Genetic and Amino Acid Analysis of the G_L Protein of Canadian, American and European Equine Arteritis Virus Isolates

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ABSTRACT

The genetic variation in equine arteritis virus (EAV) G_L protein encoding gene was investigated. Nucleic and deduced amino acid sequences from 7 different EAV isolates, including 4 eastern Canadian field isolates, were compared with those of the Bucyrus reference strain. Nucleotide sequence identities between these isolates and the Bucyrus reference strain ranged from 87.5% (Vienna isolate) to 93.9% (11958 isolate). Amino acid identities with the Bucyrus reference strain varied from 90.2% (Vienna isolate) to 95.1% (19933 isolate). A 2nd potential N-linked glycosylation site was found at position 81 in the G_L protein of all EAV isolates. Three amino acid substitutions at residue position 90 (Glu→Val), position 101 (Ala→Val or Thr), and position 119 (Val→Leu, Phe or Ser) were also found in all EAV isolates. Phylogenetic analysis showed that the North American EAV isolates, including the Canadian isolates, and the European prototype Vienna isolate could be classified in 2 distinct groups. Three putative sequential antigenic sites were predicted in EAV G_L protein. The 1st antigenic site (TAQRFT) was located at positions 24 to 29, and the 2nd antigenic site (RYDEHTA) at positions 47 to 53. The 3rd antigenic site was predicted to be located at positions 78 to 84 and showed the less conserved amino acid sequence.

RÉSUMÉ

Dans cette étude, les séquences d'acides nucléiques et d'acides aminés (AA) prédites de la protéine G_L de 7 isolats du virus de l'artérite équine (VAE), incluant 4 isolats canadiens, furent comparées à celles de la souche de référence Bucyrus. Les niveaux d'identité des bases nucléotidiques et des acides aminés ont varié de 87.5 (isolat Vienne) à 93.9 % (isolat 11958) et de 90.2 (isolat Vienne) à 95.1 % (isolat 19933), respectivement. Un deuxième site de N-glycosylation fut trouvé à la position 81 de la protéine G_L chez tous les isolats de VAE. Trois substitutions au niveau des AA aux positions 90 (Glu→Val), 101 (Ala→Leu ou Thr) et 119 (Val→Leu, Phe ou Ser) furent relevées chez tous les isolats de VAE. Une analyse phylogénétique a indiqué que les isolats nord-américains, incluant ceux isolés du Canada, et l'isolat européen Vienne pourraient être classifiés en 2 groupes distincts. Trois sites antigéniques séquentiels ont été prédits chez la protéine G_L. Le premier site antigénique (TAQRFT) fut localisé aux positions 24 à 29 et le deuxième (RYDEHTA), aux positions 47 à 53. Le troisième site antigénique qui était le moins conservé fut localisé aux positions 78 à 84.

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Equine arteritis virus (EAV), the causative agent of equine viral arteritis, was first isolated in Bucyrus, Ohio in 1953, during an abortion epidemic episode (1). EAV belongs to the recently described arterivirus group (2). The EAV genome is a polyadenylated positive single-stranded RNA of 12.7 Kb in length. Eight open reading frames (ORFs) have been identified (3). ORFs 1a/1b encode the viral polymerase (4), while ORFs 2 and 5 encode for the glycosylated 25-kDa (G_S) membrane protein and the heterogeneously glycosylated 30-to 42-kDa (G_L) large membrane protein, respectively (3,4). ORF 6 encodes for an unglycosylated membrane (M) protein of 16-kDa, and ORF 7 encodes for a 14-kDa nucleocapsid (N) protein (3,5). The ORF 3 and 4-encoded products are believed to be glycosylated nonstructural proteins (3,5).

EAV is present in horses throughout the world. Some EAV strains have been shown to exhibit different levels of pathogenicity (6–8), thereby suggesting genetic variation between EAV isolates. Indeed, genetic variation has been demonstrated by RNA oligonucleotide fingerprint analysis (9). Although it is believed that there is only one EAV serotype, minor antigenic differences among various EAV isolates have been reported (10,11). A neutralization epitope has been defined with monoclonal antibodies within the hydrophilic part of the G_L protein, and has been shown to be located from amino acid residues 19 to 115 (12,13). This region was believed to encompass overlapping or closely adjacent epitopes. Further studies have located two G_L neutralization domains between amino acid residues 55 to 104 (14–16).

Although progress has been made in recent years, the molecular basis for the biology of EAV has yet to be determined. Most information has been obtained using American and European EAV isolates. In this report, the reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify nucleic acid sequences of the G_L protein encoding gene of 4 eastern Canadian EAV field isolates for comparison with American and European EAV isolates, including the prototype Bucyrus reference strain. The deduced amino acid sequences of the G_L protein for the different isolates

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were compared and used to establish a phylogenetic relationship between the EAV isolates. The putative sequential antigenic sites within the G_L protein were also predicted.

Each EAV isolate (Table 1) was plaque-purified, propagated in rabbit kidney (RK-13) or VERO cells and purified by ultracentrifugation (17). Virion RNA was extracted from purified virus particles using the guanidium isothiocyanate method (18). The sense and antisense primers specific for nucleotides encoding the heterogeneously glycosylated 30 to 42-kDa (G_L) protein were 5′-ATGTTATCTATGATTGTATTG-3′, and 5′-ATGAATCTATGGCTCCCA-3′, respectively. These primers were selected according to the cDNA sequence of the EAV Bucyrus strain genome (3). RT-PCR assays were carried out as described previously (17). The resulting amplified cDNA fragment derived from the translation product of ORF 5 (with an expected size product of 768 bp) of each EAV isolate was cloned into PCR II TA cloning vector (Invitrogen, San Diego, California, USA) (17) and sequenced using the Sanger dideoxynucleotide chain termination method (19) with the T7 Sequencing Kit (Pharmacia, Uppsala, Sweden). Two or more independant cDNA clones were sequenced from RT-PCR products for each EAV isolate. Comparison and multiple alignments of nucleic and deduced amino acid sequences were performed using the GCG sequence analysis software within the VAX (20). Potential transmembrane segments were determined by scanning procedures using a window of 21 as described by Engelman et al (21). The probability for a region to be antigenic (sequential epitope) was evaluated by the method described by Jameson and Wolf (22). The antigenic index value of each predicted antigenic site was determined by the sum of the antigenic index values for each amino acid divided by the number of amino acids present within the predicted site (23).

The percentages of identity for nucleic and deduced amino acid sequences between each isolate and the prototype Bucyrus reference strain (3) were established (Table 2). The levels of nucleic acid identity between the Canadian isolates and the prototype Bucyrus reference strain ranged from 91.3 (T1329) to 93.9% (11958). Similar percentages of identity ranging from 90.3 to 96.2% were observed when the Canadian isolates were compared to the American EAV isolates 84KY-A1 and 86NY-A1. However, lower percentages of identity ranging from 85 to 87.5% were observed when Canadian isolates were compared to the European Vienna isolate. When the Canadian isolates were compared to each other, the T1329 and 19933 isolates showed the lowest level of identity of 90.9%. Nucleotide sequence identities of the Vienna, 84KY-A1 and 86NY-A1 isolates with the Bucyrus reference strain were 87.5, 92.8 and 91.3%, respectively. The 84KY-A1 and 86NY-A1 isolates were the most closely related to each other with nucleic acid identity of 96.6%, whereas 15492 and Vienna isolates displayed the lowest level of identity of 85%. The 86NY-A1 and T1329 isolates were found to share the same percentage of nucleotide identity (91.3%) with the Bucyrus reference strain. These 2 field isolates were closely related to each other with 95.2% identity.

No insertions or deletions of nucleic acids that might have accounted for differences in pathogenicity were found in the GL protein encoding gene for any of the EAV isolates analyzed. However, a total of 422 nucleic acid substitutions were observed. Only 35.5% of all base substitutions observed in the G_L protein encoding gene lead to amino acid changes. Most of the base substitutions observed were randomly distributed and silent, occuring mainly at wobble base. Spontaneous mutation rates of several polymerases with an average of about 10^{-4} to 10^{-5} base substitution per single base site have been frequently observed in RNA viruses (24). Such a high mutation rate may lead to a rapid evolution of RNA viruses and development of the quasispecies model (25), which describes the heterogeneity and population dynamics of viruses. This genetic plasticity of RNA viruses in combination with selective pressure by the immune response may result in the emergence of new viral phenotypes that might be associated with altered pathogenicity.

There was a high level of amino acid sequence conservation in the G_L protein between the EAV isolates, despite the high number (422) of nucleic acid substitutions observed. The most highly conserved region of the G_L protein was located in the N-terminal ectodomain of the protein from amino acid positions 15 to 60. Amino acid identities of the Canadian field isolates with the Bucyrus reference strain varied from 91.8 to 95.1% (Table 2). The overall data showed the Vienna isolate and the T1329 Canadian isolate to have the lowest levels of identity of 90.2 and 91.8% with the Bucyrus strain, respectively, whereas amino acid identities of all other isolates varied from 92.7 (86NY-A1 isolate) to 95.1% (19933 isolate). When all EAV isolates were compared to each other, the highest percentage of identity (96.7%) was observed between 84KY-A1 and 86NY-A1 isolates, with the lowest (89.4%) between T1329 and Vienna isolates. Analysis of the deduced amino acid sequence alignment (Fig. 1) of all EAV isolates revealed 2 major variable regions. These regions encompass residue positions 61 to 104 predicted to be located in the ectodomain of the protein, and residue positions 141 to 180 predicted to be located in the potential transmembrane segment (amino acid residues 115 to 200) of the G_L protein. The putative N-linked glycosylation site in

TABLE I. Characteristics of the equine arteritis virus (EAV) isolates used in this study

Isolate	Origin (Year of isolation)	Source	Passage history
T1329	Ontario, Canada (1988)	Neonatal lung[a]	RK, P5[b]
19933	Ontario, Canada (1992)	Semen	RK, P5
11958	Ontario, Canada (1990)	Semen	RK, P5
15492	Ontario, Canada (1991)	Semen	RK, P5
Vienna	Vienna, Austria (1968)	Nasal swab	ED, P1/RK, P2
84KY-A1	Kentucky, USA (1984)	Nasal swab	RK, P5
86NY-A1	New York, USA (1986)	Semen	ED, P4/V, P2

[a] EAV was isolated from 5 d old standardbred foal
[b] Cells: RK: rabbit kidney-13, ED: equine dermis, V: Vero, P: refers to passage number

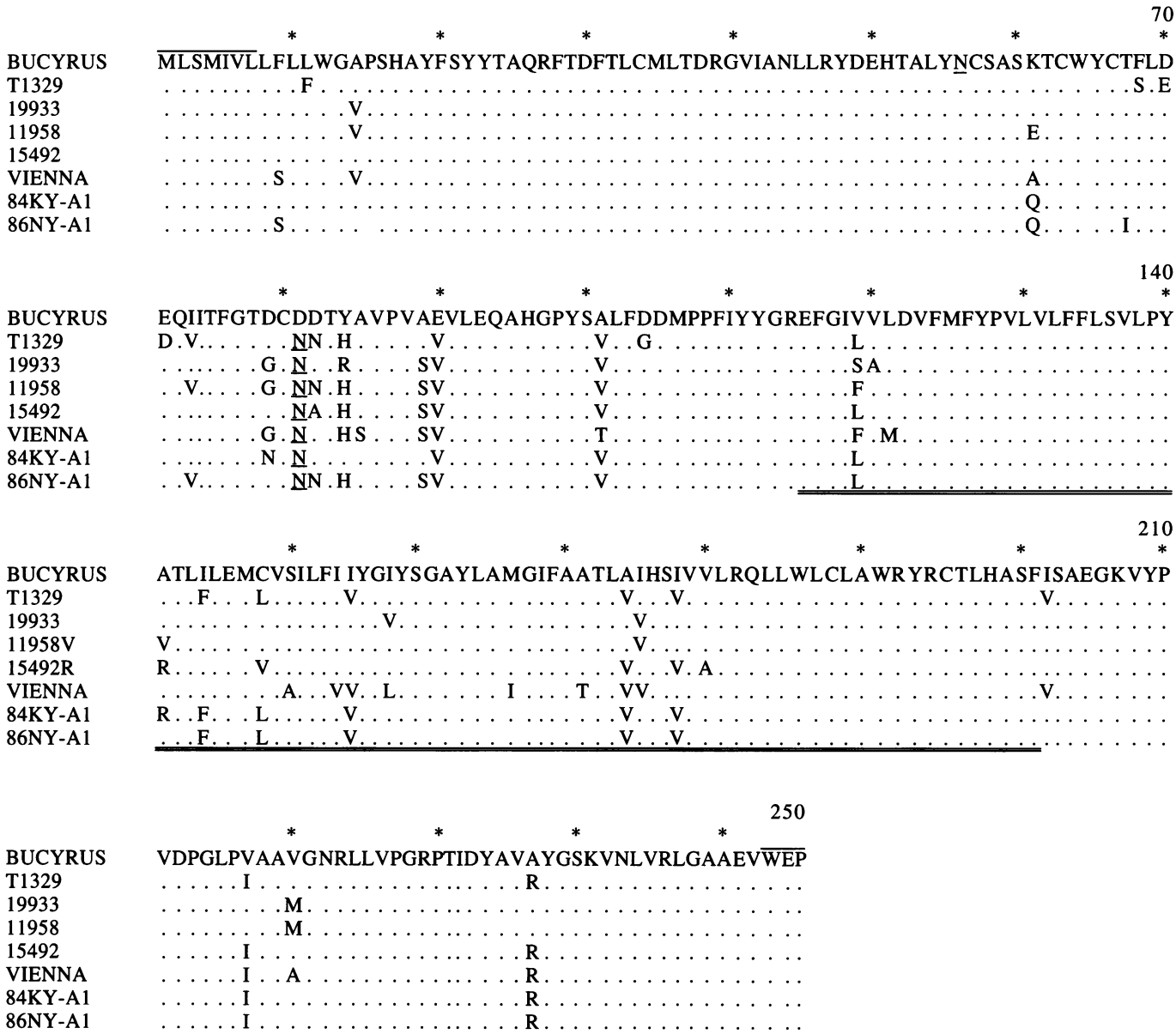

Figure 1.Alignment of deduced amino acid sequences of the G_L protein of 7 equine arteritis virus (EAV) isolates with the Bucyrus reference strain. Potential N-linked glycosylation sites are underlined. Potential transmembrane segment is double underlined. Overlined amino acid residues in all isolates except for the Bucyrus strain are primer-derived.

the ectodomain of the G_L protein at position 56 was found in all analyzed EAV isolates. The nucleic acid substitution that resulted in an aspartic acid to asparagine residue change at position 81 was predicted to introduce a 2nd potential N-linked glycosylation site in the G_L protein of all EAV isolates. Three amino acid residues were changed in all analyzed EAV isolates: glutamic acid (position 90) was changed to a valine; alanine (position 101) was changed to a valine or a threonine (Vienna isolate); and valine (position 119) was changed to a leucine or a phenylalanine (Vienna and 11958 isolates), or a serine (19933 isolate). Among the EAV isolates analyzed, only a few amino acid substitutions were observed in the C-terminal portion of the G_L protein.

In order to establish a phylogenetic relationship between the EAV isolates, a dendogram based on the deduced amino acids of the G_L protein was constructed by using the Pileup program within the GCG sequence analysis software (Fig. 2). Using the dendogram, the European prototype Vienna EAV isolate and North American EAV isolates were found to be genetically divergent such that they could be classified into 2 major distinct groups. The group A included the Canadian and American isolates which could be further subdivided into 2 subgroups: subgroup A1 included 15492, 84KY-A1, 86NY-A1 and T1329 EAV isolates, and subgroup A2 included 19933, 11958 and Bucyrus EAV isolates. The European prototype Vienna isolate was the only representative of the 2nd group E. This primary classification also shows that the North American EAV isolates examined in this study, including the eastern Canadian field isolates, are closely related to each other and constitute a

TABLE II. Nucleic and deduced amino acid sequence identities (%) of G_L gene of equine arteritis virus (EAV) isolates[a]

		Nucleic acid							
	Isolate	BUCYRUS	T1329	19933	11958	15492	VIENNA	84KY-A1	86NY-A1
Deduced amino acid	BUCYRUS		91.3	93.5	93.9	92.4	87.5	92.8	91.3
	T1329	91.8		90.9	91.4	94.0	85.4	96.2	95.2
	19933	95.1	90.2		97.0	92.0	87.5	91.7	90.3
	11958	94.3	91.4	96.7		92.4	87.2	92.2	91.7
	15492	94.3	94.3	93.5	93.9		85.0	94.7	93.2
	VIENNA	90.2	89.4	92.2	92.2	91.0		86.0	85.7
	84KY-A1	94.3	95.1	92.7	92.7	96.3	91.0		96.6
	86NY-A1	92.7	95.9	91.8	93.5	95.9	91.4	96.7	

[a] The ORF 5 nucleotide sequence data reported for these isolates have been deposited in the Genbank Database under accession numbers: U46948 (11958 isolate), U46949 (19933 isolate), U46950 (15492 isolate), U46951 (T1329 isolate), U46952 (Vienna isolate), U46954 (84KY-A1 isolate), and U46955 (86NY-A1 isolate)

relatively homogeneous group. Our results are in agreement with those of another study (26) in which a phylogenetic divergence was also observed between North American (including one Alberta isolate) and European EAV isolates on the basis of the ORF 5-gene product, and the North American isolates were classified into 2 subgroups. Our results and those of others (26) also indicate that the amino acid sequence of the EAV G_L protein includes more genetic variation than the well conserved M and N proteins (27).

As reported previously (23), data derived from computer analysis can be used to predict antigenic sites in polypeptide sequences. Computer analysis of the G_L protein amino acid sequence of all our EAV isolates predicted the presence of 3 sequential antigenic epitopes located in the ectodomain of the protein. The 1st putative antigenic site with an antigenic index value of 1.25, and 6 amino acid residues in length (TAQRFT) was found at residue positions 24 to 29. This site appeared to be well conserved among all EAV isolates. The 2nd putative antigenic site with an antigenic index value of 0.75 consisted of an amino acid sequence of 7 residues (RYDEHTA) in length (positions 47 to 53). Its amino acid sequence was also found to be well conserved among all EAV isolates under study. The 3rd putative antigenic site (positions 78 to 84) was found to be the least conserved antigenic site of the G_L protein of the EAV isolates studied on the basis of the amino acid composition and the number of residues. The amino sequences of this predicted antigenic site were TDCDDTY (positions 78 to 84, Bucyrus strain), TDCNN (positions 78 to 82, T1329 isolate), TGCNDT (positions 78 to 83, 19933 isolate), TGCNN (positions 78 to 82, 11958 isolate), TDCNA (positions 78 to 82, 15492 isolate), TGCNDTH (positions 78 to 84, Vienna isolate), TNCNDT (positions 78 to 83, 84 KY-A1 isolate), and TDCNN (positions 78 to 82, 86NY-A1 isolate). The antigenic indices of this site varied from 1.21 to 1.51.

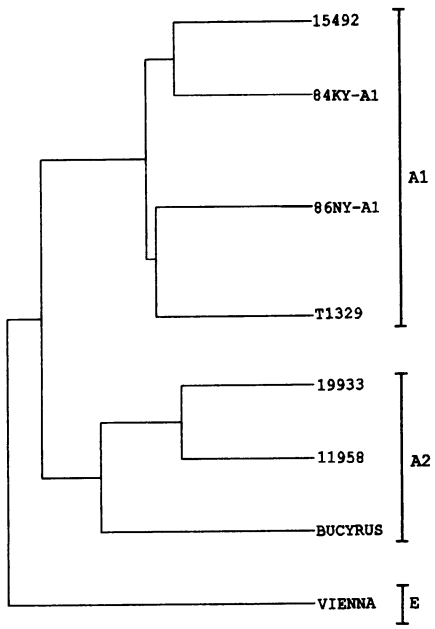

Figure 2. Phylogenetic relationship of 8th equine arteritis virus (EAV) isolates including the Bucyrus reference strain based on their G_L protein sequences.

One major feature of site 3 was the substitution of an aspartic acid to an asparagine residue at position 81. This introduces a potential N-linked glycosylation site within this putative epitope which could affect the immunogenicity of that epitope or closely adjacent epitopes (28). In a previous study, Chirnside et al (16) defined a neutralization epitope in EAV G_L protein at positions 75 to 97 which is consistent with the predicted antigenic site 3 (amino acid residue positions 78 to 84) identified in this study. Although amino acid substitutions were found within the predicted antigenic epitope (site 3) for all EAV isolates tested, it has been previously reported that antigenic amino acid single replacement may be compensated by other amino acid replacements with no change in the antigenic specificity of the epitope (29,30). Therefore, the nonadditive effect of amino acid multiple replacements observed on the antigenic specificity of the predicted antigenic epitope (site 3), or, in contrast, the relationship between this variable epitope and the minor antigenic differences among various EAV isolates reported by others (10,11) have yet to be determined.

Two other groups of investigators (14,15) identified a 2nd major sequential neutralizing epitope (whose amino acid sequence was found to be well conserved among all our analysed EAV isolates) encompassing amino acid residue positions 99 to 104 that was not predicted in our study using the algorithm of Jameson and Wolf (22). Because the additional potential N-glycosylation site in the ectodomain of G_L is very close to this major neutralization epitope, the presence of a large oligosaccharide chain might interfere with neutralization (28) and, therefore, might be related to antigenic differences between EAV strains and, perharps, to differences in EAV pathogenicity.

In summary, the results presented in this study demonstrate a high level of amino acid conservation in the EAV G_L protein among the analyzed Canadian EAV isolates. They also indicate

that the North American isolates, including all 4 eastern Canadian field isolates, and the European prototype Vienna isolate can be classified into 2 distinct phylogenetic groups. Moreover, the phylogenetic classification of the Canadian and American EAV isolates into two subgroups confirms the results of another study (26) in which several North American EAV isolates could also be classified into 2 subgroups. The data also revealed the existence of 3 putative sequential antigenic sites located in the ectodomain of the G_L protein. The 3rd putative antigenic site with the less conserved amino sequence was located into a distinct variable region of the G_L ectodomain. This variable region contains the neutralization epitopes of EAV. The significance of the amino acid substitutions and the new N-linked glycosylation site observed in the G_L protein ectodomain on the EAV antigenic diversity and/or pathogenicity has yet to be determined.

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