REPLICATION-DEFECTIVE VIRUS INFECTION OF FEATHER BUDS PRODUCES A LOCALIZED REGION OF β-GALACTOSIDASE ACTIVITY

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We are interested in using retroviral vectors to trace cell lineage and to introduce exogenous genes in chicken skin explant cultures. Here the LZ10 virus carrying the gene encoding β-galactosidase was introduced to the skin explants by two different means: a) the virus was added to the media or b) the virus was microinjected into regions of the developing feather buds. Infection by microinjection led to localized expression of β-galactosidase in the developing feather bud, while, surprisingly, infection by adding the virus to the culture media led to localized band of β-galactosidase expression in the middle of the feather filament. The significance of this finding in skin morphogenesis and as a tool for experimental embryology is discussed.

Pattern formation is crucial to embryonic development. The identification of molecules involved in pattern formation and their mechanism of action have been central interests of developmental biologists. Recent studies have shown that the localized expression of growth factors, homeobox proteins and cell adhesion molecules can cause profound structural changes in developing embryos and have brought exciting new understanding to pattern formation (Jessel and Melton, 1992). Therefore, it is important to establish the fates of cells in order to determine how they respond to localized position-specific determinants.

We have been using feather explant cultures as a model to study pattern formation (Chuong, in press). We have found highly spatial specific expression patterns of adhesion molecules (Jiang and Chuong, 1992) and position specific homeoprotein gradient in feather buds (Chuong et al., 1990, 1992). In this study, we have used the avian sarcoma retrovirus to trace the lineage of embryonic feather cells in developing chickens. The viral vectors can infect cells that may be difficult to microinject directly

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with other lineage tracers and will not be diluted by mitotic cell divisions. The viral vectors also offer the potential to introduce and express exogenous genes to localized regions in order to assess their developmental consequences.

MATERIALS AND METHODS

Chicken Skin Explant Culture

Chicken back skin was dissected from E8 embryonic chickens, placed on a Falcon culture insert (Cyclopore membranes, 25 mm diameter, 0.45 um pore size, Becton Dickenson, Lincoln Park, NJ) and grown in DMEM containing 2% fetal bovine serum. The media was changed one and three days after infection. For detail, see Jiang and Chuong, 1992.

Virus

We have obtained the avian retroviral vector LZ10 (Franke and Sanes, 1989). This retroviral vector integrates into the host cell genome and only is passed to daughter cells since viral replication is dependent on genes provided by a packaging cell line. They each contain the LacZ gene which codes for β-galactosidase which can be detected in fixed tissue with X-gal.

Infection by Incubation in the culture media

Embryonic chicken skin (E8) was infected with the replication defective avian sarcoma virus LZ10 by adding the virus at 5,000 pfu/ml to the culture media in a biological containment hood. To avoid contamination all surfaces were washed with bleach and sterilized with 70% ethanol. The cultures were grown for a total of 5 days before fixation and staining for β-galactosidase activity with X-gal.

Infection by Microinjection

Glass capillary tubes (1.0 mm outer diameter, 0.75 mm inner diameter) were formed into microinjection needles with a tip diameter of approximately 10 um using a PB-7 pipet puller (Narishige, Tokyo, Japan). The microinjection needle was moved into place to deliver injections to different locations within the feather bud with a Narishige micromanipulator. 0.1 - 0.5 ul of virus were microinjected into the feather buds of E8 chicken skin with a WPI picopump (New Haven, CT). After 5 days the skin explants were fixed and stained with X-gal.

Detection of Infected Cells with β-galactosidase In Vitro

β-galactosidase was detected in fixed tissue with the conventional X-gal substrate. Skins were fixed in 2.5% paraformaldehyde, pH 7.0 and rinsed with PBS. β-galactosidase activity was detected with 0.5mg/ml X-gal in 50mM phosphate buffer containing 5mM potassium fericyanate and 5 mM potassium ferocyanate which produces a blue color (Sanes et al., 1986).

Clearing of Back Skins

The detection of β-galactosidase was facilitated by clearing the skins. The skins were dehydrated in methanol and placed in benzyl benzoate:benzyl alcohol (2:1) (Dent et al., 1989).

RESULTS

Embryonic chicken skin (E8) was infected with the replication defective avian sarcoma virus LZ10 which encodes β-galactosidase by adding the virus at 5,000 pfu/ml to the culture media. Many feathers grown in the media containing the LZ10 virus developed a localized ectodermal band of X-gal positive cells after 5 days of culture. The band was observed midway along the growth of the developing feather bud (Fig. 1).

An alternate route of infection was by microinjection as has been used by others (Mikawa et al., 1992; Frank and Sanes, 1991; Koseki et al, 1991; Galileo et al., 1990; Gray et al., 1990; Stoker et al., 1990; Luskin et al., 1988; Turner and Cepko, 1987; Price et al., 1987; Sanes et al., 1986. One to five hundred microliters of virus at 5,000 pfu/ml were microinjected into the feather germs of E8 chicken skin. The microinjected virus produced X-gal positive cells localized within the microinjected feather buds (Data not shown).

Fig. 1. *Avian sarcoma virus placed in the culture media produced a localized ectodermal ring of infected cells in developing chicken feathers.* The LZ10 avian sarcoma virus was placed in the culture media of chicken skin explants at 5×10^3 pfu/ml. After 5 days in culture, the skins were fixed stained for β-galactosidase and cleared. β-galactosidase positive virally infected cells were visible as a narrow band in many feather buds. The band was localized to the ectoderm of the feathers. The fact that the infected cells formed a ring could be easily discerned by focussing up and down through the explant culture.

DISCUSSION

Recently replication defective retroviral vectors expressing β-galactosidase from the *E. Coli* lacZ gene have been used to infect dividing cells in order to study cell lineage in chicken and mouse (Sanes et al., 1986; Price et al., 1987). This procedure has been applied to study the lineage of cells within the brain (Frank and Sanes, 1991; Gray et al., 1990; Galileo et al., 1990; Luskin et al., 1988; Turner and Cepko, 1987), heart (Mikawa et al., 1992), and kidney (Koseki et al., 1991) as well as to determine the influence of the limb bud micro-environment on the activity of the *src* oncogene (Stoker et al., 1990).

We have tested the efficacy of using replication defective retroviral vectors as a means of determining cell lineage in developing chicken cutaneous appendages using 2 possible routes of infection. Microinjecting E8 chicken skin with replication-defective avian sarcoma virus produced localized patches of β-galactosidase activity in the mesoderm. To our surprise, infection through the culture media also produced a restricted distribution pattern. Localized rings of infected cells in the ectoderm of the growing feather buds were observed. These narrow rings suggest that a) the virus infected a limited population of cells that gave rise to a clonal band of cells or b) the virus infected a band of cells that were infectable which did not continue to divide and were simply pushed up the developing feather bud by dividing cells from below.

Whatever the mechanism is, this finding shows that this novel approach will be a very useful tool in establishing cell lineage during skin morphogenesis and in providing localized exogenous gene expression during skin development. First, the limited distribution of infected cells produced by placing the LZ10 virus in the media suggests that the virus was only capable of infecting cells for a relatively short time span. Since the retrovirus only infects cells actively dividing, this will provide a useful tool to investigate the timing of cell division within the developing feather bud and will aid in our understanding of skin morphogenesis. Second, the unique and localized β-galactosidase expression pattern we have observed also suggests that these retroviral infection routes will be a very powerful means to deliver ectopically expressed transgenes to study gene function within normal developing feather germs. This can be achieved by ectopically expressing retroviral transgenes in mesodermal or ectodermal cells where they are not normally expressed and subsequently following cell fates. Once we have established the timing of cell divisions and ultimate cell fates, it should be possible to deliver any gene of interest to any region of the feather to test its function. The detection of the infected cells in the transparent living feather explants using fluorescent substrate for β-galactosidase will also be possible. This will enable us to observe behavioral differences in infected cells overexpressing genes of interest.

We are currently investigating how infection at different developmental stages effects the distribution of infected cells and the rapidity of infection. Infection through the culture medium is rapid and easy and should complement microinjection to facilitate the investigation of gene function in embryogenesis. The combination of this transgene technology with experimental embryology will lead to new molecular understanding in the classical transplantation-recombination experiments in chicken embryos.

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