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Detection of Proviral DNA in Horse Cells Infected with Equine Infectious Anemia Virus

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Equine infectious anemia virus (EIAV) recently has been shown to possess a high-molecular-weight RNA genome and a virion reverse transcriptase. We completed the demonstration that EIAV is a retrovirus by showing the presence of proviral DNA in equine cells infected in vitro, but not in normal horse DNA. These studies were performed by using a highly representative cDNA probe synthesized by the virion polymerase. It was found that this cDNA reassociated extensively, and with high thermal stability, with either viral RNA or DNA extracted from infected cells, but showed no detectable reassociation with DNA from uninfected horse cells. Similarly, sequences related to EIAV were neither found in the DNA of four other *Equus* species, nor in a variety of other mammals including sheep, cows, pigs, dogs, cats, and humans; nor did EIAV cDNA hybridize with a variety of other retrovirus RNAs. These experiments were performed under conditions of very low stringency to enable detection of distantly related sequences, with a sufficient ratio of DNA to cDNA to allow detection of less than one viral copy per haploid genome. We conclude that EIAV is not an endogenous virus of the horse or of the other species tested.

Equine infectious anemia virus (EIAV) is a horizontally transmitted agent that establishes a persistent infection characterized by recurring acute episodes of fever, anemia, lymphoid proliferation, and various manifestations of immune complex disease (15). The virus is world-wide in distribution, appears to be infectious only for the horse, and is transmitted from mare to foal and through insect vectors (13, 23). It has recently been shown that EIAV has many of the properties of a retrovirus, including a high-molecular-weight RNA genome (1, 6, 7, 20), reverse transcriptase (1, 6), and a polypeptide composition similar to that of non-type C retroviruses (6). Our current studies show the presence of viral DNA only in infected cells of the horse, but not in normal horse or other mammalian DNA. A highly representative cDNA transcript did not detect relatedness with other retroviruses.

MATERIALS AND METHODS

Virus. The Malmquist strain of tissue-culture-adapted EIAV (16) was propagated in roller-bottle cultures of equine fetal kidney cells (EFK-2 line, more than 20 passages postinfection) grown in RPMI-1640 containing 15% fetal bovine serum, 2 mM glutamine, 100 U of penicillin, and 100 μg of streptomycin per ml. Culture fluid was collected at 5-day intervals and clarified, and virus was purified by double banding in sucrose gradients. Pelleted virus was suspended in 0.01 M Tris (pH 7.2)-0.1 M sodium chloride-0.001 M EDTA (TNE) at a 10,000-fold concentration (about 5 mg of protein per ml) relative to the culture fluid. The specific preparations from which cDNA's were synthesized reacted in complement fixation tests with sera specific for EIAV p25 but not with sera detecting mammalian type C virus p30 proteins.

Other viruses used were: Rauscher leukemia virus (R-MuLV), grown in the JLSV-9 BALB/c bone marrow cell line; the endogenous baboon virus (M7), grown in a human osteosarcoma line; rat type C virus (RPLAV), grown in Lewis rat embryo cells transformed by Rous sarcoma virus; bovine leukemia virus (BLV), grown in fetal lamb kidney cells; and mouse mammary tumor virus (MMTV), grown in the Mm5mt/c_1 line. Viruses were produced in the Viral Resources Laboratory (FCRC) and purified according to the procedure described by Johnson et al. (12). Avian myeloblastosis virus (AMV) was obtained from the plasma of infected chickens (supplied by J. Beard).

Synthesis of EIAV cDNA's. Reaction mixtures of 50 μl contained 10 to 15 μl of 10,000× virus, 1.6 to 2 mM each of dATP, dGTP, and dCTP, 0.13 to 0.45 mM [^{3}H]TTP (28 or 50 Ci/mmol), 0.02 to 0.03% Triton X-100, 0.1 M Tris (pH 8), 0.005 M magnesium acetate, 0.05 M sodium chloride, 0.01 M dithiothreitol, and in some cases, 100 μg of actinomycin D per ml. After incubation at 37°C for 2 h, reactions were stopped by addition of 0.1 volume of 10% sodium dodecyl sulfate (SDS), and samples were centrifuged for 3 h at 40,000 rpm at 10°C in an SW 41 rotor through linear gradients of 15 to 30% sucrose made up in TNE and 0.01% SDS. Aliquots of fractions were assayed for trichloroacetic

acid-precipitable radioactivity. A broad peak of material that had sedimented through one-third to one-half of the gradient was seen. Fractions in this peak were pooled, incubated for 3 h at 37°C in 0.3 N sodium hydroxide, neutralized, extracted with an equal volume of chloroform-octanol (24:1), and precipitated at −20°C after addition of two volumes of ethanol. Yields were generally about 2×10^6 cpm per reaction. Assuming 25% thymidine in the cDNA, specific activities were 1.9×10^7 or 3.4×10^7 cpm/μg.

The cDNA's purified in the above manner always exhibited considerable self-complementarity, particularly when synthesized in the absence of actinomycin D. This could be eliminated by sedimenting the cDNA through an alkaline sucrose gradient (5 to 20% sucrose in 0.8 M sodium chloride–0.2 N sodium hydroxide; sedimentation overnight in an SW 41 rotor at 36,000 to 40,000 rpm at 10°C), and discarding the discrete peak of material of about 500 nucleotides average size. Pooled fractions of the faster sedimenting material (whose average size varied from about 1,500 to 3,500 nucleotides) were neutralized, precipitated in 2 volumes of ethanol at −20°C, and suspended in TNE. These long-length cDNA's exhibited minimal self-annealing (≤8%), even when incubated for extended periods.

Preparation of viral RNA. Virus suspensions in TNE were brought to 1% SDS and 750 μg of pronase per ml, incubated for 30 min at room temperature, and extracted with a 5:1:5 mixture of chloroform, *m*-cresol, and water-saturated phenol. The aqueous phase was adjusted to contain 0.3 M sodium chloride and 67% ethanol and was stored overnight at −20°C. Precipitated material was redissolved in several hundred microliters of TNE and sedimented for 2.5 h through a 15 to 30% sucrose gradient as described above. Optical density (260 nm) of the fractions was determined, and material in the 70S region was pooled, reprecipitated in ethanol, and resuspended in TNE.

70S RNA from the Rickard strain of feline leukemia virus (FeLV) and total cellular RNA from hamster embryo cells producing the endogenous hamster type C virus (HaLV) and from RD cells producing RD-114 were gifts of Hiromi Okabe (FCRC); total RNAs from A204 cells producing the squirrel monkey virus (SMRV) or the Mason-Pfizer monkey virus (MPMV) were gifts of Gerald Schochetman (FCRC).

Preparation of DNAs. *Equus* DNAs were prepared according to a modification of the Marmur procedure (17) from spleens of animals autopsied at the San Diego Zoo; all other DNAs were prepared according to the urea-phosphate method (5). DNA was also prepared by the Hirt procedure (10) from two identical roller bottles of EIAV-infected cells to one of which was added 80,000 cpm of SV40 [^{3}H]DNA (a mixture of forms I and II; Bethesda Research Laboratories, Inc.) before lysis. Less than 1% of the SV40 DNA precipitated during storage overnight at 4°C in 1 M sodium chloride. Pelleted DNA was redissolved in 0.01 M Tris and purified by the urea-phosphate method.

All cellular DNAs and some cDNA's were sonicated (11) to an average size of about 450 nucleotides before use in hybridization experiments. Sizes of boiled DNAs and cDNA's were routinely determined by sedimentation through alkaline sucrose gradients (as described above), which included radioactive DNA of known size (450 nucleotides); size calculations were performed according to the method of Studier (24).

Hybridization. Hybridization mixtures in TNE were boiled for 2 min, sodium-phosphate buffer (PB) (pH 6.8) was added to 1 M final buffer concentration, and the mixtures were incubated at 63°C for variable times. The stringency of these conditions was about as low as could be achieved without losing specificity (22). Samples were diluted to 0.14 M PB containing 0.02% SDS, applied to hydroxyapatite (HAP) columns at 50°C, and eluted in 10°C increments. Optical densities (260 nm) of the fractions were routinely assayed to verify that cellular DNA had reassociated appropriately. Total radioactivity in the fractions was assayed after addition of 2 volumes of Aquasol II. The extent of hybridization was defined as the percentage of total radioactivity eluting from HAP above 50°C. C_0t values were corrected to the standard condition of 0.12 M PB (5).

For the thermal elution experiments shown in Fig. 1 and 2, a mixture of the hybridized sample and native HeLa [^{14}C]DNA of about 450 base pairs was added to the HAP column at 50°C in 0.14 M PB-0.02% SDS. Elution of the [^{14}C]DNA served as a control for column operation. Bound material was then eluted by a linear temperature gradient (0.8°C per 5-ml fraction), and fractions were assayed for radioactivity after addition of 2 volumes of Aquasol II.

Hybridization of samples to be treated with S1 nuclease was accomplished by boiling them in TNE, adjusting to 0.02 M Tris (pH 7.4)–1.5 M sodium chlo-

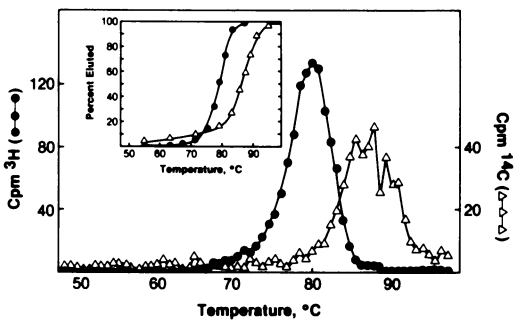

FIG. 1. *Thermal dissociation of the hybrid formed between EIAV [^{3}H]cDNA and EIAV viral RNA. A 0.044-ng (1,500 cpm) amount of EIAV [^{3}H]cDNA with an average size of 600 nucleotides was incubated with 0.21 μg of EIAV viral RNA to a C_rt 0.8 and then applied to HAP at 50°C. A total of 94% of the ^{3}H counts per minute bound. Also applied to the column was native HeLa [^{14}C]DNA, whose subsequent thermal elution served as an internal standard. Bound material was eluted during a linear temperature gradient (0.8°C per 5-ml fraction), and fractions were assayed for radioactivity after addition of 10 ml of Aquasol. ●, ^{3}H counts per minute in each fraction; △, ^{14}C counts per minute per fraction. In the inset is the integral plot of the data, showing the cumulative percentage of the bound material eluted versus temperature. Temperature at which 50% of bound ^{3}H eluted = 79.7°C; temperature at which 50% of bound ^{14}C eluted = 86.8°C.*

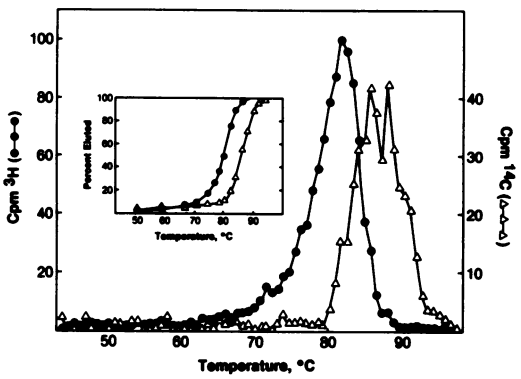

FIG. 2. *Thermal dissociation of the duplex formed between EIAV [³H]cDNA and DNA from EIAV-infected equine fetal kidney cells. A 0.04-ng (1,400 cpm) amount of EIAV [³H]cDNA with an average size of 350 nucleotides was incubated with 360 μg of denatured cellular DNA to C_0t 13,000. Subsequent treatment was as described in the legend to Fig. 1. A total of 82% of ³H- and 90% of native [¹⁴C]DNA bound to the column at 50°C. Temperature at which 50% of bound ³H eluted = 80.6°C. Temperature at which 50% of bound ¹⁴C eluted = 86.4°C. ●, ³H counts per minute per fraction; △, ¹⁴C counts per minute per fraction.*

ride, and incubating at 63°C. After incubation, each reaction mixture (about 100 μl) was added to 3 ml of a solution containing 0.1 M sodium acetate (pH 5), 0.2 M sodium chloride, and 10^{-4} M zinc chloride, and was treated with S1 nuclease at 37°C for 1 h. Trichloroacetic acid-precipitable radioactivity was then determined.

RESULTS

Hybridization of EIAV cDNA with viral RNAs. The purified product of the endogenous reverse transcriptase reaction reassociated almost completely with EIAV viral RNA and not at all with RNAs of the other retroviruses tested. A total of 95% of EIAV cDNA bound to EIAV 70S RNA at a C_rt of 1, but 3% or less bound to 70S viral RNAs of R-MuLV, M7, RPLAV, FeLV, MMTV, BLV, and AMV (Table 1). Similarly, EIAV cDNA showed no detectable reassociation with total RNA ($C_rt \geq 2{,}100$) extracted from cells actively producing the HaLV, the endogenous cat virus (RD-114), MPMV, or SMRV.

The reassociated cDNA-EIAV viral RNA duplex exhibited high thermal stability. The elution midpoint from a hydroxyapatite column of native DNA marker with average-size 450 nucleotide pairs was 86.8°C in 0.14 M phosphate buffer, whereas the cDNA-viral RNA duplex was half eluted at 79.7°C (Fig. 1). Contributing to this 7°C difference between the two profiles could be the small size of the reactants and/or a low guanine-plus-cytosine content of the viral RNA. In any case, the results indicate that the EIAV cDNA exhibits a high degree of complementarity to the viral RNA initially used as a template for its synthesis.

Detection of viral DNA in EIAV-infected cells. When EIAV cDNA was incubated with total DNA (C_0t, 2×10^4) from cultured EIAV-infected horse cells, more than 85% of the cDNA entered into a double-stranded structure capable of binding to HAP. Prior treatment of the cellular DNA with RNase (15 μg/ml) or with 0.3 N sodium hydroxide for 3 h at 37°C did not affect this duplex formation, indicating lack of contaminating viral RNA. The high thermal stability of the duplex indicates a high degree of complementarity between EIAV cDNA and the cellular DNA of infected cells (Fig. 2). Again the native [¹⁴C]DNA marker was half eluted at 86.4°C, whereas the [³H]cDNA was half eluted at 80.6°C.

Assuming that all cells in the culture are infected (see below), the average number of viral DNA sequences per cell can be deduced from the kinetics of reassociation of cDNA with cellular DNA. However, when assayed by HAP, these kinetics depend greatly on the precise size of the cDNA. The results of reassociating EIAV cDNA's of two different average sizes (350 and 1,350 nucleotides) with DNA of constant size (about 580 nucleotides) are shown in Fig. 3A. Increasing the size of the cDNA about fourfold resulted in an approximate sixfold decrease in $C_0t_{1/2}$. This effect was at least qualitatively predictable, for the longer a cDNA molecule, the

TABLE 1. *Lack of homology between EIAV cDNA and other viral RNAs*[a]

Viral RNA	C_rt	% Reassociation of EIAV cDNA
EIAV	1.7	99
EIAV	1.1	95
R-MuLV	3.3	1
M7	3.3	1
FeLV	2.7	3
RPLAV	3.2	2
BLV	2.8	1
MMTV	3.3	1
AMV	3.5	2
HaLV[b]	4,600	1
RD-114[b]	2,100	2
SMRV[b]	8,300	2
MPMV[b]	3,900	3

[a] In each experiment, 0.022 ng (400 to 750 cpm) of EIAV [³H]cDNA was incubated with 0.3 to 1.2 μg of viral RNA to the indicated C_rt. Binding of radioactivity to HAP at 50°C in 0.14 M PB was measured.

[b] In these experiments we used total RNA (130 to 300 μg) from cells producing the indicated virus.

more radioactivity will be retained on HAP as a result of a single collision with a much shorter piece of nonradioactive cellular DNA. The long unhybridized cDNA tails were scored as reassociated, resulting in an apparent increase in the rate of reaction. When these tails were eliminated by S1 nuclease digestion and only duplexed regions were scored, then the two cDNA's of disparate size reassociated with cellular DNA with similar kinetics (Fig. 3B).

Calculation of the average number of viral DNA copies per cell is based on the observed reassociation kinetics of nonrepeated cellular DNA under the same incubation conditions. The reassociation of HeLa nonrepeated [^{3}H]DNA (unreassociated at C_0t 230) with total A204(M7) DNA, using HAP, gave a $C_0t_{1/2}$ of 4,100. All incubation and assay conditions, including size of the reactants, were identical to those used in following the reassociation of the 350 nucleotide EIAV cDNA (Fig. 3a). Since a $C_0t_{1/2}$ of 1,700 was observed in the latter experiment, we conclude that there are an average of two to three viral DNA copies per haploid genome in the EIAV-infected cells. Similar results have been obtained

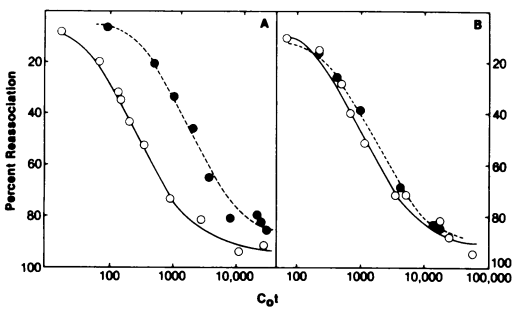

FIG. 3. *Kinetics of reassociation of EIAV [^{3}H]-cDNA with DNA from EIAV-infected equine fetal kidney cells. (A) For each datum point, 0.02 ng (700 cpm) of EIAV [^{3}H]cDNA was incubated with 180 μg of denatured DNA (average size 580 nucleotides) from EIAV-infected cells. Binding of [^{3}H]cDNA to HAP at 50°C in 0.14 M PB-0.02% SDS was measured.* ○, *cDNA with an average size of 1,350 nucleotides;* ●, *the same cDNA preparation, sonically treated to an average size of 350 nucleotides. The curves represent the least-squares best fit (5) to the datum points and yield $C_0t_{1/2}$ values of 270 and 1,700 mol·s/liter, respectively. Under the same incubation conditions, nonrepeated (unreassociated at a C_0t of 230) HeLa [^{3}H]DNA with an average size of 330 nucleotides reassociated with A204(M7) DNA with a $C_0t_{1/2}$ of 4,100 (data not shown). (B) The same cDNA's and cellular DNA as above were incubated in 0.02 M Tris (pH 7.4), 1.5 M sodium chloride for various lengths of time. Samples were treated with S1 nuclease, and trichloroacetic acid-precipitable radioactivity was scored. The best-fit curves yield $C_0t_{1/2}$ values of 1,100 and 1,600 mol·s/liter.*

in a number of exogenously infected systems (3, 20a; N. Rice and L. Allan, unpublished data).

Hybridization of EIAV [^{3}H]cDNA with cell DNA extracted according to the procedure of Hirt (10) demonstrated that the majority of this viral DNA exists in very high-molecular-weight molecules. In paired experiments cDNA was reassociated with either total cellular DNA or with Hirt pellet DNA to each of several C_0t's, and the extent of hybridization was measured. No difference was observed between the kinetics of reassociation of cDNA with cellular DNA and with Hirt pellet DNA, indicating that most of the viral DNA sequences exist in an integrated proviral state.

Absence of proviral DNA in normal horse DNA. In searching for sequences related to viral cDNA in DNAs of uninfected cells, it is imperative that the ratio of DNA to cDNA be sufficiently high that one copy per haploid genome can be detected. Since the degree to which a particular cDNA is representative of the entire viral genome determines what will be an adequate ratio, the latter must be determined for each new cDNA used. This has been done for two different EIAV cDNA preparations by incubating each with varying amounts of EIAV-infected cell DNA to a high C_0t and scoring the final percent hybridization. The results (Fig. 4) indicated that each cDNA required about 50 μg of DNA per 0.022 ng of cDNA (= 750 cpm) to reach 80% hybridization at an extended C_0t. Since this cell DNA contained about three copies of viral DNA per haploid genome, and since the amount of DNA required to hybridize the cDNA is a strict function of the number of copies per genome, we concluded that $3 \times 50 = 150$ μg of DNA is sufficient to allow 80% hybridization of 0.022 ng of the cDNA's to a single related sequence per haploid genome. All experiments reported below used this or higher levels of DNA.

Some limits can also be set on the complexities of these cDNA's. The less complex of the two (the one requiring the greater amount of DNA to achieve a given level of reassociation) had an average size just after synthesis of about 2,700 nucleotides. It represents, therefore, at least 30% of the viral genome. The more complex cDNA was synthesized under the same conditions of high substrate concentration, but in the presence of actinomycin D. Its initial size was only about 1,350 nucleotides, yet its average complexity appeared to be about twice that of the first cDNA, or at least 60% of the viral genome.

With these cDNA's we tested DNA from five species of *Equus* for sequences related to EIAV cDNA and found none. As shown in Table 2, less than 10% hybridization of cDNA was ob-

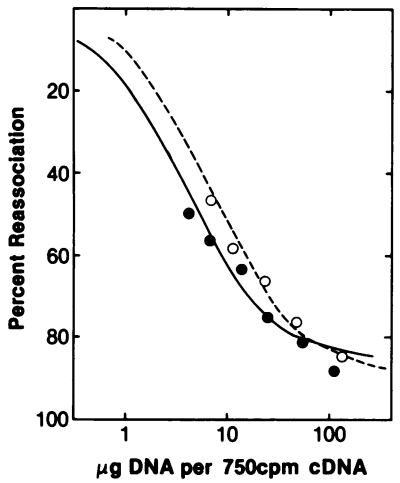

FIG. 4. *Effect of the ratio of DNA to cDNA on the final percent reassociation of cDNA. Samples with varying ratios were incubated to a C_0t of between 8,800 and 54,000, and binding to HAP at 50°C was measured. Final percent reassociation at infinite C_0t was calculated by reference to a C_0t curve at the standard ratio (180 μg of cellular DNA per 750 cpm [0.022 ng] cDNA) for each cDNA. In no case did the correction factor exceed 1.1. ●, cDNA synthesized in the presence of 100 μg of actinomycin D per ml; ○, cDNA synthesized without actinomycin D. As references we have included theoretical curves for homogeneous cDNA preparations of differing complexities.*

served in all cases, which is not significantly above background. These incubations were performed under conditions of very low stringency (high salt, relatively low temperature) to allow reassociation of even distantly related sequences. We conclude that EIAV is not an endogenous virus of the horse or other species of *Equus* tested, and that the horse does not carry sequences related, but not identical, to those represented in these cDNA's.

Under the same conditions of low stringency we tested DNAs from a variety of other animals for EIAV-related sequences and found none. These results are also shown in Table 2. We cannot exclude the possibility that there are EIAV sequences which are unrepresented in the cDNA's but which are found in normal DNA of one or more species. Such a finding would indicate the origin of a fraction of the EIAV genome, but would leave unknown the origin of at least 60% of the genome represented in these cDNA's.

TABLE 2. *Lack of homology between EIAV cDNA and DNAs from various species*[a]

DNA from:	C_0t	% Reassociation of EIAV cDNA
Horse		
EIAV-infected equine fetal kidney cell	23,000	90
Equus caballus (Clydesdale)	24,000	2
E. przewalskii	25,000	4
E. zebra hartmannae (Hartmann's Mountain zebra)	5,300	6
E. hemionus kulan (Kulan)	32,000	9
E. burchelli antiquorum (Damara zebra)	27,000	6
Cow	28,000	5
Sheep	12,000	9
Pig	19,000	6
Chicken	20,000	5
Dog	17,000	8
Cat	37,000	6
Mouse		
Mus musculus	50,000	7
M. caroli	15,000	6
M. cervicolor	28,000	6
Rat	47,000	5
Rabbit	27,000	3
Human (A204 cell line)	16,000	6
A204(M7)	27,000	6
Green monkey	45,000	5
Rhesus monkey	39,000	7
None		8

[a] A 0.012- to 0.022-ng amount of EIAV [^{3}H]cDNA was incubated with 140 to 770 μg of denatured DNA to the indicated C_0t. Binding of radioactivity to HAP at 50°C in 0.14 M PB was measured.

DISCUSSION

Only recently, with the development of a reliable tissue culture system for propagation of EIAV (15), has this virus been found to be a typical retrovirus containing both a high-molecular-weight RNA (1, 6, 7, 20) and a reverse transcriptase capable of copying either an exogenous template (1, 6) or the viral RNA itself (1). The experiments reported here complete this demonstration by showing that exogenously infected cells contain viral DNA sequences, most of which appear to be in an integrated proviral state.

We have also provided the first demonstration that EIAV is not an endogenous virus of the horse, for there was no detectable hybridization of EIAV cDNA with normal horse DNA. Thus, the EIAV system differs from those of mice, rats, hamsters, cats, baboons, and langurs (among others), where proviral DNA exists in every cell as part of the normal genetic complement. In these cases the virus, like any other genetic marker, is transmitted vertically and cannot be easily removed from the population. EIAV proviral DNA, on the other hand, is not found in normal horse DNA, indicating that infection can

only occur after environmental exposure of the horse to the virus. Since EIAV is known to be transmitted by biting flies (25), by transmission from infected mares to foals (13), or by contact with fomites contaminated with infected horse blood (23), we then asked whether the source of environmental EIAV is some other species in which the virus is endogenous. Thus, EIAV cDNA was incubated with a variety of cellular DNAs, i.e., from four other *Equus* species, and from sheep, cows, pigs, chickens, dogs, cats, mice, rats, and humans. In no case was any reassociation detectable, indicating that none of these species is the source of EIAV. Until recently the situation was similar with MPMV, isolated from, but clearly not endogenous in, a rhesus monkey (8, 21). It has now been shown (4) that MPMV is highly related to the endogenous type D virus of the langur. Similarly, the highly infectious and apparently widespread gibbon leukemia virus is not endogenous in the gibbon, but is related to an endogenous virus of Asian mice (14). Relatedness and orgin of EIAV, as in BLV (2, 19), are unknown.

Hybridization of EIAV cDNA with DNA from EIAV-infected cells revealed an average of two to three viral DNA copies per haploid genome. Similarly, exogenous infection of a variety of cell lines by M7 and by RD-114 also results in only a few ($\leq$5) proviral copies per haploid complement (3, 20a; Rice and Allan, unpublished data). Our EIAV calculation depends on the assumption that all cells in the culture were infected. In view of the fact that the cultures are relatively heavy virus producers and that the cells have been passaged more than 20 times since infection, the assumption seems reasonable. Indeed, in similar cultures, as many as 40% of the cells have been reported to contain viral antigen (18). Nevertheless, scanning electron microscopic examination of our cultures revealed only about 5% of the cells with budding particles (M. Gonda, personal communication), and direct immunofluorescent staining showed a comparably low percentage of the cells to be positive for viral antigen. We cannot rule out the possibility, therefore, that all the proviral DNA is contained in a small percentage of the cells, each of which may have as many as 100 copies per haploid genome. Such a high multiplicity has been reported to occur in sheep choroid plexus cells infected with visna virus (9). Since a cell may contain proviral DNA and not produce any viral component, the direct studies we have performed to date only provide a minimum estimate of percent infected cells.

Finally, the existence of viral DNA in infected cells may help to explain why equine infectious anemia is a persistent disease in spite of a well-developed immune response in the infected animal. An infected but nonproducing cell could escape surveillance during a given cycle of the disease but could become active at some later time, initiating another round of viremia. Experiments aimed at localizing infected cells in the diseased horse are in progress.

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