thermal stability of EHV-1 subtype 1-subtype 2 heteroduplex DNA. A 0.05-μg amount of EHV-1 subtype 1 (A and C) or subtype 2 (B and D) [$^3$H]DNA per ml was denatured and allowed to reanneal for 4 h at 65°C with a 200-fold molar excess of unlabeled heterologous viral DNA. A 2-μg amount of EHV-1 subtype 1 (A and C) or subtype 2 (B and D) [$^{14}$C]DNA per ml was denatured and allowed to reassociate at 65°C for 24 h to form homoduplex DNAs. Hetero- and homoduplexes were diluted into PB and adsorbed onto a hydroxypatite column at 40°C. The column temperature was raised stepwise to 90°C, and denatured DNA was removed at each step with 2 ml of PB containing 8 M urea. Each fraction was mixed with 10 ml of RIA-Fluor and counted for both tritium (●) and carbon-14 (○) radioactivity. Radioactivity present in each eluted fraction at the indicated temperature is plotted in (A) and (B); the cumulative radioactivity is plotted in (C) and (D).

duplexes of either viral subtype DNA possessed steep melting profiles with midpoint temperatures of 76 to 78°C in 8 M urea. EHV-1 heteroduplex DNAs, in contrast, were eluted from hydroxyapatite over a broad temperature range whose midpoint was 20°C below that of the homoduplexes.

## DISCUSSION

In this report, the extent of genetic homology between the two recognized molecular subtypes of EHV-1 was determined by a kinetic analysis of viral DNA-DNA reassociation. Results of this study indicate that the two herpesviruses share only about 17% of their genome nucleotide sequences. Similar estimates of homology between the two viral DNAs (11%) were obtained by analysis of the maximum fraction of each viral genome that could be made resistant to S1 nuclease after hybridization with a large excess of the heterologous viral DNA. These values are approximately one-third of the amount of DNA homology (47%) exhibited between the two types of herpes simplex virus (7).

Because the two EHVs, during their evolutionary descent from a common ancestor, have accumulated base substitutions (i.e., mutations) in their DNAs, heteroduplexes formed between the two viral genomes will contain non-complementary regions representing the positions at which base substitutions have occurred. These mispaired bases reduce the stability of the hybrids to an extent that a 1.5% mismatch results in a 1°C decrease in the melting temperature of the duplex DNAs (8). The extent of base-pair mismatching in the subtype 1-subtype 2 duplexes was therefore estimated by comparing their thermal stability with that of the homoduplex viral DNAs. Such heteroduplexes melted over a broad temperature range, with a midpoint 20°C below that of the homoduplex DNAs. These results indicate the presence of an average of 30% noncomplementary nucleotides within the subtype 1-subtype 2 hybrids (8), which represents, by comparison, approximately twice the amount of base-pair mismatching reported for herpes simplex type 1-type 2 heteroduplexes (7). These observations may be interpreted to indicate a longer time interval or a more rapid rate of evolutionary divergence of the two subtypes of EHV-1 than for the two types of herpes simplex virus.

The technique of hybridization of each labeled virion DNA to nitrocellulose blots of restriction endonuclease DNA fragments from the heterologous virus was employed to determine whether the observed homology between the two EHV-1 subtypes was localized to specific regions of the viral genome. Results of this procedure indicated a nonuniform distribution within the genome molecule of the sequences shared by the two virus subtypes. Based upon the restriction enzyme cleavage maps generated for EHV-1 by Whalley and co-workers (18), the evolutionarily conserved sequences of EHV-1 are located both within the unique L- and S-components and within the repeat sequences of the viral genome. Studies currently in progress to map EHV-1 proteins to specific restriction fragments should aid in determining the biological functions of the more conserved and divergent sequences between the two viruses.

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