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Gene transfer into birds using ALV-based retrovirus vectors

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The difficult access to fowl oocyte nucleus leads to consider retrovirus vectors as appropriate vehicles for gene transfer into avian germinal cells. Although replication-competent vectors have been tested with success, they are endowed with major drawbacks related in particular to their replicative potencies. Such problems can be avoided by using defective retrovirus vectors. We report here our approach to generate and to improve retrovirus vectors derived from the genome of Avian Leukosis Viruses (ALV), and preliminary studies of expression following their inoculation into chick embryos.

I-RETROVIRUS VECTORS AND PACKAGING CELL LINES

Vectors. Starting with the genome of the oncogenic retrovirus AEV (Avian Erythroblastosis Virus) [1], we have constructed a set of double expression (DE) vectors carrying and expressing two genes under control of the *cis*-acting sequences contained in the AEV LTR; one of the gene being represented by the *neo* selectable gene, and the other one being the gene of interest [2,3]. These DE vectors have been recently improved by exchanging the most part of *cis*-acting sequences of AEV by the ones from other ALVs (fig 1). We have shown (Table 1) that we could obtain a gain of one log of virus production (more than 10^6 helper-free particles/ml in the supernatants of some clonal producer cell lines) when the *cis*-acting sequences were originated from Rous Associated Viruses type 1 or 2 (RAV-1 or 2) compared to vectors bearing sequences from AEV [2]. Moreover, these new types of vectors could be characterized by an increased expression and stability of the reporter gene (i. e. *lacZ* gene) in the QT6 avian cell line commonly used as target cells for *in vitro* studies [2,3].

Helper cells. We have constructed plasmids expressing the retroviral structural genes *gag-pol* and *env*. These packaging vectors derived from the genome of RAV-1, from which both the packaging sequence located into the leader region and the 3' terminal regions (3' non coding region and LTR) had been deleted, this latter being replaced by an heterologous polyadenylation sequence. These plasmids allowed us to generate helper cell lines from which AEV-based vectors could be produced at titers of about 10^6 RFU/ml [4]. As an improvement of these cell lines, we have generated a second set of helper plasmids in which the viral coding sequences were expressed from two different plasmids and were linked to selectable markers which were expressed from the same transcriptional unit (either *gag-pol/hygro* or *phleo/env*) [5]. Compared to previous packaging cell [4], we have shown that helper cell lines generated with these two complementary plasmids could be characterized by an enhancement in vector titers [5], and by an increased stability of both expression of helper functions and of vector production (Table 1), [2]. No replication competent viruses could be detected in the supernatants of all of our helper cell lines, even if grown during continuous long-term culture.

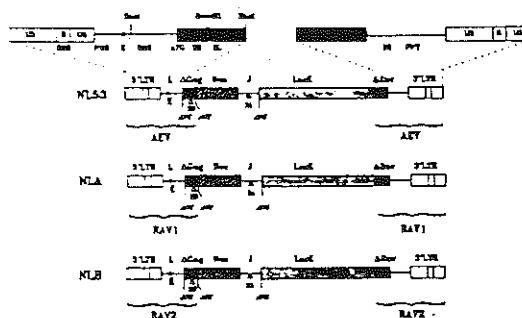


Fig. 1. Structure of NL retrovirus vectors. RBS, ribosome binding site; PBS, primer binding site; E, encapsidation sequence; ATG, *gag* gene initiator codon; SD & SA, splice donor & acceptor sequences; DL, dimer linkage sequence; DR, direct repeat sequence; PPT, polypurine track; LTR, long terminal repeat; L, leader region; J, junction fragment.

TABLE 1. Titers obtained with NL retrovirus vectors transfected into Isolde packaging cell line

| Vector ^a | Neo (RFFU/ml) ^b | Titers | | | |
|---------------------|-------------------------------|-----------------------------------|-----------------|-------------------|-----------------|
| | | <i>lacZ</i> (CFU/ml) ^c | | | |
| | | Initial | 1 mo | 4 mo | 5 mo |
| NL53 | 2×10^4 | 3×10^4 | 2×10^4 | 1.5×10^4 | 1×10^4 |
| NLA | 2×10^5 | 2×10^5 | 2×10^5 | 1.4×10^5 | 1×10^5 |
| NLB | 2×10^5 | 3×10^5 | 1×10^5 | 2×10^5 | 2×10^5 |

^a Vector structures are depicted in Fig. 1. NL vectors were introduced into Isolde packaging cells by lipofection according to the standard procedure recommended by the supplier (Lipofectin, Gibco-BRL);

^b RFFU are per milliliter of supernatant collected from pools of stable producer Nco⁺ clones of Isolde cells;

^c Viral supernatants were harvested after different growth periods (up to 5 months) of NL vector producer cells. ND, not determined. Fewer than 1 helper virus was detected at each titration per 5 ml of viral supernatants, as previously described.

II-IN VIVO GENE TRANSFER

Viral supernatants of three neo-*lacZ* vectors (NLA, NLB, and NL53, Fig. 1) were collected and were concentrated 50-100 times by ultracentrifugation. These viral stocks were inoculated to early chick embryos (before incubation) by injection with a capillary glass pipette into the germinal cavity through the blastodisc at its periphery. About 10^5 *lacZ*-CFU were inoculated per embryos, thus giving a multiplicity of infection of about 2 infectious NL virus per embryo cell. Injected eggs were incubated, and embryos were recovered at different stages of development: 18 h, 72 h, or 5-6 days of incubation. Some embryos were analysed by Southern blot to detect the exogenous *lacZ* gene, and other embryos were fixed and stained with X-GAL to study the *in situ* expression of the NL vectors.

Compared to the number of infectious NL particles injected into the embryos, the number of expression foci was very low since no more than about 30 *lacZ* positive foci could be observed in embryos stained at stage 18 h. By comparison, we have found that NL proviruses could be detected by Southern blots in the DNA of marked embryos, since NL proviruses could be detected above the limit of detection (1/75 cell) for 30% of the embryos; one strongly positive embryo displaying NL proviruses in about 5% of its cells. Hence, only a small proportion of proviruses could give rise to *lacZ* expression (no more than 0.6%, [Thomas et al., in preparation]).

From observations of either whole-mounts or serially sectioned X-GAL stained embryos, we have localized precisely the sites of expression of the helper-free vectors. No evidences for tissue-restriction were observed (Fig. 2). However, slight differences of behaviour between the 3 NL vectors were found regarding the staining of specific organs like the heart and the neural tube. Compared to the vector NLB (in which the regulating sequences originated from RAV-2), the NLA vector (carrying *cis*-acting sequences from RAV-1) was more often expressed in the heart (3 times) and less often in the neural tube (2 times). The vector NL53 in which the *cis*-acting sequences originated from AETV did not display such a preferential tropism of expression. Since variabilities can be found between the sequences of enhancer parts of the U3 regions of these different LTRs, our results suggest that the discrepancies observed between the vectors might be related to specific cellular factors interacting with the LTRs, thus regulating their activities in specific embryo cell types.

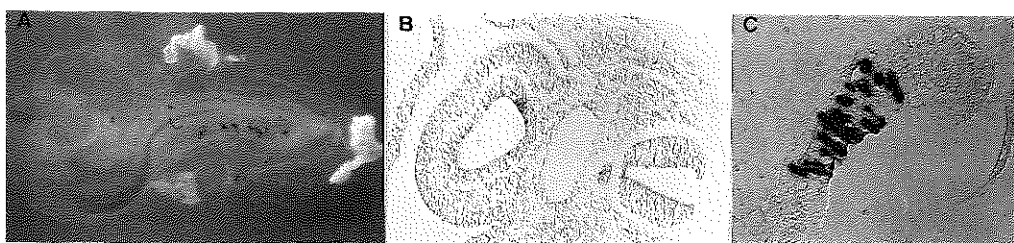


Fig. 2. X-GAL stainings of NL infected chick embryos. NLB-infected embryos, stained at 72 h (A, B) or at 5 days (C), and observed either as whole-mount (A), or after serial sections in the region of the trunk (B) or in the eye (C).

III-CONCLUSIONS AND PROSPECTS

These preliminary results demonstrate that ALV-based vectors can be useful tools for gene transfer into birds. We are currently working on two major fields of interest: germinal transgenesis and use of our vectors as vaccine vectors [Chebloune et al., Cosset et al., in preparation].

Experiments are in progress to improve both retrovirus vectors and packaging cell lines. We are trying to construct vectors with internal promoters able to integrate into target cell DNA as a disorganized proviral structure because of the presence of an internal retroviral attachment sequence within the vector. Such integrations would result in a specific expression of the internal promoter, and in the inability to obtain further virus production [Drynda et al., in preparation].

To develop packaging cell lines, we are constructing helper cells able to produce retroviral vectors pseudotyped by different retroviruses envelopes. We are also trying to modify envelopes proteins in order to increase the affinity of attachment between the virion and its receptor.

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RESUME : Les difficultés d'accès au noyau d'oocytes de volaille nous ont conduit à envisager les vecteurs rétroviraux pour transférer des gènes dans les cellules germinales aviaires. Bien que des vecteurs compétents pour la réplication aient été testés avec succès, leur utilisation est nuancée par des inconvénients majeurs et, en particulier, leur potentiel de réplication. De tels problèmes peuvent être contournés par l'utilisation de vecteurs rétroviraux défectifs. Nous présentons ici une autre approche qui consiste à générer et à améliorer les vecteurs rétroviraux dérivés du génome de virus de la leucose aviaire (ALV), ainsi que l'analyse préliminaire de l'expression consécutive à leur inoculation dans les embryons de poulet.