Rous sarcoma virus is integrated but not expressed in chicken early embryonic cells
(retroviruses/early development/gene expression)

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ABSTRACT We have developed a protocol that allows us to infect chicken early embryonic (CEE) cells with high efficiency. This was achieved by exposing the CEE cells to a semicontinuous dose of Rous sarcoma virus (RSV) for a period of 20 hr. Southern blot analysis indicated that an average of one proviral copy is integrated per embryonic cell. However, there was no production of infectious viral particles by the cells containing the proviral genome, although low levels of full-length genomic RNA could be detected by RNA transfer blot analysis. These low RNA levels contrast with the 100- to 1000-fold higher levels found in RSV-infected chicken embryo fibroblasts. We conclude that in cells derived from pregastrulating chicken embryos, RSV DNA is integrated into the cell genome but fails to be expressed in an efficient manner. These primary cells can therefore be used to identify factors involved in regulation of retroviral gene expression in normal cells. Such factors may also be instrumental in elucidating basic mechanisms involved in gene regulation during early development in higher vertebrates.

RNA tumor viruses (retroviruses) have been studied extensively because of their capacity to induce tumors, their particular replication strategies, and their wide distribution in nature as agents that can be transmitted horizontally and genetically (1). Retroviruses are now becoming increasingly useful as vectors for gene transfer into animal cells (2, 3). This capacity, however, has been severely hampered by the lack of provirus expression once introduced into early embryos or embryonic stem cells (4, 5). In the mouse, preimplantation embryos are susceptible to retrovirus integration but cannot express viral functions (6). In contrast, in postimplantation mouse embryos, retrovirus can integrate as well as express high concentrations of viral-specific RNAs in every organ (7). Attempts at understanding this block of retroviral expression in early mouse embryonic cells have led to the proposal of generalized mechanisms of gene regulation that may operate in early development, such as de novo DNA methylation (4). In addition, certain regulatory sequences of retrovirus do not function in embryonic carcinoma (EC) cells unless the enhancer sequence is deleted. It has thus been proposed that undifferentiated cells contain trans-acting regulatory factors that reduce transcription by interacting with viral enhancers (8).

In an effort to examine to what extent what has been learned about retroviral behavior in the early mouse embryo can be generalized to other early developmental systems we have now examined retroviral integration and expression in the pregastrulating early chicken embryo. The advantage of the early chicken embryo is that it contains about 2 orders of magnitude more cells at the blastula stage than the corresponding mammalian embryo. It can be operated upon and grown in ovo and in vitro, and primary cells from various stages and from different areas can be grown in culture (9–12). Salters et al. (13) have reported the stable integration of proviral Rous sarcoma virus (RSV) into chicken embryo germ cells after infection in ovo with RSV. Yet, the mechanisms of interaction between RSV and the embryonic cells and the stage at which the embryonic cells become infected have not been determined. In the present work we present data that shows that normal early chicken embryonic cells can be infected at high efficiency with RSV. We also show that in spite of this, the levels of viral mRNA produced by the infected cells are very low and are not sufficient to generate fully competent viral particles.

MATERIALS AND METHODS

Preparation, Culture, and Retroviral Infection of Chicken Early Embryo (CEE) Cells. Stage X blastoderms were isolated from commercial unincubated eggs provided by SPAFAS. Most unincubated eggs obtained were found to be at stages later than stage X. A special arrangement was therefore made with SPAFAS to obtain eggs that had been collected immediately after laying. After dissection, only those blastoderms found to be at stages X–XI (14) were used. Whole embryos were then trypsinized as described (12) and dissociated in culture medium supplemented with RSV at 10^7 focus-forming units (ffu)/ml. The cells were plated at a concentration of 1.5 × 10^5 cells per ml. Twenty-four hours later the medium was replaced with virus-free medium and the cells were incubated further for various periods of time (protocol I). When CEE cells were exposed to a semicontinuous dose of RSV (protocol II), the initial medium was replaced 12 hr later and then at 4-hr intervals, for the next 12 hr, with fresh medium containing RSV at a concentration of 10^5 ffu/ml. The strain used, Pr RSV, subgroup B (Pr B RSV), has been described (15). The stock used contained about 50% of mutants lacking part of the src gene.

DNA Analysis. At the end of the culture period, the cells were rinsed five times in phosphate-buffered saline (PBS), removed with a rubber policeman, and digested with proteinase K (200 μg/ml) at 45°C for 45 min. The cell digest was then extracted twice with phenol/chloroform. Sodium acetate to 0.3 M and 2 vol of ethanol were added to half of the material, which was stored and processed later to prepare RNA. The other half was digested with boiled pancreatic RNase (120 μg/ml) at 37°C for 2.5 hr and dialyzed extensively in 10 mM Tris-HCl/1 mM EDTA (TE), pH 8.0. The DNA was precipitated and resuspended in a small volume of TE, and

Abbreviations: RSV, Rous sarcoma virus; CEE, chicken early embryonic; EC, embryonic carcinoma; Pr B RSV, Prague RSV, subgroup B; Pr C RSV, Prague RSV, subgroup C; kb, kilobase(s).

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the concentration was determined by measuring the optical absorbance at 260 nm. The concentration was used throughout as a reference point so that quantities of RNA could be calculated as DNA equivalents. DNA was digested with Sac I restriction enzyme (Bethesda Research Laboratories). Samples (10 µg per lane) were electrophoresed on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with a 32P-labeled riboprobe complementary to bases 103–2870 of the Prague RSV subgroup C (Pr C RSV) genome (a gift from D. Hanahan).

**RNA Analysis.** Total nucleic acids obtained as described above were precipitated, resuspended in 10 mM Tris-HCl/10 mM MgCl₂, and digested with DNase I (10 µg/ml) for 30 min at 37°C. RNA was then denatured and electrophoresed on a 1% agarose/formaldehyde gel, transferred to a GeneScreen nylon membrane, and hybridized to a 32P-labeled riboprobe complementary to bases 103–255 in the leader region of the Pr C RSV genome (a gift from S. Herman).

**RESULTS**

Southern blots of DNA isolated from the RSV-treated CEE cells (protocol I) using a riboprobe complementary to bases 103–2870 of the RSV genome could only detect the endogenous proviral components. In one experiment, in which the cells were grown for 3 weeks before the DNA was processed, a faint band (not shown) was detected that corresponded to <10% of the cells being infected. This suggested that some cells were incorporating the proviral genome after "pulse" administration of RSV but that the infection did not spread through the culture as would be expected if competent particles that could infect other cells were produced. Additional experiments were thus performed in which cells were grown in the constant presence of RSV in culture to obtain a higher initial multiplicity of infection (protocol II). Under these experimental conditions, RSV does integrate into genomic DNA of CEE cells as shown in Fig. 1. Since the intensity of the band corresponding to the newly integrated provirus is roughly the same as that of each of the single-copy endogenous components, this indicates an average of about one copy of RSV DNA per CEE cell.

To understand why CEE cells did not transmit competent virus to other cells, RNA, extracted 72 hr after infection, was analyzed on RNA transfer blots using a riboprobe complementary to bases 103–255 in the leader region of the RSV genome. Lane 1 of Fig. 2A shows RNA extracted from chicken embryo fibroblasts infected with RSV. Lane 2 shows RNA from RSV-infected CEE cells. Lane 3 shows RNA from CEE cells untreated with RSV. Lane 4 shows RNA from RSV-infected CEE cells (lane 3) and from RSV-infected CEE cells (lane 4). Even at these levels of exposure, no bands corresponding to env and src mRNAs can be detected. The riboprobe also hybridizes to rRNA. The presence of more than one band in the 9-kb region (arrows) is due to heterogeneity of the viral stock, which contained mutants in the src region (9).

![Fig. 2.](image)

**Fig. 2.** RSV-infected CEE cells produce very low levels of viral RNA. RNA, extracted from cultured cells 72 hr after RSV infection, was denatured and electrophoresed on a 1% agarose/formaldehyde gel, transferred to a GeneScreen nylon membrane, and hybridized to a 32P-labeled riboprobe complementary to bases 103–255 in the leader region of the RSV genome. (A) Lane 1, RNA extracted from chicken embryo fibroblasts infected with Pr B RSV (positive control). Lane 2, CEE cells grown at high density and infected with Pr B RSV. RNA in lane 2 was extracted from 10 times as many cells as in lane 1. (B) Autoradiograph, exposed for a longer period of time, of a transfer blot of RNA extracted from untreated CEE cells (lane 1) and from RSV-infected CEE cells (lane 4). Even at these levels of exposure, no bands corresponding to env and src mRNAs can be detected. The riboprobe also hybridizes to rRNA. The presence of more than one band in the 9-kb region (arrows) is due to heterogeneity of the viral stock, which contained mutants in the src region (9). (C) Diagram of RSV spliced mRNAs.

CEE cells grown at high density and infected with RSV. Although RNA in lane 2 was extracted from 10 times as many cells as that in lane 1, the bands that hybridized to the viral probe in this lane are weaker by a factor of ∼10 than those in lane 1. Thus, RSV-infected CEE cells produce only very low levels of RNA (less by a factor of ∼100 than RSV-infected embryo fibroblasts in this case). In no case did we detect viral RNA levels higher than this in CEE cells infected with RSV. Depending on which cells were used as positive controls, the levels in CEE cells were lower by a factor of 1000 than those produced by RSV-infected embryo fibroblasts. Since productively infected chicken embryo fibroblasts contain 1–10,000 copies of virus RNA per cell (3), one can estimate that infected CEE cells contain each about 1–10 copies of viral RNA. Fig. 2B shows an autoradiograph exposed for a longer period of time from a transfer blot of RNA extracted from untreated CEE cells (lane 3) and from RSV-infected CEE cells (lane 4). Even at these levels of exposure, no bands corresponding to env and src mRNAs were detected in three different experiments in which the
cells were grown at high density and RNA was extracted after 72 hr. In another experiment in which the cells were grown at high density and RNA was extracted after 8 days, no env and src mRNA bands were detected either. The intensities of the full-length viral RNA bands were, however, considerably weaker. We did observe, nonetheless, faint (<100-fold) env and src mRNA bands in one case in which RNA was extracted after 8 days but the CEE cells were grown instead at low rather than high density. In all cases studied, however, the common feature was that CEE cells infected at high efficiency with RSV produced only very low levels of RNA.

DISCUSSION

The method described here allows one to infect CEE cells with an efficiency of about one proviral copy per cell. Rubenstein et al. (16) have been able to introduce genes into transgenic mice by infecting early embryos with defective retroviruses. They estimated, however, that only about 20% of the cells in transgenic fetuses contained the proviral genome. Even lower efficiencies of infection have been achieved when using retrovirus in other transgenic systems (17, 18).

Even though the CEE cells were infected at high efficiency, the levels of viral RNA produced in all cases studied were low and were not sufficient to generate fully competent viral particles. In most cases the low levels of RNA consisted of unspliced genomic RNA. At present we do not know whether the unspliced RNA detected is, at least in part, genomic RNA that remained in the culture from the time of infection or newly synthesized unspliced RNA. It is also possible that the expression of env and src mRNAs varies with the culture conditions. Splicing of viral RNA has been a controversial issue. It has been reported that simian virus 40 RNA is not spliced in undifferentiated murine teratocarcinoma stem cells (19), but those data have been questioned recently by others (8). In our experiments, the levels of RNA produced were clearly not sufficient to generate competent viral particles. The situation described here is similar to what has been observed in early mouse embryos and in EC cells. Other workers have found that mouse undifferentiated EC cells do produce low levels of viral RNA after retroviral infection, even though no viral particles can be detected in these cells (20).

Other groups have found that the promoter in the long terminal repeat (LTR) of murine sarcoma virus (MSV) does not function in undifferentiated EC cells. EC cells can be infected with DNA viruses such as simian virus 40 (21) and polyoma (22–25) as well as retroviruses (26–28). Yet, infected, preimplantation mouse embryos or undifferentiated EC cells (F9) are unable to efficiently express the viral genomes, although these genomes are efficiently expressed in the differentiated derivatives. The polyoma virus (Py) enhancer is not active in EC cells. A point mutant, however, of the Py enhancer has been shown to be active in undifferentiated EC cells (23) and not to be repressed by adenovirus 2 EIA products (25). It was first suggested that the lack of Moloney murine leukemia virus (MuLV) gene expression in preimplantation embryos and in EC cells was due to methylation of the integrated viral DNA (29, 30). It was then postulated (31, 32) and later shown (8) that certain regulatory MuLV sequences do not function in EC cells. The LTR promoter of MSV is not active in undifferentiated EC cells, but when the enhancer sequence is deleted expression takes place. It has thus been proposed that undifferentiated cells contain trans-acting regulatory factors that reduce transcription by interacting with viral enhancers (8). More recently, Barklis et al. (33) have suggested that although this may be one reason for inefficient retroviral expression in undifferentiated F9 EC cells, expression may be restricted at other levels as well. By infecting F9 cells with a recombinant retrovirus carrying the neomycin gene (34–36) and characterizing rare colonies that grew in the presence of antibiotic G418, they found that some proviruses were expressed as a result of being integrated close to specific cellular genomic sequences that apparently promoted proviral gene activity. An additional case was found in which the provirus was expressed due to a point mutation in the tRNA primer binding site of the proviral genome, although how this point mutation increases viral expression is not yet clearly understood (33).

Studies of interactions between retroviruses and normal cells have proved very fruitful, in particular in characterizing various oncogenes and growth factors (3, 35, 37, 38). Retroviruses are also becoming indispensable vectors for introducing foreign genes in a stable manner into various cellular and animal systems (2, 3). Yet, the way in which retroviruses interact with eukaryotic cells is poorly understood. We have shown here that in cells derived from pregastrulating chicken embryos, RSV retrovirus is integrated into the cell genome but fails to be expressed in an efficient manner. This indicates that such a pattern of behavior, previously found only in the mouse system, seems to be of a more universal nature. Furthermore, the range of viruses that may be affected by such a mechanism seems to be equally broad. Thus, we believe that CEE primary cells may be useful for defining factors that may be involved in regulation of retroviral gene expression. Such factors may also be instrumental in elucidating basic mechanisms involved in gene regulation during early development in higher vertebrates.

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