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Genetic Relatedness of Equine Herpesvirus Types 1 and 3

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The genetic relatedness of two types of equine herpesviruses (EHVs), 1 (EHV-1) and 3 (EHV-3), was determined by DNA-DNA reassociation kinetics. Denatured, labeled viral DNA probe was allowed to reassociate in the presence or absence of the second unlabeled 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 only 2 to 5% homology between the two EHV genomes. Moreover, labeled RNA extracted from EHV-3-infected cells hybridized to filter-immobilized EHV-1 DNA only 2 to 3% as efficiently as to the homologous EHV-3 DNA. These results demonstrate that the genital (EHV-3) and nongenital (EHV-1) types of EHVs exhibit very little genetic homology.

Two types of herpesviruses are associated with disease in horses. Equine herpesvirus type 1 (EHV-1; equine abortion virus) has long been recognized as the etiological agent of a respiratory and abortigenic disease of horses (5). Recently, a second species of EHV, EHV type 3 (EHV-3), has been identified as the cause of a venereally transmitted progenital disease of horses (6). Although EHV-1 and EHV-3 are morphologically indistinguishable and share many common biochemical and structural features of intracellular development (2, 18, 25), they differ widely in a number of biological, physicochemical, and immunological properties. Whereas EHV-1 strains have been isolated from aborted equine fetuses, EHV-3 is non-abortigenic for horses (5). In cell cultures, the two types of EHVs have been found to differ in their ability to multiply in cells of certain species: EHV-1 exhibits a wide in vitro host range, whereas EHV-3 replication is restricted to cells of equine origin (6, 18). EHV-1 and EHV-3 DNA species have widely different buoyant densities of 1.716 and 1.725 g/cm^3, corresponding to 57 and 66 mol% guanine plus cytosine, respectively (15, 23). Moreover, EHV-3 is not neutralized by antiserum against EHV-1, indicating the presence of unique neutralizing antigens, and also exhibits a more rapid replicative cycle than does EHV-1 (1, 5, 18).

The purpose of the studies described in this paper was to measure the extent of genetic relatedness between the genomes of EHV-1 and EHV-3. The experimental approach to this problem involved an analysis of the kinetics of viral DNA reassociation, a method that has proved to be the most reliable technique now available for the detection of base sequence homology between two viral genomes. Our results will show that the two types of EHVs share less than 5% of their genome sequences.

MATERIALS AND METHODS

Cells and virus. The conditions for propagation of EHV-1 (Kentucky A strain; 19) in Syrian hamsters and propagation of EHV-3 (1118 strain; 6) in equine fetal dermal cells (KyED) have been described elsewhere (1, 6, 19). EHV-1 was purified from the plasma of infected hamsters and EHV-3 was purified from the cell-free culture fluids of infected KyED cells by sequential filtration through 5-, 1.2-, and 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) followed by two cycles of isopycnic banding in 28 to 40% (wt/vol) potassium tartrate gradients (8, 12, 18).

Purification and labeling of viral DNA. Viral DNA was isolated from purified herpesvirions by incubation for 3 h at 37°C in TE buffer (0.01 M Tris-hydrochloride–0.001 M EDTA [pH 7.4]) containing Pronase (1 mg/ml) and sodium dodecyl sulfate (0.5%). After two cycles of extraction with phenol at room temperature, the DNA was precipitated with 2 volumes of ethanol, dissolved in 0.3 N NaOH, and degraded to fragments 400 to 500 nucleotides in length by boiling in 0.3 N NaOH for 20 min (22). The solution was neutralized by the addition of 0.2 volume of 3 M Tris-hydrochloride (pH 3) and dialyzed for 2 days against several changes of 0.1 M Tris-hydrochloride (pH 8.1)–0.015 M EDTA–0.15 M NaCl (TNE buffer). The size of the single-stranded (ss)DNA fragments was determined by sedimentation through 5 to 20% neutral sucrose gradients, using ^{3}H-labeled RNAs (4S, 18S, and 28S) as markers and utilizing the equation of Eigner and Doty (9) for conversion of sedimentation coefficients of ssDNA to molecular weights.

^{3}H-labeled EHV-3 DNA was obtained from virus purified from cultures infected in the presence of 250 μCi of [^{3}H]thymidine per ml (50 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.). EHV-1 DNA was labeled in vitro with Na^{125}I by modification of the

Commerford method (7) as described in considerable detail by Kraiselburd et al. (14). Briefly, 5 μg of denatured, fragmented EHV-1 DNA was incubated in 140 mM acetate buffer (pH 5.0) for 2 min at 100°C with 0.5 mM thallium trichloride (ICN · K & K Laboratories, Plainview, N.Y.) and 2 mCi of $Na^{125}I$ (New England Nuclear, Boston, Mass.) in a total volume of 20 μl. The labeled DNA was then purified by chromatography on hydroxyapatite and dialyzed against TNE buffer. The specific activities of EHV-3 and EHV-1 DNAs were 5×10^6 and 4×10^7 cpm/μg, respectively.

Isolation of cellular DNA. Uninfected KyED cells and hamster liver nuclei were treated with Pronase and sodium dodecyl sulfate as described above. The lysates were then extracted three times with chloroform-butanol (3:1, vol/vol), and the nucleic acids were precipitated with ethanol. RNA was hydrolyzed by incubation for 18 h at 37°C in 0.3 N NaOH. After fragmentation by boiling in 0.3 N NaOH for 20 min, the solution of DNA was neutralized and dialyzed exhaustively against TNE buffer. Calf thymus DNA was purchased from P-L Biochemicals (Milwaukee, Wis.) and treated in an identical manner.

DNA-DNA hybridization. Hybridizations were performed at 65°C in 0.1 M Tris (pH 8.1)–0.015 M EDTA–0.15 M NaCl (TNE). Reaction mixtures consisted of 80 to 150 ng of labeled viral probe DNA per ml, 0 to 15 μg of unlabeled test DNA per ml, and, when necessary, enough calf thymus DNA to give a total DNA concentration of 15 μg/ml. The mixtures (20 to 100 μl) were sealed in 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 (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.) chromatography at 60°C, as described in detail by Sharp et al. (22). The DNA eluting from the column at 0.14 M phosphate (ssDNA) or 0.4 M phosphate buffer (pH 6.8) (double-stranded [ds] DNA) was precipitated with trichloroacetic acid, collected on nitrocellulose membrane filters (type HAWP; Millipore Corp.), and counted by liquid scintillation spectroscopy (21). A total of 3% of ssDNA adsorbed to the column in 0.14 M phosphate buffer; the fraction of ss- and dsDNA observed during the renaturation was corrected accordingly.

The hybridization results were plotted by the linear transformation method of Wetmur and Davidson (28), 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 was used to determine the slopes of the plotted data.

DNA-RNA hybridization. RNA for hybridization was prepared by the method of Glisin et al. (11) from mock- or EHV-infected KyED cells that had been labeled for 8 h (2 to 10 h postinfection) with 250 μCi of [^{3}H]uridine per ml (28 Ci/mmol; Schwarz/Mann). The techniques of hybridization of [^{3}H]RNA to denatured viral DNA immobilized on nitrocellulose membrane filters and subsequent assay for DNA-RNA hybrids have been previously described in detail (1).

RESULTS

Design of the hybridization experiments. The strategy of these kinetic hybridization tests was to allow a small amount of denatured, radioactive viral "probe" DNA to reassociate in the presence of the unlabeled second viral DNA (test hybridization) or in the presence of identical concentrations of unrelated DNAs (control hybridizations). Because the rate of reassociation of any nucleotide sequence is proportional to its concentration in the hybridization mixture, homologous sequences present in the unlabeled test DNA will increase the reassociation rate of the labeled probe.

As described by Britten and Kohne (4), the reassociation of complementary DNA sequences may be described by equation 1: $1/f_{ss} = 1 + kC_0t$, where f_{ss} is the fraction of DNA remaining single stranded at time t, C_0 is the molar concentration of homologous DNA, and k is the second-order reassociation rate constant. If all the different sets of complementary sequences in the hybridization mixture are present at equal concentration, a plot of $1/f_{ss}$ versus t will be a straight line having an intercept of 1 and a slope proportional to the initial concentration of homologous DNA. The observed data would take a nonlinear form if all sets of DNA sequences were not present in equal concentration, a situation that would exist if the probe or test DNA contained reiterated sequences or if the added test DNA were only partially related to the probe genome.

Fidelity, sensitivity, and specificity of probe DNAs. The efficacy and sensitivity of the labeled viral DNAs as hybridization probes were tested by reconstruction experiments consisting of mixtures of probe DNA and sufficient unlabeled homologous viral DNA to yield total-to-probe DNA ratios (n values) of 1, 2, 4, and 10. The results (Fig. 1) demonstrated that renaturation of the probe DNAs followed second-order kinetics, indicating an absence of detectable reiterated sequences. Furthermore, addition of a 2-, 4-, or 10-fold excess of unlabeled homologous viral DNA resulted in a 2-, 4-, or 10-fold acceleration, respectively, of the rate of probe reassociation. Viral DNA chemically iodinated in vitro with $Na^{125}I$ reassociated with the same kinetics as did DNA labeled in cell culture with [^{3}H]thymidine (data not shown). We conclude on the basis of these experiments that both EHV DNA probes were adequate for kinetic hybridization tests.

DNA-DNA hybridization. In the first series of experiments, in vitro labeled EHV-1 [^{125}I]-DNA was used as the probe and allowed to reanneal in the presence of a 100-fold excess of unlabeled EHV-3 DNA or in the presence of

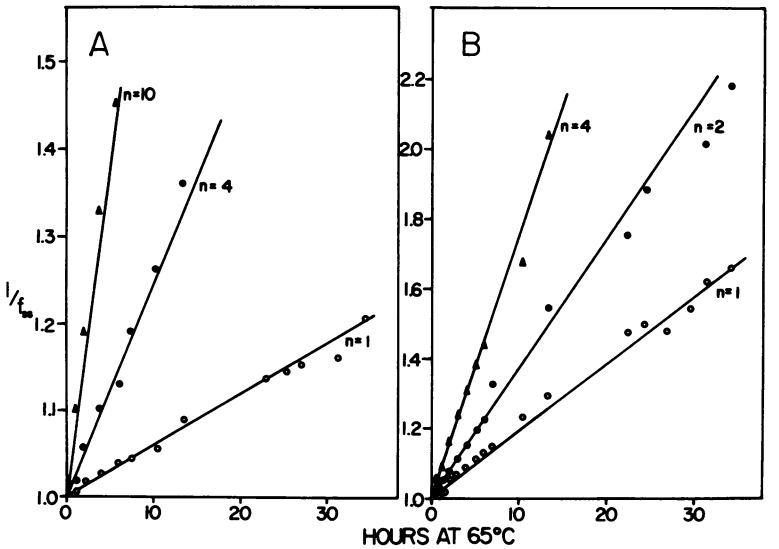

FIG. 1. *Kinetic analysis of the reassociation of denatured, fragmented EHV DNAs. Hybridizations were performed at 65°C in TNE buffer for various lengths of time, and the fraction of DNA remaining single stranded (f_{ss}) was determined by hydroxyapatite chromatography. (A) Reassociation of 80 ng of EHV-1 [^{125}I]DNA per ml (4 × 10^7 cpm/μg) in the presence of no unlabeled EHV-1 DNA (n = 1), 240 ng of unlabeled EHV-1 DNA per ml (n = 4), or 720 ng of unlabeled EHV-1 DNA per ml (n = 10). The total concentration of DNA in each hybridization reaction was adjusted to 800 ng/ml with calf thymus DNA. (B) Reassociation of 150 ng of EHV-3 [^{3}H]DNA per ml (5 × 10^6 cpm/μg) in the presence of no unlabeled EHV-3 DNA (n = 1), 150 ng of unlabeled EHV-3 DNA per ml (n = 2), or 450 ng of unlabeled EHV-3 DNA per ml (n = 4). The total concentration of DNA in each hybridization reaction was adjusted to 600 ng/ml with calf thymus DNA.*

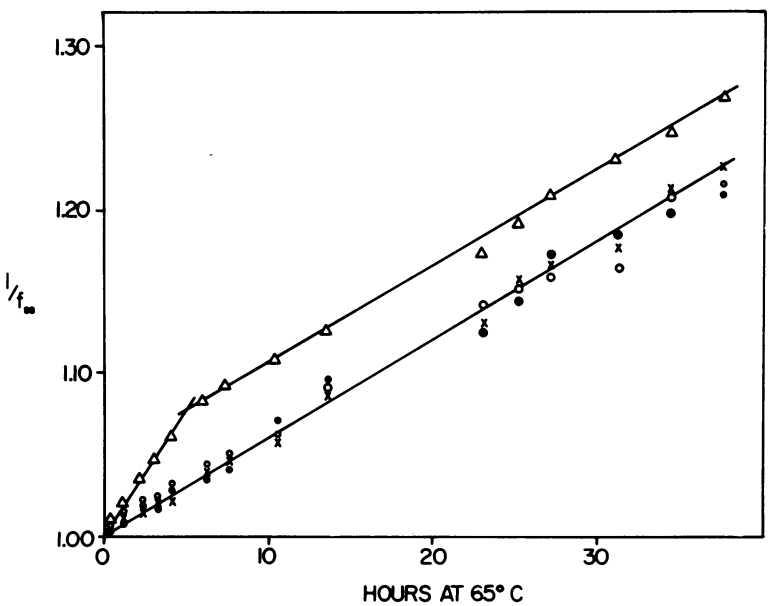

FIG. 2. *Reassociation reaction of EHV-1 [^{125}I]DNA in the presence of unlabeled DNAs. Hybridizations were performed at 65°C in 20 μl of TNE buffer for various lengths of time, and the fraction of DNA remaining single stranded (f_{ss}) was determined by hydroxapatite chromatography. A 0.08-μg/ml amount of denatured EHV-1 [^{125}I]DNA (4 × 10^7 cpm/μg) was allowed to reassociate in the presence of 8 μg (n = 100) of unlabeled calf thymus DNA (○), unlabeled hamster liver DNA (●), unlabeled KyED cell DNA (×), or unlabeled EHV-3 DNA (△) per ml.*

the same concentration of three control DNAs (calf thymus, hamster liver, or KyED DNA) (Fig. 2). The initial rate of EHV-1 probe reassociation was higher in the presence of EHV-3 DNA than in the presence of the control DNAs, indicating that EHV-3 contained sequences homologous to EHV-1 DNA. Moreover, in the presence of the control DNAs, EHV-1 DNA reannealed with second-order kinetics; however, in the presence of EHV-3 DNA, the experimental points for EHV-1 probe reassociation no longer fell on a straight line but, instead, fit a biphasic curve. Thus, the addition of the unlabeled second viral DNA (EHV-3) to the hybridization reaction increased the concentration of sequences homologous to only a restricted portion of the probe DNA, indicating that only a fraction of the EHV-1 probe sequences were present in the test DNA. Additionally, the fact that the presence of a 100-fold excess of the unlabeled EHV-3 DNA caused only a very small increase in the initial rate of probe reassociation suggested that the fraction of shared sequences between the two herpesvirus genomes was quite small.

The second series of experiments consisted of reassociation of EHV-3 [^{3}H]DNA in the presence or absence of unlabeled EHV-1 DNA (Fig. 3). Again, the presence of the unlabeled test DNA (EHV-1) in the hybridization mixture resulted in a deviation from second-order kinetics (biphasic curve) and an increase in the initial rate of reassociation of EHV-3 probe DNA. The results indicated that EHV-1 DNA contained sequences homologous to only a fraction of the EHV-3 genome.

Computation of homology between EHV-1 and EHV-3. Fujinaga et al. (10) have derived a formula for quantitating the amount of base sequence homology between two viral genomes utilizing 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 (C_{ss}/C_{ds} versus $1/t$) are shown in Fig. 4 for the reassociation of labeled EHV-1 DNA fragments in the presence of two different concentrations of unlabeled EHV-3 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 C_{ss}/C_{ds} versus $1/t$ during the initial period of reassociation of the

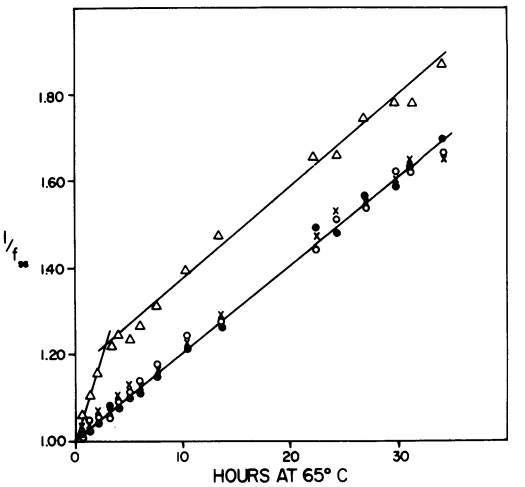

FIG. 3. *Reassociation reaction of EHV-3 [^{3}H]-DNA in the presence of unlabeled DNAs. Hybridizations were performed at 65°C in 100 μl of TNE buffer for various lengths of time, and the fraction of DNA remaining single stranded (f_{ss}) was determined by hydroxapatite chromatography. A 0.15-μg/ml amount of denatured EHV-3 [^{3}H]DNA (5 × 10^5 cpm/μg) was allowed to reanneal in the presence of 15 μg (n = 100) of unlabeled calf thymus DNA (○), unlabeled hamster liver DNA (●), unlabeled KyED cell DNA (×), or unlabeled EHV-1 DNA (△) per ml.*

probe DNA in the presence (A) and in the 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 (10): $x/f = [1 - (A/A_0)]/[n(A/A_0)]$ (equation 2). Using equation 2, the fraction of nucleotide sequences shared between EHV-1 and EHV-3 molecules has been computed in Table 1. It can be seen that the two herpesviruses share only 2 to 5% of their genome sequences.

DNA-RNA hybridization. To determine whether the small amount of homology between EHV-1 and EHV-3 could be detected also at the level of viral RNA, labeled RNA from EHV-infected KyED cells was tested for its ability to hybridize with the heterologous viral DNA. The results, illustrated in Fig. 5, demonstrate a degree of cross-hybridization (2 to 3% of control hybridization) commensurate with the small amount of homology found between the two viral genomes by DNA-DNA reassociation kinetics.

DISCUSSION

The technique of nucleic acid hybridization has become a popular and highly sensitive tool

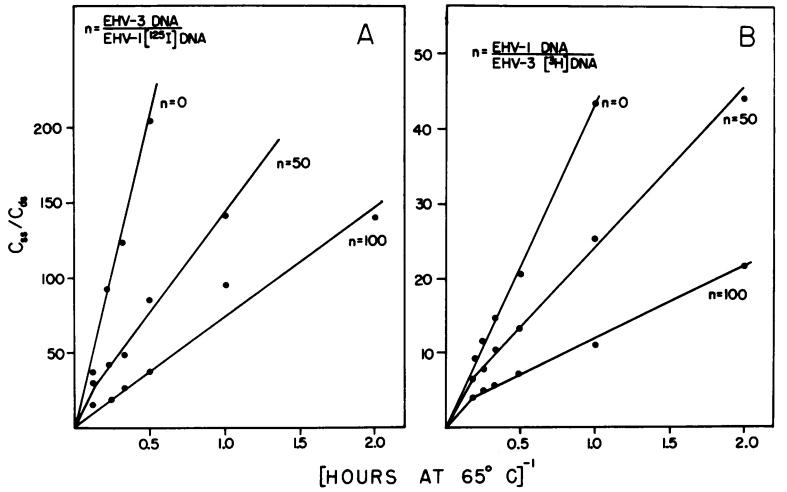

FIG. 4. *Reassociation of labeled EHV DNAs in the presence of different concentrations of the unlabeled heterologous viral DNA. Hybridizations were performed at 65°C in TNE buffer for various lengths of time, and the ratio of ss- to dsDNA (C_{ss}/C_{ds}) was determined by hydroxyapatite chromatography. (A) Reassociation of 0.08 μg of EHV-1 [^{125}I]DNA per ml (4 × 10^7 cpm/μg) in the presence of no unlabeled EHV-3 DNA (n = 0), 4 μg of unlabeled EHV-3 DNA per ml (n = 50), or 8 μg of unlabeled EHV-3 DNA per ml (n = 100). The total concentration of DNA in each hybridization mixture was adjusted to 8 μg/ml with calf thymus DNA. (B) Reassociation of 0.15 μg of EHV-3 [^{3}H]DNA per ml (5 × 10^5 cpm/μg) in the presence of no unlabeled EHV-1 DNA (n = 0), 7.5 μg of unlabeled EHV-1 DNA per ml (n = 50), or 15 μg of unlabeled EHV-1 DNA per ml (n = 100). The total DNA concentration in each hybridization reaction was adjusted to 15 μg/ml with calf thymus DNA.*

TABLE 1. *Fraction of EHV-1 DNA homologous to EHV-3 DNA*

Unlabeled test DNA/labeled probe DNA (n)	Ratio of slopes[a] (A/A_0)	Shared fraction of genome[b] (x/f)
50[c]	0.506	0.020
100[c]	0.230	0.033
50[d]	0.325	0.042
100[d]	0.188	0.043

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

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

[c] EHV-3 [^{3}H]DNA was reassociated in the presence of unlabeled EHV-1 DNA.

[d] EHV-1 [^{125}I]DNA was reassociated in the presence of unlabeled EHV-3 DNA.

for the analysis of evolutionary relationships between different types of herpesviruses. From 40 to 70% homology has been observed between the genital and nongenital types of herpes simplex virus (13, 16, 26). Evidence has also been presented that the herpesvirus isolated from a progenital disease of cattle is identical to infectious bovine rhinotracheitis virus (27). In this report, the extent of homology between EHV-1 and EHV-3 DNAs was determined by a kinetic analysis of viral DNA-DNA reassociation. The results indicate that the genital (EHV-3) and nongenital (EHV-1) types of EHVs exhibit only 2 to 5% homology. As much or more genetic relatedness has been reported between herpesviruses indigenous to different animal species: 8 to 10% base sequence homology between herpes simplex virus and pseudorabies virus (16), 6 to 8% homology between pseudorabies virus and infectious bovine rhinotracheitis virus (24), and 14% homology between herpes simplex virus and bovine herpes mammillitis virus (24). However, as pointed out by Fujinaga et al. (10), it is difficult to compare results of hybridization methods involving different degrees of stringency in estimating duplex formation. In addition to closely related sequences, there may exist partially homologous sequences, which would be scored to different extents under different hybridization conditions. The low percentage of homology we observe between EHV-1 and EHV-3 DNAs may, therefore, represent only the fraction of well-matched sequences shared by the two herpesivruses, and it is possible that less stringent hybridization conditions would reveal more homology.

That this small fraction of sequences shared by EHV-1 and EHV-3 represents genes transcribed during productive infection was demon-

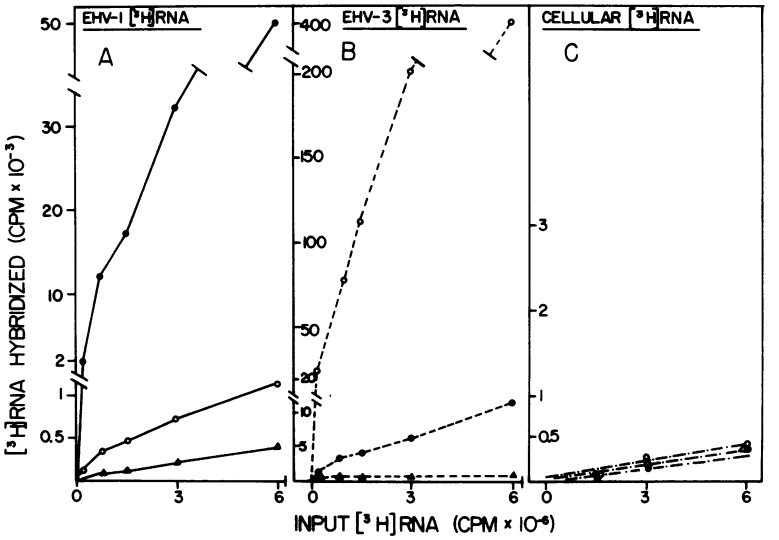

FIG. 5. *Hybridization of [³H]RNA from EHV- or mock-infected KyED cells to filter-immobilized EHV DNA. [³H]RNA was prepared by the method of Glisin et al. (11) from mock- or EHV-infected KyED cells that had been labeled for 8 h (2 to 10 h postinfection) with 250 μCi of [³H]uridine per ml (28 Ci/mmol; Schwarz/Mann). DNA-RNA filter hybridizations were performed for 48 h at 65°C in 100 μl of 4× SSC (0.15 M NaCl + 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate. Each reaction mixture contained 5 μg of denatured DNA immobilized onto 5-mm nitrocellulose membrane filters (type B6; Schleicher & Schuell, Inc., Keene, N.H.) and different concentrations of [³H]RNA. DNA-RNA hybrid formation was assayed by incubation of the filters with 50 μg of pancreatic RNase A per ml in 2× SSC for 1 h at room temperature, followed by washing in 2× SSC and counting by liquid scintillation spectroscopy (1). (A) Hybridization of increasing amounts of [³H]RNA from EHV-1-infected cells (1.6 × 10⁴ cpm/μg) to 5 μg of EHV-1 DNA (●), 5 μg of EHV-3 DNA (○), or 5 μg of calf thymus DNA (△). (B) Hybridization of increasing amounts of [³H] RNA from EHV-3-infected cells (1.2 × 10⁴ cpm/μg) to 5 μg of EHV-1 DNA (●), 5 μg of EHV-3 DNA (○), or 5 μg of calf thymus (△). (C) Hybridization of increasing amounts of [³H]RNA from mock-infected KyED cells (2.2 × 10⁵ cpm/μg) to 5 μg of EHV-1 DNA (●), 5 μg of EHV-3 DNA (○), or 5 μg of calf thymus DNA* (△).

strated by detection of cross-hybridization between ^{3}H-labeled RNA from EHV-infected cells and the heterologous viral DNA.

The virtual lack of homology between the two types of EHVs is reflected also in their widely different phenotypic characteristics: their host range in vitro and in vivo (6, 18), the enzymes they specify (3; unpublished observations), their protein and antigenic composition (4, 25; unpublished observations), and their pathogenic manifestations (5, 6, 25).

It is tempting to speculate that the genital and nongenital types of EHVs, if they evolved from a common ancestor, have undergone such divergent evolution that they are now only distantly related, to an extent that they share less than 5% of their genome sequences. However, the alternative view cannot be excluded that EHV-1 and EHV-3 originated from different progenitor viruses, a mechanism that has been proposed to account for the existence within the same species of two or more unrelated herpesviruses (17).

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