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# Systematic epitope analysis of the p26 EIAV core protein

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The major core protein of equine infectious anemia virus (EIAV), p26, is one of the primary immunogenic structural proteins during a persistent infection of horses and is highly conserved among antigenically variants of viral isolates. In order to investigate its immune profile in more detail for a better diagnostic, an epitope mapping was carried out by means of two libraries of overlapping peptide fragments prepared by simultaneous and parallel SPPS on derivatized cellulose membranes (SPOT synthesis). Polyclonal equine sera from infected horses were used for the biological assay. Particularly two promising continuous epitopes (NAMRHL and MYACRD) were localized on the C-terminal extreme of p26, region 194–222. A cyclic synthetic fragment of 29 amino acid residues containing the identified epitopes was designed and studied. A significant conformational change towards a helical structure was observed when the peptide was cyclized by a bridge between Cys198 and Cys218. This observation correlated with an improvement of its ability to be recognized by specific antibodies in an [EIA<sup>Q3</sup>](#). These results suggest that the conformationally restricted synthetic antigen adequately mimics the native structure of this region of p26 core protein. Copyright © 2007 John Wiley & Sons, Ltd.

**Keywords:** circular dichroism; conformation; equine anemia virus; p26 core protein; epitope analysis; ELISA; molecular recognition; spot synthesis

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## INTRODUCTION

Lentiviruses are a subfamily of retroviruses that infect a range of mammalian hosts. Examples of lentiviruses for which information on the genomic organization is available and for which aspects of host–virus interaction have been characterized include caprine arthritis-encephalitis virus (CEAV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and equine infectious anemia virus (EIAV). All of the lentiviral infections have several clinical symptoms in common: long

incubation periods, vigorous immune response, and multi-organ disease (Miller *et al.*, 2000).

Most of the sequence homology between highly divergent lentivirus is present in the *gag* and *pol* gene products (Olmsted *et al.*, 1989). EIAV infects horses worldwide and causes a chronic disease characterized by recurrent episodes of fever and viremia, associated with the development of antigenic variants (Payne *et al.*, 1984; Clements *et al.*, 1988; Rwambo *et al.*, 1990). The animals generally survive this disease but remain infected becoming lifelong inapparent carriers (Hammond *et al.*, 1997; Montelaro *et al.*, 1984).

EIAV contains two surface glycoproteins, called gp 90 and gp 45 and four major non-glycosylated internal proteins in the core, known as p26, p15, p11, and p9, consistent with other lentiviruses. Previous studies have shown that all four core proteins are derived from the processing of a single 55 kDa precursor (Montelaro *et al.*, 1984; Hussain *et al.*, 1988). The occurrence of specific structural variations during a persistent infection by EIAV has been found on the surface glycoproteins, gp90 and gp45. In contrast, no structural variation has been observed in the internal viral proteins p15, p26, and p9 from any of the different virus isolates (Montelaro *et al.*, 1984; Salinovich *et al.*, 1986).

The capsid protein (CA) of EIAV, p26, is one of the primary immunogenic structural proteins during a persistent infection and is highly conserved among antigenically

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**Abbreviations used:** AGID, agar gel immunodiffusion; BCIP, 5-bromo-4-chloro-indolylphosphate; BPB, bromophenol blue; CA, capsid protein; CD, circular dichroism; CTD, C-terminal domain; DIC, diisopropylcarbodiimide; DMF, N,N-dimethylformamide; EDT, 1,2-ethanedithiol; EIAV, equine infectious anemia virus; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; HPL, High-performance liquid chromatography; CAN, acetonitrile; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NMM, N-methylmorpholine; NMP, N-methylpyrrolidinone; NTD, N-terminal domain; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TMB, 3,3',5,5'-tetramethylbenzidine; TIS, triisobutylsilane; TFA, trifluoroacetic acid.

variant viral isolates. It shares 30% sequence identity with HIV-1 p24 (Stephens *et al.*, 1986). As in other retroviruses, the CA of EIAV is one of the most abundant proteins in the virus particle and is the predominant viral protein employed in commercial diagnostic tests for the detection of EIAV antibodies in animals. The agar gel immunodiffusion (AGID) test detects precipitating antibodies to the core protein p26 (Coggins *et al.*, 1972). Diagnostic assays for EIAV also include competitive and non-competitive-enzyme-linked immunosorbent assays (ELISA) using expressed EIAV Gag-derived proteins or synthetic antigens from surface glycoproteins (Bürki *et al.*, 1992; Ball *et al.*, 1994; Kong *et al.*, 1997, 1998; Soutullo *et al.*, 2000).

Different immunological cross-reactivity studies between EIAV, HIV-1, and FIV have indicated the presence of conserved interspecies determinants among the lentiviral core proteins, suggesting a similar antigenic organization and three-dimensional structure (Montelaro *et al.*, 1988; Egberink *et al.*, 1990; Grund *et al.*, 1994). Particularly, a 20 residue sequence within the carboxyl-terminal segment of the p26 capsid, designated the major homology region (MHR), which was predicted to contain a B-cell epitope, facilitates protein interactions that result in self-assembly of the Gag precursor.

The only report so far on B-cell epitope mapping of EIAV p26 by means of recombinant *gag* fusion proteins and synthetic peptides focused on the C-terminal domain (CTD) of p26 (158–227). Chong *et al.* (1991) identified an epitope with the sequence: KEPYPEFVDRLLSQI, region 158–172 by means of a monoclonal antibody (Hussain *et al.*, 1988), and three synthetic peptides from regions 202–227, 170–201, and 183–216 were shown to be the most reactive when evaluated by ELISA with sera from natural and experimentally infected horses. The general antigenic organization of EIAV CA protein, p26, includes a minimally immunogenic amino terminus, a moderately immunoreactive central domain, and an immunodominant carboxy terminus (Chong *et al.*, 1991). Nevertheless, the exact localization of continuous epitopes within the whole protein is still unknown.

The presence of multiple epitopes recognized by T helper (Th) and cytotoxic lymphocytes (CTL) in p26 EIAV has been widely documented (McGuire *et al.*, 1994, 2000, 2004; Zhang *et al.*, 1998; Lonning *et al.*, 1999; Fraser *et al.*, 2002, 2003, 2005).

The aim of this work was to identify peptide epitopes for use in peptide-based ELISA diagnostics; we were particularly interested in the major, most antigenic epitopes present in the p26 CTD; in addition, we explored the N-terminal domain (NTD) to find the most abundant antigenic regions. The epitope mapping was carried out using peptide arrays synthesized on cellulose membranes (Frank, 1992, 2002).

Two membranes, one covering the complete p26 sequence and another corresponding to the C-terminal region, were prepared. Using this information several reactive regions containing continuous epitopes could be identified in the two domains (NTD and CTD) of the protein and a synthetic cyclic antigen was designed, evaluated by ELISA and its secondary structure characterized by circular dichroism (CD).

Abbreviations for Amino acids and nomenclature of peptides follow the recommendations of the IUPAC-IUB

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1  PIMIDGAGNRNFRPLTPRGYTTW VNTIQTNGLLNEASON LFGILSVDCDS 50
                                     H1 H2
51  EEMNAFLDVVPGQAGOKOILLDAIDKIDDDNRHPLPNAPLVAPQGP 100
    H3 H4
101 PMTARFIRGLGVPRERQMEPAFDOFROTYROWIIEAMSEGIKVMIGKPKA 150
    H5 H6 H7
151 QNIRQGAKEPYPEFVDRLLSOIKSEGHPOISKFLDTLTIQNANEECRN 200
    H8 H9 H10
201 AMRHLRPEDTLEEKMYACRDIGTTKQKMMLLAKAL 235
    H11

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**Figure 1.** Amino acid sequence of p26 EIAV core protein, according to Stephens *et al.* (1986). Regions of the primary sequence corresponding to helices H1–H11, from the Protein Data Bank file for a recombinant capsid protein (code accession number: 2EIA) are in bold and underlined.

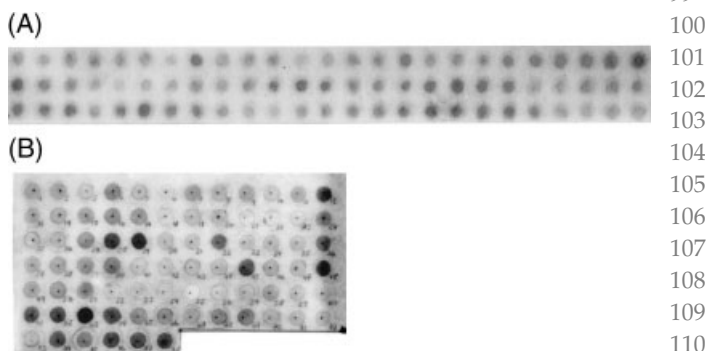
Commission on Biochemical Nomenclature [*Eur. J. Biochem.* **138**: 9–37 (1984)].

## RESULTS

Seventy five overlapping decapentapeptides were synthesized as an array on a cellulose membrane to investigate the regions of p26 from EIAV that were recognized by sera of natural infected horses. Each synthesized peptide sequence consisted of 15 amino acid residues and was offset by three amino acids from the previous peptide. The membrane was also incubated with negative control sera to evaluate non-specific antibody binding.

Figure 1 gives the entire primary sequence of p26 as reported by Stephens *et al.* (1986) where epitopes recognized by a pool of EIAV positive sera have been found to be distributed along the entire length of the sequence. Different assays varying sera dilution (1/100 and 1/200) and reaction times (30 and 40 min) were carried out to optimize signal to background of the different spots of the pentadecapeptide array (see Figure 2a). The sequences of the positive spots in the biological assays are shown in Table 1, and their relative intensities are shown in Figure 3A.

The p26 CA-NTD includes residues 1–148; the most reactive peptide fragments that were found in the



**Figure 2.** (a) pentadecapeptide array of the whole p26 (region 1–235) EIAV-CA protein; (b) hexapeptide array of the C-terminal domain of p26 (region 153–235). All peptides were synthesized directly on a cellulose membrane and incubated with EIAV positive sera diluted 1:100 in PBS buffer. Bound antibodies were detected with alkaline phosphatase (AP)-conjugated anti-horse secondary antibody followed by a color reaction with BCIP and MTT.

**Table 1. Sequences of the reactive spots scanned by pentadecapeptides spanning the complete sequence of p26**

Sequences	Spot no.	p26 (region)	SS(*)
RNFRPLTPRGYTTWV	4	10–24	$\beta$ , H1
TWVNTIQTNGLLNEA	8	22–36	H1, $\beta$
LFGILSVDCSTSEEMN	14	40–54	H2, $\beta$ , H3
ILSVDCSTSEEMNAFL	15	43–57	H2, $\beta$ , H3
VDCSTSEEMNAFLDVV	16	46–60	$\beta$ , H3, $\beta$
EMNAFLDVVPGQAGQ	18	52–66	H3, $\beta$
AFLDVVPGQAGQKQI	19	55–69	H3, $\beta$ , H4
DVVPGQAGQKQILLD	20	58–72	$\beta$ , H4
PGQAGQKQILLDAID	21	61–75	$\beta$ , H4
AGQKQILLDAIDKIA	22	64–78	H4
KQILLDAIDKIADDW	23	67–81	H4
LLDAIDKIADDWNR	24	70–84	H4
AIDKIADDWNRHPL	25	73–87	H4, $\beta$
KIADDWNRHPLPNA	26	76–90	H4, $\beta$
DDWNRHPLPNAPLV	27	79–93	H4, $\beta$
FIRGLVPRERQMEP	36	106–120	$\beta$ , H6, $\beta$
GLGVPRERQMEPAFD	37	109–123	$\beta$ , H6, $\beta$
VPRERQMEPAFDQFR	38	112–126	$\beta$ , H6, $\beta$ , H7
ERQMEPAFDQFRQTY	39	115–129	H6, $\beta$ , H7
AFDQFRQTYRQWII	41	121–135	$\beta$ , H7
QFRQTYRQWIIAMS	42	124–138	H7
QTYRQWIIAMSEGI	43	127–141	H7
RQWIIAMSEGIKVM	44	130–144	H7
IIEAMSEGIKVMIGK	45	133–147	H7, $\beta$
KVMIGKPKAQNIRQG	48	142–156	H7, $\beta$
IGKPKAQNIRQGAKE	49	145–159	$\beta$
PKAQNIRQGAKEPYP	50	148–162	$\beta$
QNIHQGAKEPYEFV	51	151–165	$\beta$ , H8
RQGAKEPYEFVDRL	52	154–168	$\beta$ , H8
AKEPYEFVDRLLSQ	53	157–171	$\beta$ , H8
EFVDRLLSQIKSEGH	55	163–177	H8
DRLLSQIKSEGHQP	56	166–180	H8, $\beta$
LSQIKSEGHQPQISK	57	169–183	H8, $\beta$
IKSEGHQPQISKFLT	58	172–186	$\beta$ , H9
NANEECRNAMRHLRP	65	193–207	$\beta$ , H10, $\beta$
RNAMRHLRPEDTLEE	67	199–213	$\beta$
MRHLRPEDTLEEKMY	68	202–216	$\beta$ , H11
LRPEDTLEEKMYACR	69	205–219	$\beta$ , H11
EDTLEEKMYACRDIG	70	208–222	$\beta$ , H11
LEEKMYACRDIGTTK	71	211–225	$\beta$ , H11, $\beta$

(\*)SS: secondary structure corresponding to each sequence, according to PDB: 2EIA; H (helix),  $\beta$  (beta strand/turn).

proximities of the N-terminus of this domain correspond to spot 4, RNFRPLTPRGYTTWV (region 10–24), spot 8, TWVNTIQTNGLLNEA (region 22–36), and spots 14–16 (region 40–60); spot 16, (VDCSTSEEMNAFLDVV) was the most reactive in the latter region. Within the NTD of p26 primary sequence, other high reactive regions were localized by spots 18–27 (region 52–93), spots 36–39 (region 106–129), and spots 41–45 (region 121–147). According to the spots-image intensities, spot 25, AIDKIADDWNRHPL (region 73–87), 37, GLGVPRERQMEPAFD (region 109–123), and 43, QTYRQWIIAMSEGI, (region 127–141) were the most reactive.

The p26 CA-CTD includes residues 153–235; many high reactive peptide fragments were found in this region by means of the pentadecapeptide array, covering spots 51–53 (region 151–171), 55–58 (region 163–186), 65 (region 193–207), and 67–71 (region 199–225). Spots 48–50 (142–162) overlap the flexible loop that interlinks the NTD and CTD of p26 and were reactive. According to the spots-image intensities spots 51, 53, 56, 67, and 70 were the most reactive (see Figure 3A).

### Hexapeptide array of the C-terminal part of p26 core protein

In order to study more precisely the presence of continuous epitopes within the CTD of p26 (region 153–235), a spot membrane containing a series of overlapping hexapeptides with an offset of only one amino acid residue was prepared. Figure 2b shows the hexapeptide membrane probed with a pool of polyclonal EIAV positive sera and Table 2 gives the corresponding peptide sequences.

According to the spots-image intensities, spots 12, 28–29, 36, 45, 48, and 61–64 were the most reactive. Other spots also considered as positives were 4, 24, 32, 40, 51, 66, and 68–69 (see Figure 3B).

Unspecific, false positive signals were detected on spots 74–78 during the evaluation of the membrane with a pool of negative sera.

The results reveal the localization of continuous epitopes in region 151–193 of p26, defined by spots 4, GAKEPY (156–161); 12, FVDRL (164–169); 24, GHPQE (176–181); 28–29, ISKFL (181–185); 32, FLTDTL (184–189); and 36, TLTIQN (188–193).

Spots 61–64 of the hexapeptide membrane were positives, with spot 63, MYACRD (region 215–220) and 48, NAMRHL (region 200–205) being the most reactive. This clearly defines two continuous epitopes in this region. The high intensity observed in spots 67–70 of the pentadecapeptide library corroborates this finding.

### Design of p26-1 antigen

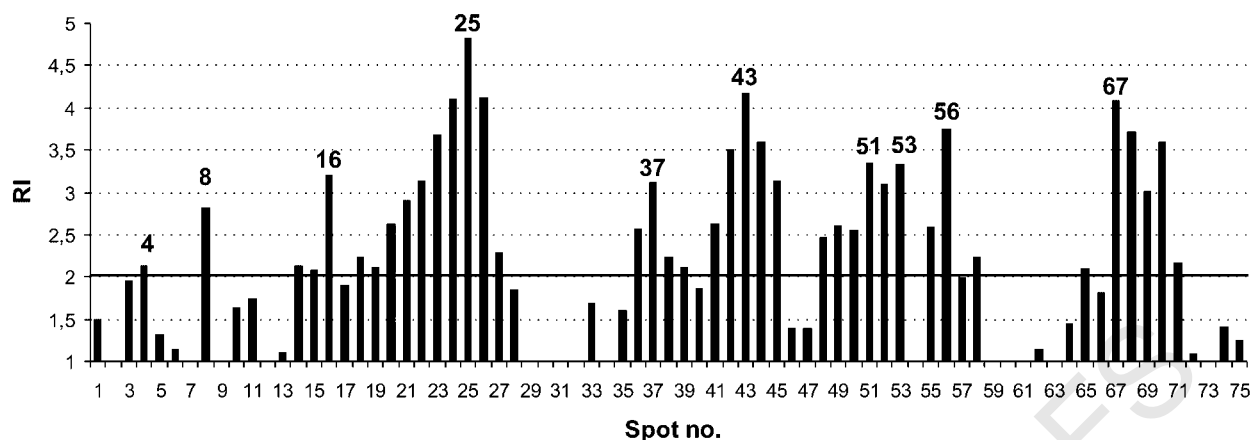
An extended part of the antigenic region covering residues 194–222 of EIAV p26 protein was synthesized in larger quantities as soluble antigen and denoted as p26-1. During high-performance liquid chromatography (HPLC) purification the main peak was collected and the identity of the product confirmed by HPLC-ESI-MS (theoretical MW: 3450.9; ESI-MS: 3449.6). Experimental values of  $M^{+2}$ ,  $M^{+3}$ , and  $M^{+4}$  were detected at  $m/z$ , 1725.8, 1148.3, and 861.7, respectively (Figure 4).

When the immunoreactivity of the linear and cyclic versions of p26-1 was analyzed by ELISA (Figure 5) it was found that the recognition by antibodies was dramatically improved when the peptide was cyclized via the two cysteine residues present in the sequence.

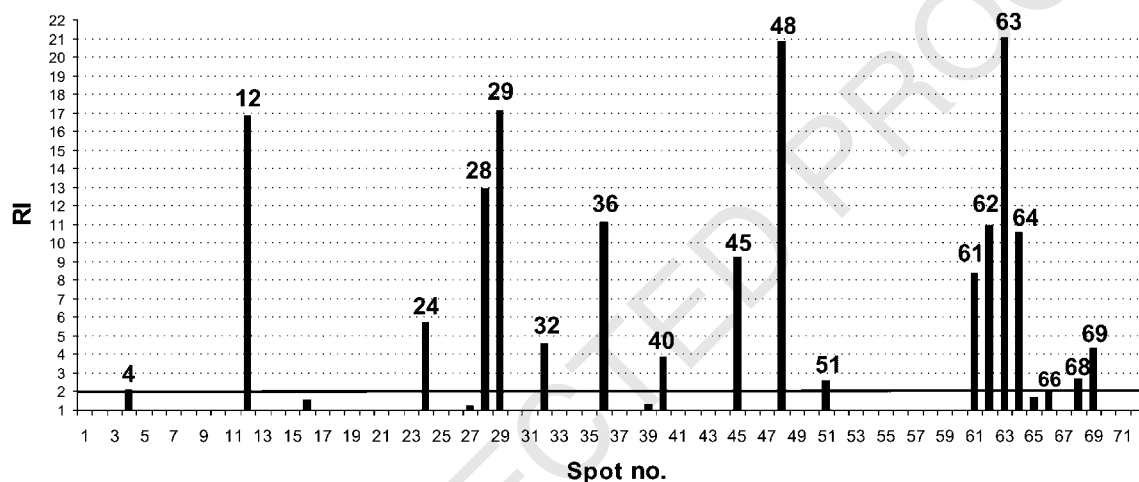
### p26-1 secondary structure

The p26-1 sequence contains a region with high propensity for an  $\alpha$ -helix (EEKMYACRD) according to prediction

(A)



(B)

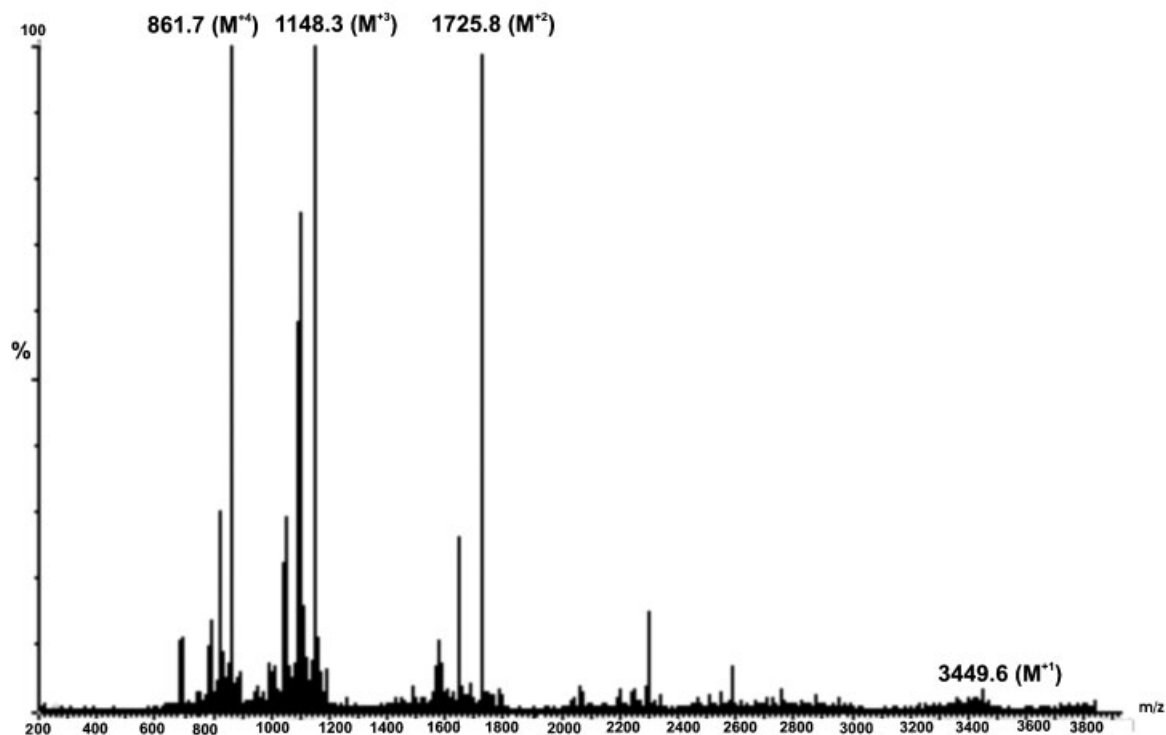


**Figure 3.** Relative Intensity (RI) of spots in the pentadecapeptide (A) and hexapeptide (B) membranes. To evaluate the antibody reactivity shown in Figure 2, the relative intensity of the signal compared to the background was calculated. A signal was scored as reactive when RI was 2 or greater.

**Table 2. Sequences of overlapping hexapeptides spanning regions 153–235 of p26 EIAV-CA**

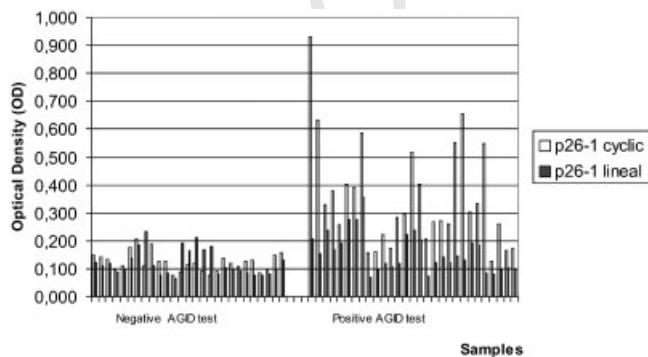
IRQGAKEPYPEFVDRLLSQIKSEGHPQEISKFLTDTLTIQNANEECRNAMRHLRPEDTLEEKMYACRDIGTTKQKMMLLAKAL						
1. IRQGAK	13. VDRLLS	25. HPQEIS	37. LTIQNA	49. AMRHLR	61. <u>EKMYAC</u>	73. KQKMMML
2. RQGAKE	14. DRLLSQ	26. PQEISK	38. TIQNaN	50. MRHLRP	62. <u>KMYACR</u>	74. QKMMLL
3. QGAKEP	15. RLLSQI	27. QEISKF	39. IQNANE	51. <u>RHLRPE</u>	63. <u>MYACRD</u>	75. KMMLLA
4. <u>GAKEPY</u>	16. LLSQIK	28. <u>EISKFL</u>	40. <u>QNAEE</u>	52. HLRPED	64. <u>YACRDI</u>	76. MMLLAK
5. AKEPYP	17. LSQIKS	29. <u>ISKFLT</u>	41. NANEEC	53. LRPEDT	65. ACRDIG	77. MLLAKA
6. KEPYPE	18. SQIKSE	30. SKFLTD	42. ANEECR	54. RPEDTL	66. <u>CRDIGT</u>	78. LLAKAL
7. EPYPEF	19. QIKSEG	31. KFLTDT	43. NEECRN	55. PEDTLE	67. RDIGTT	
8. PYPEFV	20. IKSEGH	32. <u>FLTDTL</u>	44. EECRNA	56. EDTLEE	68. <u>DIGTTK</u>	
9. YPEFVD	21. KSEGHP	33. LTDTLT	45. <u>ECRNAM</u>	57. DTLEEK	69. <u>IGTTKQ</u>	
10. PEFVDR	22. SEGHPQ	34. TDTLTI	46. CRNAMR	58. TLEEKM	70. GTTKQK	
11. EFVDRL	23. EGHPQE	35. DTLTIQ	47. RNAMRH	59. LEEKMY	71. TTKQKM	
12. <u>FVDRLL</u>	24. <u>GHPQEI</u>	36. <u>TLTIQN</u>	48. <u>NAMRHL</u>	60. EEKMYA	72. TKQKMM	

The sequences of all positive spots are underlined.



**Figure 4.** ESI-MS analysis of p26-1 synthetic antigen. Theoretical MW: 3450.9; ESI-MS: 3449.6. Experimental values of  $M^{+2}$ ,  $M^{+3}$ , and  $M^{+4}$  were detected at  $m/z$ , 1725.8, 1148.3, and 861.7, respectively.

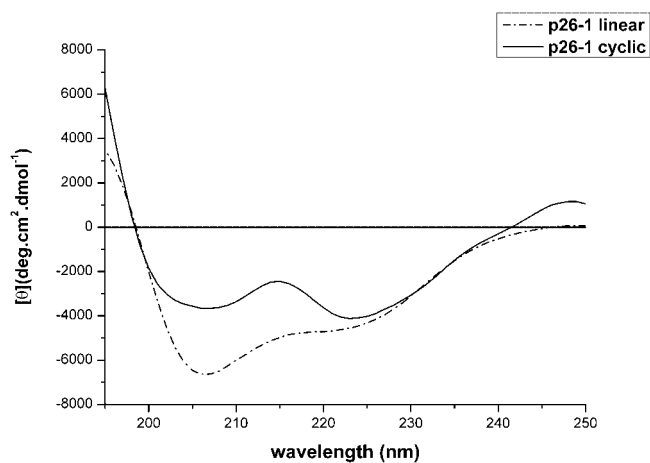
methods and the reported structural data of the p26 protein (PDB: 2EIA). The CD spectra of p26-1 in the linear and cyclic versions are shown in Figure 6. Two minima next to 206 and 223 nm were observed in both cases, typical of proteins spectra that contain an  $\alpha$ -helix in their structures. However, the more intense peak detected at 206 nm in the CD spectra of the linear version can be attributed to the presence of a short helix in the peptide and to contributions of unordered structures. CD deconvolution spectra, using Selcon-2 program, confirmed the increased in the helix content in cyclic p26-1 (10% in the linear form to 60% in the cyclic form).



**Figure 5.** A panel of 29 AGID test positive serum samples were used to evaluate the reactivity of p26-1 synthetic peptide (linear and disulfide cyclic forms) by ELISA. It was clearly distinguished that antibodies recognition was improved when the peptide was cyclized. This figure is available online at [www.interscience.wiley.com/journal/jmr](http://www.interscience.wiley.com/journal/jmr)

## DISCUSSION

Lentiviral core proteins p26 and p24 from EIAV and HIV-1 respectively exhibit a high degree of homology (30% identity) in their primary sequences and CD studies indicate a predominant  $\alpha$ -helical structure (40%  $\alpha$ -helix for p24 and 51%  $\alpha$ -helix for p26) (Hausdorf *et al.*, 1994; Misselwitz *et al.*, 1995; Birkett *et al.*, 1997). Both, HIV-1 and EIAV CAs have a similar structure consisting of two  $\alpha$ -helical domains, the NTD and the CTD interlinked by a flexible loop



**Figure 6.** CD spectra of p26-1 linear (dashed line) and cyclic (solid line) versions. Each spectrum was recorded at 25°C with a JASCO J715 instrument in a cylindrical 0.1cm path quartz cuvet.

(Figure 1). The NTD consists of seven helices (H1–H7) and the CTD is formed by four (H8–H11) bundled  $\alpha$ -helices; the last two (H10 and H11) are linked by a disulfide bond between Cys 198 and Cys218. These Cys residues are conserved in nearly all retroviruses and mutagenesis studies have revealed an essential role of the disulfide bond for HIV-1 infectivity (McDermott *et al.*, 1996).

### Mapping epitopes on EIAV-CA-NTD

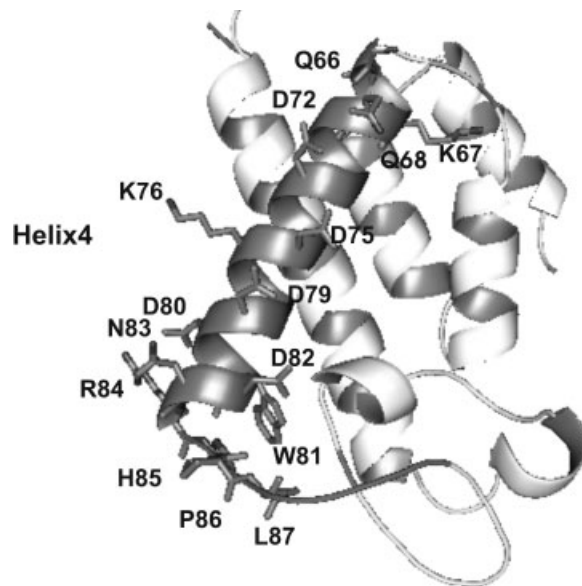
Utilizing a systematic 15 mer peptide scan of the complete p26 sequence, we have found that sera of infected animals recognize different regions in the NTD of p26 that overlap H1, H2, H3, H4, H6, and H7. The biological assays of the pentadecapeptide membrane suggest the existence of two sequences containing epitopes close to the N terminus of the protein (regions 10–24 and 22–36) that overlap H1 (region 20–29, YTTWVNTIQT) and include the surface-exposed residues T21, T22, N25, T26, Q28, and T29. The localization of an epitope in HIV-1 p24 (region 11–25) (VHQAI SPRTLNAWVK), where residues P17, R18, W23, and V24 are identical in both EIAV and HIV-1 CAs has been reported (Ferns *et al.*, 1987, 1989; Franke *et al.*, 1992). Another reactive sequence was found near the N-terminus of the protein, centered in spot 16, region 46–60 (VDCTSEEMNAFLDVV) and formed by residues of the  $\beta$ -turn that connects the two adjacent helices (H2 and H3) and covering H3. The localization of an epitope in HIV-1 p24, region 44–60 that overlaps spot 16 of our p26 pentadecapeptide membrane has been reported (Janvier *et al.*, 1990; Truong *et al.*, 1997). On the other hand, different studies have shown that peripheral blood mononuclear cells (PBMC) from infected horses reacts with overlapping synthetic peptides from p26 amino terminus (Zhang *et al.*, 1998; Lonning *et al.*, 1999).

An extended reactive region was found in the EIAV-CA-NTD from residues 52–93. This region includes H3, H4 and partially overlaps the turn connecting H4 and H5. The results suggest the presence of an epitope centered in spot 25, region 73–87 (AIDKIADDWNRHPL). The high relative intensity (RI) of spots 20 and 21 also indicates the presence of an epitope close to the N-terminal end of Helix 4 (see Figure 7).

In HIV-1-CA-NTD, the presence of a relevant epitope within H4 (region 77–85, EAADWDRHLH) has also been reported (Niedrig *et al.*, 1989; Hinkula *et al.*, 1990). Sequences containing this epitope have been used for HIV-1 diagnostic purposes (Tonarelli *et al.*, 2000; Lottersberger *et al.*, 2003, 2004).

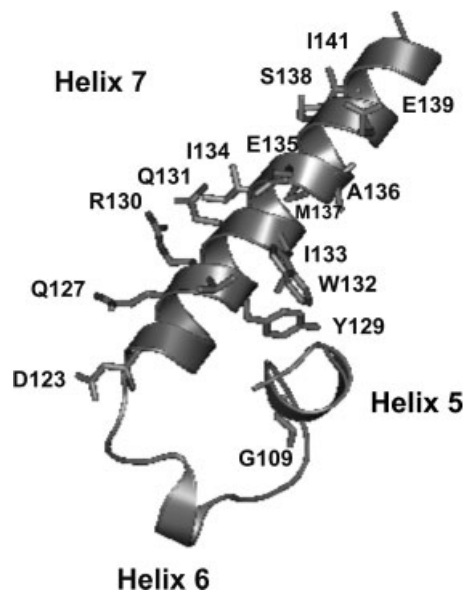
Spots 36–38 of the decapentapeptide membrane cover an exposed and unordered region of p26 which includes amino acid residues of the short H6 and the turns that connect H6 to H5 and H7; Spot 37 (region 109–123), GLGVPRERQMEPAFD was the most reactive and the results suggest the presence of an epitope in this region. The identification of a Th 1 lymphocyte epitope in region 97–121 of p26 was recently reported (Fraser *et al.*, 2002).

Region 123–144 of p26 from EIAV corresponds to a large amphipathic helix named H7 (DQFRQTYRQWIIAMSEGIGIKVM) which was covered by spots 41–45 of the pentadecapeptide membrane. Analyzing the sequences of



**Figure 7.** Overlapping decapentapeptides from spots 23–26 define an epitope centered in region 73–87 (AIDKIADDWNRHPL) that partially covers Helix 4 and the turn that connects Helix 4 to Helix 5. The side chains of the hydrophilic residues and of W81 and L87 are shown as sticks. Another epitope was found in region 60–72 (PGQAGQKQILLD) defined by the turn that connects Helices 3 and 4, and by amino acid residues of the N-terminal end of Helix 4; the side chains of residues Q66, K67, Q68, and D72 are shown as sticks.

the overlapping peptides, an epitope centered in region 127–141 (QTYRQWIIAMSEGI) of p26 can be defined, see Figure 8. This correlates with results found by mapping p24-HIV-1, where an epitope in region 120–134 (NPPIPV-



**Figure 8.** Spots 36–38 of the decapentapeptide membrane cover an exposed and unordered region of p26 which includes amino acid residues of the short H6 and of the turns that connect H6 to H5 and H7, defining an epitope in region 109–123 (GLGVPRERQMEPAFD). The overlapping pentadecapeptides of spots 41–45 define an epitope centered in region 127–141 (QTYRQWIIAMSEGI) which covers partially H7. The side chains of the hydrophilic and hydrophobic residues are shown as sticks.

GEIYKRWII) was identified (Ferns *et al.*, 1987, 1989), with W132, I133, and I134 conserved in both CAs. Additionally, a peptide that includes region 130–149 of p26 has been reported to cause proliferation of PBMC from EIAV-infected horses, and a Th 1 lymphocyte epitope was identified in region 118–137 (Zhang *et al.*, 1998; Lonning *et al.*, 1999).

### Mapping the C-terminal domain of EIAV CA protein, p26

The MHR of HIV type 1 (HIV-1), residues 153–174, is remarkably conserved among retrovirus and is essential for viral assembly, maturation, and infectivity (Mammano *et al.*, 1994).

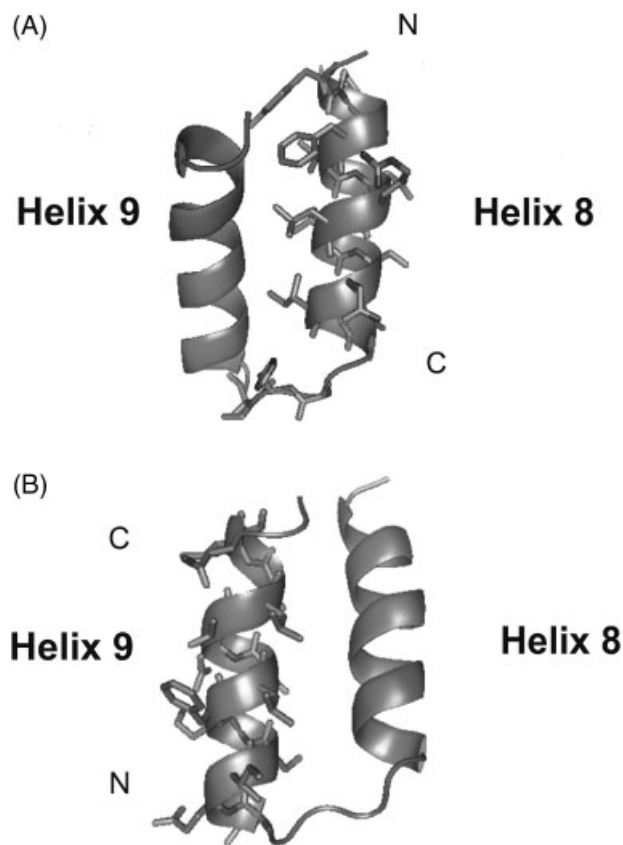
HIV-1-MHR forms a strand-turn-helix motif which does not participate directly in the dimerization interface, but may contribute indirectly to HIV-1 CA-C dimerization (Gamble *et al.*, 1997). Residues 153–160 of EIAV-MHR form part of the flexible loop that interlinks the N- and C-terminal domains of p26, and residues 161–174 define an amphipathic  $\alpha$ -helix, named H8. Spot 51 (QNIRQGAKEPY-PEFV) of the pentadecapeptide membrane overlaps the flexible loop that connects both domains and its reactivity suggests the presence of an epitope, which was also identified as GAKEPY (156–161) by the hexapeptide membrane (spot 4).

Signals from spots 52, 53, and 56–58 of the pentadecapeptide membrane indicate the presence of epitopes localized in H8 and in the  $\beta$  turn that connects H8 with H9, (see Figure 9a). These can also be correlated with the results from the hexapeptide membrane showing signals for FVDRL (spot 12; 164–169) and GHPQEI (spot 24; 176–181). These results correlate with the findings of Chong *et al.* (1991) who identified an epitope in region 158–172 (KEPYEFVDRLLSQI) of EIAV p26 core protein also using synthetic peptides.

Remarkably, for the HIV-1 p24 CA protein, the presence of B cell epitopes within MHR, regions 153–172 (IRQGPKEPFRDYVDRFYKTL), 161–170 (FRDYVDRFYK), and the successful design of synthetic antigens have been reported (Hinkula *et al.*, 1990; Robert-Hebmann *et al.*, 1992a, 1992b; Lottersberger *et al.*, 2003). This highly conserved region also contains CTL and Th lymphocyte epitopes (Nakamura *et al.*, 1997).

Homodimerization of the CA of HIV through its CTD constitutes a key step in virion assembly and a potential target for the design of antiviral inhibitors. The dimerization interface is formed by the packing of H9 from each subunit (Mateu, 2002; Garzon *et al.*, 2004). It has been demonstrated that the majority of the energetically critical residues at the interface are non-polar and almost conserved in HIV and SIV (de Alamo *et al.*, 2003). NTD and CTD homodimer interactions appear to be also involved in the assembly of EIAV CA; NTD–NTD associations form an antiparallel four-helix bundle dimer interface by H2 and H7 packing; CTD–CTD interactions form a dimeric interface through the packing of H9, that is similar to that proposed for HIV-1 CA dimers (Jin *et al.*, 1999).

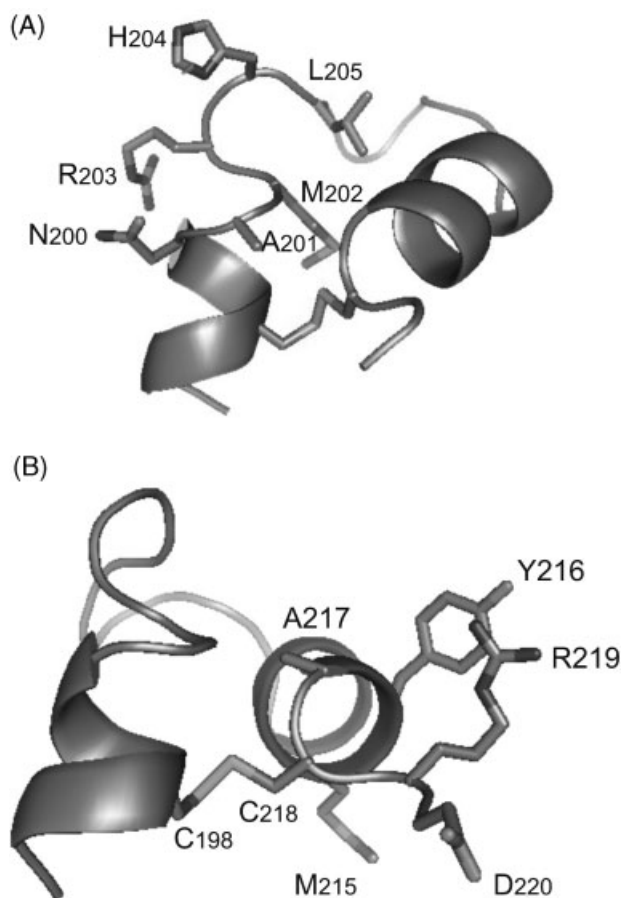
EIAV and HIV-1 CTD-dimerization sites (H9) are characterized by the presence of identical (Q179, E180, T186, T188, L189, Q192) and other chemically similar



**Figure 9.** (a) Residues 161–174 of EIAV-CA constitute the amphipathic Helix 8. The hydrophilic face is formed by residues P162, E163, D166, R167, S170, Q171, and Lys173 and the hydrophobic one by Y161, F164, V165, L168, L169, and I172. One epitope was identified within Helix 8: FVDRL (164–169). Another epitope was localized in the  $\beta$  turn that connects H8 and H9, GHPQEI (176–181); E180 and I181 are included into the first turn of Helix 9. The side chains are indicated as sticks. (b) The fine analysis by the hexapeptide membrane demonstrates the presence of surface-exposed epitopes in H9 of EIAV-CA protein, corresponding to sequences ISKFL (181–185), FLTDTL (184–189), and TLTIQN (188–193). The hydrophobic face of H9 formed by I181, F184, L185, and L189 is clearly shown. This figure is available online at [www.interscience.wiley.com/journal/jmr](http://www.interscience.wiley.com/journal/jmr)

residues. The major difference appears in residues 184 and 185 (FL for EIAV and WM for HIV-1). The fine analysis by the hexapeptide membrane demonstrates the presence of surface-exposed epitopes in H9 of EIAV-CA protein, corresponding to sequences ISKFL (181–185; spots 28–29, overlapping sequence), FLTDTL (183–188; spot 32), and TLTIQN (188–193; spot 36), see Figure 9b.

Helix 9 of EIAV-CTD (region 179–192) was covered by spots 58–63 of the decapentapeptide membrane. It is interesting to remark that during the biological evaluation of the decapentapeptide membrane it was not possible to detect any reactivity in spots 59–63, suggesting that the peptides may self-associate while being immobilized on the cellulose membrane. Correspondingly, reported works about epitope mapping of the HIV-1 gag protein with monoclonal antibodies identified immunogenic epitopes localized in region 181–190 (VKNWMTETLL) and also in the more extended region 176–190 (QASQEVKNWMTETLL) (Hinkula *et al.*, 1990; Niedrig *et al.*, 1991).



**Figure 10.** Two continuous epitopes close to the C-terminus of p26 were identified by the hexapeptide membrane, in regions 200–205 (NAMRHL) ( $\beta$ -turn) (a) and 215–220 (MYACRD) (b), which covers partially H11. The side chains of these amino acid residues are shown by sticks. The disulphide bridge between C198 and C218 is marked. This figure is available online at [www.interscience.wiley.com/journal/jmr](http://www.interscience.wiley.com/journal/jmr)

The C-terminus of p26 includes H10, H11, and a series of  $\beta$  turns that connect both helices. The hexapeptide membrane revealed the strongest signals in this part of p26: spot 48 (200–205; NAMRHL) ( $\beta$ -turn) and spot 63 (215–220; MYACRD) which partially covers H11 (see Figure 10). This region was chosen to prepare a synthetic antigen in larger scale for the development of an ELISA. The sequence of p26-1 synthetic antigen covers region 194–222 and contains these two continuous epitopes and the two cysteine residues Cys198 and Cys218. These cysteins are also conserved in nearly all retroviruses. Mutagenesis of these residues to Ser indicated an essential role of the disulfide-bonded Cys for HIV-1 infectivity (McDermott *et al.*, 1996). Additionally, it has been proposed that the formation of this disulfide bond may facilitate CA oligomerization (Worthylake *et al.*, 1999).

In addition to the localization of B lymphocyte epitopes in p26 CTD, it is important to highlight that the presence of Th lymphocyte epitopes and a CTL (cytotoxic T lymphocyte) epitope in regions 154–173 and 214–235 of p26 has also been reported (Zhang *et al.*, 1998; Lonning *et al.*, 1999).

A number of immunogenic and antigenic peptides have been shown to have conformational preferences for

structured forms. By using mainly NMR and CD spectroscopy, it has been possible to detect and quantify populations of beta-turn, helical and nascent helical conformations indicating that the presence of structured forms is correlated with the location of T cell and/or B cell epitopes in peptide sequences (Precheur *et al.*, 1994; Dyson and Wright, 1995). We have previously analyzed the conformational mobility of p26-1 antigen by molecular dynamics with GROMACS program (Garay *et al.*, 2001). It was shown that the helical structure remained stable during the dynamics simulation. The experimental secondary structure found by CD analyses agrees with that found by calculations. The stabilization of the helical structure in the cyclized version of p26-1 also improved the ability of the cyclic peptide to be recognized by antibodies in ELISA by more than two orders of magnitude in some serum samples (Figure 5). These results suggest that the conformationally restricted synthetic peptide adequately mimics the native structure of this portion of p26 core protein.

In this work, we have identified three regions of different immunoreactivities in p26 EIAV CA protein, amino terminus (1–60) being with less antigenic than the central part (61–150) and the CTD (153–235). The results of this study could allow the design of new antigens containing the identified epitopes. The potential application of p26-1 antigen for EIAV diagnosis by ELISA or by fluorescence polarization-based assays (Tencza *et al.*, 2000) should be explored more deeply by testing immune sera from EIAV-infected horses from other countries, or by means of the USDA reference serum set.

## CONCLUSIONS

Although there have been several earlier reports of antibodies binding to some regions of intact p26 or its peptide fragments, a systematic epitope mapping of the entire primary sequence of p26 has not been previously reported. The identification of conserved structural and functional motifs made in this work may provide foundation for the development of more sensible and specific diagnostic assays for EIAV infection and for vaccines design.

## MATERIALS AND METHODS

### Peptide arrays

Peptides were simultaneously synthesized by the SPOT-method (Frank, 1992) on derivatized cellulose membranes with  $\beta$ -Ala- $\beta$ -Ala linker, for the preparation of immobilized peptides. Assembly of the peptides was carried out utilizing 9-fluorenylmethoxycarbonyl (Fmoc) chemistry essentially as described (Frank and Overwin, 1996). The activation of 0.2M solutions of protected amino acids derivatives dissolved in *N*-methylpyrrolidinone (NMP) was carried out *in situ* by DICD to form the 1-hydroxybenzotriazole esters. Bromophenol blue (BPB) was used to monitor the coupling reaction procedure by color change from blue to yellow (Krcnák *et al.*, 1988). Finally N-terminal acetylated sequences were deprotected with 50% trifluoroacetic acid

(TFA) in dichlorometane with 3% triisobutylsilane (TIS) and 2% water as scavengers for two times 1 h.

Two membranes were prepared, one consisting in overlapping pentadecapeptides spanning the complete sequence of p26 (residues 1–235), with an offset of three amino acid residues and the other was a hexapeptide array with an offset of one amino acid residue, spanning the C-terminal part of p26, region 153–235.

**Antibody-binding assay.** Equine serum samples were collected from blood of naturally infected and uninfected horses from ranches in the province of Santa Fe, Argentina. Pools of either five positive or five negative polyclonal sera were used to analyze the membranes. These serum samples were selected from a panel constituted by 29 positive and 29 negative sera, previously characterized by the AGID test (Coggins *et al.*, 1972) and by ELISAs designed with synthetic-peptide antigens from gp90, gp45, and p26 proteins, following reported protocols (Soutullo *et al.*, 2000). Sera dilutions of 1/100 and 1/200 were used in the membrane antibody-binding assays. An alkaline phosphatase (AP)-conjugated secondary antibody was used for detection of bound Ab, and was visualized by a color reaction with 5-bromo-4-chloro-indolylphosphate ((BCIP)) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).

### Manual peptide synthesis

A partial sequence close to the C-terminal part of EIAV p26 core protein (region 194–222) was chemically synthesized (ANEECRNAMRHLRPEDTLEEKMYACRDIG) and denoted as p26-1.

It was prepared by the solid-phase method following Fmoc chemistry. Rink amide resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-resin) was used to prepare the C-terminal peptide amide. Couplings were performed by benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*-methylmorpholine (NMM) was used as catalysts; deblockings were achieved by 20% piperidine/*N,N*-dimethylformamide (DMF) (v/v). Ninhydrin colorimetric assay (Kaiser *et al.*, 1970) and the spectrometric measurement of piperidine-Fmoc adduct were used to monitor the coupling and deprotection steps. The following protected derivatives were used for three functionalized amino acids: Fmoc-Cys(Trt)-OH, Fmoc-Tyr(tBut)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBut)-OH, Thr(tBut)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, and Fmoc-Lys(Boc)-OH (Calbiochem-Novabiochem Corp., California, USA). Final cleavage from the resin was done by a mixture of TFA/H<sub>2</sub>O/EDT (1,2-ethanedithiol)/TIS (94:2.5:2.5:1). The cyclization between Cys198 and Cys 218 was done by air oxidation; a solution containing 0.1 mg/ml of p26-1 in an ammonium bicarbonate buffer, pH 7.9 was used. The reaction was monitored by the Ellman test (Ellman, 1959) and HPLC. It was completed in 9.5 h.

Linear and cyclic versions of p26-1 were purified by reverse phase HPLC (Gilson, France), using a Vydac 218TP1010 (C18, 10 μm, 10 × 250 mm) column with a

gradient from 20 to 40% acetonitrile (ACN) with 0.1% TFA over 30 min. The synthetic peptide was analyzed by analytical RP-HPLC and ESI-MS.

### Enzyme-linked immunosorbent assay

Comparison of the immunoreactivity of linear and cyclic versions of p26-1 was done by indirect ELISA, employing equine serum samples, collected from blood of naturally infected and uninfected horses, checked first by AGID Test and ELISA, as mentioned.

For that, peptides were dissolved in a 0.05 M carbonate/bicarbonate buffer (pH 9.6) and adsorbed to the wells of a microtiter plate (COSTAR 2580). Best results were obtained with a peptide concentration of 10 μg/ml, and 100 μl of this solution was added to each well.

The antigen-coated plates were incubated with 100 μl of serum sample, diluted 1/200 in PBS-T, for 1 h at 37°C. After washing three times with PBS-T, HRP-conjugated anti-horse immunoglobulin IgG (Sigma) diluted in PBS-T, was added and incubated for 30 min at 37°C. The unbound antibody was removed by washing and the reaction was developed by adding a solution of 3,3',5,5'-tetramethylbenzidine (TMB) (1 mg/ml) in a 0.05 M citrate buffer (pH 4.0) with 0.05% (v/v) H<sub>2</sub>O<sub>2</sub>. Color development was stopped by the addition of 2.5 M H<sub>2</sub>SO<sub>4</sub>. Absorbance measurements were made at 450 nm using a Multiskan Automatic ELISA Plate Reader (Labsystem).

### Far-UV circular dichroism measurements

Far-UV CD measurements were performed using 0.15–0.8 mg/ml, peptide solutions in ultra-pure water (Milli Q). Each spectrum was recorded at 25°C with a JASCO J715 instrument (JASCO Corporation, Japan) in a cylindrical 0.1 cm path quartz cuvet and represents the accumulation of 16 runs. Data were reported in millidegrees and converted to molar ellipticity (θ).

### Secondary structure estimation

Analyses of the secondary structure were performed using the Self Consistent Method (SELCON) developed by Sreerama and Woody (1993), with a root mean square (RMS) less than 1% for all deconvolutions.

Figures in this work were produced with DeLano Scientific Software, PyMOL, version 0.97, 2004 (DeLano, 2004), and structural data were obtained from the Protein Data Bank file for p26-EIAV. Code accession number: 2EIA.

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