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Original Article

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ABSTRACT **Defective avian leukosis-based vectors expressing the bacterial lacZ gene were used as helper-free preparations to infect early stage Brown-Leghorn embryos. Both *in toto* X-gal staining and DNA analysis using Southern blot technique were applied to detect virus integration and expression. Our results demonstrate a low efficiency of *in vitro* infection in early stages of embryonic development. Southern blot analysis reveals that only 1% of embryonic cells integrate the vector genome after infection using 2 to 12 virus particle per embryonic cell. *In situ* expression of the lacZ marker gene was detected in only 0.06% of embryonic cells. These results lead us to conclude that only 6% of infected cells express efficiently the lacZ marker gene. This low level of expression could result from avian leukosis virus LTRs inhibition in chicken embryonic cells at an early stage of development. In spite of the low effiency of infection, no evidence for tissue restrictive expression was observed. However, vector containing LTRs from RAV-2 virus allows preferential expression of provirus vector in neural tube tissue, whereas cardiac localization of the preferential expression was observed using vector containing the RAV-1 LTRs. The chronological analysis of the marker gene expression in terms of location of expression foci and sizes of these foci, lead us to hypothesize the putative regulation of retrovirus expression linked to embryonic development.**

KEY WORDS: *retroviral vector, chick embryo, lacZ, expression, ALSV*

Introduction

Nowadays *in vivo* gene transfer in rodents is widely used and represents a major technique for studying several aspects of gene biology. Gene transfers have been successfully performed either by microinjecting plasmid DNA into cells (Palmiter *et al.*, 1982; Lacy *et al.*, 1983; Hammer *et al.*, 1985), or by infecting cells with recombinant vectors produced as helper-free or replication-competent virus particles (Jaenisch *et al.*, 1981; Van der Putten *et al.*, 1985; Rubenstein *et al.*, 1986; Soriano *et al.*, 1986; Stuhlman *et al.*, 1989). In contrast, gene transfer into birds has still not reached the same level of accomplishment. This is due in part to the position of embryo in oocyte possessing a great mass of vitellus, and second to the difficulty arising from zygote access in the female genital tract. Moreover, the chick ovum is fragile to manipulate outside the shell (Freeman and Messer, 1985). Somatic gene transfers in birds have been obtained with retroviral infection (Shuman and Shoffner, 1986; Salter *et al.*, 1987; Hippenmeyer *et al.*, 1988; Lee and Shuman, 1990) and also with DNA microinjection (Sang and Perry, 1989; Naito *et al.*, 1990; Perry and Sang, 1990). Several authors have reported somatic gene transfer into birds by using either REV- or ALV-based vectors. Souza *et al.* (1984) have provided evidence for somatic expression of chicken growth hormone (cGH) in blood of 7-day-old chickens after hatching by infecting 9-day-old embryos with a Rous sarcoma virus (RSV) vector carrying a cGH gene. Using a RSV vector in which the *src* gene was replaced by the *neo* gene, Hippenmeyer *et al.* (1988) have studied the sites of expression of the vector by performing biochemical titrations of the NPT-II enzyme (neomycin phosphotransferase) encoded by the *neo* gene in various organs. Bosselman *et al.* (1990b) have reported the expression into 15-day-old embryos of a cGH gene inserted into a reticuloendotheliosis virus (REV)-based defective vector. However, little is known on precise sites of cell or tissue expressions since

Abbreviations used in this paper: ALV, Avian Leukosis Virus; RAV-1, Rous Associated Virus type 1; cGH, chicken growth hormone; RSV, Rous sarcoma virus; REV, reticuloendotheliosis virus; lacZ-EFU, lacZ-expression forming unit; PGC, primordial germinal cell.

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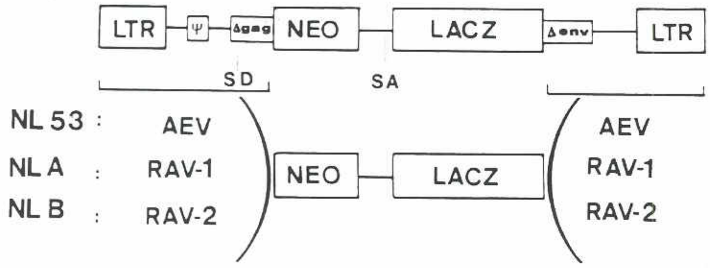


Fig. 1. Structures of the lacZ-retroviral vectors. *These vectors (Cosset et al., 1991) carry and express both the neo^r (conferring resistance to G418) and the lacZ gene (coding for E.Coli ß-galactosidase). The neo^r gene is expressed from a genomic RNA and the lacZ gene is expressed from a subgenomic RNA processed from genomic RNA by splicing of sequences between SD (splice donor site) and SA (splice acceptor site) sequences. The 3 different vectors share the same neo-lacZ fragment whereas the cis-acting sequences LTRs, packaging sequence (), gag and env residues are of various origins: from ALV for NL53 vector, from RAV-1 for NLA vector and from RAV-2 for NLB vector.*

expression of introduced gene was most often monitored after biochemical titrations of lysates of organs. Data on tissue expression of retroviral vectors have been reported from experiments related to some aspects of cell lineage during embryogenesis by using the E. Coli lacZ gene allowing an accurate localization of the sites of vector expression after histochemical techniques (Gray *et al.*, 1988; Galileo *et al.*, 1990; Stoker *et al.*, 1990).

Concerning germinal gene transfer, successful results were reported from approaches using retroviral infections. Some transgenic chicken lines have been obtained by infection of 0-h-old embryos with replication-competent recombinant Avian Leukosis Viruses (ALV) carrying the coding sequences of RAV-1 (Rous Associated Virus type 1) which bears subgroup A envelopes (Crittenden *et al.*, 1989; Salter and Crittenden, 1989). Some of these chicken lines were protected against infection with an ALV of subgroup A, thus demonstrating the efficient expression of the exogenous *env* gene inserted in the germ line. Similarly, Bosselman *et al.* (1990a) have obtained germinal gene transfer with a helper-free REV-based vector leading to an average rate of 2-8% transmission from mosaic males of G^0 generation to G^1 generation. Lee and Shuman (1990) have also reported successful gene transfer into quails by using helper-free REV-based vector, but with a lower efficiency than that above mentioned (0.06% G^1 quail were found to be transgenic by using REV-based vectors). Until now, no reports of germinal gene transfer using defective ALV-based vector have been published.

Whatever is the goal of these studies, the authors have focused on the need for an efficient system for gene transfer: vectors and packaging cell lines. Authors working with REV-based vectors (Shuman, 1984; Shuman and Shoffner, 1986; Bosselman *et al.*, 1990a,b; Lee and Shuman, 1990) use the canine D17-C3 packaging cell line established by Watanabe and Temin (1983), and more recently, a new packaging cell line (DSN) for REV-based vectors (Mikawa *et al.*, 1991). ALV-based vectors were produced from a QT6-derived packaging cell line (Gray *et al.*, 1988; Stoker and Bissel, 1988; Galileo *et al.*, 1990; Stoker *et al.*, 1990). We have previously described the generation of ALV-based vectors (Benchaibi *et al.*, 1989), of the corresponding helper cell lines (Savatier *et al.*, 1989; Cosset *et al.*, 1990), and of their improvement (Cosset *et al.*, 1991). The resulting Isolde packaging cell line produces high levels of defective vectors completely free of any helper particles (Fuerstenberg *et al.*, 1990).

In the work reported here, we have studied the expression of three ALV-based retroviral vectors in chick embryos after inoculation of helper-free virus stocks at early stages of embryogenesis. Our results show that the efficiency of expression was very low in spite of the high multiplicity of infection (more than 1 viral particle per cell). Every embryonic tissue could express the three vectors. However, some preferential tissue expression seemed to be observed in relation to the viral origin of regulating sequences carried by the different vectors tested.

Results

Choice of an inoculation protocol

Twenty-five 24 h embryos (E1 embryos) were infected with 6×10^5 NLA lacZ-EFU (lacZ-expression forming unit); then 22 embryos were recovered after 48 h of incubation and were found to be grown to developmental stage 17 following Hamburger and Hamilton (1951).

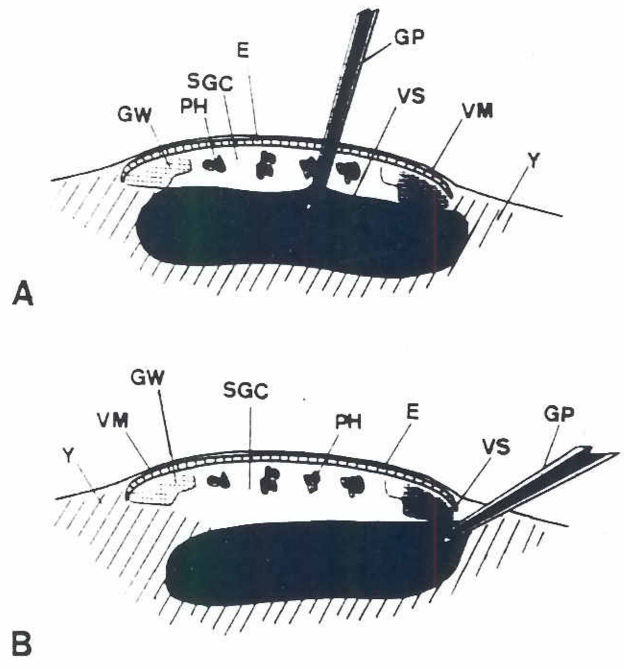


Fig. 2. Procedure of injection of viral suspensions in E0 embryos. (A) *Intrablastodermic injections which cause a tear in the epiblast cell-layer and compromise the survival of embryos when 20 µl of viral stock is injected,* **(B)** *periblastodermic injection, immediately along the side of germinal wall. In this latter case the liquid did not always spread well under the blastoderm, but this allowed the best survival ratios. E: epiblast; GP: glass pipette; GW: germinal wall; PH: primary hypoblast; SCG: subgerminal cavity VS: Viral Stock; VM: vitelline membrane; V: vitellus; Y: yolk.*

After X-GAL staining, 9/22 embryos were found positive for either embryonic or extra-embryonic tissues (40%). Among these 9 embryos, 7 were also positive for embryonic tissues. LacZ-positive foci were found into the trunk, the head, and the ectoderm (data not shown).

Since the efficiency of infection was very low (no more than 100 lacZ-foci were found for $6x10^5$ lacZ-EFU injected in the best marked embryos), we sought to determine whether a higher number of ß-gal-positive cells could be obtained by modifying the ratio between infectious particles and target cells. Therefore, a similar experiment was attempted by injecting retroviral vectors at an earlier stage of development (0 h), corresponding to about $5x10^4$ cells. In a first experiment, 20 µl of concentrated viral suspensions of either NLA or NLB vectors supernatants were injected through the blastodisc (see Fig. 2A) of 0 h embryos. In these conditions no more than 20% of the embryos were still alive at the 72 h stage. Death of embryos occurred very early in their growth, likely as a result of a traumatic injection and possible disruption of the pellucida membrane. Among the X-GAL stained recovered embryos, no more than 2/4 for NLA vector and 1/4 for NLB were shown to be positive (data not shown). Higher rates of survival and positive embryos were obtained 72 h postincubation after NLA, NLB or NL53 virus inoculation in the vicinity of the blastodisc (Fig. 2B). Moreover, a broad distribution of the lacZ-foci was observed, also related to an increased number of stained cells.

Having chosen the best injection protocol, we next tested the effect of inoculation of unconcentrated viral supernatants (titrating at about 105 lacZ-EFU/ml). 20 µl of either NLA or NLB viral stocks (containing approximately $2x10^3$ lacZ-EFU) was then injected at the periphery of the blastodisc. Only a low proportion of embryos was shown to be marked. Moreover, the positive embryos displayed only few numbers of marked foci, which were of a small size compared to the latter experiments (data not shown). From these results, we concluded that 10^3 lacZ-EFU injected was the minimal amount of vector required to observe at least one marked cell.

Experiments with direct injection of NL-producer cells were also conducted. 20 µl of suspensions containing either 10^5 or 10^4 mitomycin-treated NL-producer cells was injected into 0 h embryos at the boundary of the blastodisc, as described in Fig. 2B. These injections of cells severely impaired embryo development. X-GAL staining of the embryos at 72 h showed that only the extra-embryonic tissues displayed lacZ-positive cells having a particular morphology not typical of embryonic cells but instead resulting from an encystment of the producer cells within the extraembryonal membranes, hence giving a positive staining (data not shown).

NL vectors are expressed in a great variety of embryonic tissues

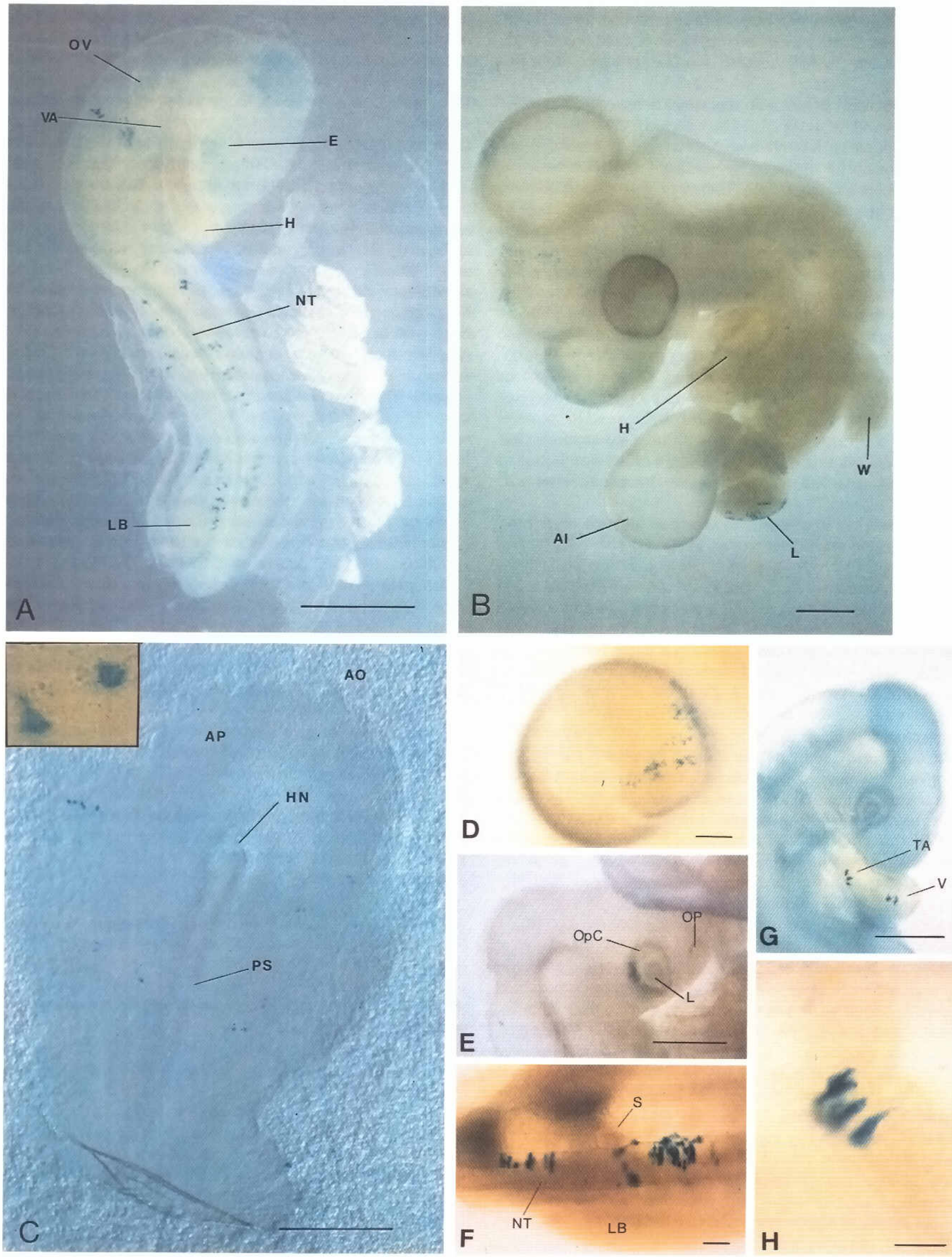
These experiments were performed in order to gain information on the distribution of lacZ-positive cells in the embryo body following inoculation of viral suspensions at early stages of development. Optimal conditions of inoculation allowing the greatest number of lacZ-positive cells as determined in the previous section were used: i) injection at 0 h of development, ii) inoculation of 20 µl of concentrated NL virus stocks, iii) injection at the periphery of the blastodisc to avoid mortality. Injected embryos were then incubated, recovered at different stages of development (Hamburger and Hamilton, 1951): stages XVI or XVII (72 h of incubation), 26, and 29 (5 and 6 days post incubation), and analyzed after X-GAL staining either from *in toto*, or after serial sections of interesting embryos as judged from *in toto* analyses.

Detailed observations of embryos allowed us to establish that there was no evident restriction of expression to a particular embryonic tissue (Fig. 3A, Table 1). Extra-embryonic tissues were found to be marked on both the seroamniotic and the splanchnopleur membranes. In embryonic tissues of *in toto* preparations stained at 72 h, lacZ-positive foci were found widely dispersed: marked cells were found in the head and the trunk. A preliminary histological study of transverse sections of the head of some marked embryos revealed that the neural ectoderm (Fig. 4A,B,C,D) as well as mesenchyme cells (details not shown) could be marked. In the trunk, most tissues or organs seemed to display the potential for being marked. For the NLB vector, these tissues include surface ectoderm (Fig. 4E,F), somitic mesoderm (Fig. 4G,K), vascular endothelia (Fig. 4H,I), mesoderm in the periphery of the gonadal ridge displaying epithelial cylinder cells bordering the upper angle of the coelom which participates in the differentiation of the gonadal ridge (not shown), the mesonephros duct of mesodermic origin (Wolffian duct), the nephrogenous mesoderm (Fig. 4J), and for NL53 vector, the intestinal endoderm (Fig. 4M). The eyes were rarely marked. From observations of serial sections of these eyes marked with NLB vector (Fig. 4A and B), we have noticed that only the nervous part of this organ was marked. Marked cells were also found on the pathway of migration of cells of the neural crest (Fig. 4K,K',L) as described by Rickman and Fawcett (1985). If it could be confirmed that these marked cells were of neural crest origin, it would be of great interest since these latter cells have a great pluripotentiality of differentiation (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Le Douarin and Smith, 1988; Noden, 1988).

Embryos recovered after 5 or 6 days of incubation were shown to be developed at stages 27 and 29 respectively (Hamburger and Hamilton, 1951). Results are presented in Table 2, and photographs are shown in Fig. 3 for NLA- or NLB-injected embryos. Obviously, compared to analyses performed at 72 h (see above), lacZ positive foci were found to be greater in number of cells, but equivalent or lower in number of foci. Although the total number of 5 and 6 day-old positive embryos was relatively small for drawing conclusions, positive cells were frequently found in the head: in the eye (Fig. 3D), the cephalic mesenchyme (Fig. 3B), and the encephalic neurectoderm (Fig. 4O). In this latter tissue, the aspect of the positive foci as a radial cluster could be the result of the radial growth described by some authors (in the mouse, Luskin *et al.*, 1988; Gray *et al.*, 1988; Galileo *et al.*,. 1990). Less frequently, other parts of the embryos were found to be positive: the face of the head of one embryo (not shown), the leg ectoderm (Fig. 3B) and the cardiac cell wall (Fig. 3E). From these studies, we concluded that the 3 initial cell layers could be infected since organs derived from these cells displayed positive foci.

RAV-1 and RAV-2 LTRs display a preferential tropism of expression in heart and neural tube

From observations of whole mounts, we found that neural tube and the heart were frequently but differently marked at 72 h post-incubation according to the type of NL vector injected (Table 1). These results, obtained from several series of injections, were confirmed by histological sections (Figs. 3 and 4). Moreover, depending on the nature of the NL vector injected, different frequencies could be evaluated since 26% (5/19, Table 1) of marked NLA-injected embryos displayed positive cells in the neural tube, whereas the frequencies were twice as high (48%) in the NLB-injected embryos (12/25) as well as for NL53-injected embryos (4/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8). Conversely, 73% (14/19) of NLA-marked embryos displayed lacZ-positive cells in the heart, whereas the frequencies were three times lower in NLB-injected embryos (24%, 6/25) and 50% (4/8) in NL53-injected embryos. These results suggest that discrepancies between the behavior of NL vectors might be related to the origins of their LTR, rather than to differences of tropism of infection of these vectors, since all of them were enveloped in the same subgroup A coat (provided by Isolde cells).

A limited number of cells express the NL vectors at 24 h (stage 5)

Regarding the high number of lacZ-EFU inoculated per embryo, it was striking to notice that only few ß-gal foci could be observed at 72 h or later stages of development. In order to determine whether this low number of positive cells was the result either of a progressive suppression of vector expression between the stage of inoculation and staining, or of a weak susceptibility of E0 embryos to infection, we have stained the infected E0 embryos after only 24 h of incubation to minimize this eventual suppression by a short period of development. An example of such *in toto* observations is presented in Fig. 3C. The proportions of positive embryos were similar to those evaluated for 72-h-old embryos (at 72 h, 40% (Table 1), and 50% at stage 5 (data not shown)). In these positive embryos, compared to X-GAL stained embryos at 72 h, the average number of lacZ-foci was slightly lower. The *area opaca* was always found to display positive cells, whereas stained clusters were also found in the area pellucida for 50% of the marked embryos. Rare positive foci were found in the vicinity of the primitive streak. Some positive embryos displayed lacZ-foci near the Hensen's node (Fig. 3C). These foci were composed of either one positive cell, or two cells likely resulting from the division of one infected cell (Fig. 3C: detail). Unfortunately, these observations of whole-mount embryos did not allow us to determine the accurate nature of the infected tissues (i.e., endoderm, mesoderm, or ectoderm), but allowed us to conclude that the low number of positive cells found at later stages of development (3, 5, and 6 days) was not the result of a progressive suppression of LTR activity, but rather, the result of either a weak efficiency of infection of E0 embryos, or of a repression of retrovirus promoter upon being integrated. To address these questions, we have quantified the number of proviral insertion by Southern blotting experiments.

Only a low proportion of infected cells express retroviruses

13 surviving embryos at 6 days of incubation, resulting from inoculation with 20 µl of concentrated suspension of NLB vector, were isolated with their extra-embryonic tissues. As a control of infection, the extra-embryonic tissues were stained with X-GAL, whereas the embryonic tissues were used to extract cellular DNA. Twelve of these 13 embryos displayed lacZ-foci in the extra-embryonic tissues although in different proportions: about 10 LacZ-foci were obtained in the splanchnopleur near the amnios for 4 embryos, whereas more than 100 LacZ-foci were found in the sero-amnios and the splanchnopleur for the other embryos (Table 3). Southern Blot analyses of embryo DNA were realized by using a LacZ-specific probe (Fig. 5A). The DNAs of 4 embryos out of 8 displaying the largest number of LacZ-foci in their extra-embryonic tissues were found to be positive for the presence of vector provirus (Fig. 5B). From these data, we have attempted to determine the proportions of embryonic cells which had integrated the vector sequences in their genome. Since NL vectors were prepared as helper-free preparations, once integrated, the proportion of NL proviruses in the total number of embryo cells should remain stable at later stages. An internal standard was made by diluting LacZ-positive DNA (obtained from NLB-infected chicken embryo fibroblasts), with control embryo DNA originating from non infected embryos. 30µg of such a mixture containing 1/5, 1/10, 1/25, 1/50, 1/75, 1/100, 1/500 or 1/1000 NLB-positive DNA, were analyzed by Southern Blot to compare with 30µg of the DNA of NLB-injected embryo for comparisons. After hybridization to the LacZ probe and autoradiography, the lowest limit of detection of this analysis was obtained with the 1/75 dilution corresponding to 0.4 µg of LacZ-positive CEFs DNA (Fig. 5C). Compared to the signal intensities of the dilutions of internal standard, the intensities of the 4 X-GAL-positive embryo DNAs were found to correspond respectively to about 1/20, 1/25, 1/50 and 1/75 dilutions. Thus it appeared that for these 4 embryos at the minimum, more than 1 cell/75 had integrated on average the vector provirus in its genome.

These results led us to conclude that the low number of lacZ positive cells was not only due to a weak efficiency of integration, but also to a specific blocking of retrovirus LTR, immediately after proviral insertion.

Discussion

Transgenic animals have become invaluable tools for a variety of studies (Church *et al.*, 1985; Wagner, 1985). Due to the particular physiology of the development of birds, germinal transgenesis in chickens has only been achieved very recently (Crittenden *et al.*, 1989; Bosselman *et al.*, 1990a) since introduction of recombinant DNA into the germ line involves a technology different from that used for insertion of genes into mammalian embryos, because of the high number of cells that form the embryo at oviposition.

In the previous works, studies of transgenesis in birds using avian retroviruses were essentially analyzed by Southern blot method detecting the provirus of recombinant viruses (Shuman and Shoffner 1986; Salter *et al.*, 1987; Bosselman *et al.*, 1990a,b).

Fig. 3. X-gal stained embryos infected with NL vectors. E0 embryos were injected with concentrated helper-free viral stock. (A) *72-h-old embryo injected with 2.105 lacZ-EFU of NLB vector.* **(B)** *5-day-old embryo injected with 2.10^5 lacZ-EFU of NLB vector.* **(C)** *Stage 5 (Hamburger and Hamilton, 1951) embryo injected with 8.104 lacZ-EFU of NLA vector. In detail, a magnification of the lacZ-foci encircled.* **(D)** *Eye of a 5-day-old embryo injected with 8.104 of NLA vector.* **(E)** *Eye of a 72-h-old embryo injected with NLB vector. The stainings in the retina are also represented in paraffin section of Fig. 4A.* **(F)** *Neural tube of a 72-h-old embryo injected with about 10^5 lacZ-EFU of NLB vector. The cell marked with an arrowhead may be of a neural crest origin.* **(G)** *Stained heart of a 72-h-old embryo injected with 1.10^5 lacZ-EFU of NLA vector.* **(H)** *Detail of the* truncus arteriosus *of a 72-h-old embryo injected with 2.10^5 lacZ-EFU of NLB vector. The arrowhead shows the nucleus. AL: allantoid, AO: area opaca, AP: area pellucida, E: eye, H: heart, HN: Hensen's node, L: leg, LB: leg bud, Ln: lens, NT: neural tube, OP: olfactory pit, OpC: optic cup, OV: otic vesicle, PS: primitive streak, S: somite, TA:* truncus arteriosus, *V: ventricle, VA: visceral arch, W: wing (A) and (G): bar, 1mm, (B): bar, 2 mm, (C) and (D): bar, 500 µm, (E) and (H): bar, 400µm, (F): bar, 100 µm.*

TABLE 1

FREQUENCIES OF DISTRIBUTION OF THE STAINED FOCI IN 72-H-OLD EMBRYOS

	NLA N° (%)	NLB N° (%)	NL53 N° (%)
Injected embryos	50 (100)	83 (100)	24(100)
Survival	30 (60)	41 (50)	10 (47)
Positive[a] samples	23 (77)	36 (88)	8 (80)
Positive[b] embryos	19 (63)	25 (61)	8 (80)
Positive[b] embryos	19 (100)	25 (100)	8(100)
Head	7 (36)	11 (44)	5 (62)
Trunk	14 (73)	17 (68)	7 (87)
Heart	14 (73)	6 (24)	4 (50)
Eye	0 (0)	2 (8)	0 (0)
Leg bud	3 (16)	1 (4)	1 (12)
Neuro-ectoderm	5 (26)	12 (48)	4 (50)
Surface ectoderm	8 (42)	5 (20)	3 (37)

NL vectors have been injected at 0 h stage. Frequencies were estimated following *in toto* observations.
a: taking into account both extra-embryonic and embryonic tissues.
b: positive for embryo cells.

Works showing the detection of retrovirus expression using biochemical methods (Souza *et al.*, 1984; Hippenmeyer *et al.*, 1988; Briskin *et al.*, 1991; Federspiel *et al.*, 1991) and more recently in situ visualization of vector expressions (Stoker *et al.*, 1990; Mikawa *et al.*, 1991; Reddy *et al.*, 1991) have been reported. In the present study, we show that the *lacZ* gene used as marker makes it possible to pinpoint the expression sites in different embryonic tissues of the used NL vectors. Analysis by combining the histological methods for detection of vector expression and the Southern blot technique for estimation of integrated provirus number, made it possible to reveal a particular restriction of retrovirus infection and expression in early chicken embryonic tissues. These observations were shown by using the 3 types of NL vectors, in which only the LTR_s and flanking regions were originated either from RAV-1 (NLA) or RAV-2 (NLB) or finally AEV ES4 (NL53).

Spatio-temporal regulation of vector-expression in embryo cells depends on origin of their cis-acting sequences

Results reported in Tables 1 and 2 show clearly that the NLA vector compared to NLB or NL53 vectors seemed to be expressed more frequently in the heart, whereas lacZ-positive cells were more often observed in the neural tube following infection with the NLB vector. The NL53 vector did not display such a specificity since these two tissues or organs were equally marked. Although the size of the sample was too small for a definitive conclusion, these differences could not be related to distinct tropisms of viral envelopes, since all three vectors were produced in particles of subgroup A (from Isolde producer cells), but rather must be related to the nature of *cis*-acting sequences of the NL vectors. Indeed, some differences can be found in the U3 region of the LTRs of NLA (originated from RAV-1), NLB (from RAV-2) (Majors, 1990) and from NL53 (from AEV). More precisely, structural differences have been found in the enhancer parts of U3 which are composed of several transcription factor-responsive elements (Laimins *et al.*, 1984; Cullen *et al.*, 1985; Sealey and Chalkley, 1987; Goodwin, 1988; Gowda *et al.*, 1988; Ryden and Beemon, 1989; Boulden and Sealy, 1990). Therefore it is possible that specific factors expressed in some cell types might interact specifically with the different parts of the enhancers, thus resulting in different regulations of NL vectors, and could account for the discrepancies observed between the vectors.

Other particular examples of striking host/vector interactions have been observed, especially in the eyes and heart. Embryos stained at 72 h rarely displayed lacZ-positive cells in the retina for 2/25 (8%) and 0/19 (0%) of NLB- and NLA-infected blastodiscs, respectively. By contrast, these proportions of positivity were respectively 2/5 (40%) and 2/9 (22%) for similarly infected embryos when they were stained at 5 days. Although we cannot exclude the possibility that cells from the other part of the embryo could have colonized the eye, these results might suggest that a specific derepression of NL vector expression might have occurred in the retina between 3 and 5 days of incubation. Moreover, the discrepancies of positivity at both 3 and 5 days between NLA and NLB vectors would confirm the above-mentioned putative «neural» specificity of RAV-2 cis-acting elements inserted into NLB compared to NLA, taking into account the fact that retina is a diencephalic derivative (Romanoff, 1960).

As discussed above, the heart was frequently stained with the NLA vector, since at 72 h, 73% (14/19) of the NLA-marked embryos

Fig. 4. Paraffin sections of 72-h- and 5-day-old stained embryos. E0 Embryos were injected with NL53 and NLB vectors, X-gal stained, included into paraffin before sections. (A, B, B') *Embryos injected with 6.10⁴ lacZ-EFU of NLB vector.* **(A)** *The retina and the diencephal are positive (embryo of Fig. 3.E) and* **(B, B')** *in another embryo positive cells can be seen in the optic stalk.* **(C)** *Positive cells in the neural tube and in the splanchnopleural endoderm.* **(D)** *Detail of the positive neural tube.* **(E)** *Positive cells in surface ectoderm.* **(F)** *Positive surface ectoderm at the otic vesicle level.* **(G and G')** *Positive somitic mesoderm and nephrogenous tissue.* **(H and I)** *Positive vascular endothelium at an intermediate level above the omphalomesenteric arteries junction (single aortic branch) and at the upper level including the lung bud.* **(J)** *Stainings in the trunk at the level below the omphalomesenteric arteries showing positive cells in the Wolffian duct. This embryo shows additional stainings in neural tube and splanchnopleural endoderm (see C and C').* **(K, K')** *Embryo with stained somites and potentially neural crest cells (arrowhead, region below the omphalomesenteric arteries junction).* **(L)** *Embryo seen in Fig. 8G with marked cells located on the neural crest cells migration pathway at the level of the lung bud.* **(M, N)** *Embryo injected with the NL53 vector showing positive endodermal cells in the anterior intestine and in the heart ventricle (arrowhead).* **(O)** *NLB vector markings in the external mesencephalic wall of a 5-days-old embryo injected with 2.10^5 lacZ-EFU of NLB vector (embryo shown in Fig. 3B (arrowhead)). The radial disposition of the clusters is in agreement with the stainings observed by Gray* et al. *(1988) in the optic tectum of the chicken embryo. Bar in A, B, C, F, 100 μm; bar in B', D, G' details in H, I, and J, 20 μm; bar in E, G, H, I, J, K, K', L and N, 200 μm; bar in O, 50 μm. A: aorta; AI: anterior intestine; Die: diencephal; Ect: ectoderm; End: endoderm; LB: lung bud; Mes: mesoderm; Mese: mesencephal Mye: myelencephal; NT: neural tube; O: oesophagus; OpS: optic stalk; OV: otic vesicle; R: Retina; S: somite; SV: sinus venosus; V: ventricle; WD: Wolffian duct.*

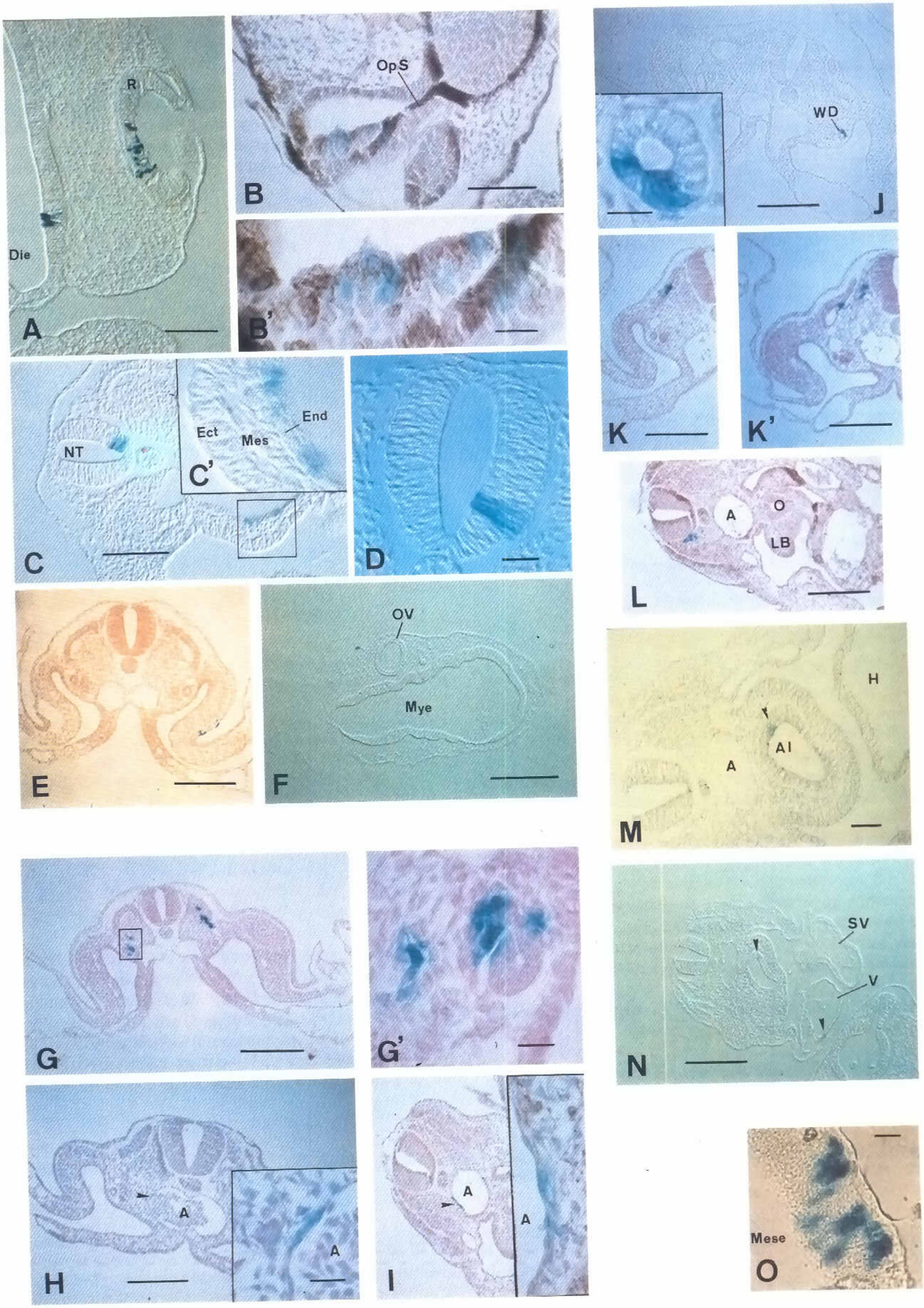
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TABLE 2

FREQUENCIES OF DISTRIBUTION OF THE STAINED FOCI IN 5-DAY-OLD EMBRYOS

	NLA N° (%)	NLB N° (%)
Injected embryos	65 (100)	51 (100)
Survival	22 (33)	20 (39)
Positive[a] samples	13 (59)	8 (40)
Positive[b] embryos	9 (41)	5 (25)
Positive[b] embryos	9	5
Head	2	1
Trunk	3	3
Heart	1	2
Eye	2	2
Wing	0	1
Leg bud	1	1
Neuro-ectoderm	2	1
Surface ectoderm	0	1
Seroamnios	6	4
Allantoid	0	0
Splanchnopleur	8	8

NLA and NLB vectors have been injected in E0 embryos. Frequencies were estimated following *in toto* observations.
a: taking into account both extra-embryonic and embryonic tissues.
b: positive for embryo cells.

displayed positive cells in this organ (Table 1). Comparatively, only about 10% (1/9) of marked embryos displayed positive foci in the heart at 5 days (Table 2). These results are striking since one would expect that the numerous stainings observed at 72 h in the heart would have led to a high positivity of this organ at 5 days because of the divisions of the marked cells, as observed for the marking of eye. However, there were no more positive cells at 5 days than at 72 h, and also, the positive cells were often represented by individual blue cells (Fig. 3). This could be explained i) by migrations of the cells out of the heart during its development, and thus, a decrease in the proportion of positive cells during morphogenesis; ii) a specific regulation of the expression of the vector in the heart that would inhibit the ß-gal phenotype. As previously suggested, kinetic study combined with the observation of serially sectioned hearts would be necessary to answer these questions.

Efficiencies of NL vector integration and expression: implication for cell lineage and for germinal transgenesis

Our results also provide evidence that only a small proportion of cells integrating a provirus could give rise to expression of NL vector. The proportion of cells harboring a provirus among total embryo cells ranged from 1/20 to 1/75 for 4 strongly X-GAL-positive 6-day-old embryos examined. If we consider together that the most infected E0 embryos contain approximately one proviral copy per 20 cells (i.e., 2.5×10^3 «provirus-positive» cells among 5×10^4 blastodermic cells exposed to infection at 0 h) and the most marked embryos (when observed at stage V) displaying a maximum of 30 lacZ-positive foci (as visualized from *in toto* observations, see Fig. 3C), with a multiplicity of infection of about 1:1, the expression efficiency can be evaluated at 0.06% ($30/5 \times 10^4$), whereas at most 1.2% ($30/2.5 \times 10^3$) of the infected cells that harbor a NL provirus can give rise to *lacZ* expression. It should be noted that this last value is probably underestimated since the *in toto* observation cannot allow the visualization of the totality of lacZ-positive cells, especially

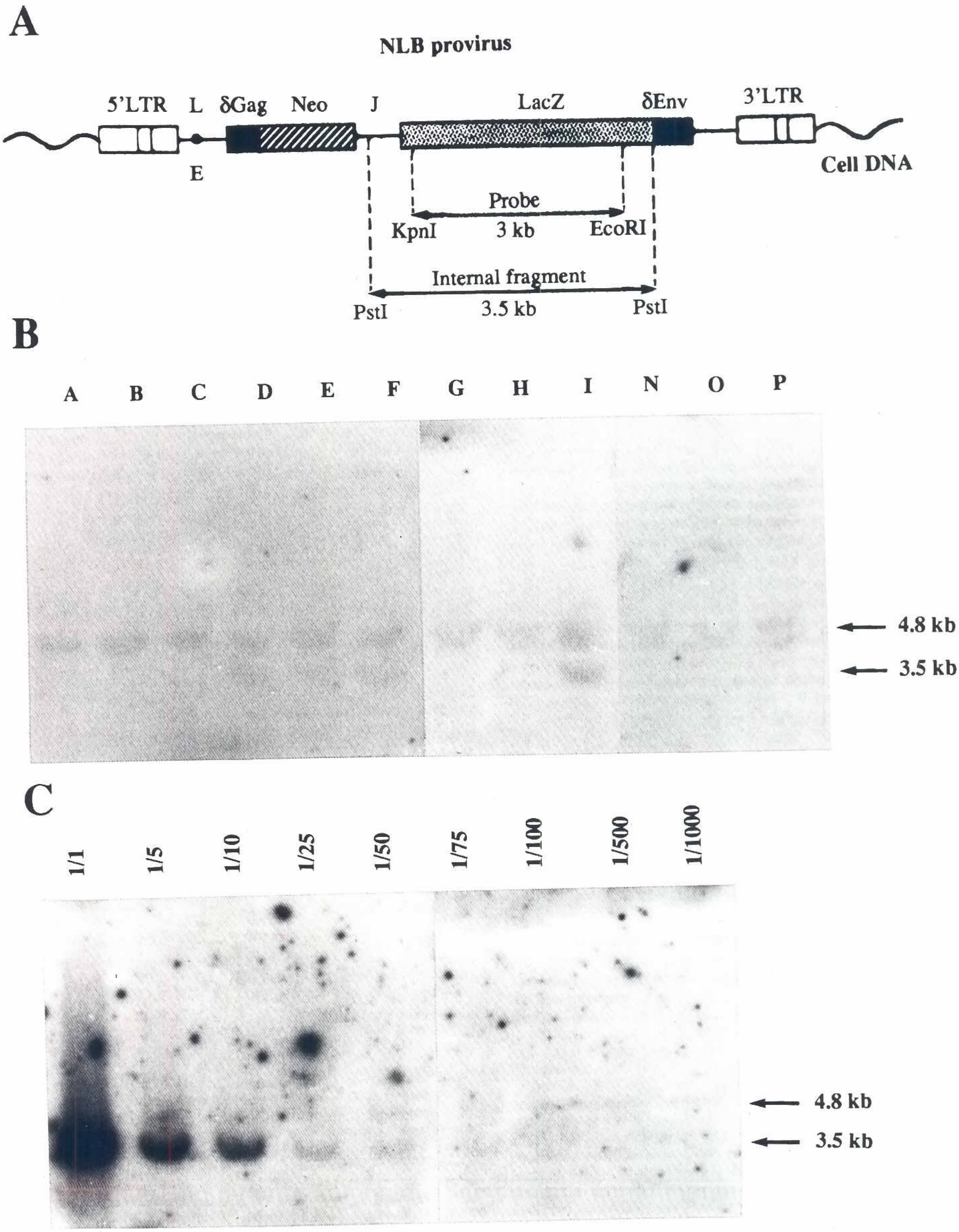
TABLE 3

RELATION BETWEEN PROVIRAL INTEGRATIONS AND *LacZ*-EXPRESSION

embryo	*lacZ* expression[a]	detection of proviruses[b]	intensity of signal[c]
A	+++	-	
B	+	-	
C	+	-	
D	+++	+	1/50
E	++	+	1/75
F	+++	+	1/25
G	-	-	
H	+	-	
I	+++	+	>1/20
J	++	-	
K	+	-	
L	+++	-	
M	++	-	

a: *LacZ* expression in extra-embryonic tissues after staining with X-Gal.
- no LacZ-foci.
+ about 10 LacZ-foci in the splanchnopleur near the amnios.
++ about 100 LacZ-foci in the sero-amnios and the splanchnopleur.
+++ more than 100 LacZ-foci in the sero-amnios and the splanchnopleur.
b: Detection of proviruses in embryo DNA by Southern Blot analysis with a *LacZ* probe. negative for provirus integration.
+ positive for provirus integration.
c: Proportion of embryonic cells having integrated the vector sequences in their genome in comparison with an internal standard (cf. Fig. 5).

Fig. 5. Embryo DNA Southern Blot analysis. (A) *Detection of NL proviruses by Southern Blots. The 3.5 kb PstI internal fragment of NL vector was detected after hybridization of PstI-digested embryo DNA to a* lacZ *probe. L: Leader region, E: packaging sequence, J: junction sequence from AEV.* **(B)** *Analysis of proviral integration. 25μg of Pst1 digested embryo DNAs was electrophoresed on agarose gels and transferred to cellulose-nitrate filters by the procedure of Southern. The filters were hybridized to a* LacZ *probe. Lane A, B, C, G and H: LacZ-negative embryo DNA; Lane D, E, F and I: LacZ-positive embryo DNA; Lane N, O and P: control embryo DNA. The 4.8 kilobase-pair hybridizing fragment of DNA corresponds to a* LacZ *cross-hybridization with genome of embryos.* **(C)** *Determination of the proportion of embryonic cells which had integrated the vector sequences in their genome. An internal standard was made by diluting LacZ-positive DNA (from NL-infected CEF) with control embryo DNA. 30μg of total DNA containing 1/5, 1/10, 1/25, 1/50, 1/75, 1/100, 1/500 and 1/1000 NL-positive DNA, was analyzed by Southern Blot comparatively to 30μg of NL-injected embryo DNA.*


A
NLB provirus
5'LTR
L
δGag
Neo
J
LacZ
δEnv
3'LTR
E
Cell DNA
Probe
KpnI
3 kb
EcoRI
Internal fragment
PstI
3.5 kb
PstI
B
A B C D E F G H I N O P
4.8 kb
3.5 kb
C
1/1 1/5 1/10 1/25 1/50 1/75 1/100 1/500 1/1000
4.8 kb
3.5 kb

in the *area opaca*. Our data indicate that 30% of the injected embryos displayed the presence of provirus though at different levels (ranging from 1% to 5% of positive cells), but these results are also probably underestimated since the 1/75 dilution is the lowest limit under which no provirus signal can be detected.

Several factors would account for the low efficiencies of infection and expression of the embryonic cells: i) trivial problems like either lack of accessibility of virions to viral receptors because of the compactness of the embryo cells, or spreading of the viral supernatant in yolk rather than in the embryo, or degradation of the viruses; ii) lack of susceptibility of the embryonic cells to ALV infection because of the absence of receptors at early stages of development; iii) presence of specific factor either inhibiting the viral replication in the infected cells or inhibiting the transcription of the provirus, or conversely, absence of stimulating transcription factors. This latter hypothesis is reminiscent of the observations of several authors on murine retroviral-mediated transgenesis. Taken together, their data indicate that the expression of the MLV-LTR depends on the murine embryonic developmental stage (Jaenisch *et al.*, 1975; Jaenisch, 1980; Jähner *et al.*, 1982; Niwa *et al.*, 1983; Jaenisch and Jähner, 1984; Loh *et al.*, 1988; Stewart *et al.*, 1987; Feuer *et al.*, 1989; Tsukiyama *et al.*, 1989; Savatier *et al.*, 1990). In the case of birds, Mitrani *et al.* (1987) have reported that early embryo cells cultured *in vitro* were not very susceptible to RSV infection.

Our results agree with those reported by Mitrani *et al.* (1987) and Reddy *et al.* (1991) concerning the efficiency of blastodermic cell infection with ASV from subgroup A and B. However, we have not studied quantitatively the transcriptional efficiency of our vectors in early blastodermic cells in order to compare these findings with the results reported by Reddy *et al.* (1991). We just notice that no significant difference in the intensity of the staining was observed in positive cells in early embryonic tissues (0 to 3 days) in 9-day- old CEF_s.

One of the goals of numerous laboratories including our own is to develop methods of obtaining transgenic chickens, in particular by using retrovirus vectors in order to infect primordial germinal cells (PGCs) with a high efficiency. In this work, we have obtained results—albeit not convincing enough—concerning *lacZ* retrotransfer into PGCs (data not shown). However, our results on somatic transgenesis provide evidence that germ line transmission through retrotransfer by using ALV-based vectors might be feasible. Assuming that the PGCs—approximately 200 per stage XIII embryo, Cuminge and Dubois, (1989)—are as permissive to infection as other embryonic cells, one should expect to obtain 2 to 10 PGCs positive for integration among 30% of infected embryos. If we admit that the sex-ratio is 50%, and that the survival of the PGCs is not impaired by the insertion of the NL-provirus, the theoretical transmission rate of NL provirus for male progenitors should therefore range between 0.5% and 2.5%, which is similar to values obtained by Bosselman *et al.* (1990a, b) with defective REV-based vectors.

Even if the direct inoculation of retroviral vectors into early embryos seems the most efficient method to obtain transgenic lines of birds (Salter *et al.*, 1986, 1987; Salter and Crittenden, 1989; Bosselman, 1990a; Lee and Shuman, 1990) its efficiency is low. Other methods have recently been approached by some authors. Several techniques of introducing exogenous genes into explanted blastodermic cells or PGCs, including microinjection (Naito *et al.*, 1990; Perry and Sang, 1990), transfection (Verrinder Gibbins *et al.*, 1990) or retroviral infection (Simkiss *et al.*, 1990) have been tested followed by re-implantation into recipient embryos. Experiments are in progress to infect explanted PGCs with our ALV-based vectors, and to study their transmission into gonadal ridge after re-implantation.

Materials and Methods

lacZ retroviral vectors

Three ALV-based retroviral vectors were used in this work. These defective vectors carry two markers driven by avian LTRs: the neo^R selectable gene expressed from a genomic mRNA and the bacterial *lacZ* gene expressed from a subgenomic mRNA processed by splicing of the full-length mRNA (Fig. 1). Structural differences in these three viruses (called NL vectors) were related to the origin of their *cis*-acting sequences including LTRs, leader region, both *gag* and *env* gene residues, and the 3' non coding regions. In the NL53 vector, these *cis*-acting sequences originated from AEV-ES4 (Vennström *et al.*, 1980), whereas for NLA and NLB vectors, the neo^R and lacZ genes were under control of the regulating sequences of RAV-1 and RAV-2, respectively. Details of the constructions were published elsewhere (Cosset *et al.*, 1991). Helper-free NL vectors were produced from our Isolde packaging cells and titrated as previously described (Cosset *et al.*, 1990). Titers expressed as lacZ-CFU/ml of supernatants ranged from 10^5 to $3x10^5$ lacZ-CFU/ml for vectors NLA and NLB, whereas the NL53 vector was produced at lower titers: $5x10^4$ lacZ-CFU/ml. No replication-competent viruses were detected in vector supernatants as determined by a standard assay previously described (Savatier *et al.*, 1989). These viral supernatants were concentrated by ultracentrifugation (4°C; 30 min; 33,000 RPM) in a 50.2 TI Beckman rotor. Sedimented virions were resuspended in 1/100 of initial volume. Concentrated titers were found 50 fold higher than native titers ($5x10^6$ to $3x10^7$/ml according to the NL vector tested).

Infection of embryos

A Brown Leghorn strain of chickens selected by us for its pattern in endogenous proviruses (Ronfort *et al.*, 1991) was used for *in vivo* experiments.

Infections of the embryos were performed at 0 h (E0 embryos) or 24 h (E1 embryos) after laying. The E0 embryos were theoretically at stage XI according to Eyal-Giladi and Kochav (1976). Eggs were laid blunt end up for one hour before inoculation. Then, a triangular window (1 cm each side) was performed with an abrasive diamanted disc through the shell in the center of the blunt end. 20 µl of concentrated viral supernatants was inoculated into the blastodisc by using a glass capillary of 30 to 50 µm diameter at the tip (Fig. 2). Inoculations of E1 embryos—corresponding to stage V according to Hamburger and Hamilton (1951)—were performed by injection between the *area opaca* and the *area pellucida*.

The shell opercula was then replaced on the egg and maintained with adhesive tape (Scotch 3M) during incubation (38°C; 60% hygrometry). Percentages of survivors were related to the egg storage conditions and were found to depend on the time between laying and incubation. Best results were obtained with eggs kept at 15°C and injected before the seventieth day of storage.

X-GAL staining

After various periods of incubation, injected embryos were fixed and the ß-*gal* activity was checked. Embryos were washed with phosphate buffer (PBS) 0.1 M, pH 7.4 at 4°C, and fixed at 4°C with formaldehyde 4% in PBS-$MgCl_2$ (2 mM) for 15 min for embryos of stage V and XVI, or for 30 min for embryos of later stages. The embryos were then rinsed with PBS-MgCl2 for 20 min at 4°C, and incubated into the following X-GAL solution in PBS (0.1 M, pH 7.4): $MgCl_2$, 2 mM; potassium cyanoferrate 5 mM; potassium cyanoferride, 5 mM; sodium desoxycholate, 0.01%; Triton X-100, 0.01%; DMSO (DiMethylSulfOxide), 3%; and 1 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-ß-galactopyranoside, Boehringer Manheim, France). Incubations were performed at 37°C for less than 5 h in order to detect only the NL proviral ß-galactosidase activity. In some cases, a chicken endogenous a-gal activity can be detected in some embryos, mainly in the neural tube and in the chord at stages 16-17, and in the liver at stages 27-29. X-GAL stained embryos were then incubated at 4°C overnight with 100mM EDTA, 3% DMSO in PBS

0.1 M (pH 7.4) in order to stop the ß-galactosidase reaction. Embryos were post-fixed with 4% formaldehyde in PBS before analyses. Macrophotographs were made with a SMZ-10 Nikon binocular microscope and Kodak Ektachrome EPY-135 film.

Inclusions in paraffin and sections

Embryos were rinsed in PBS 0.1 M (pH 7.4) for one h, then post-fixed in Carnoy's fluid (30 min) at room temperature, dehydrated 10 min with absolute ethanol, incubated in ethanol/toluene (1/1) for 30 min, and in toluene for one h. They were embedded in two paraffin waxes for one h each at 60°C. After cooling, the included embryos were serially sectioned at 6 fm. Some sections were counterstained with Mayer's hematoxylin (with 1 N HCl) which stains the nucleus pink. The sections were mounted in DEPEX (Gurr), observed with a light microscope (Nikon optiphot-2 or Olympus BH-2 microscopes), and photographed with Kodak Ektachrome EPY-135 film.

Southern analyses

Six-day-old embryos were homogenized in buffer containing 10 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl (PH:8) and 0.3% sodium dodecyl sulfate. Samples were incubated at 65°C for 2 h with 500 µg of proteinase K per ml. RNA was digested with 500 µg of RNase per ml at 25°C for 2 h and RNase was eliminated with proteinase K (500 µg/ml, 2 h, 65°C). DNA was then extracted twice with phenol and chloroform-isoamylalcohol (24:1, v:v) and once with chloroform-isoamylalcohol. DNA was precipitated with 2 volumes of ethanol and dissolved in 0.5 or 1 ml of water overnight at 4°C. Purified embryo DNAs were digested with Pst1 endonuclease in its specific buffer (Boehringer, Manheim) for 2 h at 37°C. Samples (30 µg) of digested DNA were electrophoresed on agarose gels and transferred to cellulose-nitrate filters by the procedure of Southern (Southern, 1975). The filters were prehybridized at 65°C for 15 min in 3xSSC (1xSSC is 0.15 M NaCl and 0.015 M TriSodiumCitrate), 0.1% SDS (Sodium Dodecyl Sulfate) and for 2 h in 3xSSC-0.1% SDS/1xDenhart solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% ficoll) containing 100 µg/ml of salmon sperm DNA. Hybridization to a *lacZ*-specific probe (see below) was done in the same mixture containing 10^9 cpm of the radioactive probe for 36 h at 65°C. Filters were washed at 65°C for 1 h in 3xSSC-0.1% SDS, for 30 min in 1xSSC-0.1% SDS and for 30 min in 0.1xSSC-0.1% SDS. Filters were then rinsed in 3xSSC, dried and exposed to Kodak X-Omat AR films at -80°C with an intensifying screen.

LacZ gene specific probe was isolated from pNL53 plasmid (fig 5.A) containing the genome of the NL53 vector. After digestion with EcoR1 and Kpn1 endonucleases and electrophoresis on a 0.8% agarose gel, the *lacZ* probe (corresponding to a 3 kilobase-pair fragment) was purified by electroelution as described by Maniatis *et al.* (1982). The lacZ probe was then P32-labeled by random primed synthesis (Feinberg and Vogelstein 1983). Specific activities higher than 109 dpm/µg were obtained.

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