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Molecular epidemiology of Lyssaviruses: focus on the glycoprotein and pseudogenes

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ABSTRACT

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The use of the polymerase chain reaction and sequencing of portions of lyssavirus glycoprotein and pseudogenes are discussed in attempts to better understand the epidemiology of rabies in Africa, the Americas and Europe.

INTRODUCTION

In the polymerase chain reaction (PCR) method originally described for rabies (Sacramento, Bourhy & Tordo 1991), particular attention was paid to simplifying and standardizing methods for usage in diagnostic and/or reference laboratories. Because lyssavirus replication produces RNA transcripts exclusively, the total RNA is extracted from brain samples and then reverse transcribed into complementary DNA (cDNA) that is amplified by PCR. The amplified products can be successively analyzed (Tordo, Bourhy & Sacramento 1994) by:

- Southern or dot blots using either radio-active or nonradio-active probes for diagnosis (Sacramento *et al*. 1991; Kamolvarin, Tirawatnpong, Rattanasiwamoke, Tirawatnpong, Panpanich & Hemachudha 1993).
- Restriction fragment length polymorphism (RFLP) using specific panels of restriction enzymes for typing (Sacramento *et al*. 1991; Smith, Fishbein, Rupprecht & Clark 1991; Bourhy, Kissi, Lafon, Sacramento & Tordo 1992; Nadin-Davis, Casey & Wandeler 1993).
- Direct sequencing of the fragment excised from Nu-Sieve GTG agarose gels, without additional purification (Benmansour, Brahimi, Tuffereau, Coulon, Lafay & Flamand 1992; Bourhy *et al*. 1992; Sacramento, Badrane, Bourhy & Tordo 1992; Smith, Orciari, Yager, Seidel & Warner 1992; Bourhy, Kissie & Tordo 1993; Nadin-Davis *et al*. 1993). In this case, the sensitivity of the PCR method allows the study of the sequence of the infecting lyssavirus without previous adaptation to either mouse brain or cell culture, which could introduce non-specific mutations.

Several variants of this method were tried (Tordo *et al*. 1994). For example, the cDNA synthesis and the PCR amplification steps can be performed in succession in the same tube. This method, which limits handling, is under development for diagnostic purposes. Either T7 or Taq polymerases can be used for sequencing. The choice of primer is strategically

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important. The cDNA step may use either specific or random priming. Specific priming allows selection of either the positive or the negative RNA strand as target, an important point for sequencing purposes. However, the subsequent pair of PCR primers must be located at the same level or downstream (3' side) of the cDNA primer.

The classic approach to PCR is to design primers capable of detecting the greatest number of lyssaviruses by the selection of stable sequence regions (Tordo *et al.* 1994). It is particularly important to have stringent conservation near the 3' end of the primer. This is achieved by selecting amino acids with stringent codes (tryptophane, methionine, etc.) rather than those encoded by multiple codons (wobble positions). In addition, preference should be given to amino acids with biased conservation in a typical protein, for example, glycine and basic residues in the polymerase (L) (Poch, Blumberg, Bougueleret & Tordo 1990) and cysterines in the glycoprotein (G) (Tordo, Bourhy, Sather & Ollo 1993a). In the non-coding regions, functionally conserved genomic signals (Tordo & Poch 1988) are convenient targets, although inefficient for differentiating between genes because the same consensus pair of signals flanks each gene. These rules have been partially observed in designing the primer sets used so far.

IMPORTANCE OF LYSSAVIRUS GENOME REGIONS FOR DIAGNOSIS, TYPING AND MOLECULAR EPIDEMIOLOGY

The nucleoprotein (N) gene is highly conserved and strongly expressed (Tordo & Kounetzoff 1993b). For this reason it is a more convenient target for routine diagnosis where the detection of minimal traces of infectivity by a broad spectrum of lyssaviruses is required. Therefore, the N gene (specifically the conserved central region) has been targeted in all the diagnostic trials using PCR (Kamolvarin *et al.* 1993; Sacramento *et al.* 1991). The N gene is also a convenient candidate for comparison of molecular and antigenic evaluation in taxonomic studies, because antigenic classification of lyssaviruses has been based on patterns of reactivity with anti-N monoclonal antibodies (Dietzschold, Rupprecht, Tollis, Lafon, Mattei, Wiktor & Koprowski 1988; King & Crick 1988; Smith 1989; Rupprecht, Dietzschold, Wunner & Koprowski 1991).

The extreme stability of several motifs within the L protein (Poch, Sauvaget, Delarue & Tordo 1989; Delarue, Poch, Tordo, Moras & Argos 1990; Poch *et al.* 1990), designates the L gene, makes it a convenient target for diagnosis when the detection of all members of the *Lyssavirus* genus and even other rhabdoviruses is required. However, a very sensitive amplification procedure needs to be developed first to compensate for the fact that the L gene is poorly expressed.

For characterization of the infecting viruses, the most appropriate genomic region upon which to focus depends both on the method used for identification (differential hybridization with specific probes, RFLP, sequencing) and on the evolutionary distance between the viruses to be compared—the more closely related the isolates, the more variable the genome region examined and *vice versa*. Indeed, the lyssavirus genome can be considered as a microscope with variable magnifications, the selection of the magnification depending on the object which needs to be observed. The large non-protein coding regions, such as the pseudogene, which are subject to mutation free of selective pressure, are most appropriate for differentiating closely related isolates (same genotype, neighbouring biotype) (Sacramento *et al.* 1992; Sacramento *et al.* 1991). There is a range of more conserved protein-coding regions which are appropriate for differentiating more distantly related lyssaviruses (different genotypes) (Bourhy *et al.* 1992; Bourhy *et al.* 1993; Tordo *et al.* 1993a; Tordo *et al.* 1994).

Besides the aspect of sequence conservation, it may be necessary to analyze regions of the genome because of their functional attributes. For example, the G ectodomain and the N protein are interesting because of their involvement in the immune response and may be used to examine the molecular basis of antigenicity and crossprotection (Wunner, Dietzschold & Smith 1988; Celis, Rupprecht & Plotkin 1990).

ANALYSIS OF LYSSAVIRUS DIVERSITY USING THE GLYCOPROTEIN AND THE PSEUDOGENE REGIONS

To date, studies on the genetic variability of wild isolates of lyssaviruses have been essentially focused on the N, G and pseudogenes (Ψ) (Tordo *et al.* 1994). The most important results obtained with the N gene are presented in accompanying articles by Bourhy, Kissi & Tordo (1993b), Smith, Yager & Orciari (1993) and Nel, Thomson & Von Teichman (1994). To avoid repetition, the following discussion will focus on the results obtained with the G-Ψ region, approximately 2 500 nucleotides in length (Fig. 1). It is interesting to compare variability in a region of immunological importance with that of a non-protein coding region that is not subject to selective pressure. Two M2 and L primer sites were found in highly conserved domains of the flanking M2 and L genes (Sacramento *et al.* 1991; Tordo, Bourhy & Sacramento 1992; Tordo *et al.* 1994). One is located near a conserved region at the COOH end of the M2 protein-coding region of rabies and Mokola viruses. The other corresponds with the first conserved motif

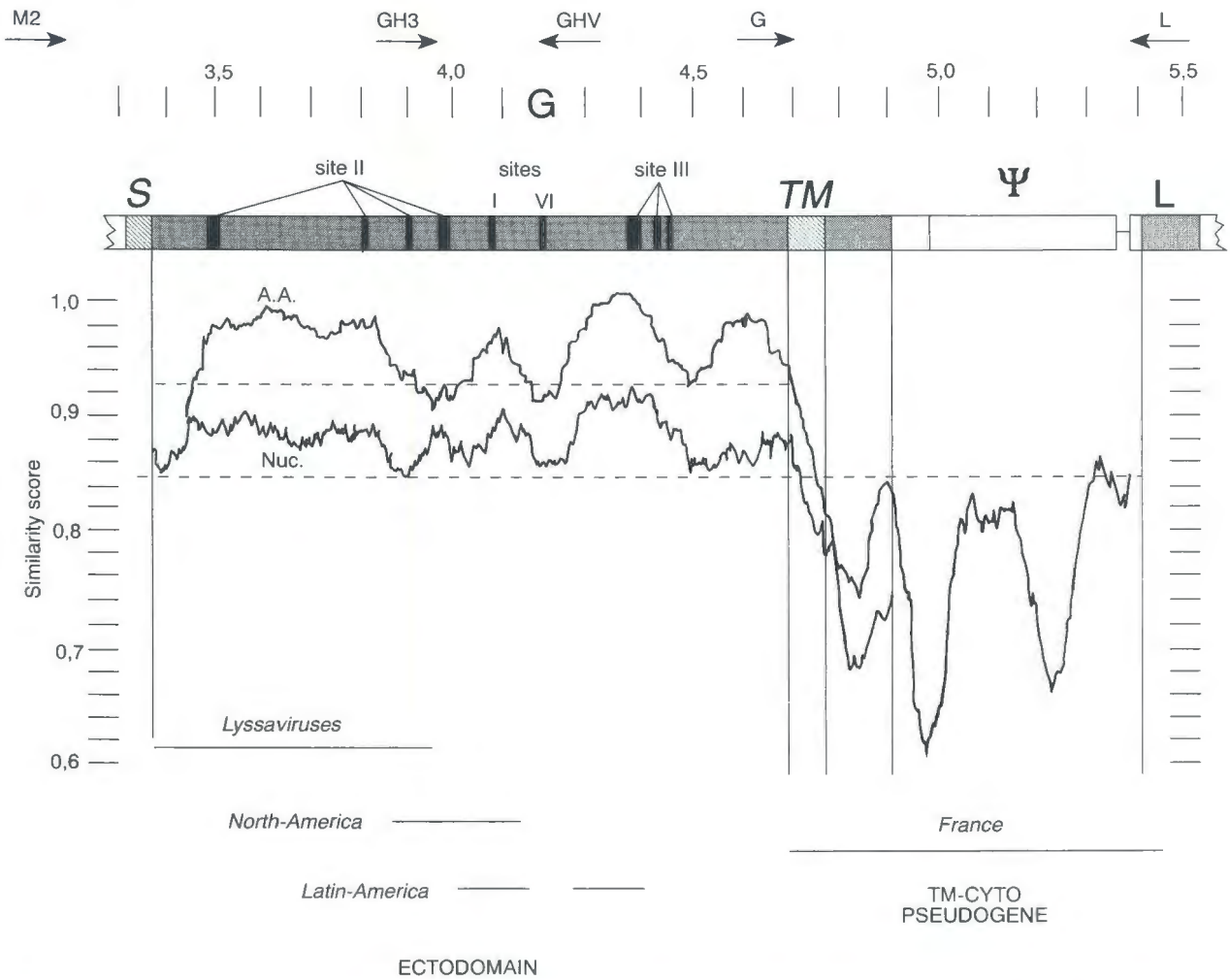

FIG. 1 Similarity profile along the G-Ψ region resulting from the comparison of about 50 sequences of rabies isolates (genotype 1 of the *Lyssavirus* genus) obtained from various parts of the world. The different primer sets mentioned in the text are indicated as are the different domains of the G glycoprotein (signal peptide, ectodomain, transmembrane domain, cytoplasmic domain). At the bottom of the figure is an indication of the limited regions that were used in more extensive analyses addressing specific epidemiological questions

at the NH2 end of the L proteins of rhabdoviruses and paramyxoviruses (Poch *et al.* 1990). Both primers were first checked as a single pair and it was found that difficulties in amplification may be experienced because they are far apart (2 500 residues).

Alternatively, smaller overlapping amplification with intermediate primers may be used (Tordo *et al.* 1992; Tordo *et al.* 1994), for example, the M2-GHV (1 200 residues) and GH3-L (1 600 residues) sets. Also, the G-L primer set is efficient at specifically amplifying the highly variable genomic area encompassing the *TM* and *cyto* domains of the G protein as well as the following pseudogene in all lyssaviruses checked so far (Sacramento *et al.* 1991; Sacramento *et al.* 1992). This is despite the fact that these regions show no significant conservation. The G primer is located within the conserved ectodomain of the G protein with moderate similarity between rabies and Mokola genomes (five mismatches in 23 residues), but terminating in five hyperconserved nucleotides. Beside the primer sets for amplification, numerous other nested primers have been designed for sequence analysis. Indeed, the direct sequencing of Nu-Sieve excised fragments allows either flanking or internal primers to be used with equal efficiency.

WORLDWIDE VARIABILITY IN THE RABIES VIRUS (GENOTYPE 1)

Rabies is maintained and transmitted worldwide by animal species that serve as reservoirs of infection as well as vectors of the disease (Baer 1991). An efficient virus-vector combination results from the mutual adaptation at the molecular, physiological and behavioural levels. This equilibrium can be broken by natural or artificial selection pressure, resulting in

the (dis)appearance of vector species. The epidemiology of rabies in Europe in the 20th century illustrates these points (Kauker & Zellt 1963; Sureau & Bourhy 1990; Blancou, Aubert & Artois 1991): the systematic elimination of stray dogs led to the eradication of canine rabies in the 1920's. After the second World War, rabies spread in the red fox (*Vulpes vulpes*) population but recently the virus has become established in the eastern European raccoon dog (*Nyctereutes procyonoides*) originally imported from Asia (Artois 1985).

Today, in most parts of the world rabies exists in one of two forms: urban rabies, in which dogs are the principal vector and sylvatic rabies, in which one or two principal vectors may be associated with disease in a particular geographical area. Urban (dog) rabies is mostly prevalent in developing countries. The principal wildlife vectors are: In Europe the red fox, the raccoon dog and the wolf (*Canis lupus*); in North America, the arctic fox (*Alopex lagopus*), the red fox, the raccoon (*Procyon lotor*), the skunk (*Mephitis mephitis*) and a number of insectivorous bat species, chiefly *Eptesicus, Lasionycteris* and *Myotis* spp.; in Mexico/Central/South America, the mongoose (*Herpestes* sp.) (Caribbean) and insectivorous as well as vampire bats (*Tadarida* and *Desmodus* spp.); in Asia, the arctic fox (Siberia) and red fox and wolf and in Africa the jackal (*Canis* spp.), bat-eared fox (*Otocyon megalotis*) and several mongoose species.

Before RT/PCR/sequencing methods became available, studies on the molecular genetics of rabies viruses were limited to laboratory vaccine strains. These are, however, derived from isolates made more than 50 years ago, most from Pasteur's cow isolate (Pasteur 1884; Sacramento *et al.* 1992). In order to investigate the natural genetic variability of rabies viruses, we studied the G-Ψ region of 47 isolates (genotype 1) derived from different hosts and countries. Multiple alignment of the sequences was obtained with the Clustal programme (neighbour joining method), and a radial phylogenetic tree was constructed (Fig. 2). Although this study is not exhaustive and needs to be completed, notably by studies on isolates from Asia and far-eastern Europe, several tentative conclusions can be drawn based on clusters which can be related to geographic location, historical relationships, or host species:

- Isolates from Europe (fox, raccoon dog, wolf) and the Middle-East (fox, wolf, jackal) are closely related, although it is possible to sub-classify some isolates on a geographical basis. There is little host-species distinction other than where certain regions coding for the N protein were examined (see Bourhy *et al.* 1993b).
- In Africa and Asia two groups of canid viruses are found: one (Africa 1, China 1) joins the Europe/Middle-East cluster and may be linked by historical relationships established during the colonial period; the other (Africa 2, China 2, S.E. Asia) branches distinctly and could correspond to a different lineage of rabies virus. Africa 2 is mostly found in west-central countries, while Africa 1 occurs throughout the continent, (see Bourhy *et al.* 1993b).
- In North America (USA, Mexico), terrestrial rabies variants (dog, skunk) cluster with those of Europe Middle/East Africa 1.
- Several virus variants seem to have adapted very well to their host: the arctic fox variant which is similar from Alaska to Siberia; the yellow mongoose variant from South Africa (not shown; see Nel *et al.* 1993); the bat variants also form typical clusters with vampire bat isolates being closely related while viruses from insectivorous bats are heterogeneous.
- Vaccine strains form a rather heterogeneous group more closely related to the Europe/Middle-East/Africa 1/American terrestrial cluster. The intrinsic conservation of the G protein ectodomain is 90 % (nucleotide) and 93 % (amino acid). This differs significantly from conservation exhibited by bat viruses where the respective figures are 80 % and 87 %. Here, important antigenic sites can be altered and this may possibly explain some vaccination failures. In any case, it is obvious that the vaccine strains are not at the centre of the genotype I phylogenetic tree.

Fig. 1 shows the similarity profile along the whole G-Ψ region (about 2 500 residues), when the sequences of the 47 wild rabies isolates were compared. In the G gene, the ectodomain is more conserved than the *TM* and *cyto* coding regions which are almost as variable as the pseudogene. It is interesting to note that basically the same trees were obtained when the whole G-Ψ region, or internal parts of it, were used for sequence comparison (not shown). This confirms that the different areas of the lyssavirus genome differ only in terms of sensitivity.

We have used this opportunity to address more detailed molecular epidemiological studies on limited geographical areas. Fig. 1 shows the genome regions that were compared in the following discussion:

- for lyssavirus taxonomy, 500 nucleotides at the conserved NH2 end of the G ectodomain
- to differentiate epidemiological cycles in South and North America between 300 and 500 nucleotides encompassing important antigenic sites of the G ectodomain
- to distinguish very closely related isolates from France over the very variable *TM-cyto*-Ψ region (850 nucleotides)

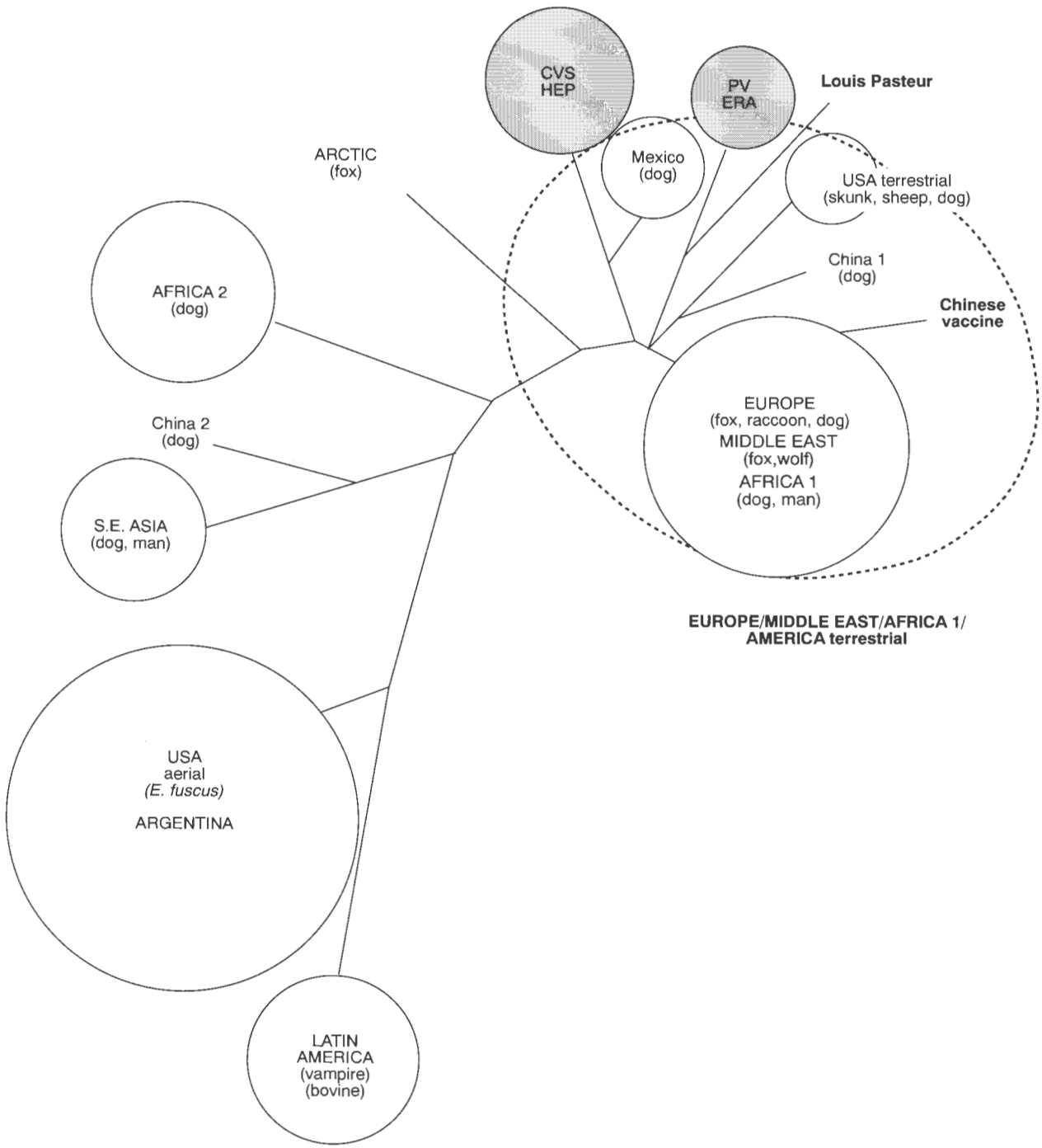

FIG. 2 Radial phylogenetic tree illustrating the genetic variability of rabies isolates (genotype 1) obtained from different countries and different hosts. The tree was calculated by the CLUSTAL program (neighbour joining method) by comparing the sequence of the whole M2-L amplicon (2 500 residues). It is unrooted. The divergence between two samples is proportional to length of their joining branch. The vaccine strains are noted in bold characters or in grey circles

MOLECULAR TAXONOMY OF THE LYSSAVIRUS GENUS

Lyssavirus taxonomy has been re-investigated on the basis of genetic variability either of the whole N gene (Bourhy *et al.* 1992; Bourhy *et al.* 1993a) or of the 500 NH2 nucleotides of the G gene. Both concur in distinguishing six genotypes (Fig. 3). The first four genotypes correspond with the four serotypes previously characterized using immunological techniques (cross serum neutralization and reactivity with monoclonal antibodies) (Dietzschold *et al.* 1988; King

& Crick 1988; Smith 1989; Rupprecht *et al.* 1991; WHO 1992). Their prototype viruses are (1) rabies, (2) Lagos bat, (3) Mokola, (4) Duvenhage. The last two genotypes (5 and 6) correspond to the European bat lyssaviruses (EBL1 and EBL2), which have not been classified by immunological methods. In addition, the radial tree displays the relationships between genotypes more accurately. For example, genotypes 2 and 3 are related and very distant from the others. Also, genotypes 4 and 5 are closely related, explaining *a posteriori*, why EBL1 isolates were initially classified as serotype 4 but later left unclassified. These results demonstrate that molecular analysis is more sensitive and subtle than immunological analysis for taxonomic purposes. Genotype 1 are rabies viruses and members of genotypes 2–6 are so-called rabies-related viruses.

An important but as yet unresolved question concerns the limits of a genotype. The grey circle around genotype 1 (Fig. 3), by far the most extensively studied, gives an idea of the maximum intragenotype diversity (80 % amino acid conservation in the G protein ectodomain between American insectivorous bat isolates and vaccinal strains). Presumably a similar diversity exists within each genotype. Comparatively, the two most related genotypes (4 and 5) have about 75 % and the two most divergent (1 and 3) about 60 % amino acid conservation in the G protein ectodomain.

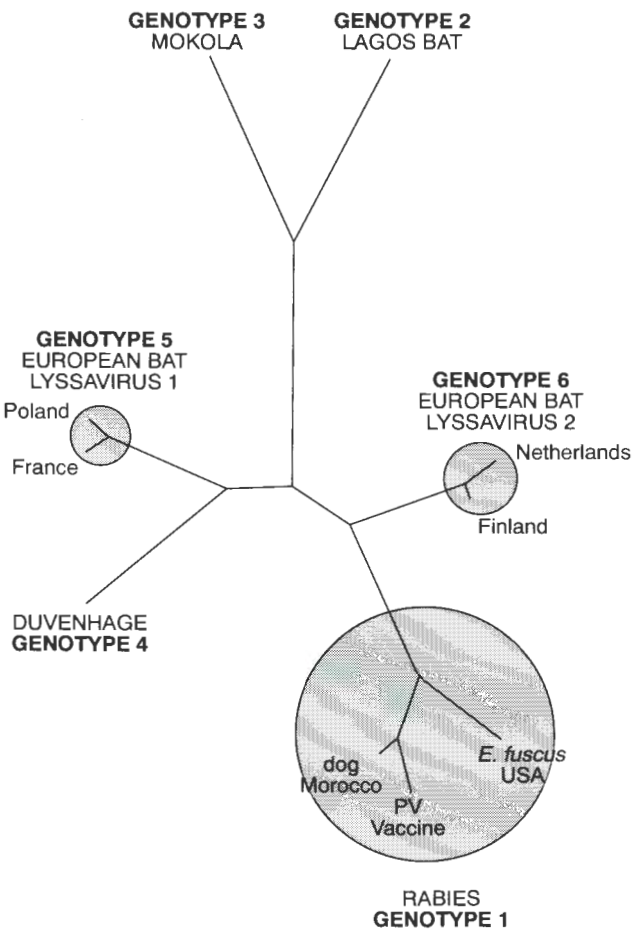

FIG. 3 Radial phylogenetic tree of the *Lyssavirus* genus by comparison of 500 nucleotides in the G ectodomain gene using CLUSTAL program (neighbour joining method). The length of the horizontal branches are indicative of evolutionary distance. The circles estimate the intragenotype variability

RABIES IN LATIN AMERICA

Latin America is unique in terms of rabies prevalence because haematophagous bats, in addition to mammalian carnivores, are responsible for transmission (Baer 1991; Flores-Crespo & Arellano-Sota 1991). There are two distinct epidemio-logical cycles: urban and sylvatic with stray dogs and vampire bats (mostly *Desmodus rotundus*) respectively the principal vectors. Urban rabies is a major public health concern because of the close relationship between people and dogs. In Brazil, despite extensive control programmes which have been in operation since 1972 (compulsory vaccination, control of stray dogs), the incidence or rabies in humans remains high in the poor northern regions. Sylvatic rabies is also a serious economic problem. It is transmitted from vampire bats, in which it is endemic, to cattle resulting in paralytic disease. Since 1903 (Carini 1911), outbreaks in cattle or vampires have been periodically reported as well as rare human cases. Accurate statistics are unavailable due to the lack of efficient surveillance and diagnostic facilities. In Brazil, about 10 000 cases are reported annually (more than 200 000 estimated) in herbivores, but less than 10 % are confirmed in the laboratory. Their distribution reflects the distribution of diagnostic facilities rather than disease distribution. Most cases occur along the sea shore and the larger rivers which have high population densities. Numerous cases are also reported from the Atlantic forest, a region with constant high temperatures, high humidity and numerous caves. No government program exists for the prevention of sylvatic rabies although farmers sponsor control actions (vaccination of cattle with local vaccines, elimination of vampires with anticoagulant, etc.).

Limited epidemiological studies with monoclonal antibodies have identified two major antigenic reaction patterns among Brazilian rabies virus isolates corresponding to the urban and sylvatic cycles (Smith 1989; Baer 1991; Rupprecht *et al.* 1991). In view of this and the recent increase in cattle rabies, we investigated the molecular approach to rabies epidemiology.

We focused mostly on the Brazilian state of Sao Paulo where survey and diagnostic facilities are available. Between 1987 and 1992, 32 isolates were identified by immunoflourescence, histology and

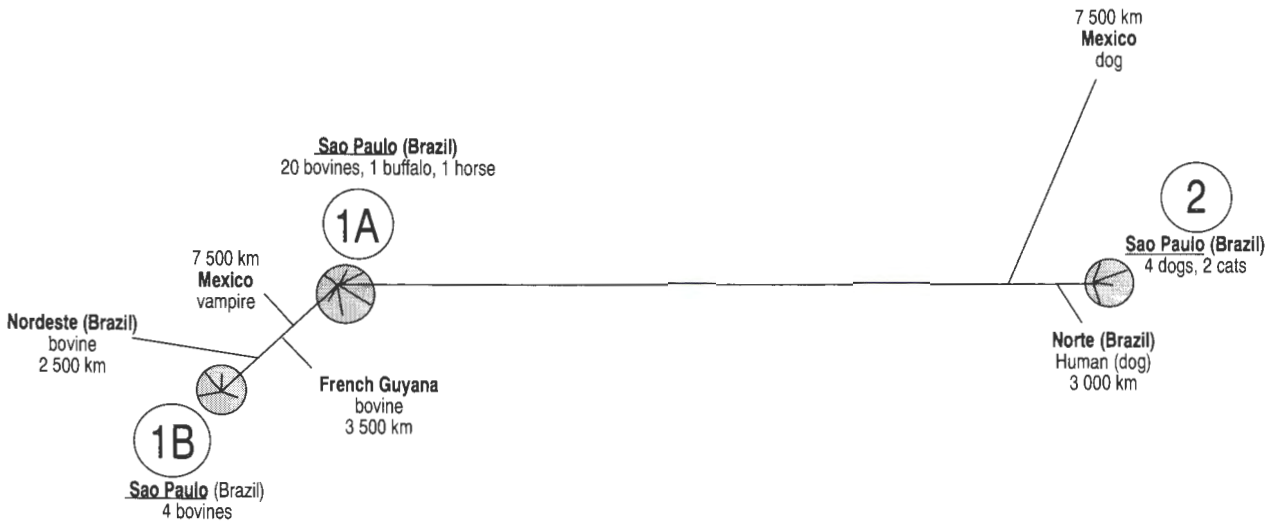

FIG. 4 Radial phylogenetic tree of rabies isolates from Latin America. Approximately 400 nucleotides encompassing important antigenic sites of the G ectodomain are compared using the CLUSTAL program (neighbour joining method). The length of the horizontal branches mimics evolutionary distance

mouse inoculation and a portion of the G protein ectodomain was amplified and sequenced. The isolates reflected the species distribution of rabies cases in the state of Sao Paulo: 24 were from cattle, one from a buffalo, one from a horse, four from dogs and two from cats. In addition, isolates obtained from further afield but from the same animal species, were included in the study:

- one bovine from the north-east of Brazil (2 500 km)
- one bovine from French Guyana (3 500 km)
- one *Desmodus rotundus* from Mexico (7 500 km)
- one human (bitten by a dog) from northern Brazil (3 000 km)
- one dog from Mexico (7 500 km)

Specific mutations were observed depending on whether the specimens were of "sylvatic" or "urban" origin. The radial tree presented in Fig. 4 clearly distinguishes two sequence clusters. Group 1 comprises mostly cattle isolates. In the state of Sao Paulo it is, however, possible to distinguish two subgroups—1A and 1B related to geographic distribution. 1B isolates were derived from areas south of Sao Paulo city, a region with a high prevalence of sylvatic rabies while 1A are mostly from areas north of Sao Paulo city. Group 2 included dog and cat isolates from the state of Sao Paulo. Whatever their geographical origin (separated by up to 7 500 km), Latin American isolates were classified either as Group 1 or 2, depending on whether their origin was bovine/vampire or dog/cat.

These results show that the two overlapping epidemiological cycles of northern Mexico and southern Brazil are associated with genetically distinct variants maintained by either domestic carnivores (urban cycle) or vampire bats (sylvatic cycle). The nucleotide conservation in the region in question is greater than 93 % within groups and less than 80 % between groups.

RABIES IN NORTH-AMERICA

Early in 1990 there was a debate among Canadian rabies researchers as to whether Ontario skunk rabies was a self-sustaining enzootic, or whether it was dependent on rabies in red foxes. A collection of Ontario isolates (from Dr Ken Charlton) obtained in 1990 and representative of local geographic and host diversity, was studied using a limited portion of the G protein ectodomain encompassing important antigenic sites. The viruses comprised five striped skunk isolates, four from red foxes, one from a coyote (*Canis latrans*), one from a raccoon and one dog isolate. For comparison, isolates from Montana obtained from terrestrial species in 1981 to 1982 (from Dr D. Lodmell) were included, viz. eight from skunks, one dog and one sheep isolate. In addition, one arctic fox isolate from the American arctic circle (3 000 km to the north), and three insectivorous bat isolates obtained from *Eptisicus fuscus* in the vicinity of Hamilton, Montana, completed the study.

The radial phylogenetic tree obtained (Fig. 5), grouped terrestrial isolates from Ontario and Montana into two distinct clusters, irrespective of the host animal. Those from Ontario were closely related to the arctic fox isolate, while those from Montana clustered closer to the isolates from the USA. On the other hand, the *E. fuscus* isolates showed an extreme intrinsic heterogeneity similar to that observed within genotype 1 as a whole (see Fig. 2). This is

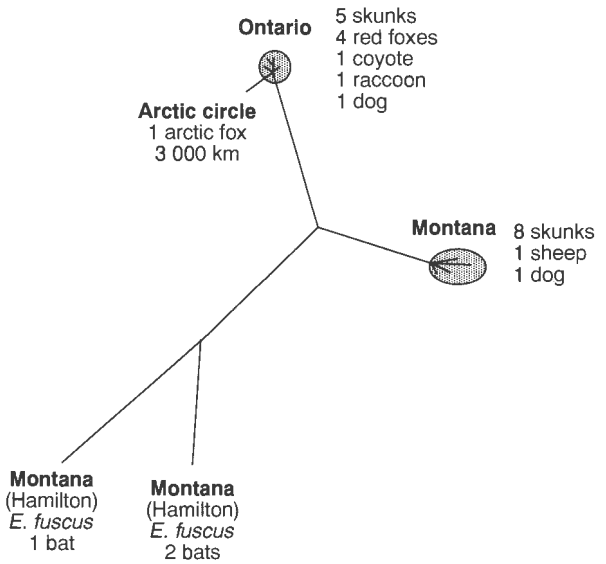

FIG. 5 Radial phylogenetic tree of rabies isolates from North America. Approximately 400 nucleotides encompassing important antigenic sites of the G ectodomain are compared using the CLUSTAL program (neighbour joining method). The length of the horizontal branches mimics the evolutionary distance

compatible with data from antigenic studies (Baer & Smith 1991).

These results clearly indicate that although spreading in similar hosts (skunks), rabies variants in Ontario and Montana are fundamentally different. The former correspond to arctic fox rabies while the latter are more closely related to the Europe/Middle-East/Africa 1 cluster and to vaccine strains. As to the question concerning skunk rabies in Ontario, a more precise analysis of the sequences indicated that isolates are grouped according to geographic distribution but not with the host animal. This suggests that a single vector such as the red fox (Nadin-Davis *et al.* 1993) is probably responsible for the persistence of the disease in Ontario.

This, in addition to the findings from South America, confirms that throughout the Americas, isolates from bats and terrestrial animals are genetically distinct, indicating the existence of overlapping epidemiological cycles in different vectors with no apparent interference. Among the variants from bats, vampire bat viruses form a relatively homogeneous group, whereas a single insectivorous bat species (*Eptisicus fuscus*), can be infected by heterogeneous viruses.

RABIES IN FRANCE

Because of the extreme similarity of French rabies isolates, our analysis focused on the pseudogene which is the most sensitive genomic region in which to measure virus evolution (Sacramento *et al.* 1992). Twelve isolates obtained in 1989 to 1990, showed an association between genome sequence and geographic locality. No variation was found between hosts (cat, sheep, stone-marten, red fox) in France, supporting the suggestion that the fox a unique reservoir and vector. This suggests slow evolution of the virus in parallel with the spacio-temporal progression of the epizootic.

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