

Expression of Rous sarcoma virus-derived retroviral vectors in the avian blastoderm: Potential as stable genetic markers

(embryonic development/replication-defective virus/lineage marker/*lacZ*)

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ABSTRACT Retroviruses are valuable tools in studies of embryonic development, both as gene expression vectors and as cell lineage markers. In this study early chicken blastoderm cells are shown to be permissive for infection by Rous sarcoma virus and derivative replication-defective vectors, and, in contrast to previously published data, these cells will readily express viral genes. In cultured blastoderm cells, Rous sarcoma virus stably integrates and is transcribed efficiently, producing infectious virus particles. Using replication-defective vectors encoding the bacterial *lacZ* gene, we further show that blastoderms can be infected in culture and *in ovo*. *In ovo*, *lacZ* expression is seen within 24 hr of virus inoculation, and by 96 hr stably expressing clones of cells are observed in diverse tissues throughout the embryo, including epidermis, somites, and heart, as well as in extraembryonic membranes. Given the rapid onset of vector expression and the broad range of permissive cell types, it should be feasible to use Rous sarcoma virus-derived retroviruses as early lineage markers and expression vectors beginning at the blastoderm stage of avian embryogenesis.

The use of retroviruses to transfer genetic information into vertebrate embryo cells has enhanced our understanding of gene regulation and function and cell lineage relationships during development (1–5). Although the retroviral approach offers high efficiency for introducing foreign genes into target cells at many developmental stages, viral expression can be blocked in very early development. This block to expression has been shown clearly in preimplantation mouse embryos and also in murine embryonal carcinoma cells (6–8). In postimplantation mouse embryos, however, where the differentiation program of the embryo has already started, retroviral expression is not blocked in any of the infected tissues (8, 9).

In the absence of a readily available transgenic model, genetic manipulation of avian embryo cells has relied principally upon the use of retroviruses. Our own studies have shown that Rous sarcoma virus (RSV) is expressed in differentiating limb cells *in ovo* after infection on day 4 of development (10–12). Replication-defective avian retroviruses have also been used by ourselves and others for lineage marking and genetic manipulation of chicken embryo cells, starting as early as day 2 of development (13, 14). The feasibility of using retroviruses for similar studies in the very early avian embryo, in the blastoderm stage for example, is less clear. One study addressing this question concluded that RSV could infect and stably integrate in cultured chicken blastoderm cells but was expressed very inefficiently (15). The apparent inactivity of RSV in blastoderm cells suggested that early chicken embryos behaved analogously to murine embryonal carcinoma cells (6–8). However, we obtained

preliminary evidence that cultured chicken blastoderm cells could express viral proteins upon RSV infection. Because the resolution of this question has important implications for the use of retroviruses in studies of early avian development, we have now addressed directly the permissivity of the chicken blastoderm toward viral expression.

We have asked specifically whether or not a block to viral expression occurs in cultured cells isolated from chicken blastoderms, and whether replication-defective vectors can be used to mark cells genetically at the blastoderm stage *in ovo*. Using cultured chicken blastoderm cells, we demonstrate that RSV not only infects and integrates but, in contrast to previous data (15), is also expressed well in these cells. Using RSV-derived replication-defective vectors, we show further that retrovirus-mediated genes can be expressed as early as 24 hr after infection of stage X blastoderm cells in culture and *in ovo*. These results indicate that replication-defective expression vectors could potentially be used in very early chicken embryos for cell lineage studies and for ectopic gene expression.

MATERIALS AND METHODS

Culture of Blastoderm Cells and Chicken Embryo Fibroblasts (CEF). Stage X blastoderms were isolated from uninoculated White Leghorn chicken eggs (SPAFAS, Norwich, CT). The blastoderms were mechanically dispersed into a loose cell suspension with a Pasteur pipette. The cells were then plated at a concentration of $\approx 1 \times 10^5$ cells per 15-mm culture well (Nunc) in Eagle's minimal essential medium/10% fetal calf serum. Culture wells were precoated with air-dried rat tail collagen (16). The medium was changed each day. The following viruses were added to culture medium 4–6 hr after cell plating: Prague A strain RSV ($1-2 \times 10^5$ focus-forming units per culture well); Rous-associated virus 1 ($1-2 \times 10^5$ infectious virus per culture well); replication-defective virus RDlac1 [$\approx 5 \times 10^3$ infectious virus per culture well, unless stated otherwise; virus titered with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) histochemistry as described (13)]. After 4 days, RNA and DNA were isolated from some RSV-infected cultures, and all remaining cultures were fixed in 2% paraformaldehyde for 20 min. The fixed, RSV-infected cultures were processed for immunofluorescence, and the fixed, RDlac1-infected cultures were treated with X-Gal. CEF were made from 10-day-old chicken embryos as described (17).

Microinjection of Blastoderms. Eggs were first incubated horizontally with respect to their long axis for 1 hr at 39°C. Egg shells were cleaned with 70% ethanol, and a 8- to 10-mm hole was made with sterile forceps in the upper shell surface. Virus was injected directly beneath each blastoderm. One hundred to two hundred virus particles were injected per

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Abbreviations: RSV, Rous sarcoma virus; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; CEF, chicken embryo fibroblasts; LacZ, β -D-galactosidase.

blastoderm in a volume of 0.5–1.0 μ l. Eggs were resealed with sterile tape and incubated further at 39°C. Embryos were later removed and fixed in 2% paraformaldehyde for 20 to 30 min, followed by washing in phosphate-buffered saline (PBS)/0.1 M glycine/0.1% Triton X-100 for 20 min. The whole embryos were then treated with X-Gal overnight at room temperature, followed by several washes in PBS and equilibration in 15% sucrose/PBS followed by 30% sucrose/PBS. Embryos were embedded in OCT compound (Miles), frozen in liquid nitrogen, sectioned (5–7 μ m thick) by using a Leitz cryostat 1720, and collected on gelatin-coated coverslips.

Construction of Replication-Defective Retroviral Vectors. Vector RDlac1 was constructed as follows. Viral sequences between base pair (bp) 630 (*Xho* I site) and bp 9238 (*Eco*RI site) were removed from a molecular clone of RSV (clone A11; ref. 13) and replaced with a 3' segment of Rous-associated virus 1 spanning the region between the *Sal* I site in the *env* gene to the *Eco*RI site in the long terminal repeat (equivalent to bp 6059–bp 9238 of the RSV sequence); the resulting vector was named RD1. A single *Bam*HI site (at bp 532 in residual viral *gag* sequences) in vector RD1 was used to insert the bacterial *lacZ* gene (5), to give the final vector RDlac1. Vector RDlac1, thus, has a long terminal repeat-driven *lacZ* gene, and the resulting protein was a gag–LacZ fusion protein (containing only a small portion of the gag protein). The construction of Blacsrc2 has been described (13).

Production of Infectious Replication-Defective Virus. Replication-defective virus was obtained after transfection of the packaging cell line Q2bn (13) with uncut plasmid vectors, and virus was titered as described (18). Q2bn cells produce subgroup A-specific virus. Virus was concentrated in Centricon-30 microconcentrators (Amicon) by centrifugation at 5000 \times *g* for 30 min at 4°C. The immunocytochemical expression focus assay, as described (18), was used to establish RSV virus titers.

Nucleic Acid Analysis. DNA and RNA were isolated and analyzed by standard Southern and Northern transfer and hybridization methods (19). Briefly, RNA and DNA were run on 1% agarose gels and then transferred onto Hybond-N (for RNA) and Hybond-N-plus (for DNA) membranes (Amersham). The membranes were hybridized to a 600-bp *Pst* I *v-src* probe radiolabeled by using the random-primer reaction method (20). The 1.7-kilobase (kb) probe was obtained by digesting clone A11 (13) with *Bam*HI. Densitometric scans of the autoradiograms were done with an LKB model 2202 Ultrascan laser densitometer.

Immunofluorescence. Frozen sections and fixed cultures were washed twice in PBS/0.1% bovine serum albumin for 5 min followed by incubation with primary antibody for 1 hr at room temperature in PBS/1% bovine serum albumin. The samples were washed for 30 min in PBS/1 mM EDTA/0.5% Triton X-100/0.1% bovine serum albumin and then incubated with a secondary antibody (anti-mouse IgG fluorescein) for 1 hr followed by washing as above. In all experiments nuclei were stained (15 min) during the final wash with 4',6-diamidino-2-phenylindole (DAPI; Sigma) at 0.5 μ g/ml. The samples were then mounted in buffered glycerol containing *p*-phenylenediamine (Sigma) at 1 mg/ml and were analyzed using a Zeiss photomicroscope III. Mouse monoclonal antibodies anti-pp60^{src} (JB327) and anti-p19^{gag} were gifts from J. Brugge (University of Pennsylvania, Philadelphia) and T. Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada), respectively. These antibodies were used at dilutions of 1:50 (JB327) and 1:1000 (anti-p19^{gag}). Mouse monoclonal anti-myosin was from R. Strohman (University of California, Berkeley) and used at a 1:100 dilution.

RESULTS

Integration of RSV into the DNA of Cultured Blastoderm Cells. The ability of RSV to infect and integrate stably in cultured blastoderm cells was addressed initially. Stage X blastoderms were isolated and placed in culture, as described. RSV was added 4–6 hr after plating, and high-molecular-weight genomic DNA was isolated after 4 days in culture. The DNA was cut with restriction enzyme *Eco*RI and, after Southern transfer, was hybridized with a *v-src* probe. The viral 3-kb *src* fragment was detected in the DNA of infected cells and not in the DNA of control cells (Fig. 1A). To test whether the viral DNA was integrated into the genomic DNA of the blastoderm cells, the *Eco*RI-cut high-molecular-weight DNA was also hybridized with a 1.7-kb fragment that spans both the 5' and 3' regions of the viral 5' *Eco*RI site. The 1.7-kb probe detected a single 3.8-kb fragment in the infected cells (data not shown), indicating that RSV was stably integrated into high-molecular-weight genomic DNA of the blastoderm cells. Genomic DNA isolated from CEF, cultured and infected in the same way as the blastoderm cells, was used as a positive control (Fig. 1A).

Expression of RSV in Cultured Blastoderm Cells. To determine whether or not the viral genome was transcribed and correctly spliced in the blastoderm cells, total cellular RNA was isolated from infected and uninfected blastoderm cultures and hybridized with a *v-src* probe. Viral transcripts were readily detected in the blastoderm RNA. The blastoderm cultures generated the three transcripts expected from RSV-infected cells (Fig. 1B), indicating that normal regulation of viral RNA splicing occurred.

Having established that RSV infects and expresses in cultured blastoderm cells, we next asked whether viral proteins were expressed. Blastoderm cultures infected with RSV were examined 4 days after infection for the presence of viral protein p19^{gag}, by using immunofluorescence. About 30% of the cells expressed the viral protein (Fig. 2), and this number increased to >80% in cultures grown until day 12. Percentages were determined after 4',6-diamidino-2-phenylindole staining of the nuclei (see *Materials and Methods*). When blastoderm cultures were infected with the transformation-defective Rous-associated virus 1, expression of p19^{gag} followed a similar pattern (data not shown). The increasing percentage of p19^{gag}-expressing cells over time in RSV cultures is, therefore, not due to a growth advantage provided by the oncogenic virus.

Supernatants from infected blastoderm cultures were collected and shown to contain infectious viral particles at titers

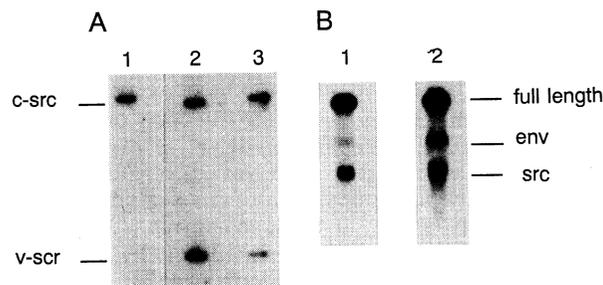


FIG. 1. Integration and expression of RSV in blastoderm cells. (A) Southern analysis of genomic DNA from uninfected blastoderm cultures (lane 1), RSV-infected CEF (lane 2), and RSV-infected blastoderm cultures (lane 3). DNA was hybridized with a 600-bp *v-src* probe. Top band is the cellular *src* gene; lower band represents viral *v-src* of the expected 3-kb size. (B) Northern analysis of total RNA from RSV-infected blastoderm cultures (lane 1) and RSV-infected CEF (lane 2) hybridized with *v-src* probe. RNA was loaded equally in both lanes, and lane 2 was exposed one-third as long as lane 1. The three viral transcripts are indicated.

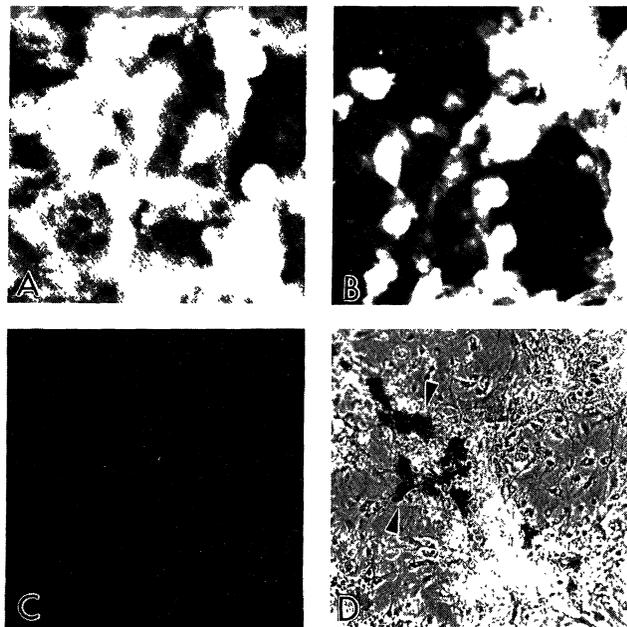


FIG. 2. Expression of viral proteins in blastoderm cultures. (A–C) Immunofluorescence analysis of viral p19^{gag}: RSV-infected blastoderm culture (A), CEF infected and cultured under conditions identical to the blastoderm cultures (B), and uninfected blastoderm culture (C). (D) Blastoderm cells in culture, fixed 4 days after infection with vector RDlac1 and treated with X-Gal. Arrowheads indicate LacZ-positive cells.

$>4 \times 10^3$ per ml. Blastoderm cells could, thus, sustain the full replicative life cycle of RSV.

Comparison of RSV Expression in Cultured Blastoderm Cells and CEF. CEF and blastoderm cells, infected and cultured under identical conditions, were compared for viral integration and viral transcription on day 4 in culture (Fig. 1 A and B). With the given virus dose, CEF showed higher amounts of integrated viral DNA as well as transcribed viral RNA (≈ 5 fold more in each case). To gauge the average efficiency of viral expression in each cell type, the ratio RNA expressed/DNA integrated was calculated by using densitometric values taken from autoradiographs. In two independent experiments, the ratio obtained from CEF was compared with that of blastoderm cells, and these ratios differed by $<10\%$ within each experiment. Thus, although the efficiency of viral integration varied significantly between CEF and blastoderm cells, RSV sequences were transcribed at similar average efficiency in both cell types. Stated differently, these data also suggested that there was no significant difference between the cell types in the amount of integrated, nonexpressed virus present.

Expression of Replication-Defective Retroviral Vectors in Blastoderm Cultures. One aim of this study was to establish the feasibility of using retroviruses as lineage markers and to express genes in early avian embryo cells. Because of its replication competence, RSV cannot be used *in ovo* for cell lineage analyses. We, therefore, took advantage of a replication-defective retroviral system using RSV-based vectors (13). A vector containing the marker gene *lacZ* (encoding bacterial enzyme β -D-galactosidase), RDlac1, was used here. The expression assay for *lacZ* gene was unequivocal due to the lack of endogenous β -galactosidase activity in blastoderm cells (data not shown).

Blastoderm cultures were infected with vector RDlac1 4–6 hr after plating, and 4 days later these cultures were fixed and treated with X-Gal. Numerous foci of stained cells were observed (Fig. 2D). Expression could be seen as early as 24

hr after infection (data not shown). Replication-defective virus can thus infect early blastoderm cells in culture and rapidly initiate the expression of foreign genes in the absence of RSV proteins. CEF cultured similarly and infected with the same numbers of replication-defective virus were used as positive controls. The number of LacZ-positive foci were higher in the CEF cultures than in the blastoderm cultures, consistent with the relatively higher levels of integrated viral DNA observed in RSV-infected CEF (Fig. 1A).

The number of RSV-expressing blastoderm cells increases with time in culture. This result could be from secondary viral infections or the progressive activation of silent proviruses. To distinguish between these possibilities, we examined replication-defective vector expression over time in culture, thus avoiding horizontal viral spread. Parallel blastoderm cultures were infected at the same time with identical doses of vector RDlac1 and assayed for the number of LacZ-expressing foci at progressive time points (Fig. 3). There was no consistent increase in the number of LacZ-expressing foci over an 11-day period, indicating that there are no significant numbers of silent proviruses being activated during this time.

Expression of Replication-Defective Vectors *in Ovo*. Having established that vector RDlac1 can infect and be expressed in cultured blastoderm cells, we tested the same vector *in ovo*. Stage X blastoderms were microinjected with replication-defective virus and analyzed 36 and 96 hr later for the expression of LacZ. Fifty percent of the blastoderms survived the injection; of these, 50% had LacZ-positive clones. When <100 virus particles were injected per blastoderm, LacZ-positive clones were rarely seen. Marked clones were identified in a variety of tissues by 96 hr, including epidermis, somites, brain, and heart (Table 1). With a second replication-defective vector, Blacsrc2 (13), LacZ-positive clones were again found in several tissues; Figs. 4A and B show a discrete Blacsrc2 clone in the somite region. This clone was located in the dermatome adjacent to the myosin-expressing myotome.

From the RDlac1 data it is of interest to note the high frequency of clones in heart tissue ($\approx 70\%$ of 96-hr embryos; Table 1). Several of the clones found in the embryos fixed 36 hr after infection were also localized in presumptive heart tissues (Fig. 4C). At both 36 and 96 hr, numerous marked clones were also seen in extraembryonic membranes (Fig. 4

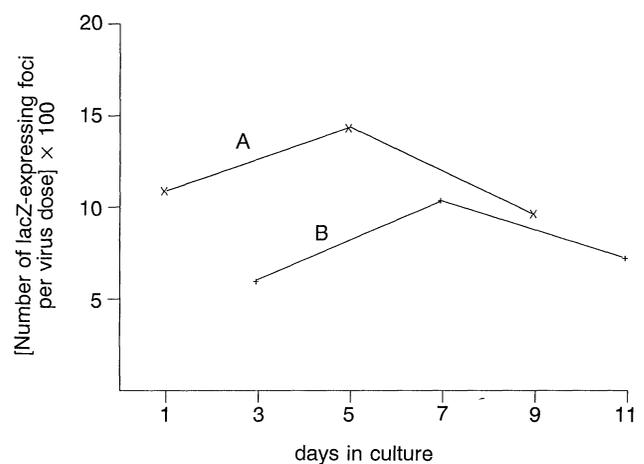


FIG. 3. Expression of vector RDlac1 over time in culture. Multiple blastoderm cultures were infected at one time (6 hr after plating) with the same dose of virus (500 virus particles per culture in experiment A; 100 virus particles per culture in experiment B). Cultures for experiment A were fixed on days 1, 5, and 9 and cultures for experiment B were fixed on days 3, 7, and 11. All cultures were treated with X-Gal, and LacZ-positive foci were counted. The number of LacZ-positive cell foci is given as a percentage of initial virus dose.

Table 1. Tissue distribution of retrovirally marked cell clones *in ovo*

Embryo group	Tissue location of infected cells				Embryos per group, no.
	Heart	Somite	Ectoderm	Brain	
A	+				3
B			+		2
C		+			2
D	+	+			4
E	+		+		1
F	+	+	+		1
G	+	+		+	1
Total clones per tissue	11*	8	4	1	

A total of 64 blastoderms were injected with vector RDlac1. Embryos were analyzed after 96 hr, and of 30 surviving embryos, 14 had LacZ-positive cell clones. Embryos were classified into groups based on tissue distribution of marked clones. Average number of virus particles injected per embryo was 150. + Indicates presence of infected cells.

*Each tissue had a single apparent clone within it, except for one embryo in group D that had two clones in the heart.

D). The earliest time point tested for LacZ expression was 24 hr after infection, when LacZ-positive cells were already identifiable (data not shown).

DISCUSSION

We have examined the expression of RSV and derivative replication-defective vectors in avian blastoderm cells, both in culture and *in ovo*. It is shown that these viruses infect and are expressed in blastoderm cells in culture and that RSV replicates and produces infectious virus particles. Our data also show that replication-defective vectors infect blastoderm cells *in ovo* and maintain stable expression in diverse tissues.

Previous studies have concluded that RSV expression in early chicken embryo cells is very inefficient (15); we have now shown that this is not the case. We cannot easily explain the previous findings, although we have considered some of

the differences between our experimental protocols. (i) The strain of virus used by Mitrani and coworkers (15) was Prague subgroup B RSV, whereas we used subgroup A virus. We have since repeated our studies with subgroup B RSV (PrB and Schmidt Ruppin B). Our conclusions are unchanged (unpublished data). (ii) The reason why Mitrani and coworkers (15) were unable to detect any viral mRNA could be due to their culture conditions, a point also mentioned in their discussion. In our initial characterization of culture conditions for blastoderm cells, several substrata were tested, and collagen I was chosen as the best matrix for sustaining cell proliferation. On collagen the growth rate, as measured by tritiated thymidine labeling index, was very high, equivalent to that of CEF on the same substrate (data not shown). In the same set of experiments we also observed that cells grown directly on plastic were often unhealthy and proliferated poorly. The cells were grown on plastic in the previous studies (15), a condition not conducive to viability and, hence, not optimal for studies of virus expression.

The ratio of viral RNA expressed/viral DNA integrated was found similar for RSV-infected blastoderm cells and CEF, although the absolute amounts of viral RNA and DNA were both significantly higher for CEF. Furthermore, the number of infections that resulted in LacZ expression, per replication-defective virus dose, was also higher for CEF, but the resulting LacZ expression (as judged by X-Gal stain intensity) was similar in both CEF and blastoderm cells. Taken together, these data indicate that (i) cultured blastoderm cells are initially more refractory to stable infection than day 10 embryo mesenchymal cells, such as CEF, and (ii) once each cell type is infected, the efficiency of viral RNA transcription is comparable. There are at least three possible explanations for the observed stringency of blastoderm infection. First, a lack of dividing cells in the early blastoderm cultures may prevent stable infection. However, we have discussed above that cell division rates for CEF and blastoderm cells were similar, making this explanation unlikely. Second, although many cells may become infected, there could be a widespread block or delay in the expression of integrated provirus. If this were the case, we would expect to see a much greater amount of transcriptionally silent, integrated DNA, reducing the RNA

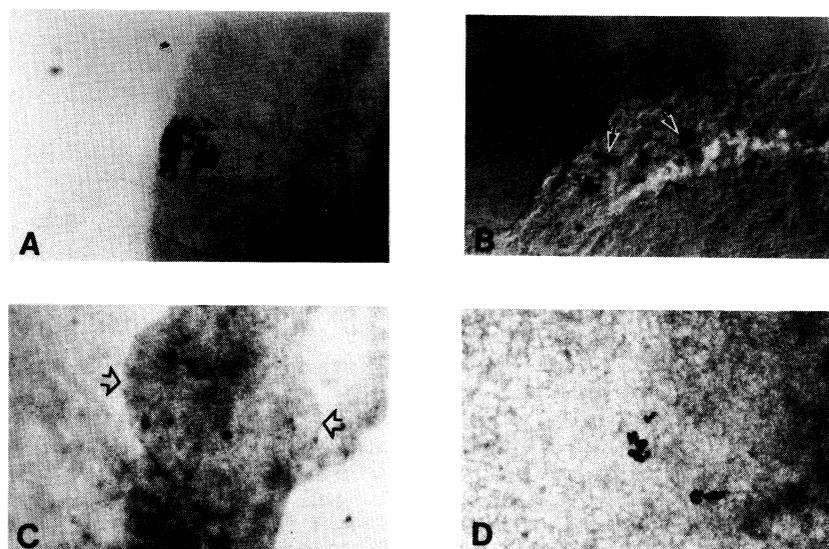


FIG. 4. Expression of replication-defective vectors *in ovo*. (A) Whole-mount view of an embryo injected with vector Blacsrc2 at stage X, fixed after 96 hr, and treated with X-Gal. LacZ-positive cells in the somite region are shown. (B) The embryo in 4A was sectioned and stained by immunofluorescence for myosin heavy chain. The LacZ-expressing cells (white arrowheads) were located in the dermatome, adjacent to the myosin-positive myotome (large arrowhead). (C) Embryo fixed 36 hr after injection of a blastoderm with vector RDlac1. Arrows indicate boundaries of presumptive heart tissue, within which the dark, LacZ-positive cells are seen. (D) LacZ-positive clones in extraembryonic tissue of the specimen in C.

expression/DNA integration ratios, but this is not observed. Also, delayed onset of proviral expression appears not to occur readily, as demonstrated using replication-defective vectors (Fig. 3). The third, and most plausible explanation is that a proportion of blastoderm cells have low levels of cell-surface virus receptors or cannot complete postadsorption stages of virus infection efficiently. It is also possible that some cells completely lack receptors. It should be noted here that the above could not explain the lack of viral expression reported by Mitrani *et al.* (15): In our hands after 12 days in culture, the length of time these workers also maintained their cells, >80% of our cells expressed viral proteins. Selective overgrowth of cells expressing oncogenic RSV does not appear a significant factor because similar data were obtained by using nononcogenic Rous-associated virus 1 (data not shown). These data also indicate that in productively infected blastoderm cultures, the stringency for infection is overcome as the cultures mature.

Replication-defective viruses can be used to infect blastoderms *in ovo*, and expression of retroviral genes is seen as early as 24 hr after virus inoculation. Although it is difficult to determine the efficiency of infection *in ovo* accurately, this efficiency appears only slightly lower than that seen in cultured blastoderm cells. Replication-defective reticuloendotheliosis virus vectors have recently also been shown to transfer genes into the unincubated chicken embryo, although expression of viral genes has yet to be tested before day 15 of development (21, 22). The rapid onset of expression seen with replication-defective RSV vectors will facilitate experimental expression of foreign genes early in embryogenesis. Although retroviruses cannot be targeted to specific cells for precise fate-mapping purposes, their stability as genetic markers could be used in studying the long-term distribution and differentiation of cell clones marked in the blastoderm. Whether or not all cells of the blastoderm are infectable with these viruses also remains to be determined. The bias toward heart-specific clones seen with the replication-defective vector suggests either that selective infectability can occur or that the inoculation site used favors virus access to certain cells.

In conclusion, our study has shown that RSV-based viruses can be used to infect early blastoderm cells in culture and *in ovo* and that viral expression is maintained as development proceeds. Given that dissociated blastoderm cells have recently been used as donor cells to create chicken chimeras (23), the genetic manipulation of such cells in culture by using retroviral expression vectors may offer another approach for creating transgenic strains. Finally, if avian embryo stem cells become available, they, too, may be open to genetic engineering with RSV-based vectors.

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