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CONTRIBUTION OF MOLECULAR BIOLOGY TO VACCINE DEVELOPMENT AND MOLECULAR EPIDEMIOLOGY OF RABIES DISEASE*

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I. RABIES DISEASE: AN HISTORICAL EXAMPLE OF INTERNATIONAL COOPERATION, NOTABLY BETWEEN BRAZIL AND FRANCE

In order to build an establishment devoted to the treatment of rabies disease based on Louis Pasteur's famous "method of prevention of rabies disease after biting" [45], a worldwide foundation was organized. Besides numerous anonymous contributors, the four first donators were the Tsar of Russia, the Sultan of Turkey, Madame Boussicot and the Emperor of Brazil, Dom Pedro II who crystalized by this action, a constant friendship with Louis Pasteur. This first authentic example of international financial and scientific collaboration resulted in the founding of the Pasteur Institute of Paris inaugurated in 1888, and inspired numerous other projects throughout the world to fight the rabies disease raging at the end of the 19th century. As early as 1888, Dom Pedro II organized the building of a Pasteur Institute in Rio de Janeiro directed by Dr Ferreira dos Santos who had studied the method of rabies prophylaxis in Paris [46]. Today, the brazilian production of anti-rabies vaccines is dispersed in four places including the Butantan Institute of São Paulo, which celebrates its 90th Anniversary in 1991.

II. CAUSES AND CONSEQUENCES OF RABIES DISEASE WORLDWIDE TODAY

Human deaths by rabies disease are estimated as at least 50,000 per year, although the WHO records less alarmist statistics due to the difficulty of collecting data from some

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* *Summary of two conferences during the symposium.*
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countries (World survey of rabies 1988 no 24, WHO/Rabies/91.202). China and India are probably the most contaminated (25,000 cases each) followed by the Philippines, Thailand and Indonesia in Asia, most of Africa, Mexico and Central America. In South America, besides human cases (200 to 500 in Brasil) rabies disease is also an economical problem, responsible for considerable losses of cattle, approximately 500,000 per year[1]. Indeed, the only continent to remain totally rabies-free is Oceania together with several island countries such as Japan, United Kingdom, Ireland, as well as certain Antillas.

The perenniality of the disease is ensured by several wild animal species serving as natural virus reservoirs. These reservoir species, also acting as vectors, are characterized by their high susceptibility to the virus and their propensity to transmit the infection before dying. They must be distinguished from the other species (cattle, human, etc...) that constitute a "cul de sac" for the infection.

The vector species are susceptible to considerable variation depending both on geographic and temporal criteria[10, 58]. Today, for example, the dog is the main reservoir in Asia, Africa and Latin-America, the raccoon and the skunk in North-America, the vampire bat in Central- and South-America, the red fox in Europe. Fox rabies is relatively recent in Western-Europe where it penetrated from the far East (USSR). It first appeared in France in 1968, replacing the dog or "street" rabies contemporary of Louis Pasteur which disappeared in 1920-1930[60]. Another recent european vector is the insectivorous bat, initially described in the North of Europe and invading progressively towards the South. The first french case was diagnosed late 1989[61], others being reported in Spain. Bat rabies is today a major problem because of its divergence from the "classical" rabies viruses (see § VI.1).

As reflected by the great variability of the host, rabies disease is caused by a group of different agents describing the *Lyssavirus* genus of the *Rhabdoviridae* family (figure 1)[4].

RHABDOVIRIDAE FAMILY

LYSSAVIRUS GENUS

serotype	geographic distrib.	animal species
1. Rabies	*world* except Oceania, U.K., Japan, islands...	carnivores cattle, bats human
2. Lagos bat	*Africa:* Nigeria, Zimbabwe Cent. Afri. Rep., South-Africa, Senegal	frugivorous bats cats
3. Mokola	*Africa:* Nigeria, Zimbabwe Cent. Afri. Rep., Cameroon	shrews, rodents cats, dogs, human
4. Duvenhage	*Africa:* Zimbabwe South-Africa	insectivorous bats human
unclassified		
Obodhiang	*Africa:* Sudan	Mansonia
Kotonkan	*Africa:* Nigeria	Culicoids
EBL (European Bat Lyssavirus)	*Europe:* Finland, France Poland...	insectivorous bats human

Figure 1 — Classification of the *Lyssavirus* genus.

These are classified on the basis of serological and antigenic relationships into four serotypes[13, 32]. Serotype 1 comprises the vaccinal strains and the "classical" wild rabies viruses. Serotype 2, 3 and 4 are the so called "rabies-related" viruses because of their distant relationship with serotype 1; serotype 3 corresponds to Mokola virus; serotypes 2 and 4 group are bat viruses, represented by Lagos bat and Duvenhage isolates, respectively. The recent european bat isolates form the European Bat Lyssavirus (EBL) group, as yet unclassified[61].

III. BRIEF DESCRIPTION OF THE DISEASE

Rabies is a viral infection of the nervous system affecting particularly mammals where it causes an acute encephalitis[4]. The virus generally penetrates by effraction (essentially biting) although some unclassical routes of infection by aerosols or licking of mucous membranes have been described[19]. At the site of the bite, generally in the external tissue, a local multiplication is believed to occur but not obligatory[56]. The virus is clearly neurotropic and tends to infect neurons of the peripheral nervous system[71]. The putative receptor of the rabies virus is as yet unknown. On the basis of sequence homology between the external glycoprotein of the virus and the receptor binding site of venom snake neorotoxins, it was postulated that the nicotinic acetylcholine receptor could be the rabies receptor [7, 39]. But if this is possible in muscular cells, it seems that at the level of fibroblastic and above all neuronal cells, the rabies receptor(s) is(are) more complex, also involving oligosaccharides or lipoproteinic elements such as the sialic acids of gangliosides[71, 78].

Once in the nerve, the virus replicates and ascends to the central nervous system by retrograde axoplasmic flow. Some regions of the central nervous system are preferentially infected such as the cortex, the pons or the thalamus[71]. Late in infection, all the central nervous system is infected as well as certain external tissues, such as the salivary glands, that allow the perenity of infection. Rabies disease is characterized by a variable incubation period (generally 1-3 months), contrasting with a short and violent symptomatic period (less than 1 week) leading invariably to death in the absence of any possible therapy[4]. Generally no evident histopathological perturbation accompanies death, as if the virus killed the organism without killing the cell. Several electrophysiological dysfunctions, notably at the level of the paradoxical sleep, have been noted in infected mice[29].

IV. STRUCTURAL AND FUNCTIONAL STUDY OF THE VIRION (FIGURE 2)

1. Morphology, structure

If Pasteur was already certain that rabies disease was caused by a virus, over 80 years of technical progress was necessary, in tissue culture and electron microscopic domains, before observing the first rabies particule in 1963, notably at the Pasteur Institute[3, 21]. The virion has a bullet-shaped form with a round extremity and a flat base. The unique single-stranded RNA genome and the five viral-encoded proteins are distributed in two structural and functional units[67]: a central dense helical cylinder corresponding to the ribonucleocapsid surrounded by a lipoproteinic envelope obtained from

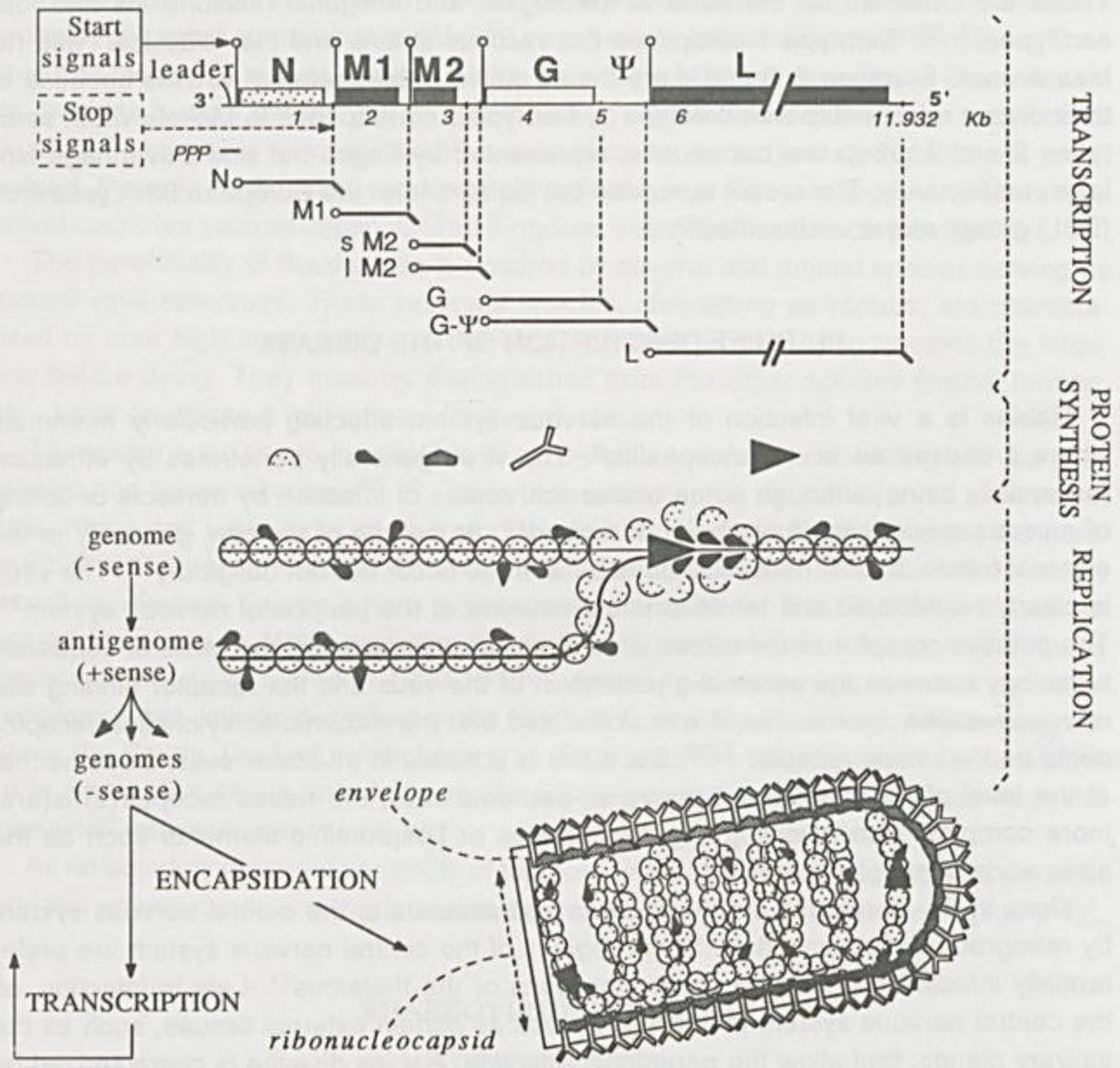

Figure 2 — Structure, transcription and replication mechanisms of rabies genome.

the host-cell surface during the budding of the virion. The ribonucleocapsid is constituted by the RNA genome tightly associated with the N nucleoprotein, and less stringently with the M1 phosphoprotein and the L polymerase. The RNA-N protein association is so intimate that the genome is completely insensitive to RNase. The envelope carries the M2 membrane or matrix protein on the inner side and is traversed by the G glycoprotein which forms spike-like glycosylated projections towards the outside. As is evident from their names, the G glycoprotein is glycosylated and the M1 phosphoprotein is phosphorylated. The N nucleoprotein is also phosphorylated.

The rabies genome is an unsegmented RNA molecule of negative polarity. Negative means that it is not directly translatable by the cell machinery, but forced to assure an autonomous transcription step by producing positive stranded mRNAs, as soon as it penetrates the cytoplasm. Unsegmented means that cistrons are juxtaposed, limited by start and stop transcription signals and separated, by intergenic regions. This genomic organization is shared by the *Rhabdoviridae* and *Paramyxoviridae* families which have consistently envolved an identical multiplication strategy.

2. Multiplication Strategy

Once fixed on its putative receptor, the virus penetrates the cell by pinocytosis and fuses its envelope with that of the lysosomal vacuole. The ribonucleocapsid is thereby liberated into the cytoplasm to serve as a template for transcription and replication mechanisms in this genuine form, without any decapsidation. These mechanisms have been largely inspired from the vesicular stomatitis virus (VSV) model, far the more extensively studied unsegmented negative stranded RNA virus[5, 26, 73, 74].

The transcription occurs from the 3' to the 5' end of the genome, producing monocistronic transcripts. First a small noncapped, nonpolyadenylated leader RNA, then five capped and polyadenylated messenger RNAs (mRNAs) successively corresponding to the N, M1, M2, G and L proteins. This transcription is sequential and of decreasing efficiency, meaning that a messenger is always transcribed after the 3' proximal one and at lower rate. Consequently, the extent of gene expression is directly related to the genomic location. It appears that the transcription complex stops at the end of each cistron, and reinitiates only partially at the beginning of the following one. This sequential progression is dictated by the ten nucleotides long start and stop transcription signals recognized by the running transcription complex.

It is only after the translation of the mRNAs into the corresponding proteins that the switch to replication step occurs. This suggests that at least one of the viral protein is involved in this switch. The N nucleoprotein ratio is currently thought to be the key point, the replication beginning only if sufficiently large amounts of N protein are available for encapsidation. The replication leads to the synthesis of a full length positive stand antigenome that, in turn, will serve as a template to amplify the negative strand genomes. These will be either encapsidated in the progeny virions or submitted to a secondary transcription step.

Although not yet studied as completely, the rabies genome expression is coherent with the VSV model but shows typical features:

1) the presence of very variable intergenes both in size and nucleotidic composition, notably between G and L cistrons (423 bases)[67, 69];

2) the presence of two consecutive stop signals for the G and M2 cistrons, alternatively used to produce either a large or a small messenger. Because the transcription complex is thereby released more or less far upstream from the distal start signal, alternative termination influences the extent of distal gene transcription. It is a typical regulatory mechanism since the ratio between both messengers varies during the course of infection, and differently in fibroblastic or neuronal cells[64, 65, 66].

3. Functional Role of the Viral Proteins

The ribonucleocapsid structure (N protein coated RNA genome, M1 and L proteins) is a functional entity autonomous in transcription and replication. Typically, the N encapsidated genome is recognized as a template by the viral polymerase composed of two functional elements. While the L protein is the actual RNA-dependent RNA polymerase carrying most of the required activities (RNA synthesis, capping and polyadenylation), the M1 seems more devoted to regulatory functions. It ensures notably a local decapsidation of the template upstream from the running L protein, by displacing the N proteins to leave the template accessible for the polymerase. The affinity of the N protein molecules for the M1 phosphoprotein results from the extreme electronegativity of the latter

that mimics the RNA backbone. This electronegativity is due both to the richness of the M1 protein in acidic amino-acids and to the presence of the phosphate residues. Downstream the polymerase complex, the N nucleoproteins immediately re-encapsidate the genome, suggesting an exact coordination between polymerization and encapsidation.

The M2 membrane protein, occupying an intermediate position between the envelope and the ribonucleocapsid, interacts with both units. Thereby, it plays a capital role during the maturation step which precedes the budding of the virion out of the cell. At the ribonucleocapsid level, it inhibits the transcription and replication mechanisms and catalyses a strong condensation in an helical structure[40]. At the membrane level, it designates the places where the virus will bud by facilitating local concentration of the G glycoprotein on the cell surface. The external spike-like glycosylated projections of the G protein constitute the major viral antigen and are likely to mediate the binding to the target cellular receptor although the binding site remains uncharacterized.

V. RABIES VACCINES: A CENTENARY OF CONTINUAL TECHNICAL PROGRESSES FROM PASTEUR'S "DESSICATED SPINAL CHORDS" TO GENETIC ENGINEERING

1. Classical Vaccines

To fight the disease, the first serious scientific approach was undertaken by Pierre-Victor Galtier and subsequently Louis Pasteur, to find an effective vaccine. Since the famous and successful Pasteur's injections of dessicated spinal chord of rabid rabbits[45], considerable efforts were undertaken to increase the efficiency and safety of vaccines. They were subsequently prepared from infected brains of adult animals, then of suckling mice to avoid risks of encephalopathy, and finally from infected cell cultures[17, 34, 59]. In parallel, performing methods to purify (zonal centrifugation) and inactivate (UV, B-propriolactone) the virus were developed.

2. Subunit Vaccines without DNA Technology

The project of producing rabies subunit vaccines consists of using purified viral polypeptides or part of polypeptides for immunization. Biochemical techniques to purify viral polypeptides and to restructure them in a convenient form were first assayed. The purified glycoprotein was anchored either on an oligosaccharide bone composed of glycoside Quil A, or on a lipidic membrane, giving rise to "rabies iscoms" and "rabies immunosomes", respectively[48]. Even though both products showed a high protective activity in pre- or post-exposure tests, the difficulties inherent to glycoprotein purification rendered impossible the development of a vaccine for commercial reasons. DNA-recombinant technology could bypass this difficulty by producing substantial quantities of viral polypeptides. Alternatively, a vaccinal approach by synthetic peptides corresponding to B- or T-cell epitopes coupled to "carrier" molecules was investigated[17].

3. Contribution of the DNA-recombinant Technology to Rabies Vaccine

The different steps for the general strategy to produce a rabies vaccine by DNA-recombinant technology are (figure 3): 1) The choice of the viral antigen eliciting the

best immune response; 2) The choice to work either with the corresponding messenger RNA or the total RNA genome; 3) After the reverse transcription into complementary DNA (cDNA) and the cloning, the choice of the expression system, including the vector, the promotor and the host-cell system.

GENERAL STRATEGY FOR A DNA-RECOMBINANT VACCINE

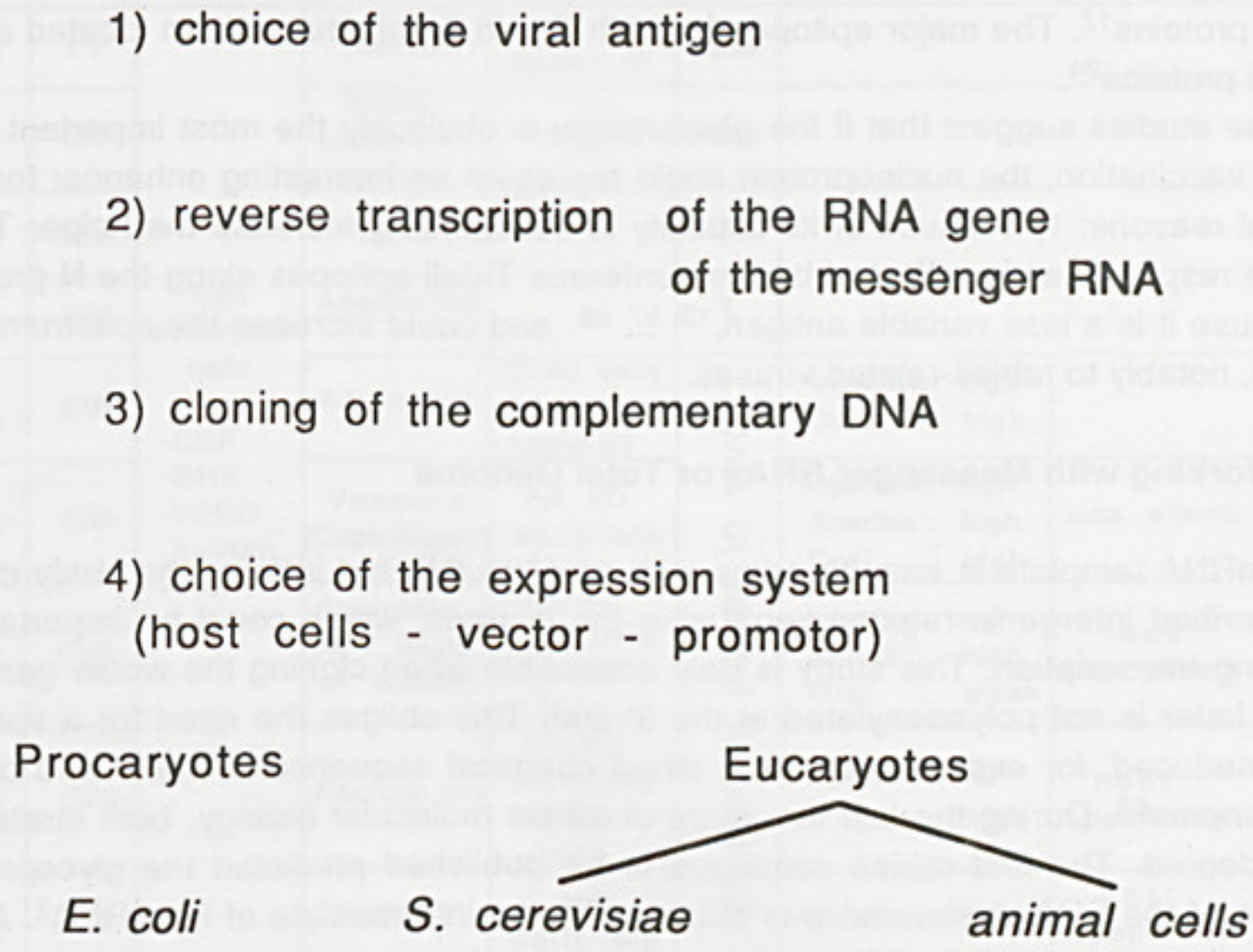

Figure 3 — Strategy towards a genetic engineered vaccine.

Assuming that expression is good, then the recombinant protein has to be purified, except when using virus vectors able to multiply in the target animal itself, such as vaccinia vectors. Finally, the last and crutial step is to test the protective power of the recombinant on animals.

A. Choice of the Viral Antigen

Although all viral proteins show antigenicity, they do not all play the same role in protection[17]: the purified G protein has a protective effect against an intracerebral challenge with rabies virus while the purified ribonucleocapsid is only protective against a less stringent peripheral challenge (intramuscular)[25]. Consistently, the glycoprotein is the only viral protein having the capacity to elicit virus neutralising antibodies[75]. This property is mainly dependent on the preservation of its three dimensional structure, although a linear neutralising epitope was recently characterized[16]. On the other hand, it shares the capacity to induce a cellular immune response involving both helper and cytotoxic T lymphocytes with other proteins of the ribonucleocapsid, namely the N and the M1 proteins[17]. The major epitopes for both B and T cells have been located along the viral proteins[24].

These studies suggest that if the glycoprotein is obviously the most important antigen for vaccination, the nucleoprotein could represent an interesting enhancer for two principal reasons: 1) because of its capacity to substantially increase the helper T cell immune response as is reflected by the numerous T-cell epitopes along the N protein; 2) because it is a less variable antigen,[13, 67, 68] and could increase the spectrum of a vaccine, notably to rabies-related viruses.

B. Working with Messenger RNAs or Total Genome

An mRNA template is easy to prime with an oligo (U), but impairs the study of the untranscribed intergenic regions separating the cistrons, which could be important in regulating transcription. This study is only accessible when cloning the whole genome but the latter is not polyadenylated at the 3' end. This obliges the need for a specific primer deduced, for example, from the direct chemical sequence of the 3' end of the RNA genome[68]. During the last ten years of rabies molecular biology, both strategies were adopted. The first rabies sequence to be published predicted the glycoprotein structure of the ERA strain, and was obtained by the intermediate of its mRNA[2]. Alternatively, the genome of the PV strain was the first to be cloned to completion by using three consecutive specific primers, and the sequence of its 11932 nucleotides determined[68, 69, 70].

Today, the structure of numerous rabies mRNAs from different strains are known[64, 78]. The genome of the AvO1[49] and SAD B19[20] viruses, mutants of the CVS and SAD strains, respectively, as well as the genome of the rabies-related Mokola virus[15] have been cloned and totally or partially sequenced. All these cloned genes were then available for expression. However, for historical reasons, almost all the expression assays were tempted on the ERA and CVS glycoproteins, the two first genes to be cloned[2, 79]. Now, the tendency is more towards diversity both at the level of the nature (G or N protein) and serotype (strains of rabies or rabies-related viruses) of the expressed antigen.

C. Choice of the Expression System

The key point is then to select an expression system able to perform the required post-translational modifications to make the recombinant polypeptides identical to the authentic protein.

Antig.	Rabies strain	Host	Vector	Promotor	%	Notes	Ref.
G	ERA	E. Coli	plasmid	lac UV5	2-5%	-inducible promotor -unglycosylated -unmatured (expression without signal peptide) -denatured (reduced) -non protective	42
G	CVS		pBR 322	tryp	2-3%		79
G	ERA		plasmid	M13 phage	25%		36,37
G	ERA		M13 phage	lac (B-Gal fused)	?		38
G	ERA	S. Cerevisiae	2 microns plasmid		weak	-glycosylated	31,37
G	PV	high eucaryote cells -CEF -BHK -VERO -human -etc...	plasmid	SV40 late Herpes TK	weak		64
G	ERA		Bovine papilloma replicon	TK gene	weak		31,37
G	ERA		SV40		?		37
G	ERA		Adenovirus		?		37
G	CVS		Adenovirus	SV40 early Adeno late Adeno E3	protective power in vivo	Injection: high Oral : high	52
G	ERA		Vaccinia (Copenhagen)	7,5 KD early-late		Injection: high Scarific : high Oral : high side effects	30
G	CVS		Vaccinia (N. Y. Board of Health)	7,5 KD early-late 11 KD late		Injection: high Scarific : high Oral : weak less side effects	27
G	CVS		Raccoon pox	7,5 KD early-late 11 KD late		Injection: high Oral : high host specificity	28,41
G	ERA		Fowlpox	H6 early-late		Injection: high host specificity	62,63
G	CVS	Spodoptora Frugiperda	Baculovirus	polyedrin	good	Protective power by Injection: high	51
G	MOK		Baculovirus	polyedrin	good	Protective power by Injection: high	14
N	CVS	high eucaryote cells	Vaccinia (Copenhagen)	7,5 KD early-late			9
N	CVS		Raccoon pox	7,5 KD early-late 11 KD late	high	Dose dependent protection by injection or tail scratching	41
N	CVS	Spodoptora Frugiperda	Baculovirus	polyedrin	high	Products for diagnosis reagents	8,50,53

Figure 4 — Summary of the systems employed to express the G and N genes of Lyssaviruses.

The glycoprotein is a classic transmembrane protein with four typical domains from the NH2 to the COOH end[67, 78]: 1) a 19 residues-long hydrophobic signal peptide; 2) a hydrophilic and glycosylated external ectodomain, approximatively 440 residues long; 3) an 22 residues-long transmembrane peptide; 4) an 85 residues long hydrophilic cytoplasmic domain. During the G mRNA translation, the first synthesized signal peptide traverses the endoplasmic reticulum towards the lumen, initiating a translocation process of the whole ectodomain. The translocation stops when the transmembrane peptide reaches the membrane where it stays anchored. A maturation process then cleaves the signal peptide and induces the glycosylation of the ectodomain. Finally, the mature protein appearing at the cell or virion surface is composed of the internal cytoplasmic domain, the transmembrane peptide and the glycosylated ectodomain protruding toward the outside.

The N mRNA translation is more simple, occuring totally in the cytoplasm, and producing a 450 amino-acid long polypeptide which is then phosphorylated at precise sites[23].

Figure 4 summarizes the numerous procaryotic and eucaryotic expression systems that were assayed.

D. Expression in Bacteria and Yeast

E. coli was extensively employed at the beginning, using several plasmid or virus vectors and inducible promotors, but was rapidly discarded because of its inability to glycosylate and cleave signal peptides. This required additional molecular biological tinkering to delete the signal sequence before expression. Although a heat inducible M13 phage promotor led to an important proportion of recombinant protein, approximatively 25% of the total proteins[36, 37], the resulting unglycosylated and denatured polypeptide showed absolutely no protective power in vivo.

In yeast, the 2 microns plasmid expressed a correctly glycosylated recombinant protein, but the production was too weak to envisage a genetic engineered vaccine[31, 37].

E. Expression in Animal Cells

In animal cells, several system were assayed, using either plasmid or viruses as vectors (Bovine papilloma virus, SV40 virus, adenovirus) but the production of the glycoprotein remained weak. Only a recent trial in adenovirus using three different promotors, allowed a substantial production of glycoprotein with a high protective power either by injection or by the oral route[52].

But the real first success was obtained with the Copenhagen strain of the vaccinia virus using the 7,5 Kd promotor[30]. The success of this system was both in expression and protective effects obtained by injection, by scarification, or by oral route[11, 37, 54, 76, 77]. Basically, a non-essential gene (here the TK gene), and a strong promotor (the 7,5 Kd) are isolated from the vaccinia virus genome. The rabies glycoprotein gene is placed under the control of the promotor and this construction is inserted into the middle of the TK gene on a convenient plasmid vector. After cotransfection of the vector with wild vaccinia genome in cells, double reciprocal recombination events using the TK gene flanking sequences insert the rabies glycoprotein in the middle of the vaccinia genome. This recombinant vaccinia virus is interesting because it is able to grow either in cell culture, or directly in animals to be vaccinated.

It was shown that laboratory animals injected intradermally or inoculated by the parenteral route with the recombinant virus develop high titer of virus neutralising antibodies and resistance to an intracerebral lethal challenge[76, 77]. Injection with the vaccinia wild type has no effect. Adult fox vaccinated by the oral route develop a neutralising antibody titer still detectable between 1-1.5 years after inoculation and are consistently protected against a lethal challenge[11, 22]. Since a fox lives approximately less than two years in the wild, this type of vaccination appeared particularly adapted. Oral vaccination campaigns by baits has been undertaken in Europe in the aim of eradicating the wild- (essentially fox-) rabies[47].

However, the Copenhagen strain of vaccinia virus that was initially used, was suspected of inducing side effects for human and animals, as previously observed during mass vaccination campaigns against smallpox [27, 28]. This was, from the beginning, a subject of controversy leading Copenhagen partisans to multiply the safety tests for an incredible number of animal species[22, 54], and Copenhagen detractors to look for new vaccinia strains or new poxviral vectors, with limited host range. Several alternatives were assayed. The New York Board of Health strain showing remarkably less side effects was used with a more powerful promotor, the 11KD[27]. However, its protective effect by the oral route appeared weak, perhaps because it is less invasive than the Copenhagen strain. The racoonpox virus was also tested to improve the host-specificity for the vaccine[28, 41]. Finally, the fowlpox virus, pathogen for poultry only, is still under development[62, 63]. Its interest as a vector is that it is unable to make a productive infection in non-permissive cells or non-permissive host. This greatly limits the possible side effects, notably a generalized infection to the vector in immunocompromised individuals. Despite this non-productive infection, fowlpox vector is able to express foreign genes, and particularly the rabies glycoprotein, at the surface of non permissive cells and thereby induces interesting levels of neutralizing antibodies by inolucation of several non permissive mammalian hosts.

More recently, expression of the glycoprotein gene in the baculovirus system was performed for CVS strain rabies virus[51] as well as for the rabies-related Mokola virus[14]. In this latter case, baculovirus appeared as a particularly convenient vector because, in contrast to rabies virus, Mokola virus grows efficiently in insect cells and was essentially isolated from insectivores in Africa. It was notably responsible for human encephalitis, death of domestic animals vaccinated against rabies, and for a limited epizooty in Zimbabwe[13]. The absence of cross protection by the classical rabies vaccines being verified in the laboratory, a specific vaccine against Mokola virus is necessary to protect exposed populations. The low growth efficiency of the virus in cell culture hypothesized the initial project of a classical cell culture vaccine and favours the search for an alternative baculovirus recombinant vaccine (unpublished results).

The technique used (figure 5) was basically similar to vaccinia virus. The Mokola glycoprotein gene was isolated, placed under the control of the polyhedrin promotor in a pEV55 transfer vector. Here again, the polyhedrin is not essential for the baculovirus and a cotransfection of the pEV55-GMok construct with the wild virus in SF9 insect cells, exchanges the polyhedrin gene with the Mokola glycoprotein in the recombinant virus.

When cells are infected with the recombinant virus, they synthesize a substantial quantity of a 56 kd recombinant protein similar both by electrophoresis and immunoblotting techniques to the native glycoprotein. In the presence of tunicamycin, which inhibits the N glycosylation, the apparent molecular weight of both recombinant and native protein are reduced to a similar extent, suggesting their effective glycosylation. By

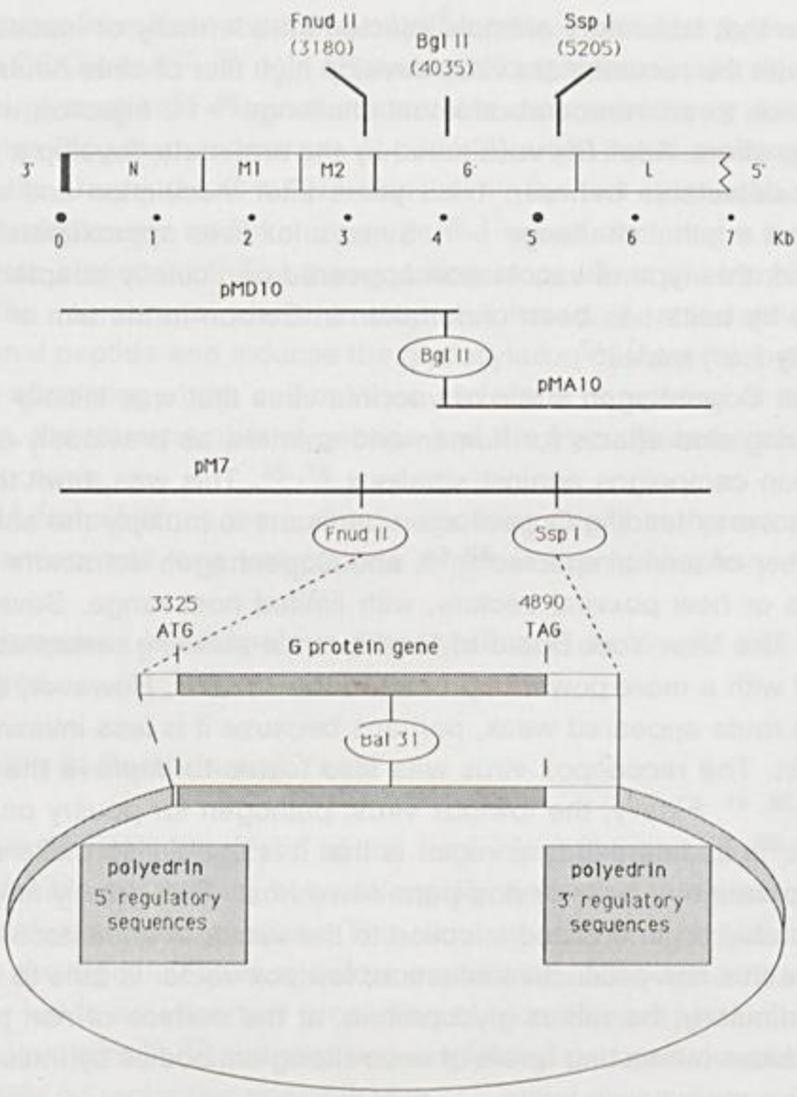

Figure 5 — Cloning of the coding sequence of the Mokola glycoprotein in a baculovirus vector. The cleavage sites of the restriction enzymes BglII, FnudII and SspI are indicated. pMD10 and pMA10 cDNAs are ligated by their common BglII site to form the pM7 cDNA. The FnudII-SspI internal fragment is excised, trimmed at the G protein NH2 side by the Bal31 exonuclease, and placed under the control of the polyedrin promotor in a pEV55 transfer vector.

treating the infected cells with a glycoprotein-specific monoclonal or a polyclonal antibody, the recombinant protein is shown to be expressed at the cell surface, as expected from its transmembrane nature. The protective power of the recombinant protein was analyzed. Mice injected intraperitonealy with insect cells expressing the recombinant glycoprotein resist an intracerebral challenge performed three weeks later. The protection is clearly due to the recombinant protein, since insect cells expressing the wild baculovirus are inefficient. It is specific for Mokola virus since mice remain sensitive to a challenge with the rabies CVS strain. The baculovirus recombinant Mokola glycoprotein is thus protective *in vivo* and constitutes the first experimental vaccine against a rabies-related virus with an excellent protective index. Additional purification and restructuration of the recombinant protein is now necessary before proposing a vaccine for human or veterinary use.

The expression of the N nucleoprotein gene was also performed in vaccinia[9, 41] and baculovirus sectors [8, 50, 53].

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VI. ARE THE CURRENT VACCINAL STRAINS ADAPTED TO THE CURRENT RABIES DISEASE?

1. The Vaccinal Strains

In contrast to the continual technical progresses in vaccine developments, very little was attempted to concomitantly adapt the vaccinal strains to rabies virus evolution. Consequently, the strains used for medical or veterinary vaccines, derive from isolates obtained between 50 and 100 years ago, following a complex history summarized in figure 6 and described in references[18, 35].

Most of them derive from the original Pasteur's isolate collected from a rabid cow in the suburb of Paris in 1882. They are therefore reminiscent of the dog-rabies that overran Western-Europe at the end of the 19th century and ignore the recent switch to the fox-rabies (see § II). The Pasteur's isolate was serially passaged on rabbit brains to give rise to the first "fixed" rabies strain (L. Pasteur strain) characterized by a constant and species-dependent incubation period. The L. Pasteur strain is still traditionally maintained at the Pasteur Institute of Paris (2074 passages) and has been adapted to cell culture. It was largely disseminated among the scientific and industrial communities to generate numerous "fixed" strains serving both for molecular studies and vaccine development. In 1940, the L. Pasteur strain penetrated the USA where it was maintained on rabbit brain and adapted to mouse brain by Karl Habel, resulting in the PM and CVS strains, respectively. It came back to Paris in 1965, after an obscure period in the Cepanzo of Buenos Aires, and gave rise to the PV strain.

Other classical strains alternatively derived from different isolates. In the USA, a rabid dog originated the SADs, SAG1, ERA and Vnukono32 strains, while a human isolate adapted to chick brain resulted in the Flury LEP and HEP strains. The Beijing31 and Kelev strains were isolated in China and Israel, respectively.

The letigimate question arising from figure 6 is the ability of current vaccinal strains to protect against the current rabies virus. Cross-protection studies have established that current rabies vaccines (serotype 1): 1) are globally efficient against members of the homologous serotype 1, although isolated cases of vaccination failures were reported in Africa[12]; 2) offer an imperfect protection dependent on the vaccinal strain used against serotypes 2 and 4[35]; 3) are ineffective against Mokola virus that constitutes the most divergent serotype 3[33].

This strongly plaids for the need for considerable efforts either to extend rabies vaccine potency towards a polyvalent Lyssavirus vaccine, or to propose new specific vaccines, particularly for viruses recognized as potentially dangerous for public health, such as Mokola virus[13]. As a logical first step in that goal, an intensive molecular epidemiological study of rabies virus appears urgent in order to appreciate the worldwide viral evolution and the antigen divergence at the genetic level.

2. Polymerase Chain Reaction (PCR) as an Alternative Tool for Diagnosis, Typing and Molecular Epidemiology of Rabies Virus

We have recently developed a very simple method based on PCR amplification of infected brain material which appears as a hopeful alternative for routine diagnosis, typing or precise molecular epidemiological studies[55]. At the epidemiological level, this method is advantageous to classical approaches based on antigenic differences with

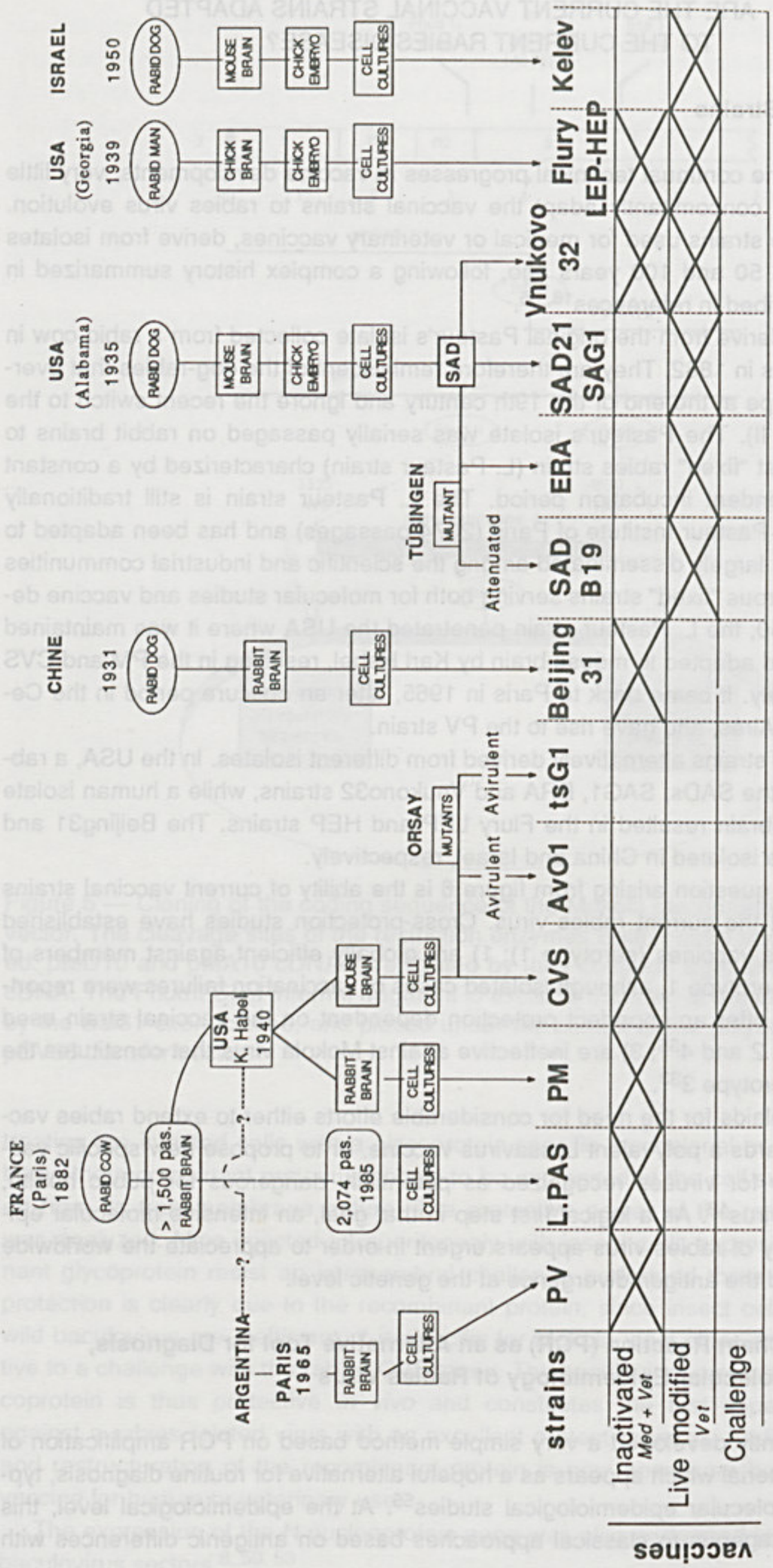

Figure 6 — Conspectus of the schematic history of the principal rabies virus strains used for vaccine development.

anti-N or -G monoclonal antibodies[24, 43, 57, 58, 72] because it does not require a previous cell culture adaptation setp, presumed to be possible source or selector of mutations. Using a disposable plastic pipette, brain samples are internally collected via the occipital foramen[6] or the retroorbital route[44] to avoid risks of contamination. Total RNA is extracted and reverse transcribed into cDNA which is consecutively amplified. The rabies specific primer used for reverse transcription can be one element of the couple used for amplification or different. One can amplify either genomic (minus sense) or messengers (plus sense) RNAs by selecting the convenient polarity for the reverse transcription primer. The amplified fragment can be progressively processed for:

- *diagnosis,* by electrophoresis on ethidium bromide agarose gel,
- *diagnosis or typing,* by differential hybridization with internal probes of divergent strains, either on dot or on Southern blots,
- *typing,* by restriction polymorphism with a limited panel of endonucleases,
- *molecular epidemiology,* by direct nucleotide sequencing of the fragment excised from agarose gel (Nusieve), without any additional purification, using the dideoxy technique (T7 Sequencing Kit, Pharmacia),
- *cloning and expression* in convenient vectors for fundamental or vaccinal purpose.

3. The Rabies ψ Pseudogene: The Best Clock of Evolution

The genomic areas targeted for amplification are different for diagnosis or epidemiological purposes. Conserved regions are more suitable for diagnosis which looks for minute quantities of viral sequences, whatever the infecting Lyssavirus. Highly variable regions are the more convenient for typing or molecular epidemiological studies where the important point is to find sensitive criteria to differentiate isolates.

From a comparison between rabies and Mokola genomes[15, 55, 68, 69, 70], which represent the two most divergent serotypes of the *Lyssavirus* genus, the N gene and the G-L intergenic regions were targeted for diagnosis and epidemiological studies, respectively (figure 7). The latter corresponds to a remnant protein gene, baptized ψ for pseudogene, placing the rabies virus in an intermediate position of unsegmented negative stranded RNA virus evolution, between the *Rhabdoviridae* and the *Paramyxoviridae* families[67, 69]. As a non protein coding region greatly susceptible to mutations, it is more likely to represent the natural evolution of the virus outside any external selective pressure and therefore the most suitable target for epidemiological studies.

4. Towards a Worldwide Molecular Epidemiological Study of Lyssaviruses

Using primers located in conserved places of the flanking G and L, the gene of worldwide fixed or wild Lyssaviruses has been successfully amplified and sequenced. The totality of the G and N genes of several isolates were also studied. In terms of divergence, the isolates rank in the same order, although at different rates, by considering either the coding G and N genes of immunological importance, or the non-coding pseudogene, respectively. This assesses "a posteriori" the significance of our rational approach of the viral divergence by the gene study, confirming this rapid method as particularly adapted for rabies epidemiological studies.

Although the results are still under exploitation, several major observations summarized in table I can be already shown (unpublished results):

1) The "fixed" strains used in vaccines form a dispersed group showing up to 18%

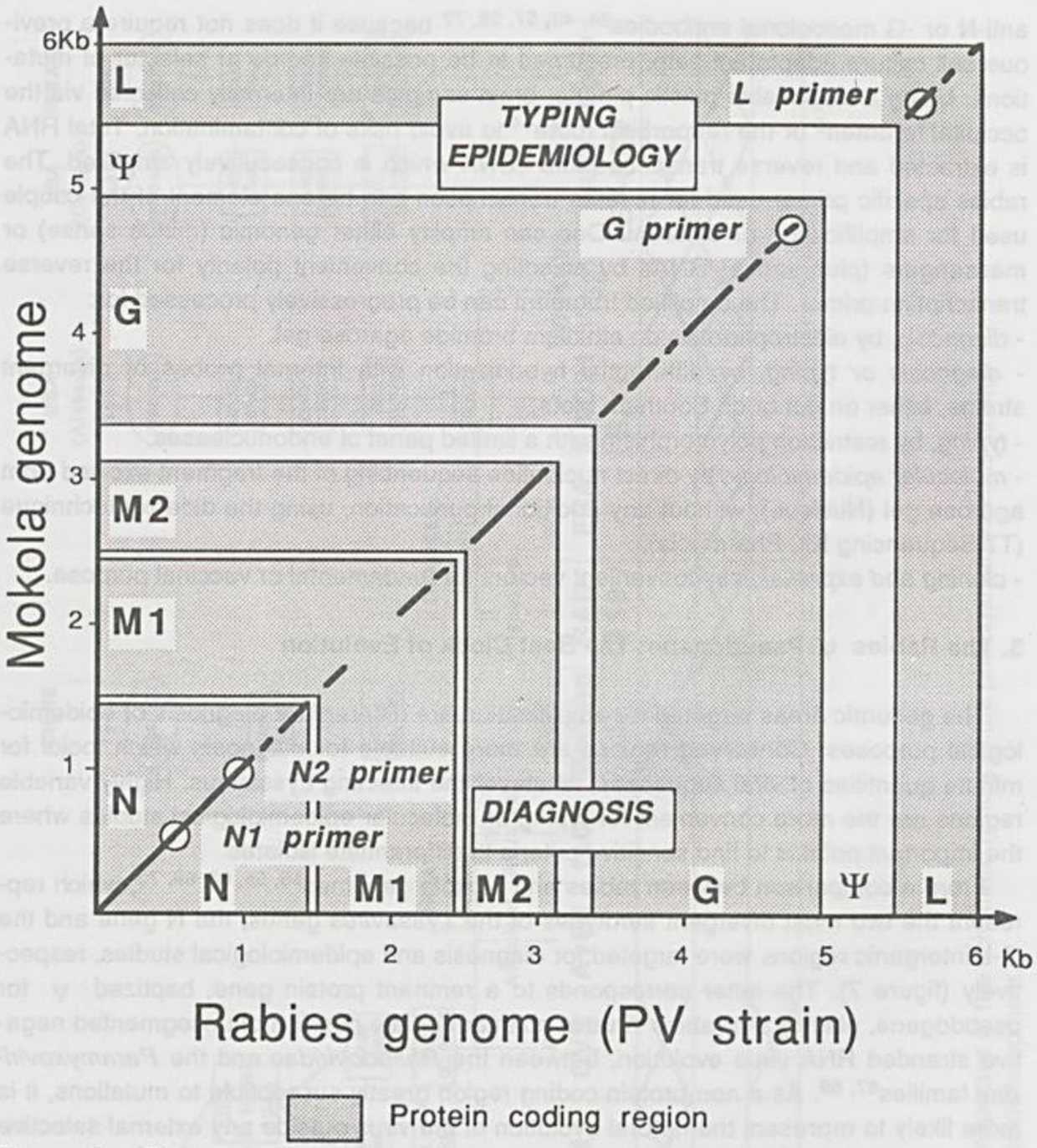

Figure 7 — Sequence comparison of the rabies (PV strain) and Mokola genome sequence. Diagonal lines indicate homologous areas. Regions convenient for diagnosis or typing-epidemiology purposes are noted.

internal divergence in the ψ gene (not shown).

2) The wild isolates of geographic proximity from relatively homogeneous groups. However, groups are substantially divergent from each other as well as from the vaccinal strains.

3) The West-African isolates, notably those suspected of vaccination failures, are consistently more divergent from the vaccinal strains than the French isolates against which the vaccines are clearly effective.

4) Bat isolates recently invading Europe or already present in Latin-America (Brasil, Guyana, not shown) exhibit a divergence almost as important as Mokola virus against which the rabies vaccines are clearly ineffective.

TABLE I

Divergence (%) between wild Lyssavirus groups and the classical vaccinal strain PV: France (12 isolates); West-Africa (Ivory Coast, Cameroon, Niger, Guinea, Marocco, etc...); European bat (France, Poland, Finland).

	ψgene/vaccine	Ggene/vaccine	ψ/group
France	14-15%	ND	2.5%
West-Africa	25-30%	15%	5%
Europ. bat	high	30%	ND
Mokola	high	40-45%	ND

VII. MAIN CHALLENGES FOR THE FUTURE

The recent progress in the molecular understanding of the rabies virus have not provided, so far, pertinent responses to the major enigmas of the disease itself. The reasons for the rabies virus neurotropy are still not understood, despite numerous hypotheses attempting to correlate the susceptibility of each cell type with the presence of specific receptors. Perhaps, the secret of neurotropy will reside in the study of more distal infection events, such as the influence of tissue-specific transcription factors on the mechanism of rabies genome expression. Furthermore, the mystery remains cloudy as to the nature of neuronal dysfunctions resulting in lethality, although their understanding would be a capital step towards an effective therapy.

Despite these numerous unsolved questions, the availability of any viral gene for studying and expression, is the most impressive contribution of the last ten years. In the near future, examination of viral isolates from various regions of the world will permit an evaluation of the spacio-temporal evolution of the virus and the influence of the host. It will be interesting to understand the basis of the cross-protection at the sequence level, in order to decipher whether the current vaccinal strains are sufficient for animal and human health, or if additional specific vaccines are required. These could be performed by DNA-recombinant techniques, taken into account the progress in rabies virus immunology. For example, synthetic structures carrying a recombinant glycoprotein(s) anchored on their surface, and containing a recombinant nucleoprotein(s) (or T-peptides) should be promising in the goal of a genetic engineering vaccine against all (or most of) the members of the *Lyssavirus genus.*

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