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# Genetic and Biological Variation in Equine Infectious Anemia Virus Rev Correlates with Variable Stages of Clinical Disease in an Experimentally Infected Pony

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## Abstract

Genetic and biological variation in the regulatory protein Rev of equine infectious anemia virus (EIAV) were examined throughout a clinically dynamic disease course of an experimentally infected pony. Following infection with the virulent EIAV<sub>Wyo</sub>, the pony underwent a variable disease course, including an acute fever episode at 12 days postinfection (DPI), multiple recurrent fever episodes until 135 DPI, a prolonged subclinical period, and two late fever episodes. Viral RNA was isolated from the inoculum and sequential sera samples, and the *rev* exon 2/*gp45* overlapping ORFs were amplified, cloned, and sequenced. Novel variants were found throughout infection, and genetic analyses indicated that both the Rev and *gp45* ORFs were under selective pressure. The Rev variant predominant in the inoculum, R1, remained predominant during the early periods following infection (until 35 DPI); however, R1 was replaced by new predominant variants during the recurrent fever period (67–135 DPI). R1 reemerged as the predominant variant during the afebrile period, but a new predominant variant, R93, was associated with the late fever episodes. Rev variants predominant during recurrent febrile and late-febrile periods had significantly higher Rev-mediated nuclear export activity than the variants predominant during the acute and afebrile periods. Statistical correlation was found between Rev activity and different stages of clinical disease. Together, these results suggest that genetic and biological variation in *rev* may be a contributing factor in EIAV disease progression.

**Keywords:** EIAV, Rev, persistence, quasispecies, variation, evolution

## Introduction

Equine infectious anemia virus (EIAV) possesses common features of the *Lentiviridae* subfamily of retroviruses, including a complex genome organization, tropism for cells of the monocyte/macrophage lineage, and establishment of a persistent, life-long infection. Many lentivirus infections are characterized by a slow, chronic disease, but infection of horses with EIAV can result in an acute, dynamic disease course characterized by recurring cycles of viremia, fever, and thrombocytopenia, with a possible development of anemia (reviewed in Issel and Coggins, 1979). Fever cycles are typically irregular and may recur for up to 1 year, despite the onset of a neutralizing- antibody response. Horses which survive these initial clinical episodes become life-long, inapparent carriers of the virus. The variable nature of clinical disease and the fact that most infected horses are able to control virus replication and remain life-long inapparent carriers make EIAV infection an excellent model for studies of virus and host factors which contribute to lentivirus persistence and pathogenesis.

Virus persistence over a life-long infection is dependent on both host and viral factors. Genetic variation, the result

of errors produced by reverse transcriptase, is a well-studied mechanism of lentivirus persistence (Hubner *et al.*, 1992; Ji and Loeb, 1994; Nowak, 1990; Patel and Preston, 1994). The result of the genetic variation is the accumulation of nonidentical, yet related, variants commonly referred to as a *quasispecies* (Domingo *et al.*, 1996; Eigen, 1993; Holland *et al.*, 1992). Selection acts on the quasispecies population, and the diversity within the population allows the virus to adapt to changes in selective pressures. The quasispecies nature of EIAV is largely the result of three regions of hypervariability: the viral long terminal repeat (LTR), the surface envelope (SU), and a region where the major exon of Rev is completely overlapped by the transmembrane protein (TM). Variation in SU, resulting from mutations, insertions, or duplications within the principal neutralization domain or hypervariable region, can alter antigenicity and facilitate evasion of host immune surveillance (Payne *et al.*, 1984, 1987, 1989; Kono, 1969, 1972; Salinovich *et al.*, 1986; Perryman *et al.*, 1988; Montelaro *et al.*, 1990; Issel *et al.*, 1990; Kono *et al.*, 1973; Zheng *et al.*, 1997). Studies examining the immune response of EIAV-infected horses indicate that both cellular and humoral immune responses contrib-

ute to suppression of virus replication during inapparent stages of infection (Salinovich *et al.*, 1986; Hammond *et al.*, 1997). No subset of the immune response, however, was ever previously shown to specifically correlate with control of virus replication during long-term persistence (Hammond *et al.*, 1997).

Lentiviruses have evolved complex mechanisms to regulate viral gene expression at the transcriptional and post-transcriptional levels. Variation in regulatory elements such as the LTR and Rev may alter the level of virus gene expression and, subsequently, viral replication (Carpenter *et al.*, 1991; Alexandersen and Carpenter, 1991; Maury *et al.*, 1997). We and others described genetic variation in the second exon of the EIAV regulatory protein Rev (Alexandersen and Carpenter, 1991; Belshan *et al.*, 1998; Leroux *et al.*, 1997), but the biological significance of Rev variation *in vivo* has not yet been explored. Rev functions to facilitate the nuclear export of the incompletely spliced viral RNAs during the late phase of virus replication. These viral RNAs encode the structural proteins and serve as progeny RNA molecules. Rev-dependent RNA export pathways have been described for numerous lentiviruses (reviewed in Cullen, 1992; Hope, 1997), and the human immunodeficiency virus type 1 (HIV-1) Rev-mediated export pathway is the best characterized. HIV-1 Rev binds a structure in the viral pre-mRNA, called the Rev-responsive element (RRE) (Zapp and Green, 1989), multimerizes (Olsen *et al.*, 1990), then utilizes the CRM1 nuclear export pathway to redirect movement of incompletely spliced viral RNA from the nucleus (Fornerod *et al.*, 1997; Stade *et al.*, 1997; Bogerd *et al.*, 1995; Stutz *et al.*, 1995). Discrete functional domains within Rev that mediate its interaction with cellular proteins and viral RNA are required for nuclear localization, RNA binding, multimerization, and nuclear export. EIAV Rev is a 165 amino acid protein functionally homologous to HIV-1 Rev (Fridell *et al.*, 1993). The major exon encodes for the nuclear export signal of EIAV Rev, amino acids 2–25 (Fridell *et al.*, 1993) and was previously shown to be able to substitute for the HIV-1 or visna virus effector domains (Fridell *et al.*, 1993; Mancuso *et al.*, 1994). A putative nuclear localization signal was previously mapped to the arginine-rich C-terminus of EIAV Rev (Harris *et al.*, 1998).

Rev is absolutely required for expression of viral structural genes and production of new virus. Therefore, factors which modulate Rev activity and, consequently, alter levels of viral gene expression may be important in regulating virus replication *in vivo*. Rev-attenuated phenotypes were previously identified during asymptomatic stages of HIV-1 infection, suggesting that variation in Rev could alter virus replication levels *in vivo* and contribute to the clinical outcome of infection (Iversen *et al.*, 1995; Hua *et al.*, 1996). We previously identified extensive nucleotide substitutions in the second exon of EIAV *rev* in virus obtained from an experimentally infected horse (Alexan-

dersen and Carpenter, 1991). The coexistence of putative Rev-competent and Rev-deficient phenotypes suggested that variation in Rev may contribute to virus persistence through regulation of virus replication. Subsequent *in vitro* analysis indicated that limited amino acid variation in Rev could alter Rev-mediated nuclear export activity (Belshan *et al.*, 1998), further suggesting that variation in Rev may modulate virus replication during disease.

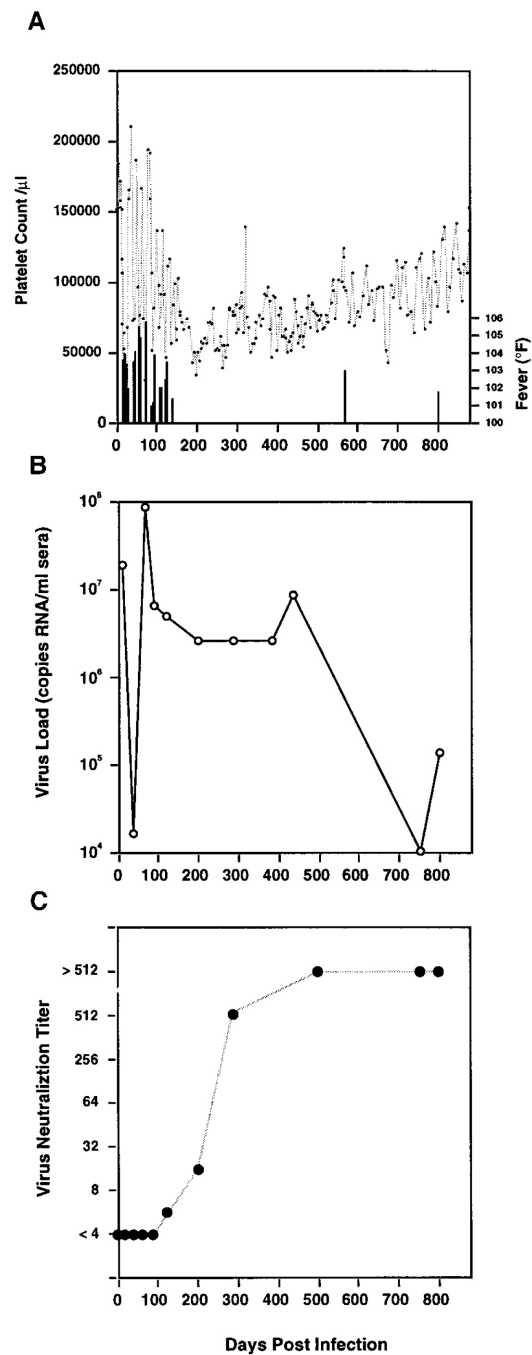
In the present study, we undertook extensive genetic and biological analyses of Rev quasispecies during long-term persistence in a single experimentally infected pony. Consistent with previous studies (Leroux *et al.*, 1997), novel *rev* variants were detected throughout infection. Significantly, changes in the genotype and phenotype of Rev quasispecies correlated with changes in clinical stages of EIAV infection. These findings indicate that genetic and biological changes in Rev contributed to the selection of EIAV variants *in vivo*, and may be important factors in lentivirus persistence and pathogenesis.

## Results

### Clinical profile of EIAV<sub>Wyo</sub>-infected pony 524

To accurately reflect the genetic diversity of an *in vivo* infection, pony 524 was inoculated with the highly virulent Wyoming strain of EIAV (EIAV<sub>Wyo</sub>), which has been maintained by serial *in vivo* passage (Oaks *et al.*, 1998). This inoculum represents a heterogeneous population of virus, similar to a natural infection. After inoculation, pony 524 experienced a clinical disease course characterized by recurring fever cycles interspersed with afebrile periods ranging from days to months (Figure 1A). The initial acute episode included a biphasic febrile response and thrombocytopenia from 10 to 22 days postinfection (DPI). The pony was then afebrile until 39 DPI. Thereafter, pony 524 underwent a period of seven recurrent fever episodes, which ranged from 2 to 6 days in length. The last fever episode ended after Day 135, and pony 524 remained afebrile except for two late fever episodes at Days 565 and 799. Febrile episodes were defined as rectal temperature greater than or equal to 101°F. Thrombocytopenia (platelet count less than 105 platelets/ml) was also observed concurrent with the initial fever cycles. After the initial fever cycles subsided, pony 524 remained persistently thrombocytopenic throughout the experiment. Except for the fever and thrombocytopenia, no other clinical signs were observed.

Semiquantitative RT-PCR was used to monitor virus levels at various DPI (Figure 1B). Consistent with previous reports, high virus loads were found in the initial fever period at 12 DPI but fell to a nearly undetectable level during the afebrile interval at 35 DPI. The highest virus load,  $9.08 \times 10^7$  copies RNA/ml, was detected during the



**Figure 1.** Clinical and serological profile of pony 524. (A) Clinical profile of EIAV<sub>Wyo</sub>-infected pony 524, including platelet counts (gray line, primary y-axis) and fever episodes (columns, secondary y-axis). Fever was defined as rectal temperature greater than or equal to 101°F. (B) Virus load, as measured by copies of RNA/ml serum, was determined by semi-quantitative RT-PCR for the days indicated by open circles. (C) Virus neutralizing (VN) antibody titer of pony 524 to EIAV<sub>WSU-5</sub>. VN titers were determined as described under Materials and Methods from serum samples obtained at 0, 11, 35, 67, 89, 118, 201, 289, 498, 754, and 800 DPI.

recurrent febrile period at 67 DPI. Levels of plasma viral RNA decreased somewhat in the subclinical period and were barely detectable at 754 DPI. However, virus re-

bounded to  $1.38 \times 10^5$  copies/ml at 800 DPI. Virus neutralization assays to EIAV<sub>WSU-5</sub>, a cell-culture-derived strain of EIAV<sub>Wyo</sub> (O'Rourke *et al.*, 1988), indicated that broadly reacting neutralizing antibodies appeared after the chronic fever cycles subsided (201 DPI) (Figure 1C). Pony 524 maintained elevated neutralizing antibody to EIAV<sub>WSU-5</sub> throughout the remainder of the experiment.

**Quasispecies distribution of the inoculum and statistical analysis of sampling procedure**

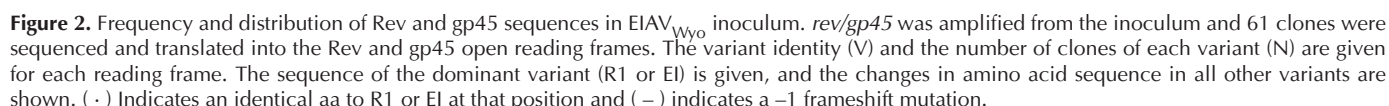
A potential problem with current methods used to characterize viral variants is the possible selection of a subpopulation of variants that may not be reflective of the population present *in vivo*. To confirm that the selected primer pairs did not bias the distribution of observed variants, statistical analyses were performed on the distribution of variants obtained by RT-PCR amplification of the inoculum. Sequences were amplified using four different sets of primer pairs (Table 1), designed from conserved regions flanking exon 2 of *rev*. Chi-square analyses indicated that the frequency of observed variants in the inoculum was not systematically biased during PCR amplification (not shown). Thus, the observed quasispecies population is likely to be reflective of variant frequencies *in vivo*.

A total of 61 individual clones, at least 10 from each primer pair, were sequenced from the EIAV<sub>Wyo</sub> inoculum. At the nucleotide level, 39 different genotypes were detected and translated into both the Rev and the gp45 ORFs. A total of 25 different Rev amino acid variants, designated R1 through R25, were observed in the Rev ORF (Figure 2). Five genotypes were observed at a frequency greater than 1, and two clones, R1 and R2, accounted for 30 and 25% of the sample population, respectively. Translation in the gp45 ORF identified 30 different variants, designated E1–E30 (Figure 2). The increased number of variants in gp45 as compared to Rev was primarily the result of an R to Q change at position 129 of gp45, which

**TABLE 1.** Primer Pairs Used for Amplification of EIAV Rev Sequences

Primer pair	Primer name <sup>a</sup>	Primer sequence (5'–3')
1	EM 7066	CAATTTGGCACAATCCATGA
	EM 7674C	GCGAGAGTTCCTTCTTGGGC
2	EM 7066	CAATTTGGCACAATCCATGA
	EM 7680CRI	<u>GGAATTC</u> CGAGAGTTCCTTCTTGG
3	EM 7160	GGATTGGGAGCTTCCATTAT
	EM 7674C	GCGAGAGTTCCTTCTTGGGC
4	EM 7160	GGATTGGGAGCTTCCATTAT
	EM 7680CRI	<u>GGAATTC</u> CGAGAGTTCCTTCTTGG

<sup>a</sup> C indicates the primer is on the complementary (antisense) strand, and R1 indicates the presence of an *EcoRI* restriction site (underlined) at the 3' end.



To analyze variation throughout infection, RNA was isolated from the inoculum and from sera samples collected at various stages of clinical disease, including: acute febrile (12 DPI), first afebrile interval (35 DPI), recurrent febrile (67, 89, and 118 DPI), afebrile (201, 289, 385, and 437 DPI), and late febrile (754 and 800 DPI) periods. The *rev* exon 2/*gp45* overlapping region of EIAV was amplified using primer pairs 2 and (Table 1), and 22 to 26 in-

The amplified region contains overlapping reading frames for two viral proteins, and we used a variety of genetic analyses to evaluate the potential significance of genetic changes in each reading frame. Consistent with observations of other retroviruses (Kim *et al.*, 1996), we observed a high ratio of transition (Ts) to transversion (Tv) at each time point. There was also a high ratio of nonsyn-



**Table 2.** Genetic Analysis of Variation in *rev* and *gp45* ORFs

		Days postinoculation (DPI)												Total
		Inoc.	12	35	67	89	118	201	289	385	437	754	800	
Total clones <sup>a</sup>		61	24	23	23	24	24	25	22	26	23	24	23	322
NT <sup>b</sup>	Variants <sup>c</sup>	39	13	12	14	20	15	20	9	15	12	11	13	145
	New variants <sup>d</sup>	39	10	7	9	17	12	15	4	10	6	7	9	
Rev <sup>b</sup>	Variants <sup>c</sup>	25	7	11	13	18	11	16	8	12	6	7	8	101
	New variants <sup>d</sup>	25	6	7	9	14	8	12	3	7	1	5	4	
	N/S <sup>e</sup>	1.0	0.9	7	2.2	2.6	1.9	2.4	1.8	2.1	1.5	2.0	1.2	
gp45 <sup>b</sup>	Variants <sup>c</sup>	30	7	10	14	16	11	15	8	10	12	8	11	105
	New variants <sup>d</sup>	30	5	4	11	12	7	10	2	4	6	5	9	
	N/S <sup>e</sup>	2.3	1.2	2.2	4.0	1.8	1.9	4.2	3.2	2.1	14	3.4	7.7	

<sup>a</sup> Number of clones sequenced at each time point.

<sup>b</sup> Analysis of variation in nucleotide sequence (NT) or amino acid sequence in the Rev or gp45 open reading frame.

<sup>c</sup> Number of distinct genotypes present at each time point.

<sup>d</sup> Number of new genotypes not previously observed.

<sup>e</sup> Ratio of nonsynonymous to synonymous mutations in that ORF.

onymous (N) to synonymous (S) mutations in both ORFs of the overlapping region (Table 2). At most time points, the N/S ratio was similar for both reading frames, although disproportionate values were sometimes observed. At Day 437, for example, there were 15 nt changes in all the sequences: in the Rev ORF, there was one change in the first codon position, seven in the second codon position, and seven in the third codon position. One of the changes in the third codon occurred concurrent with a second codon change, resulting in six synonymous changes in Rev and nine nonsynonymous changes, or an N/S of 1.5. In the gp45 reading frame, the codon positions change with respect to Rev, resulting in seven changes in the first codon position, seven in the second codon position, and one in the third codon position, resulting in an N/S ratio of 14/1 for Env. The overall N/S ratio was greater than 1 for both Rev (1.54) and gp45 (2.15), suggesting that variation in this region was the result of selective pressure. Application of the Tajima test (Tajima, 1989), which is a widely used procedure to determine whether nucleotide variation within a region was the result of either selective pressure or neutral mutations, confirmed that this region was indeed under selective pressure. Together, the results of the genetic analyses indicated a continuing evolution in Rev and gp45 throughout infection, with changes resulting from selective pressure in both reading frames.

### Rev variation throughout clinical disease

The genetic analyses suggested that variation in Rev was the result of selection rather than neutral mutations. We previously showed that genetic variation could alter

Rev activity, and we focused our subsequent analyses on variation in the Rev ORF. Rev amino acid sequences were examined from the inoculum (Figure 2) and from each of the 11 time points following infection (Figure 3). As described above, the inoculum was predominated by two Rev amino acid variants, R1 and R2, which accounted for 30 and 25% of the sample population, respectively. The consensus sequence for the inoculum was identical to the sequence of R1. The genetic variation observed was not distributed uniformly across Rev, and there were two main regions of variability. Few changes were observed in the nuclear export sequence, which was previously mapped to aa 2–25 (Fridell *et al.*, 1993; Mancuso *et al.*, 1994). However, amino acid position 25, located at the C-terminus of the nuclear export signal, was highly variable, with either a consensus serine or a leucine mutation at that position (S25L). At positions 104–108, there was a GDYQQ consensus sequence, with **DGYQR** appearing in a small number of variants. The C-terminal region of EIAV *rev* is highly basic, and a putative nuclear localization signal was previously mapped to this region (Harris *et al.*, 1998). There were only two changes in the C-terminal region, and both changes retained or increased the basic character of this region. Changes in the Rev quasispecies were examined at varying stages of clinical EIAV.

**Acute stage.** The consensus sequence of R1 was used as the standard by which the subsequent sera samples were compared. During the early period after infection, there were no changes in the consensus sequence (Figures 3 and 4). The Rev quasispecies found in the initial febrile episode at 12 DPI was genetically very homogeneous. The variant R1 remained the predominant variant, accounting

**12 DPI**

	N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
18	1	<b>R1</b>	DPQGPLESDQ	MC	RVLRQSLPEEK	ISSQ	TCIARRHLGPGPTQ	HTPSRRDWIRBQILQAEVLQERLEWRIRGVQQA	KELGEVNRGINRELHFR	EDQRGDFSAWGDYQQA	ERRWGBQSSPRVLRPGD	SKRRRHIL*			
1	1	R26	.....	.....	.....	.....	.....	.....G.....	.....	.....	.....	.....	.....	.....	.....
1	1	R27	.....	.....	.....	.....	.....	.....	.....	.....V.....	.....	.....	.....	.....	.....
1	1	R28	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....N.....
1	1	R29	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....+
1	1	R30	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....K.....
1	1	R31	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....K.....P.....

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**35 DPI**

		N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
6	1	<b>R1</b>	DPQGPLESDQ	MC	RVLRQSLPEEK	ISSQ	TCIARRHLGPGPTQ	HTPSRRDWIRBQILQAEVLQERLEWRIRGVQQA	KELGEVNRGINRELHFR	EDQRGDFSAWGDYQQA	ERRWGBQSSPRVLRPGD	SKRRRHIL*				
2	1	R2	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R14	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	1	R17	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	1	R32	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
4	1	R33	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	1	R34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R35	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R36	G.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R37	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R38	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

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**67 DPI**

		N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
0	1	<b>R1</b>	DPQGPLESDQ	MC	RVLRQSLPEEK	ISSQ	TCIARRHLGPGPTQ	HTPSRRDWIRBQILQAEVLQERLEWRIRGVQQA	KELGEVNRGINRELHFR	EDQRGDFSAWGDYQQA	ERRWGBQSSPRVLRPGD	SKRRRHIL*				
1	1	R12	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	1	R17	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10	1	<b>R32</b>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R33	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R39	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R40	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R41	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R42	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R43	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R44	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R45	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R46	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R47	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

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**89 DPI**

		N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
0	1	<b>R1</b>	DPQGPLESDQ	MC	RVLRQSLPEEK	ISSQ	TCIARRHLGPGPTQ	HTPSRRDWIRBQILQAEVLQERLEWRIRGVQQA	KELGEVNRGINRELHFR	EDQRGDFSAWGDYQQA	ERRWGBQSSPRVLRPGD	SKRRRHIL*				
2	1	R12	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R17	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
4	1	R32	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
3	1	R42	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R48	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R50	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R51	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R52	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R53	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R54	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R55	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R56	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R57	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R58	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R59	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R60	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R61	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

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**118 DPI**

		N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
0	1	<b>R1</b>	DPQGPLESDQ	MC	RVLRQSLPEEK	ISSQ	TCIARRHLGPGPTQ	HTPSRRDWIRBQILQAEVLQERLEWRIRGVQQA	KELGEVNRGINRELHFR	EDQRGDFSAWGDYQQA	ERRWGBQSSPRVLRPGD	SKRRRHIL*				
2	1	R12	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	1	R26	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
12	1	<b>R53</b>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R52	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R63	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R64	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R65	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R66	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R67	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R68	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	1	R69	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

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**Figure 3.** Alignment of Rev aa sequences obtained from sera of pony 524 at 12, 35, 67, 89, 118, 201, 289, 385, 437, 754, and 800 DPI. The variant identity (V) and number of clones of each variant (N) are given for each time point, as well as the total number of clones sequenced (below the N column). Dominant variants are highlighted in bold. Sequences are compared to R1, which is listed at the top of each time point. For individual sequences, (·) indicates an identical aa to R1 at that position, (\*) indicates a premature stop codon in the Rev ORF, and (+) indicates a +1 frameshift.



**201 DPI**

N	V	10	20	30	40	50	60	70	80	90	100	110	120	130
3	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
4	R2		L											
1	R17											DG	R	
1	R32								A			DG	R	
1	R70		L						D		K			
2	R71							A	D		K	DG	R	
4	R72		P						D		K			
1	R73		P						D		K	R	Q	
1	R74			I										
1	R75								D		K	HR		
1	R76			G		G			D		K	R		
1	R77								D		K	G	R	
1	R78								D		K	R		
1	R79								D		K			
1	R80		L					A	D		K	DG	R	*
1	R81	G	P				G		A	D	K	R		

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**289 DPI**

		10	20	30	40	50	60	70	80	90	100	110	120	130
9	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
4	R2		L											
2	R17											DG	R	
1	R32								A			DG	R	
3	R33								A		E	DG	R	
1	R82	G	P			G		A						
1	R83										N			
1	R84										E			

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**385 DPI**

		10	20	30	40	50	60	70	80	90	100	110	120	130
11	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
4	R2		L											
1	R5					G								
1	R14		L	L										
1	R32								A			DG	R	
1	R85											D		
2	R86	G	P			G			I	Y		G	R	L
1	R87	G	P			G			I	Y		G	R	L
1	R88	S						K						
1	R89			Y										
1	R90		I									DG	R	
1	R91							V						

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**437 DPI**

		10	20	30	40	50	60	70	80	90	100	110	120	130
11	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
4	R2		L											
1	R17											DG	R	
4	R32								A			DG	R	
2	R33								A		E	DG	R	
1	R92		P											

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**754 DPI**

		10	20	30	40	50	60	70	80	90	100	110	120	130
4	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
6	R26					G								
10	R93		P					A	D		K	G	R	H
1	R94		P	A				A	D		K	G	R	H
1	R95		P					V	A	D	K	G	R	H
1	R96		P			R		A	D		K	G	R	H
1	R97		P					A	D		*K	G	R	H

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**800 DPI**

		10	20	30	40	50	60	70	80	90	100	110	120	130
0	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGGPTQHTPSRRDRWIREQILQAEVLQERLEWRIRGVQVAKELGEVNRGIMRELHFREDQRGDFSAWGDYQQAQERRWGBQSSPVLRFPGDSKRRRKH*												
1	R86		G	P		G			I	Y		G	R	L
16	R93		P					A	D		K	G	R	H
1	R96		P			R		A	D		K	G	R	H
1	R97		P					A	D		*K	G	R	H
1	R98		G	P		G			I	Y		R	G	R
1	R99		P					A	D		L	K	G	R
1	R100		G	P		G			A	D		K	G	R
1	R101		P			I		A	D		K	G	R	H

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Figure 3, continued.

DPI	V	10	20	30	40	50	60	70	80	90	100	110	120	130
12	R1	DPQGPLESDQWCRVLRQSLPEEKISSQTCIARRHLGEGPTCHTPSRRDWIRBQILQAEVLQERLEWRINGVQVAKELGEVNRGIWRELHFRDQRGDFSAWDYQQAQERRWAGESSPRVLRPGDSKRRPKHL*												
35	R1													
67	R32													
89	R12*								A			DG		R
118	R53											G		R
201	R78*										K		G	R
289	R1								D		K			R
385	R1													
437	R1													
754	R93			P					A	D		K	G	R
800	R93			P					A	D		K	G	R

**Figure 4.** Consensus Rev aa sequences at each time point (DPI). The variant identity (V) and sequence, relative to R1, are given for each time point. At two time points, 89 and 201 DPI, no variant was predominant and the consensus sequence was represented by a minor variant, indicated by (\*).

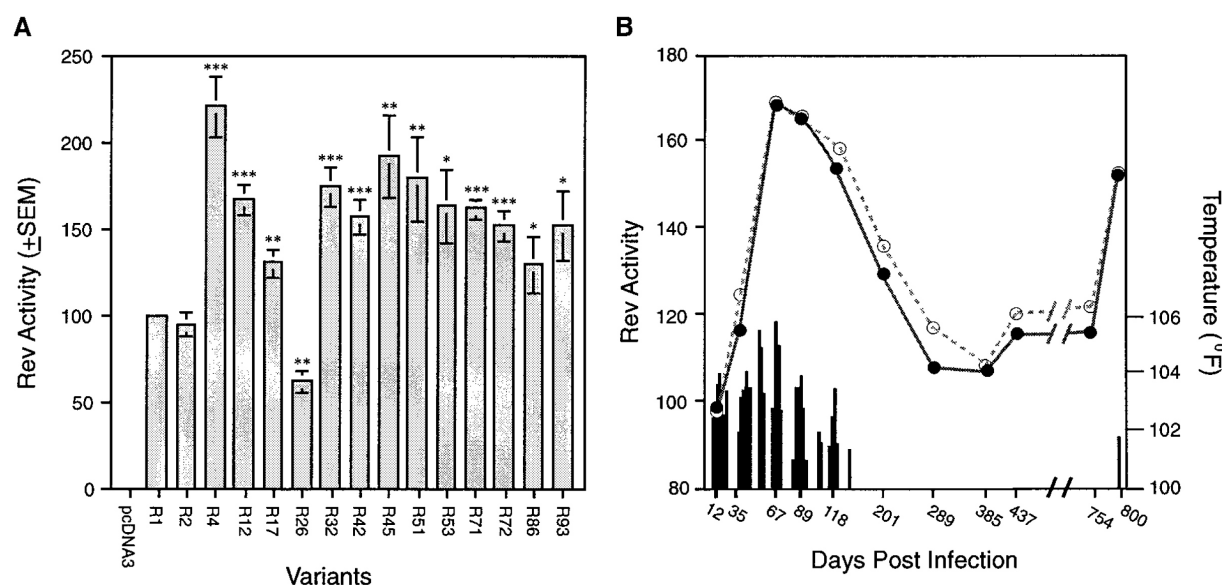
for 75% of the sample population. The remaining six variants had not been observed in the inoculum, and each differed from R1 by a single amino acid. None of the variants contained changes at aa 25 or in the GDYQQ region at 104–108. In the intervening afebrile period at 35 DPI, the quasiespecies resembled the inoculum. Both R1 and R2 variants were detected, and the former was again the predominant variant. Also, we observed two minor variants which had been present in inoculum (R14, R17), and genetic variability was detected at position 25 (S or L) and in the **DDGYQR** motif at aa 104–108.

*Recurrent febrile stage.* The Rev quasiespecies observed in the recurrent febrile period (67, 89, and 118 DPI) differed markedly from the previous populations, as reflected in the appearance of a new consensus sequence at each time point sampled (Figure 4). Notably, the variant R1, which predominated both the inoculum and the samples from 12 and 35 DPI, was not detected in any of the three samples taken during the recurrent febrile stage. Variants and/or amino acid changes present as minor populations in earlier time points grew in frequency to predominate during the recurrent febrile period. For example, variant R32 was only a minor variant at 35 DPI but represented 43% of the population at 67 DPI. The consensus sequence at 67 DPI was identical to that of R32 and had an alanine at aa 82 as well as the **DDGYQR** motif in region 104–108. At 89 DPI, R32 had decreased to 14% of the population, and no variant accounted for more than 20% of the population at this time point. The consensus sequence at 89 DPI contained a **GGYQR** motif and was represented by the variant R12, a minor variant which was also present in the inoculum and 67 DPI. R53 was present as a minor variant at 89 DPI and grew to predominate the quasiespecies population at 118 DPI. R53 shared the consensus sequence at 118 DPI, which contained an R97K substitution and a **GGYQR** motif in region 104–108. Throughout the recurrent febrile period, minor variants were observed which contained amino acid substitutions in the NES, although none contained the R2-like S25L. In summary, the recurrent febrile period was characterized by continual changes in the consensus sequence, with minor variants growing to predominate at subsequent time points.

*Afebrile stage.* The first serum sample analyzed after the recurrent febrile episodes subsided was obtained at 201 DPI, and no variant accounted for more than 20% of the population at this time point. Surprisingly, after going undetected throughout the recurrent febrile period, R1 was observed as a minor variant. Although R2, R17, and R32 were also present as minor variants, the consensus sequence at 201 DPI was unlike those previously observed. The sequence of R78 was identical to the consensus sequence at 201 DPI and consisted of G80D, R97K, and Q108R. Position 25 was highly variable, with several variants containing a proline at that residue. At aa 104–108, both the GDYQQ and the **DDGYQR** motifs were detected in addition to the consensus sequence containing GDYQR. After 201 DPI, the frequency of R1 grew to predominate each time point analyzed during the remainder of the afebrile period (289, 385, and 437 DPI), and the consensus sequence was identical to that of R1 at each of these time points. Overall, the identity of the minor variants was not highly variable during the afebrile stage, with R17, R32, and R33 observed in most of the samples. In contrast to the continual appearance and growth of minor variants in the recurrent febrile stage, the afebrile stage was relatively stable genetically and predominated by R1.

*Late febrile stage.* Two late fever episodes occurred at 565 and 799 DPI. Analysis of *rev* variation included samples collected at 754 and 800 DPI. R1 was observed as a minor variant at 754 DPI but was not detected at 800 DPI. A new variant, R93, arose as the predominant variant and the consensus sequence at 754 and 800 DPI and differed from R1 by seven amino acids. Changes not observed in previous consensus sequences included L25P, V75A, and R112H. Other changes from R1 found in the consensus sequence for 754 and 800 DPI were G80D, R97K, D105G, and Q108R, which had been part of earlier consensus sequences.

In summary, the dynamics of the Rev quasiespecies evolution were reflected by the changes in the consensus sequences during the course of disease (Figure 4). These changes were exemplified by the disappearance and re-appearance of R1, which was identical to the consensus sequence in the acute fever episode, the first afebrile interval, and most of the afebrile period. The replacement



**Figure 5.** Variation in Rev-mediated nuclear export activity correlates with temperature and clinical disease. (A) *In vitro* biological activity of Rev variants from pony 524. Rev exon 2 variants were inserted into a eukaryotic expression vector and transient transfection assays were performed in 293 cells as described under Materials and Methods. CAT assays were normalized to the activity of R1 and the results are presented as a percentage of activity compared to R1. Error bars denote the standard error of the mean. Variants that differed significantly from the activity of R1 are indicated, with *P* values represented by (\*) *P* < 0.05, (\*\*) *P* < 0.005, and (\*\*\*) *P* < 0.00005. (B) The predicted (open circles, dotted line) and experimental (closed circles, solid line) quasispecies Rev activity at different stages of EIAV. The rectal temperature of pony 524 is indicated by solid bars.

of R1 by novel variants during both the recurrent and late febrile periods is reflected in changes in the consensus sequence during those clinical stages of EIAV infection. The finding that changes in *rev* consensus sequences coincided with changes in the clinical disease suggested differences in selective pressures at sequential stages of disease. Surprisingly, these results suggested that the host environment in the acute and afebrile stages selected for similar Rev variants.

### Biological activity of Rev quasispecies

Genetic analysis of *rev* variation suggested differences in selective pressure at different stages of clinical disease. Therefore, it was important to determine the biological activity of Rev variants observed in this study. Because it was not practical to biologically test each clone, Rev variants which were predominant or representative of consensus sequences at any time point were cloned into a eukaryotic expression vector containing *rev* exon 1. The Rev-mediated nuclear export activity was determined in transient transfection assays as previously described (Belshan *et al.*, 1998) and results were normalized to R1 (Figure 5A). R2, which contained the single S25L change from R1, exhibited a level of activity similar to that of R1. The variant R26 (R52G), which was detected at 12, 118, and 754 DPI, was the only variant tested that had significantly lower Rev activity (63%) than did R1. Interestingly, all other variants assayed showed levels of nuclear export activity that were significantly higher than that of R1 (*P* < 0.05). These included variants that were identical to

the consensus sequences during the recurrent febrile and late febrile stages of disease (67, 89, 118, 754, and 800 DPI). The consensus sequences represented by the variants R12, R32, R53, and R93 all exhibited approximately 165% activity relative to that of R1. Other variants tested from the recurrent febrile stage, R42, R45, and R51, also had Rev activity approximately 175%. Thus, the recurrent and late febrile periods were predominated by Rev variants that had significantly higher nuclear export activity than that of R1, which predominated the acute and afebrile periods. Interestingly, 201 DPI was characterized by the coexistence of Rev variants with low activity (R1 and R2) and variants which had relatively higher activity (R71 and R72). Similarly, at 754 DPI, we observed a high frequency of R26 (63% activity) coexisting with the predominant R93 (153% activity). It is possible that 201 and 754 DPI reflect quasispecies transitions between different stages of clinical disease.

### Rev quasispecies activity correlates with clinical stage of disease

Our analysis of selected Rev variants indicated that changes in Rev phenotype were associated with changes in clinical disease. As our data show, however, *rev* variants existed as a quasispecies population, and no one variant can define the phenotype of the quasispecies. To determine whether the Rev quasispecies activity was statistically correlated with different parameters of clinical disease, we attempted to approximate the average phenotype for the Rev quasispecies at each time point. The

**Table 3.** Experimental and Predicted Quasispecies Rev Activity

Stage	Rev variant DPI <sup>b</sup>	R1	R2	R4	R12	R17	R26	R32	R42	R45	R51	R53	R71	R72	R86	R93	Tested/ total <sup>c</sup>	(Exp.) EQRA <sup>d</sup>	(Pred.) PQRA <sup>e</sup>
		100	96	222	169	131	63	176	158	193	180	165	162	154	131	153			
Acute	12	18 <sup>f</sup>	0	0	0	0	1	0	0	0	0	0	0	0	0	0	19/24	98	98
	35	6	2	0	0	2	0	2	0	0	0	0	0	0	0	0	12/23	117	124
Recurrent	67	0	0	0	1	2	0	10	1	1	0	0	0	0	0	0	15/23	169	170
	89	0	0	0	2	1	0	4	3	0	1	1	0	0	0	0	12/24	166	165
	118	0	0	0	2	0	2	0	0	0	0	12	0	0	0	0	16/24	153	158
Afebrile	201	3	4	0	0	1	0	1	0	0	0	0	2	4	0	0	15/25	129	136
	289	9	4	0	0	2	0	1	0	0	0	0	0	0	0	0	16/22	108	117
	385	11	4	0	0	0	0	1	0	0	0	0	0	0	2	0	18/26	107	108
	437	11	4	0	0	1	0	4	0	0	0	0	0	0	0	0	20/23	116	120
Late febrile	754	4	0	0	0	0	6	0	0	0	0	0	0	0	0	10	20/24	115	122
	800	0	0	0	0	0	0	0	0	0	0	0	0	0	1	16	17/23	152	151

<sup>a</sup> The nuclear export activity level of each variant tested ( $A_i$ ), relative to the activity of R1, which is set at 100.

<sup>b</sup> The days postinfection from which the variants were isolated.

<sup>c</sup> The number of clones experimentally tested and the total number of clones at each time point.

<sup>d</sup> The experimental quasispecies Rev activity calculated for each time point.

<sup>e</sup> The predicted quasispecies Rev activity calculated for each time point.

<sup>f</sup> The number of times that variant was detected at that time point ( $v_i$ ).

variants experimentally tested accounted for at least 50% of all clones present at each time point. Using the activity of each tested variant and its frequency in the population, we calculated a weighted average to derive an experimental quasispecies Rev activity at each time point (Table 3). Using the phylogenetic relatedness among the variants (not shown), the untested Rev variants were each assigned the same Rev activity as the nearest tested variant, and a predicted quasispecies activity was calculated. At each time point, the calculated experimental and predicted quasispecies Rev activities were nearly identical, differing at most by 8% (Table 2). Comparison of quasispecies Rev activity with febrile responses of pony 524 indicated that the quasispecies Rev activity was highest during the recurrent febrile and late febrile stages of EIA

(Table 3, Figure 5B). These findings further suggested a correlation between Rev activity and clinical stage of disease. Therefore, Pearson correlation coefficients were calculated to test for correlation between the quasispecies Rev activity and various clinical parameters, including virus titer, platelet counts, and temperature of the horse (Table 4). No significance was detected in comparisons of experimental or predicted quasispecies Rev activity and virus titer ( $P > 0.15$ ) or platelet count ( $P > 0.45$ ). Analysis by Pearson correlation coefficients found that the temperature of pony 524 was statistically correlated with the experimental ( $P = 0.007$ ) and predicted ( $P = 0.011$ ) quasispecies Rev activity. In addition, the results of the correlation analyses indicated that phenotypic variation in Rev was correlated with changes in clinical stages of EIAV dis-

**Table 4.** Correlation between Quasispecies Rev Activity and Clinical Parameters of EIAV Infection

		Experimental quasispecies Rev activity	Predicted quasispecies Rev activity
Clinical parameter	Virus titer	0.157 <sup>a</sup>	0.195 <sup>a</sup>
	Platelet count	0.453 <sup>a</sup>	0.504 <sup>a</sup>
	Temperature	0.007 <sup>a</sup>	0.011 <sup>a</sup>
Clinical disease stage	Acute vs. recurrent	0.013 <sup>b</sup>	0.014 <sup>b</sup>
	Recurrent vs. afebrile	0.010 <sup>b</sup>	0.015 <sup>b</sup>

<sup>a</sup>  $P$  values calculated using Pearson correlation coefficients.

<sup>b</sup>  $P$  values using general linear models.

ease. The experimental and predicted quasispecies Rev activities in the recurrent febrile stage were significantly higher than the quasispecies Rev activities in the acute period ( $P = 0.013$ ,  $P = 0.014$ ) and in the afebrile period ( $P = 0.010$ ,  $P = 0.015$ ).

## Discussion

Antigenic variation in the surface envelope glycoprotein has long been recognized as an important mechanism of immune evasion and lentivirus persistence. Recent studies suggested that variation in other regions of the viral genome, including the LTR and regulatory genes, may also contribute to the clinical disease course (Carpenter *et al.*, 1991; Maury *et al.*, 1997; Leroux *et al.*, 1997; Belshan *et al.*, 1998; Hua *et al.*, 1996). We and others have observed a high degree of genetic variation in the *rev* exon 2/*gp45* region of EIAV (Belshan *et al.*, 1998; Leroux *et al.*, 1997), and we previously demonstrated that limited amino acid variation in EIAV Rev alters biological activity (Belshan *et al.*, 1998). It was proposed that attenuation of the Rev phenotype may allow EIAV to persist through down-regulation of viral gene expression and reduced replication, enabling the virus to evade an effective immune response (Belshan *et al.*, 1998).

To delineate the significance of *rev* variation *in vivo*, we examined both genetic and biological variation of Rev in an experimentally infected pony. Genetic variation observed in *rev* during sequential stages of EIAV infection clearly demonstrated that *rev* variants exist as a quasispecies population. Genetic analyses indicated that the Rev ORF evolved throughout infection and that genetic variation was the result of selective pressure rather than neutral mutations. The frequency at which new variants were detected was greatest during clinical periods, concomitant with higher levels of virus replication at these times. Genetic and phenotypic changes in the Rev quasispecies coincided with changes in clinical disease. Rev variants which were predominant during the recurrent febrile period had significantly higher nuclear export activity than the variants predominant during the acute or afebrile periods. Statistical analyses found that quasispecies Rev activity was correlated with temperature of the infected pony but not with virus titer or platelet count. Additionally, the quasispecies Rev activity in the acute and afebrile stages was significantly lower than the quasispecies Rev activity in the recurrent febrile stage of disease. Together, these findings indicate that variants with high Rev activity have a selective advantage during the recurrent and late febrile stages of EIA infection. Therefore, variation in *rev* may be a contributing factor of EIAV disease progression.

The dynamic evolution of Rev quasispecies during sequential stages of clinical EIA is exemplified by changes

in the frequency of R1. This variant, and the closely related R2, clearly represented the predominant genotype and phenotype in the inoculum and the early time period following infection. During the subsequent recurrent febrile period, R1 was replaced by a series of closely related Rev variants with significantly higher biological activity. Surprisingly, R1 reappeared at 201 DPI and predominated during the prolonged afebrile period. Yet, R1 was replaced by the more biologically active R93 during the late febrile period. These genetic changes in the Rev quasispecies were also reflected in the phenotypic changes in the quasispecies Rev activity, which was highest during the recurrent febrile and late febrile stages of disease. In total, these findings indicate that there were changes in selective pressure at sequential stages of clinical disease. Specifically, our results suggest that changes in the host environment occurred during the recurrent febrile period and, further, that these changes selected for variants with significantly higher Rev activity. At present, the selective pressures present during the recurrent febrile period are not yet known. Increased Rev activity did not correlate with virus titer, as might be expected if the selective advantage was solely dependent on the virus replication rate. It is possible that a Rev-specific host immune response selects for different Rev variants; however, immune evasion does not explain the significant differences in the biological activity of selected Rev variants. Ongoing studies to identify factors important in selection of Rev variants may increase our understanding of mechanisms of EIAV pathogenesis.

Viremia has long been associated with clinical episodes in EIAV-infected animals (Issel and Coggins, 1979). The abrogation of recurrent clinical episodes is typically coincident with the maturation of the host immune response and decline in virus replication (Hammond *et al.*, 1997). The appearance of broadly acting neutralizing antibody in pony 524 at 201–289 DPI was associated with the resolution of the recurrent febrile period, although virus load remained high through most of the afebrile period. Our model predicted that Rev variant that predominated during febrile periods would differ phenotypically from variants predominant during afebrile periods. Indeed, the quasispecies Rev activity was significantly higher in the recurrent febrile period than in the afebrile period. However, our model did not predict that quasispecies activity in the acute period would differ significantly from that in the recurrent febrile period, nor that the acute and afebrile periods would be so genetically and phenotypically similar. The predominance of R1 during the acute period indicates that R1 is a robust phenotype, capable of supporting high levels of virus replication. In fact, the predominance of R1 throughout the afebrile period was associated with relatively high levels of viremia and thrombocytopenia that persisted until late in the afebrile stage of disease (754 DPI). If pony 524 had resolved to a



clinically inapparent state, in which virus load decreased to nearly undetectable levels and platelet counts returned to normal levels, it is possible that the quasispecies Rev activity would have been statistically correlated with virus load, or that Rev variants less active than R1 would have been predominant during the afebrile period. In support of this, the least-active variant, R26, comprised 25% of the observed population at 754 DPI, where virus loads were barely above the threshold of detection and platelet counts were near normal levels.

The second exon of Rev is completely overlapped by a portion of the cytoplasmic tail of the EIAV transmembrane protein gp45. Thus, nucleotide substitutions in this region may alter Rev and/or gp45. Because of the constraints of maintaining functional proteins in both reading frames, it might be expected that variation in this region would be biologically significant. However, it is not immediately obvious which reading frame determines selective advantage. Previous studies of variation in *rev/gp41* of HIV-1 suggested that both genes evolved independently and that the changes were the result of selection in only one ORF (Martins *et al.*, 1991). Our data would argue that EIAV *rev/gp45* evolved as a result of selective pressures in both reading frames. The overall complexity (number of aa variants at each time point) of Rev and gp45 was approximately the same throughout infection, and out of 146 nt variants, there was a similar number of total Rev and gp45 aa variants (101 and 105, respectively). This suggests that changes in gp45 also contribute to the selective advantage of the observed *rev/gp45* variants. The cytoplasmic tail of TM plays important and varied roles in lentivirus replication, and studies are ongoing to characterize the effect of gp45 variation on EIAV replication. It is probable that variation in both ORFs contribute to virus pathogenicity *in vivo*.

The underlying assumptions of the RT-PCR method are that all viral quasispecies are amplified with equal efficiency and that the resulting proportions are representative of the starting population. Although these assumptions have generally been accepted, some reports suggest that PCR amplification might result in selective amplification of individual templates, especially in reactions containing a heterogeneous population of templates (Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1999; Barnard *et al.*, 1998; Suzuki *et al.*, 1998). Previous studies suggested that interactions between primers and templates might alter the efficiency of template amplification and, thus, alter the sample population (Barnard *et al.*, 1998; He *et al.*, 1994; Suzuki and Giovannoni, 1999), or that primer pairs may systematically bias PCR amplification (Suzuki and Giovannoni, 1999; Polz and Cavanaugh, 1998; Barnard *et al.*, 1998). However, these studies have generally involved two or three different templates of known concentrations. The complex nature of viral quasispecies *in vivo* can further complicate sequencing studies. Our statistical

analyses of primer-pair bias in the inoculum indicated that the frequency of observed variants was not systematically biased during PCR amplification, so the random samples were likely reflective of variant frequencies *in vivo*. The risk of bias may have been reduced by designing primers to conserved target sequences and using primers with similar GC content and optimal annealing temperatures. Taken together, our results suggest that randomly sampled viral populations obtained by RT-PCR can reflect *in vivo* populations. This offers confidence in the characterization of viral quasispecies present *in vivo* and strengthens interpretations of studies examining the role of virus variation in immune evasion and disease progression.

Most studies of EIAV persistence *in vivo* used a cloned, cell-culture-propagated virus as an inoculum and were limited to analysis of febrile periods. Inocula comprised of a homogenous population of virus have been useful in identifying genetic changes that arise during an *in vivo* infection. In this study, our intention was to better reflect a naturally occurring infection in an experimentally infected animal. Therefore, the inoculum used was obtained from a donor pony undergoing its first fever episode and represented a heterogeneous population of virus rather than a single clonal population. Although this method required a more extensive analysis, we believe it provided an accurate representation of the complex nature of EIAV evolution throughout disease within a single infected pony. The heterogeneous nature of the inoculum, in combination with the extensive sampling and genetic analysis at sequential time points, allowed us to examine the genetic variants that made up the *rev* quasispecies and the biological phenotype of the Rev quasispecies that were selected at variable stages of clinical disease. It is clear that genetic and phenotypic changes in the Rev quasispecies were associated with changes in clinical stages of disease in this pony. While analysis of additional EIAV-infected ponies is needed to fully elucidate the biological significance of Rev variation, the findings presented here indicate that variation in virus regulatory proteins can contribute to lentivirus persistence.

## Materials and Methods

### Experimental infection

The experimental infection of pony 524 was previously reported (Oaks *et al.*, 1998). The pony was infected intravenously with  $10^3$  horse infectious doses of the highly virulent Wyoming strain of EIAV (EIAV<sub>Wyo</sub>). Physical examinations, rectal temperatures, hemograms, and platelet counts were performed daily during clinical episodes and intermittently during chronic clinical disease or subclinical infection. Sera and plasma were collected, processed, and stored at  $-80^{\circ}\text{C}$  until analyzed.



### Virus neutralization assay

Assays were performed using equine dermal (ED) cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, penicillin, and streptomycin as previously described (Carpenter *et al.*, 1987). ED cells were seeded 1 day prior to infection at  $10^5$  cells/well in a six-well dish. Serum from pony 524 was heat-inactivated at 60°C, to destroy complement, and was serially diluted twofold in supplemented media. Five hundred focus-forming units of EIAV<sub>WSU-5'</sub>, a cell culture-derived strain of EIAV<sub>Wyo</sub> (O'Rourke *et al.*, 1988), in a 100- $\mu$ l volume was incubated with 100  $\mu$ l of serially diluted pony 524 serum at 37°C for 1 h. Duplicate wells of ED cells were inoculated with 100  $\mu$ l of virus-serum mixture in the presence of 8  $\mu$ g/ml polybrene, and the media was changed the following day. Cells were incubated for an additional 72 h and fixed with 100% methanol, and immunocytochemistry was performed using convalescent anti-EIAV horse sera to detect foci of virus-infected cells (Carpenter *et al.*, 1987). Results are expressed as the serum neutralization titer, defined as the reciprocal of the highest serum dilution that gave an 80% reduction in foci as compared with that of preimmune and negative control serum.

### Amplification and sequence analysis of viral variants

EIAV<sub>Wyo</sub> inoculum was obtained as a serum sample collected from an experimentally infected foal experiencing its initial fever episode at 7 days postinfection (DPI). To obtain viral RNA, 100  $\mu$ l of EIAV<sub>Wyo</sub> inoculum or pony 524 serum was pelleted by centrifugation at 93,000g for 1 h at 4°C. Viral RNA was isolated from these pellets by guanidine thiocyanate lysis and acid phenol-chloroform extraction using a commercially available kit (Ambion, Austin, TX) and resuspended in 24  $\mu$ l RNase-free glass-distilled water containing 0.1 mM EDTA. RNA samples were DNase I treated using the methods of Huang *et al.* (1996). Briefly, two units of DNase I (Ambion) were added to 3  $\mu$ l of viral RNA, 20 mM MgCl<sub>2</sub>, 1 mM of each dNTP, 1 $\times$  PCR buffer II (Perkin-Elmer, Branchburg, NJ), 20 units of RNase inhibitor, and 2.5  $\mu$ M of random hexamers in a total volume of 20  $\mu$ l. The reaction was incubated at 37°C for 30 min and heated to 75°C for 5 min to inactivate the DNase I. After cooling to 4°C, 50 units of Moloney murine leukemia virus reverse transcriptase were added. Reactions were incubated at 42°C for 45 min, heated to 99°C for 5 min to inactivate the reverse transcriptase, and then cooled to 5°C for 5 min. The reaction was brought up to 100  $\mu$ l in 1 $\times$  PCR buffer II with 2 mM MgCl<sub>2</sub>, 0.2  $\mu$ M of each dNTP, 2.5 units *Taq* polymerase (Perkin-Elmer), and 1  $\mu$ M of each primer.

Primers were designed based on conserved regions flanking the second exon of *rev* (Table 1). PCR amplification conditions consisted of 37 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min, and extension

at 72°C for 1 min. The initial and final cycles contained a prolonged extension at 72°C for 5 min. A 2- $\mu$ l aliquot of PCR product was TA-cloned into pGEM-T vectors as recommended by the manufacturer (Promega, Madison, WI) and transformed into *Escherichia coli* DH-5 $\alpha$ . Positive clones were identified by colony blot hybridization using a subgenomic fragment of *rev* labeled with [<sup>32</sup>P]dTTP by random-primed labeling (Roche Molecular Biochemicals, Indianapolis, IN). Plasmids were isolated from positive clones using a commercially available kit, Wizard Plus Miniprep (Promega), and the *rev* inserts were sequenced bidirectionally with vector primers flanking the insert. All sequencing was performed by the Iowa State University DNA Synthesis and Sequencing Facility using an automated DNA sequencer. Sequences were aligned by MacVector and AssemblyLIGN software (Oxford Molecular, Beaverton, OR). To estimate the RT-PCR error rate, viral mRNA was isolated from a clonal cell line stably transfected with an EIAV proviral DNA clone, reverse transcribed and amplified with *Taq* polymerase. The amplicons were cloned and 17 individual clones were sequenced. The error rate of the RT-PCR amplification procedure was determined to be 0.025%, or three substitutions per 11,991 base pairs sequenced.

### Construction of Rev expression vectors

*rev* variants were subcloned into pERevWT, a Rev expression vector previously described as pcH21 (Belshan *et al.*, 1998). This plasmid contains Rev cDNA in the pCR3 background (Invitrogen, Carlsbad, CA). pERevWT was digested with the restriction endonuclease *Apal* to remove *rev* exon 2. The digested plasmid was gel-purified, dephosphorylated, and ligated with the exon 2 sequence variants. Two methods, based on the direction of the inserts in pGEM-T, were employed to move variant exon 2 sequences into the pERevWT background. Inserts in the "forward" direction were digested with *Apal*, gel-purified, and ligated into the digested pcRevWT background. Inserts in the "reverse" orientation were PCR-amplified with the EM7160 primer and a 3' EIAV primer containing an *Apal* site (underlined): 5'-CTGGGCCCTCATAAATGTTTCCTCCTTCGC. The PCR products were purified, digested with *Apal*, and ligated into the pcRevWT background. All clones were verified for directionality by restriction mapping and confirmed by sequencing.

### Statistical analyses

A two-dimensional chi-square test was used to test the null hypothesis that the frequency distribution of observed variants and the choice of primer pairs was independent. The chi-square statistic was calculated, and the *P* value was determined for each contingency table. The statistical threshold used in this study was 95% confidence. Subset pairwise analyses were also performed on the data, and the results were interpreted with Bon-

ferroni's correction (Miller, 1981). For the Monte Carlo simulation (Rubinstein, 1981) comparing the four primer pairs, the chi-square distance was calculated between the four sets of simulated data. The data were simulated 10,000 times, and experimentally observed chi-square distances were compared with the 10,000 simulated distances to find the percentile ranking, which correlates to the  $P$  value. For the pairwise analyses of primer pairs, 10 variants were randomly created for each set of the two simulated primer pairs. The remainder of the simulation was the same as just described, except the Kullback distance (Bishop *et al.*, 1975) was calculated rather than chi-square distance.

The transition to transversion ratio and the nonsynonymous-to-synonymous mutation ratio were calculated using the program SITES, version 1.1 (Hey and Wakeley, 1997). The Tajima test (Tajima, 1989), which determines whether nucleotide variation within a region was the result of selective pressure or neutral mutations, was also performed using SITES.

The experimental quasispecies Rev activity (QRA) for each time point was calculated as the average Rev activity of tested variants, weighted by frequency of the tested variants, using the following equation:

$$\text{QRA} = \left[ \frac{1}{\sum_{i=1}^n v_i} \right] \sum_{i=1}^n v_i A_i$$

where  $n$  is the number of different variants tested,  $v_i$  is the number of times variant  $i$  was detected at that time point, and  $A_i$  is the Rev activity level for variant  $i$ . A phylogenetic tree was constructed, and untested variants were assigned the same activity as the nearest tested variant in the tree. These values were used to estimate the predicted quasispecies Rev activity in a manner similar to the calculation of the experimentally Rev quasispecies activity. The statistical package SAS, version 6.03 (SAS Institute, 1988), was used to examine possible correlation between the experimental and predicted quasispecies Rev activity and different parameters of clinical disease. Pearson correlation was used to test virus titer, platelet counts, and temperature of the infected pony. A quadratic model was used to fit the relationship between quasispecies Rev activity and temperature, and analysis of the studentized residuals was used to test the quadratic model. Comparison of quasispecies Rev activity between different stages of disease was done using a general linear models procedure, adjusted for multiple comparisons by Tukey-Kramer correction.

### Quantification of virus load

RNA quantification standards were synthesized by *in vitro* transcription from a linearized plasmid containing a 450-base pair fragment of EIAV *gag* (pEIAp26.1). The RNA

was purified by extraction with Trizol reagent, treated with 6 U of DNase I (Ambion) for 45 min at 37°C to remove plasmid DNA, and then extracted again with Trizol reagent. Final RNA preparation was quantified by spectrophotometry, aliquoted, and stored at -80°C. The absence of contaminating plasmid DNA was verified by PCR without reverse transcription.

To create standards, known copy numbers of RNA standards were assayed in triplicate as described below; these included  $2 \times 10^7$ ,  $2 \times 10^6$ ,  $2 \times 10^5$ ,  $2 \times 10^4$ , and  $2 \times 10^3$  copies of RNA. Reaction products were visualized in ethidium bromide-stained agarose gels, and their densities quantified using a commercial digital imaging system (IS1000; Alpha Innotech, San Leandro, CA). The mean density for each copy number was plotted against the  $\log_{10}$  of the copy numbers to construct a standard curve.

The one-tube, semiquantitative RT-PCR protocol was modified from that described by Hamel *et al.* (1995). The reaction buffer contained 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, and 2.0 mM  $\text{MgCl}_2$ , and each reaction contained 0.4 mM deoxyribonucleotide mix (Roche Molecular Biochemicals), 1 U of RNase inhibitor (Roche Molecular Biochemicals), 20 U of Moloney murine leukemia virus reverse transcriptase (SuperScript II; Gibco BRL, Rockville, MD), 1.5 U of *Taq* DNA polymerase (Gibco BRL), and 90 pmol of the forward primer (5'-ACTACTTGGGTGAATACCAT) and 90 pmol of the reverse primer (5'-TCTGCCTAACTGATCAAAA) in a final volume of 25  $\mu\text{l}$ . These oligonucleotides prime both reverse transcription as well as the subsequent PCR reaction, and amplify a 322-base pair segment of the EIAV capsid protein gene (*p26*) (nts 897-1199). Amplification was performed as follows: 42°C for 40 min, DNase I inactivation at 95°C for 3 min, and 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Each RT-PCR run included positive and negative controls. Complete removal of all DNA from samples was confirmed by performing a duplicate reaction without reverse transcriptase. Following amplification, 13  $\mu\text{l}$  of the PCR reaction was analyzed by electrophoresis through 2.0% agarose and visualized by ethidium bromide staining and ultraviolet light. Serum samples from each time point were assayed in duplicate, and their mean density values were used to calculate copy numbers of viral RNA from the standard curve. RT-PCR reactions for standards and samples were performed simultaneously using a reagent-master mix, and were analyzed simultaneously in a single agarose gel.

### CAT assays

293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Transient transfections and CAT assays were performed using 293 cells as previ-

ously described (Belshan *et al.*, 1998). Briefly, 1 µg of either pcDNA3 or Rev variant plasmid was transfected by calcium phosphate coprecipitation with 0.2 µg of pERRE-All reporter plasmid, 0.2 µg of pCH110 (Amersham Pharmacia Biotech, Piscataway, NJ), and 0.6 µg of pUC19. pERRE-All, a derivative of pDM138 containing EIAV nts 5280–7534, was described previously (Belshan *et al.*, 1998). Each experiment also included a sham group, which contained no reporter plasmid, but an additional 0.2 µg of pUC19. Two days posttransfection, cells were harvested, resuspended in 0.3 ml 0.25 M Tris (pH 7.5), lysed by freeze/thawing, and assayed for β-galactosidase activity, to normalize CAT assays for transfection efficiency. Normalized lysates were assayed for CAT activity in a 0.1 ml volume with 3 µl [<sup>14</sup>C]chloramphenicol and 1 mM acetyl CoA. Acetylated products were separated by thin-layer chromatography and the percentage of acetylation was determined by quantification by phosphorimager. Experiments were performed in triplicate and results summarize a minimum of nine independent transfections. Statistical significance was determined by analysis of variance.

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