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Technical Note

Virofection: a new procedure to achieve stable expression of genes transferred into early embryos

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ABSTRACT A new procedure, virofection, designed to stabilize the expression of transfected DNA has been developed. It exploits the capacity of retroviruses to integrate their genome into the chromosomes of host cells. The co-transfection of two plasmids, one carrying the genome of a defective retrovirus vector, the other one encoding all the retroviral proteins, results in a transient production of infectious virus particles. These particles can infect the neighboring cells and this leads to the stable integration of the vector genome. This procedure is time-saving and appears to be quite efficient. When applied to chicken embryonic fibroblasts cultured *in vitro*, it resulted in the stable expression of the lacZ gene in more than 30% of the cells, and did not induce chronic viremia. Stable lacZ expression was also achieved in chicken embryos *in ovo*. Virofection appears to be a promising and generally applicable method for implementing stable, safe and efficient gene transfer *in vitro* and *in vivo*.

KEY WORDS: *gene transfer, chicken, retrovirus*

Introduction

The avian embryo is a favoured model to study vertebrate embryonic development. The use of quail-chick chimeras has provided a great deal of information on the cellular interactions that regulate tissue morphogenesis. However, a more precise understanding of how gene expression regulates embryonic development could come from the transfer and expression of exogenous DNA into the embryo during morphogenesis. For this purpose, efficient gene transfer protocols are required, ideally providing low mosaicism and germ line transmission. Direct transfer of naked DNA into avian embryos can be achieved by micro-injection into the cytoplasm of one-cell embryo (Perry *et al.*, 1991), graft of transfected blastoderm cells (Reddy *et al.*, 1988; Carsience *et al.*, 1993) or *in ovo* DNA transfection (Demeneix *et al.*, 1991, 1993). All these protocols are of limited use, since the spontaneous integration of exogenous DNA into chromosomes is a rare event and, therefore, transgene expression is usually transient. Germ line transmission has been reported only once after one-cell embryo micro-injection (Love *et al.*, 1994).

The use of retrovirus vectors is able to overcome these restrictions because vector DNA is efficiently integrated by the viral integrase into the genome and permanently expressed from the virus long terminal repeat (LTR). Both replication competent and replication defective vectors have been derived from avian leukosis viruses. Although replication competent vectors provide very efficient means of transferring genes (Hughes *et al.*, 1987; Hughes, 1988; Petropoulos *et al.*, 1992; Fekete and Cepko, 1993), they have various limitations: first they cannot accommodate exogenous sequences larger than 2 kb; second they induce chronic viremia; third, because recombination and point mutations occur at a high rate during reverse transcription, multiple rounds of virus replication are likely to mutate the transferred sequences. To our knowledge, these vectors have not yet been used to transfer a non-viral functional transgene through the avian germ line.

All the limitations of replication-competent vectors mentioned above can be circumvented by using helper-free preparations of replication-defective retrovirus vectors (Cosset *et al.*, 1990, 1991; Thomas *et al.*, 1992; Stocker *et al.*, 1993). Such vectors are unable to synthesize certain viral proteins but can replicate if the missing proteins are supplied *in trans* by a helper cell. Virus obtained from helper cells execute only one round of reverse transcription and integration. Therefore, the probability of mutating the exogenous sequences is reduced and, moreover, viremia does not occur. However, as retrovirus particles are highly unstable, replication-defective vectors are difficult to produce at high titre and have led to only a few cases of germ line transmission when injected into numerous early embryos (Bosselman *et al.*, 1989).

Abbreviations used in this paper: LTR, long terminal repeat; Xgal, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside; ßgal, ß-galactosidase; lac cells, cells with ß-galactosidase activity.

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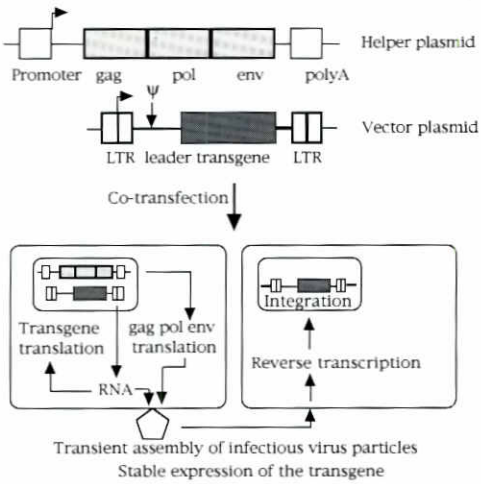

Fig. 1. Principle of virofection. *Two plasmids are transfected into the cells: the helper plasmid encodes all the gag, pol and env products necessary for the assembly of retrovirus particles. In these virus particles the only RNA molecules that can be efficiently packaged are those transcribed from the upstream LTR to the downstream LTR of the defective retrovirus vector (avian retroviral sequences are boxed by thick lines). Within a few days of transient expression, infectious virus particles are assembled and can infect the neighboring cells where the defective viral genome is reverse transcribed and integrated by the viral integrase. Non-integrated DNA molecules will be gradually lost but the transgene expression will continue, ensured by integrated provirus.*

The procedure we present here is an original combination of DNA transfection and infection by a defective retrovirus vector. The protocol called "virofection" is straightforward and, as it leads to stable gene expression, it is a promising method for both *in vitro* and *in vivo* applications.

Results

Principle of virofection: a combination of DNA transfection and retrovirus infection (Fig. 1)

Virofection requires the co-transfection of two plasmids. The first, or "vector", plasmid carries the genome of a defective retrovirus vector into which the DNA sequences to be transferred are inserted. The second, or "helper", plasmid is an expression vector encoding the gag, pol and env genes of an avian leukosis virus. Transfection results in the transient expression of both plasmids within the same cells. The helper plasmid expression produces retrovirus particle proteins. Because the RNA transcribed from the vector plasmid carries a complete retrovirus leader sequence, it is packaged into these particles. Consequently, infectious particles can bud out of the transfected cells and infect the neighboring cells where the vector genome is reverse transcribed and integrated into the chromosomes by the highly specific retroviral integrase. After several days, DNA that is not integrated is degraded by the cells or diluted during successive cellular divisions. Thus the stable expression of the vector genome is mainly a result of the retrovirus-mediated integration.

Construction of helper and vector plasmids (Fig. 2)

A conceivable pitfall in our approach could result from the extreme ability of retroviruses to recombine (Hu and Temin, 1990). Indeed, recombination between the helper and vector genomes could rescue a replication-competent retrovirus, which would quickly contaminate all the cells. Therefore, we designed a helper plasmid and a vector plasmid displaying minimal sequence homologies. The helper plasmid Cistorav was constructed by inserting the avian gag, pol, env sequences into an expression plasmid with a murine leukemia virus promoter that is active in avian cells. This construct fulfils the conditions necessary to function as a safe helper plasmid, since it does not carry the avian viral non-coding sequences which are necessary at several steps of the avian virus life cycle (reviewed in Varmus and Swanstrom, 1984), i.e. leader packaging sequence, primer binding site, and long terminal repeats. We constructed pBlagy3, a defective vector carrying a lacZ-NeoR fusion gene, which confers both ß-gal activity and G418 resistance to eukaryotic cells. In this vector, only 298 nucleotides of the avian gag coding sequence and 83 nucleotides of the avian env-coding sequence are homologous, but not identical, to Cistorav sequences. We also used pLZ10, a defective vector encoding a gag-lacZ fusion protein (Gray *et al.*, 1988) which contains slightly larger gag and env sequences.

In vitro virofection leads to stable lacZ expression (Fig. 3)

To test the ability of virofection to stabilize the expression of a reporter gene, we used Transfectam to introduce pLZ10 alone or together with Cistorav into cultured CEF. We then followed the expression of the defective genome LZ10 during the next 16 days by assaying cell fractions for ß-gal activity *in situ*. When pLZ10 was transfected alone, the frequency of the cells displaying lacZ expression (lac+ cells) was high (15%) by day 2 and then quickly dropped. This decrease resulted from poor integration and further dilution of transfected DNA. When Cistorav was added in the transfected DNA, the resulting lacZ expression was similar for the first days but a slow increase in lac+ cells frequency was observed subsequently. This stabilization of lacZ expression was presumed to be the consequence of the virofection process described above. It also indicated that lacZ expression did not impair cell proliferation. As no virus was recovered from the cell supernatant after 16 days, the

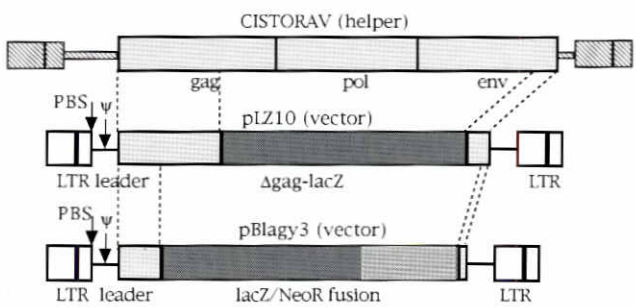

Fig. 2. Structure of the helper and vector plasmids. *Cistorav is composed of an assembly of the non-coding sequences of a murine retrovirus (hatched boxes) unrelated to avian leukosis viruses, and the coding sequences of the Rous associated virus type-1. The defective retrovirus genome LZ10 derived from Rous sarcoma virus is shown below. pBlagy3 was constructed by replacing most of the coding sequences of Rous associated virus type-2 by a lacZ-NeoR fusion gene. Dotted lines correspond to the homologies found between Cistorav and the vector genomes, in the gag coding sequence and at the end of the env sequence.*

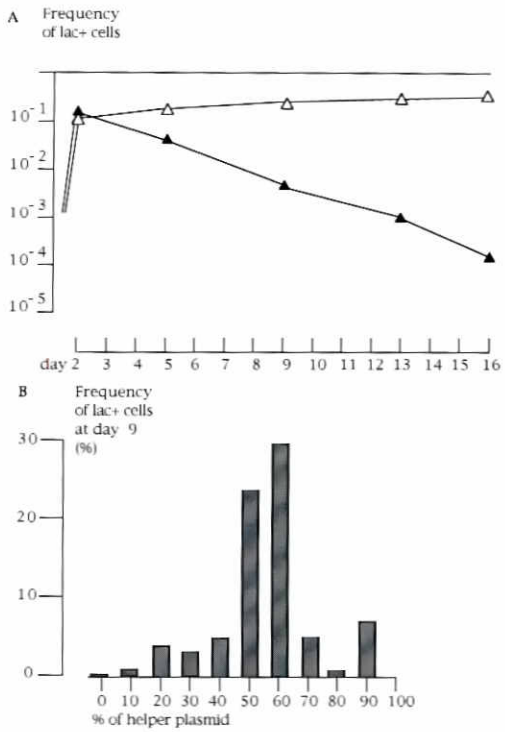

Fig. 3. Virofection of the lacZ gene in CEF *in vitro* . (A) *Time course: white triangles: co-transfection of a 60/40% mixture of Cistorav (helper plasmid) and pLZ10 (vector plasmid carrying the lacZ gene). Black triangles: transfection of pLZ10 alone. The frequency of CEF displaying lacZ expression in situ (lac+ cells) is plotted on a logarithmic scale.* **(B)** *Influence of the relative ratio of pLZ10 and Cistorav on the frequency of lac+ cells by day 9.*

stabilization of lacZ expression was not associated with the rescue of a replication-competent recombinant virus. The efficiency of stable gene transfer was dependent on the relative ratio of Cistorav to pLZ10 in the transfected DNA (Fig. 3b). A maximum of 30-40% lac+ cells was reached when nearly equimolar amounts of helper and vector plasmids were co-transfected.

This final ratio is also dependant on initial transfection efficiency. As shown on table I variations in transfection efficiency (i.e. ratio of lac+ cells by day 2) are mainly related to Transfectam batch-to-batch variation, whereas the occurrence of a stabilization in lacZ expression is highly reproducible (day 9/day 2= 2.0±1). Therefore, when DNA concentrations are precisely determined, virofection is as reproducible as transfection can be.

The same experiments were repeated with pBlagy3. In this case the highest frequency of lac+ cells was 6% (data not shown). Co-transfected cells were also seeded at low density into selective medium (250 µg/ml of G418) on day 2 and ß-gal activity of resistant cell clones was assessed on day 10. Only 15% of the G418-resistant clones contained lac+ cells. Staining was uniform in each clone, suggesting that the defect in ß-gal activity observed in the majority of the cells originates from the initial integration event. Despite the fact that Blagy3 provide less lacZ expression than LZ10, as its structure may be often rearranged during virus replication, it was used in further experiments when G418 selection was required.

Stabilization of lacZ expression results from retrovirus mediated integration

The supernatant of CEF was assayed for the presence of infectious LZ10 virus 2 days after transfection of Cistorav and pLZ10, by infecting QT6 cells. Less than 100 lac+fu/ml was detected. No infectious virus was detected beyond day 4. This low titre indicates either that the virus production is very low, or that free virus particles have a short half-life, as they can quickly infect neighboring cells. To confirm that this transient virus production is responsible for the observed stabilization of lacZ expression, we included pA0nlslacZ, a non-retroviral expression vector encoding a nuclear targeted ß-gal (Bonnerot *et al.*, 1987) into the transfection mixture, together with Cistorav and pLZ10 (Fig. 4). Two days after transfection, cells with stained cytoplasm and/or nucleus were observed. This corresponded to the expression of pLZ10 and pA0nlslacZ respectively. However, 6 days later, only cytoplasmic staining was observed, confirming that virofection only stabilizes the expression of virus-borne sequences. A Southern blot was also

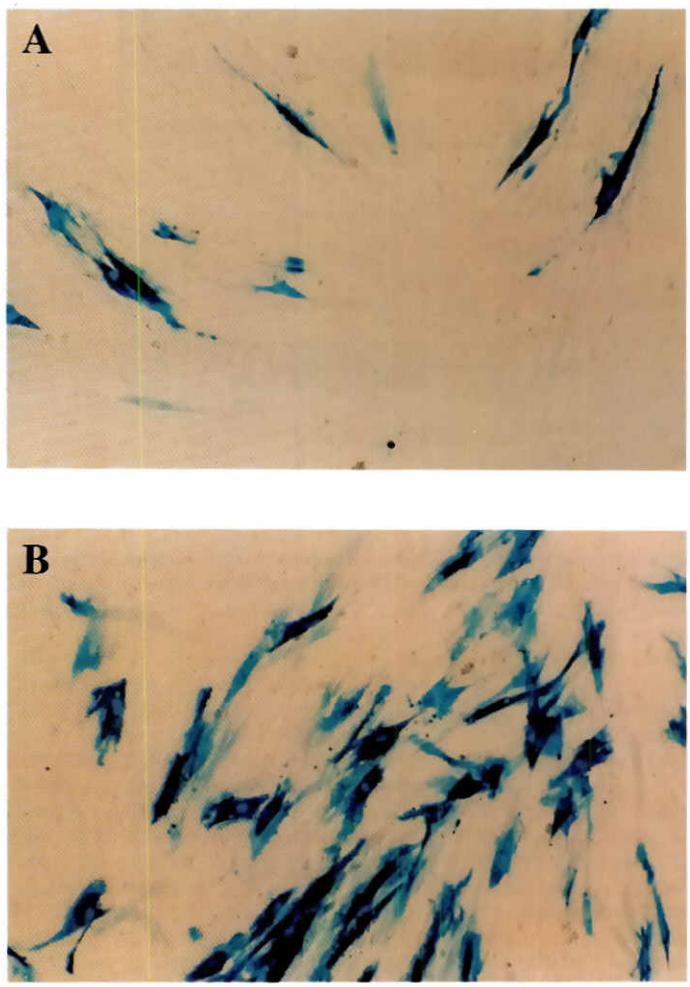

Fig. 4. Virofection stabilizes the expression of only virus-borne sequences. *CEF were co-transfected with pLZ10, Cistorav and pA0nlslacZ, a non-retroviral expression plasmid encoding a nuclear targeted ß-galactosidase.* **(A)** *By day 2, cells with cytoplasmic and/or nuclear staining were observed.* **(B)** *By day 6, cytoplasmic staining was observed almost exclusively, resulting from the stabilization of only LZ10 expression.*

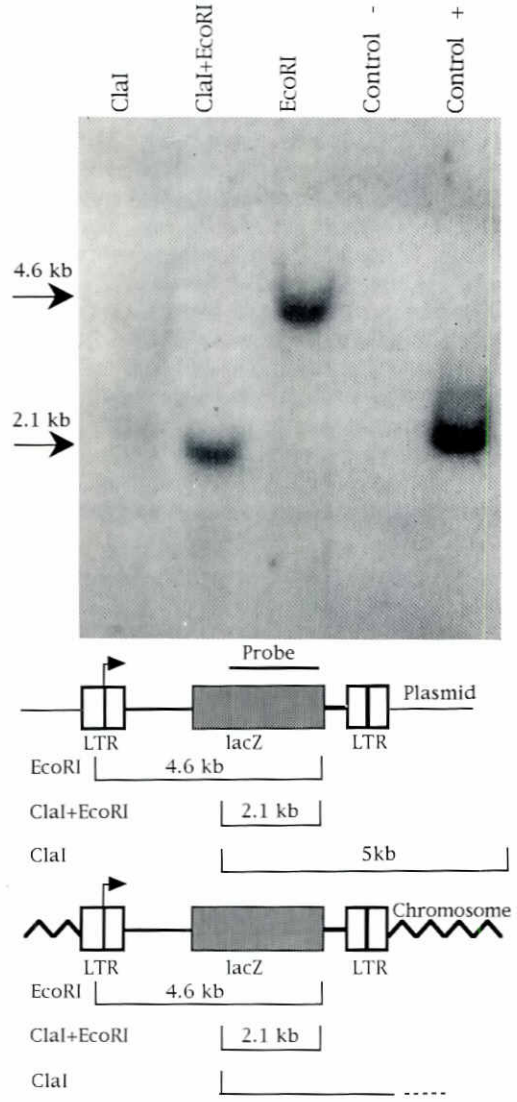

Fig. 5. Structure of the lacZ gene integrated in CEF after virofection is reminiscent of a retrovirus-mediated integration process. *CEF DNA extracted 16 days after in vitro virofection. Aliquots of the same DNA sample were digested with EcoRI, ClaI, or both, and hybridized to a lacZ probe. Control-: DNA from non-transfected fibroblasts, digested with EcoRI. Control+: DNA from a cell line carrying one copy per genome of lacZ, digested with ClaI. Ethidium bromide staining was used to control equal loading of the wells and homogeneous transfer on nylon membrane. EcoRI digestion reveals that the lacZ gene is probably present in a large fraction of the cells (compare with control +). EcoRI+ClaI digestion confirms correct digestion by ClaI. Digestion by ClaI alone makes it possible to distinguish between randomly integrated or non-integrated copies and copies integrated by the retrovirus integrase (the two possible restriction maps are depicted below). Copies of lacZ integrated by a random process are likely to integrate together with plasmid-flanking sequences and to yield a 5 kb ClaI fragment. If the integration is mediated by retrovirus integrase, the plasmid sequences are lost and the size of the ClaI is dependant on the integration site. As many independent integration events occur, no defined bands are expected.*

performed with the DNA extracted from cells 16 days after co-transfection of pLZ10 and Cistorav (Fig. 5). The ClaI restriction pattern revealed that the sequences flanking the LTR in pLZ10 had been lost during the integration process, a characteristic of specific retrovirus-mediated vector integration. As expected, no signal was observed when the same blot was hybridized with a probe covering the murine sequences of Cistorav (data not shown). Although no selection was made for lacZ expressing cells, no rearrangements were detected within the viral LZ10 structure in this experiment.

In vivo virofection leads to stable lacZ expression (Fig. 6)

To illustrate the potential of virofection for a stable gene transfer *in vivo*, pLZ10 and Cistorav were co-transfected into chicken embryos after 40 h of incubation, according to a methodology previously shown to induce the expression of several reporter genes including lacZ. In the original protocol, gene expression was transient and disappeared within 3 days after transfection (Demeneix *et al.*, 1993). In the present experiments 1 to 100 lac+ cells were observed in the majority of embryos and their extra-embryonic membranes, when whole-mount Xgal staining was performed 24 h after transfection. At least one lac+ cell focus was present in the majority of the embryos stained 5 or 6 days later. In several cases, a large number of lac+ foci was observed. The lac+ cells could be found in any part of the embryo, with a marked preference for the heart. Histological sections revealed that lac+ cells were present in several tissues including ectoderm, limb mesenchyme (Fig. 6D), neural tube, and myocardium (Fig. 6B and 6C). As whole-mount Xgal staining cannot be performed later in development, we prepared primary cell cultures from 7-day-old transfected embryos to address the stability of lacZ expression into the embryonic cells. Primary cultures from two out of six embryos contained lac+ cells. In one case lac+ cells belonged to a cell type that was quickly overgrown by fibroblasts. In another case lac+ fibroblasts were found and maintained within the cell population for 2 weeks. The frequency of lac+ cells (10^{-4}) and the intensity of the staining did not decrease during these two weeks. No replication-competent virus was found in cell supernatant at the end of the experiment.

To substantiate that virofection stabilizes gene expression *in vivo* we repeated these experiments with pBlagy3 instead of pLZ10, because this vector allows the selection of cells expressing the transgene by exploiting their resistance to G418. One out of three embryos displayed significant ß-gal activity in cardiac cells when whole-mount Xgal staining was carried out at embryonic day 8. Primary cultures were prepared from ten other embryos. Although no lac+ cells were observed in the primary cultures (<1/10^7), many G418-resistant colonies could be selected in seven cases (Table 2). These G418-resistant cells did not display any ß-gal activity, although they grew for several weeks when kept under selective pressure. Thus vector expression lasted for at least one month and was clearly stable. At this late stage, cells were still free of replication-competent virus. Southern blotting and PCR revealed the presence of lacZ sequences in these cells in only one out of five cases, and the presence of NeoR sequences in all cases (data not shown). It is therefore likely that, as in the *in vitro* experiment, the discrepancy between lacZ and NeoR function results from large deletions occurring during the replication of Blagy3.

Taken together, these data clearly demonstrate that virofection can operate *in vivo* to stabilize the expression of vector-borne sequences. As the initial transient expression is variable, the rate of stable expression is also variable (Table 2). However, the virofection process itself seems to be highly reproducible.

Comparison between virofection and helper-free infection

The prevalent way to use a replication-defective vector is to prepare helper-free virus supernatant after the stable transfection

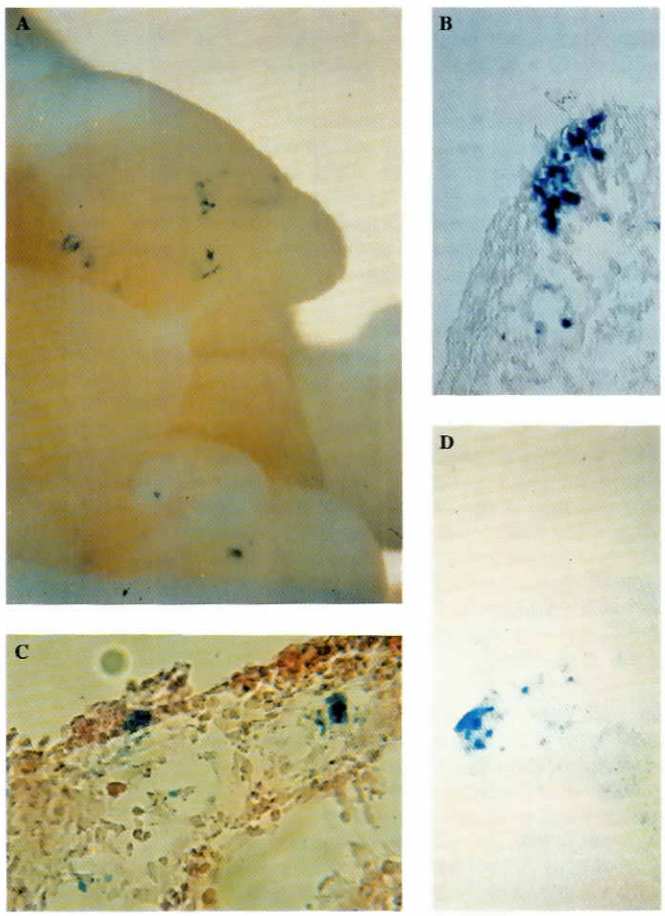

Fig. 6. Expression of lacZ six days after *in vivo* virofection. *Chicken embryos incubated for 40 h were co-transfected with* **(A)** *Cistorav+pBlagy3 (50/50%) or* **(B-D)** *Cistorav+pLZ10 (50/50%) and stained with Xgal 6 days later. A) Whole-mount. Lateral view showing lac+ foci on heart and limb bud.* **(B)** *Section of heart (magnification, x200).* **(C)** *Section of myocardium counter-stained with eosin (x800).* **(D)** *Section of a limb bud (x130).*

of helper cells. Such preparation can be injected into early embryos to obtain a stable expression of the vector genome. When an ALV vector carrying the lacZ gene was injected into the subgerminal cavity of several thousands of unincubated embryos, a single germ line transmission was observed (P. Thoraval, personal communication). Therefore, it seemed interesting to compare helper-free infection with *in vivo* virofection. High titre preparations of LZ10 ($2x10^6$ lac+fu/ml) or Blagy3 (10^5 lac+fu/ml) were injected into the subgerminal cavity of unincubated embryos or deposited on 40-hour embryos. Xgal staining was performed 2 or 4 days later. Primary cell cultures were prepared from 8-day embryos. The observation of 31 inoculated embryos can be summarized as follows: a) When LZ10 was injected into the subgerminal cavity of unincubated embryos, up to 100 lac+ foci were found in the extra embryonic membranes. However less than 5 foci were observed on the embryo. b) When LZ10 was inoculated to 40-hour embryos, a maximum of 13 lac+ foci was observed on the embryo. c) When Blagy3 was used instead of LZ10 only one lac+ was present on one of the embryos, whereas less than 20 lac+ foci were on extraembryonic membranes. d) Cells cultured from 3 embryos inoculated with Blagy3 were selected with G418. Respectively 2, 5 and 6 G418-resistant colonies were rescued (compare with Table 2). These data show that, at least in our hands, virofection can be more efficient than helper-free infection for a stable expression of foreign sequences in chicken embryos.

Discussion

The principle of virofection combines the advantages of highly efficient transfection techniques providing transient plasmid expression, with the capacity of retroviruses to integrate into the chromosomes during viral infection. As it is a one-step protocol where the virus producing cells are also the target cells, it is totally different from the production of virions by transient plasmid expression described for murine retrovirus vectors (Landau and Littman, 1992). Virofection also contrasts with standard transfection protocols as the expression of the sequences of interest is expected to be stable. More than 30% of the cells can integrate the transgene after *in vitro* virofection. In many situations this ratio is sufficient to dispense with subsequent selection of cells that stably express the transferred sequences.

TABLE 1

REPRODUCIBILITY OF *IN VITRO* VIROFECTION

Experiment	Transfectam batch	DNA batch	%lac+ cell (day 2)	%lac+ cell (day 9)	day 9/day 2 ratio
1	1	1	10	16	1.6
2	2	1	2	8	4.0
3	3	2	15	40	2.6
4	4	2	10	10	1.0
5	5	2	9	12	1.3
6	5	3	7	11	1.6

Virofection has a number of important advantages over both the use of replication-competent avian retrovirus vectors and the infection with helper-free avian retrovirus preparations. First, with virofection one avoids the two main restrictions of replication-competent vectors, i.e. a 2 kb size limit for transferred sequences and a persisting virus infection. Second, virofection does not require the selection of vector-producing cells, a time consuming part of the preparation of replication-defective vectors. Third the short half-life of virus particles does not impair gene transfer efficiency, as virus production and infection are closely associated. Fourth, there is no need to insert a selection gene within the vector, making the design of efficient constructs easier. In fact, constructs can be easily made by inserting cDNA into the polylinker of an available vector plasmid. Finally, since the time consuming and highly variable procedures associated with virus titration are not required, many constructs can be rapidly tested and compared *in vitro*.

The experiments presented here illustrate the potential of virofection as a general method for obtaining stable expression of transfected genes both *in vitro* and *in vivo*. Stable expression should occur in any population of proliferating cells, since retrovirus can only integrate in replicating cells. In this respect, we have found that Transfectam does not affect cell proliferation and yields higher rates of stable expression than other transfection reagents. We

TABLE 2

RESCUE OF G418 RESISTANT CELL CLONES FROM CEF PREPARED FROM EMBRYO 6 DAYS AFTER VIROFECTION WITH BLAGY3

Embryo	Frequency of G418 resistant cells ($\times 10^{-7}$)
A	45
B	12
C	>100
D	5
E	44
F	>100
G	24
H	0
I	0
J	0

have repeatedly observed that the constructs used here do not recombine to produce a replication-competent virus. However we have sometimes observed the rescue of replication-competent viruses with other vectors carrying larger sequences homologous to Cistorav.

Virofection has been more efficient in our hands than helper-free infection for *in vivo* applications. As it is difficult to control all the experimental parameters, this observation should not be considered as fully conclusive. Nevertheless, the difficulty of infecting early embryos with helper-free virus preparations has been described by others (Reddy *et al.*, 1991). This may reflect the instability of retrovirus particles, and perhaps a low number of receptors on early embryonic cells. Virofection could overcome these problems by producing a limited number of virus particles in the close vicinity of the target cells. Avian species have proved to be very refractory to germ line transgenesis and direct transfer of DNA has failed to provide transgenic birds until now. Therefore, it would be interesting to adapt the principle of virofection to chicken oocyte micro-injection (Love *et al.*, 1994) or grafting of transfected blastoderm cells (Fraser *et al.*, 1993). As these two protocols are performed at a very early stage of development, they would favour germ line transmission of the vector. We propose that a similar approach might also be beneficial to transgenesis in non-avian species.

Materials and Methods

Plasmids

Cistorav was constructed by inserting the large SacI fragment of pHF13 (Savatier *et al.*, 1989) into Cistor (Sorge *et al.*, 1984) at a ClaI site located between two LTR from the amphotropic murine retrovirus 4070A. pBlagy3 carries the genome of a replication-defective retrovirus vector. It was constructed by replacing the coding sequences of the Rous associated virus type-2 by a lacZ/NeoR fusion cassette (Friedrich and Soriano, 1991) using the XhoI site of gag and an AccI site located upstream to the 3' LTR. pLZ10 carries a defective avian retrovirus vector derived from the Schmidt-Ruppin (A) strain of Rous sarcoma virus and encodes a gag-lacZ fusion protein (Gray *et al.*, 1988). pA0nlslacZ is a non retroviral expression vector encoding a nuclear targeted ß-galactosidase (ß-gal) driven by an SV40 promoter (Bonnerot *et al.*, 1987). pKoNeo is an SV40-derived expression vector carrying the NeoR gene.

In vitro transfection of chicken embryonic fibroblasts (CEF)

Cells were prepared from C/O SPAFAS embryos as described previously (Janpas and Demayers, 1990; Flamant *et al.*, 1993) and split every three days (1/3). Transfection with Transfectam (Promega) was carried out by adding a mixture containing 5 µg of DNA and 400 µl of Dulbecco's Modified Eagle Medium and 10 µl of Transfectam to 10^5 CEF. Medium was changed after 1 h, and cells were then kept in exponential growth.

In vivo transfection of chicken embryos

Embryos were transfected after 40 h of incubation, mainly as described previously (Demeneix *et al.*, 1991, 1993). Usually 5 µg of DNA in 20 µl of 150 mM NaCl was mixed with 0.5 µl of Transfectam (40 mM in 100% ethanol), then 4 µl of this mixture was deposited on the embryo with a glass capillary pipette. Eggshells were sealed with transparent tape and the eggs returned to a 38°C humidified incubator.

Assaying for virus expression

Histochemical detection of ß-gal activity was performed on CEF and whole-mount embryos by overnight staining with 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Xgal) at 30°C (Sanes *et al.*, 1986; Price and Thurlow, 1988). We verified that CEF reached confluency at 1.6×10^5 cells per cm^2. Therefore we were able to calculate the ratio of vector expressing cells by counting only lac+ cells on a defined surface of confluent cultures. Several hundreds of lac+ cells were counted in each dish. To control the accuracy of this method, we plated several cell samples at low density and counted both lac+ and lac- cells and obtained identical results. Cells were assayed for the production of replication competent virus by laying 0.2 µm filtered supernatant on QT6 cells (Moscovici *et al.*, 1977). Two days later, these QT6 cells were assayed for gag production by an *in situ* immunoassay (Savatier *et al.*, 1989).

Production of helper-free virus preparation

pBlagy3 or pLZ10+pKoNeo were transfected into Isolde helper cells (Cosset *et al.*, 1990) and submitted to G418 selection. Cell clones expressing high levels of ß-gal in a stable manner were further selected. Medium of sub-confluent cells was replaced by serum-free Dulbecco's Modified Eagle Medium and the cell supernatant was harvested 16 h later. After filtration on a 0.4 µm membrane, virus was further concentrated on Centricon30 (Amicon). Titration was performed on QT6 cells by end point dilution, using the *in situ* Xgal assay.

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