NOTES

Genetic Relatedness of the Genomes of Equine Herpesvirus Types 1, 2, and 3

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Received 6 July 1982/Accepted 25 October 1982

Genomic DNAs of equine herpesvirus type 1 (EHV-1), EHV-2 (equine cytomegalovirus), and EHV-3 were examined by reassociation kinetic and thermal denaturation analyses to determine the extent and degree of homology among the three viral DNAs. Results of reassociation analyses indicated a limited homology among the three EHV genomes. Homologous DNA sequences equivalent to 1.8 to 3.7 megadaltons between EHV-1 and equine cytomegalovirus, 7.6 to 8.2 megadaltons between EHV-1 and EHV-3, and 1.3 to 1.9 megadaltons between equine cytomegalovirus and EHV-3 were detected. Examination by thermal denaturation of the DNA homoduplexes and heteroduplexes formed during reassociation revealed a high degree of base pairing within the duplexes, suggesting that closely related sequences may be conserved among the genomes of EHV.

Recent reports from our laboratory have established the utility of employing equine herpesviruses (EHVs) as a model system for studying the varied biological consequences of herpesvirus infections (10; D. J. O'Callaghan, G. A. Gentry, and C. C. Randall, in H. Fraenkel-Conrat and R. R. Wagner, ed., *Comprehensive Virology, series 2, The Herpesviruses*, in press). The EHVs have common biochemical and structural features (1, 1a, 3, 15). The genome of EHV type 1 (EHV-1; equine abortion virus strain Kentucky A) is a 92-megadalton (md) DNA molecule that is comprised of a 71.6-md long region and a 20.4-md short region which contains inverted repeat sequences that bracket a unique region (U_S) segment (7, 17) and has a buoyant density of 1.716 g/cm³ (7, 11, 17, 19). Similarly, EHV-3 DNA has a buoyant density of 1.727 g/cm³ and is a 96.2-md molecule that is comprised of a 73- to 76-md long region and a 21- to 23-md short region which contains 8-md inverted repeat sequences that bracket a U_S segment (2). EHV-2 (equine cytomegalovirus [ECMV]) DNA has a buoyant density of 1.716 g/cm³ (21) and is a 126-md molecule which appears to contain 3- to 5-md inverted repeat sequences bracketing a 5.8-md segment (J. Staczeck, W. T. Ruyechan, and D. J. O'Callaghan, unpublished data). Therefore, in pursuit of a better understanding of the biochemical relationships among the EHVs, reassociation kinetic analyses and thermal denaturation of DNA het-

eroduplexes were employed to ascertain the extent and degree of homology among the three viral DNAs.

EHV-1 was propagated in L-M cell suspension cultures, and EHV-1 virions were purified as described previously (14). ECMV was propagated in RK-13 cell cultures (21), and EHV-3 was propagated in equine transitional cell carcinoma cell cultures (2). Nucleocapsids isolated from ECMV- and EHV-3-infected cultures were obtained by the procedure of Heilman et al. (5) and were purified as described previously (2, 14). Purified virions or nucleocapsids were digested with 1 mg of pronase per ml in a buffer containing 10 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, and 1% sodium dodecyl sulfate. The viral DNAs were extracted twice with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and were subjected to two sequential isopycnic centrifugations in CsCl (2, 6, 21). Each of the EHV DNAs has a buoyant density in CsCl greater than that of cellular DNA and was found to band as a homogeneous species when analyzed by CsCl analytical ultracentrifugation (2, 6, 11, 21). Digestion of the viral DNAs with several different restriction endonucleases produced representative fragment patterns in agarose gels, indicating the absence of defective viral DNAs.

The quantitation of homologous DNA sequences among the three viral genomes was made by DNA-DNA reassociation kinetic analy-

FIG. 1. $[^{32}\text{P}]$ EHV-1 (A), $[^{32}\text{P}]$ ECMV (B), and $[^{32}\text{P}]$ EHV-3 (C) were permitted to reanneal in the presence of EHV-1 ($\blacklozenge$), ECMV ($\circ$), and EHV-3 ($\blacktriangle$). Double-stranded viral DNAs were radiolabeled by the procedures of Maniatis et al. (8). The radiolabeled DNAs (specific activity, 1.0×10^8 to 6.0×10^8 cpm/ μg) were mixed with an excess of cold viral DNAs (molar ratio = 500 to 5,000) in 500 μl of hybridization buffer (0.1 M Tris-hydrochloride [pH 8.1]–0.015 M EDTA–1.2 M NaCl–20% formamide). Calf thymus DNA was added to the reaction mixtures so that the final DNA concentration in each reaction set was identical. The DNAs were sonicated to 300 to 600 nucleotides in length, denatured at 110°C for 10 min, and permitted to reanneal at 51°C . Samples were removed at the times indicated and quick-frozen until analyses for single-strand and double-strand content by differential elution from hydroxylapatite columns at 64°C (see reference 18). The samples were monitored for radioactivity by Cerenkov counting in a Beckman scintillation counter.

sis as described previously (18). Briefly, 26 to 150 pg of ^{32}P -labeled genomic EHV DNA (specific activity, 1.0×10^8 cpm/ μg) was mixed with an excess of the appropriate partial DNAs (molar excess = 500 to 5,000) in 500 μl of hybridization buffer (0.1 M Tris-hydrochloride [pH 8.1]–0.015 M EDTA–1.2 M NaCl–20% formamide). DNAs within the reactions were sonicated to a length of 300 to 600 nucleotides, and the fragments were denatured at 100°C for 10 min and permitted to reanneal at 51°C .

In the first series of experiments, single-stranded ^{32}P -labeled EHV-1 DNA was permitted to reanneal in the presence of a 5,000 M excess of either ECMV DNA, EHV-3 DNA, or calf thymus DNA or a 100 M excess of EHV-1 DNA. The rate of reannealing of the EHV-1 probe was accelerated in the presence of EHV-1, ECMV, and EHV-3 DNA but not in the presence of calf thymus DNA (Fig. 1A). Since neither the $[^{32}\text{P}]\text{EHV-1} \times \text{ECMV}$ nor the $[^{32}\text{P}]\text{EHV-1} \times \text{EHV-3}$ reannealing rates showed second-order kinetics, only partial homology between the EHV-1 genome and the ECMV or EHV-3 genome was indicated. Extrapolations of the probe-to-probe reannealing in the heterologous reaction mixtures indicated that ECMV was homologous to 2.0% (1.8 md) of the EHV-1 genome and that EHV-3 was homologous to 8.9% (8.2 md) of the EHV-1 genome (Fig. 1A).

Conversely, when ECMV was used as a probe, it was determined that EHV-1 hybridized to 2.9% (3.7 md) of the ECMV genome (Fig. 1B), and when EHV-3 was used as a probe, EHV-1 had sequences that hybridized to 7.8% (7.6 md) of the EHV-3 DNA (Fig. 1C). Furthermore, it was determined that EHV-3 hybridized to 1.0% (1.3 md) of the ECMV sequences (Fig. 1B) and, conversely, that ECMV hybridized to 2.0% (1.9 md) of the EHV-3 genome (Fig. 1C). These data indicated that the heterologous DNA sequences shared by the EHV-1 and ECMV totaled 1.8 to 3.7 md between EHV-1 and ECMV, 1.3 to 1.9 md between ECMV and EHV-3, and 7.5 to 8.2 md between EHV-1 and EHV-3.

To determine the extent of homology between the EHV homoduplexes and heteroduplexes, thermal dissociation profiles were obtained by procedures outlined previously (9). The thermal denaturation profiles of the heteroduplexes closely paralleled each other, indicating that, under the conditions of hybridization used, the degree of base pairing within all heteroduplexes was very similar (Fig. 2). The 50% melting temperature for the EHV-1 $\times$ EHV-3 heteroduplexes was 75 to 76°C , and the 50% melting temperature for the EHV-1 $\times$ ECMV heteroduplexes was 77 to 82°C . The 50% melting temperature for the ECMV $\times$ EHV-3 heteroduplexes was 78 to 78.5°C . These data, together

FIG. 2. Unlabeled EHV-1 (●), ECMV (○), and EHV-3 (×) were permitted to anneal with $[^{32}\text{P}]\text{EHV-1}$ (A), $[^{32}\text{P}]\text{ECMV}$ (B), and $[^{32}\text{P}]\text{EHV-3}$ (C) (specific activities, 4.6×10^8 to 6.8×10^8 cpm/ μg) in 0.1 M Tris-hydrochloride (pH 9.1)–0.15 M EDTA–1.2 M NaCl–20% formamide at 51°C for 4 days. Single-stranded probe was separated from the homo- and heteroduplexes by elution from hydroxylapatite columns with 0.14 M sodium phosphate buffer (pH 6.8) at 64°C . The duplex molecules were eluted with 0.4 M sodium phosphate buffer (pH 6.8). The duplex samples were diluted to 0.10 M sodium phosphate and loaded onto water-jacketed hydroxylapatite columns at 55°C . As the temperature of the bath water was increased at 2°C increments, the columns were allowed to equilibrate, and the single-stranded DNA was eluted at each temperature increment with 0.14 M sodium phosphate (pH 6.8).

with those of the liquid hybridization results, indicated that EHV-1 and EHV-3 DNAs are more homologous to each other than to ECMV DNA but that all three genomes have limited regions of very high homology.

It is tempting to speculate that the limited sequence homology among the EHV DNAs is indicative of the presence of an identical or very similar subset(s) of viral DNA sequences that have been conserved within the individual herpesvirus genomes. Possession of common sequences among the three EHV-1s could reflect the evolution of the three species from a common ancestor or the exchange of genetic information by recombination events. It has already been demonstrated that EHV-1 and EHV-3 have a similar genomic organization characterized by the presence of inverted repeat sequences (2, 7, 17), and preliminary findings suggest that ECMV DNA may have inverted repeat sequences (Staczek et al., unpublished data). Furthermore, the EHV-1s possess oncogenic potential (4, 12, 16; O'Callaghan et al., in press), and EHV-1- and ECMV-transformed hamster cells have been shown to retain viral sequences during tumorigenesis (O'Callaghan et al., in press; R. A. Robinson and D. J. O'Callaghan, Cell, in press; J. Staczek and D. J. O'Callaghan, preliminary findings presented at the Sixth Cold Spring Harbor Meeting on Herpesviruses, Cold Spring Harbor, N.Y. 1982). It will be of interest to determine whether the conserved sequences are those associated with viral oncogenesis, whether they are retained as a subunit within the inverted repeats, and whether they map at similar positions within the unique regions of each viral genome. Experiments using the Southern blotting technique (20) are now in progress to determine the areas of homology within the physical maps of the EHV-1, ECMV, and EHV-3 genomes. Once these areas of homology are identified, restriction enzyme fragments representing these shared sequences can be isolated and subjected to DNA sequences analysis to determine precisely the extent of nucleotide sequence conservation.

We thank Katie Carey for excellent technical assistance and Linda Devine and Marcie Minyard for typing the manuscript. This work was supported by National Science Foundation grant PCM 78-22700, Public Health Service grants AI-02032 and S-507-RR05386 from the National Institutes of Health, and an Institutional Grant from the American Cancer Society.

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