Genomic plasticity of the Lucké renal carcinoma: a review

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ABSTRACT The differentiation potential of the Lucké renal carcinoma of the northern leopard frog, *Rana pipiens*, can be characterized by the nuclear transplantation procedure. Transplantation of tumor nuclei into activated and enucleated ova results, in the best of cases, in swimming larvae which fail to feed. The larvae die in about 10 to 14 days. Rescue of tumor nuclear transplantation tadpole tissue, destined to die, has been accomplished by allografting fragments of that tissue to normal hosts. The allografts persist and differentiate a diversity of tissues which cannot be distinguished by histological analysis from allografted normal control tissue. Allografts are an imperfect mode of assay for histological competence because of the immune response of the host. Lymphocytes and eosinophils invade the grafts in about 40 days. The host immune response occurs in both experimental and control allografts. Consequently, we believe that added histogenetic potential exists in the genome of the Lucké renal carcinoma. We propose that unexpressed differentiative potential of the grafted tissue can be extracted by abrogation of the immune response of the host. A herpesvirus is the etiological agent of the Lucké renal carcinoma. We currently seek to detect viral DNA in tissue derived from tumor nuclear transplant embryos. The presence of the viral genetic material in normal mitotic progeny of Lucké tumor cells, if demonstrated, raises the question of the long-term stability of differentiated cells derived from a virus tumor. Alternatively, absence of viral DNA in the tumor nuclear transplant tissue would suggest that normal differentiation ensues after elimination of the oncogenic DNA from that tissue. Loss of viral DNA may prognosticate stable differentiation.

KEY WORDS: *Lucké renal carcinoma, nuclear transplantation, differentiation, Lucké herpesvirus DNA, Rana pipiens*

Introduction

A tenet of modern molecular biology is that specialized cells retain a genome identical with that of the zygote nucleus. Cell specialization, it follows, is not due to alterations in the genome itself but to a selective reading of the genome. The most rigorous test available for examination of this fundamental tenet is the nuclear transplantation procedure developed by Briggs and King (1952). The procedure in its simplest form involves the insertion of a somatic nucleus into an egg that was previously activated and enucleated (King, 1966, 1967; McKinnell, 1978). Early embryonic nuclei from amphibians have been shown to be competent to substitute for the zygote nucleus so that after transplantation the recipient egg will develop normally to the adult stage and become sexually mature (Gurdon, 1962; McKinnell, 1962). The nuclear transplantation procedure was extended to a number of other amphibian species (McKinnell, 1978) and more recently has been adapted for use in mammalian reproduction and genetic studies (Prather *et al.*, 1989; Bondioli *et al.*, 1990; First and Prather, 1991; Kanka *et al.*, 1991).

A notable and unexpected outcome of these studies is that while early embryonic nuclei of a diversity of species are totipotent (as would be inferred from the tenets of molecular biology), the genome of adult cells has yet to exhibit totipotency. Nuclei from adult donors have limited competence to promote embryonic development (DiBerardino, 1987). For example, a small proportion of adult skin cell nuclei, 99.9% of which react with an antibody to keratin, have been shown to have the competence to program for the formation of larvae when inserted into enucleated ova. The most advanced of these larvae survived for about 1 week, when they became edematous and died without feeding (Gurdon *et al.*, 1975). These and many other experiments clearly reveal that while nuclei from juvenile and adult amphibians are pluripotent, «*to date, no nucleus of a documented specialized cell nor of an adult cell has yet been shown to be totipotent*» (DiBerardino, 1987).

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0214-6282/93/$03.00

Fig. 1. Chromosomes from a triploid (3n=39) virus-induced Lucké renal adenocarcinoma. *Arrows indicate silver-stained nucleolar organizer regions which are found on chromosome 10q.*

Transplantation of the Lucké renal carcinoma nuclei

The nuclear transplantation procedure was developed initially in the North American leopard frog, *Rana pipiens* (Briggs and King, 1952). The leopard frog is vulnerable to a herpesvirus-induced renal carcinoma (Lucké, 1934; McKinnell *et al.*, 1991). Since amphibians are no exception to the generalization that euploidy is a prerequisite for normal development (Boveri, 1902; Fankhauser, 1945; Briggs and King, 1959), if one were to seek to characterize the genomic potentiality of a malignancy by the nuclear transplantation procedure, a euploid or near euploid chromosome constitution would be required. Fortunately for this purpose, the Lucké carcinoma has the euploid chromosome number (2n=26). DiBerardino and Hoffner (1969), who studied spontaneously arising renal carcinomas, concluded «this malignancy is not characterized by significant deviation from the diploid number.» Because the nuclear transplantation procedure was developed in *Rana pipiens*, because there is a naturally occurring renal carcinoma with a known etiology, and because that neoplasm has a euploid chromosome constitution, the Lucké tumor was an ideal candidate for the characterization of a cancer genome by nuclear transplantation. The rationale for such characterization relates to two fundamental issues in cell biology, *viz.*, what is the developmental potential of the genome of a somatic nucleus derived from a juvenile or adult animal, and to what extent can the nucleus of a neoplastic cell be reprogrammed to direct the development of a multiplicity of normally differentiated cell types?

The nuclear transplantation studies of the Lucké renal carcinoma were begun a third of a century ago. Those early studies indicated that indeed the procedures developed for embryonic nuclei could be adapted to studies of the Lucké renal carcinoma. Activated and enucleated ova became the recipients of primary- or anterior eye chamber-cultured renal carcinoma nuclei. Blastulae, gastrulae, neurulae and two postneurulae ensued from these transplantations (King and McKinnell, 1960).

Inasmuch as we were well aware of the limited development programmed from nuclei of relatively young embryos (McKinnell, 1972), we were extraordinarily concerned with providing assurance

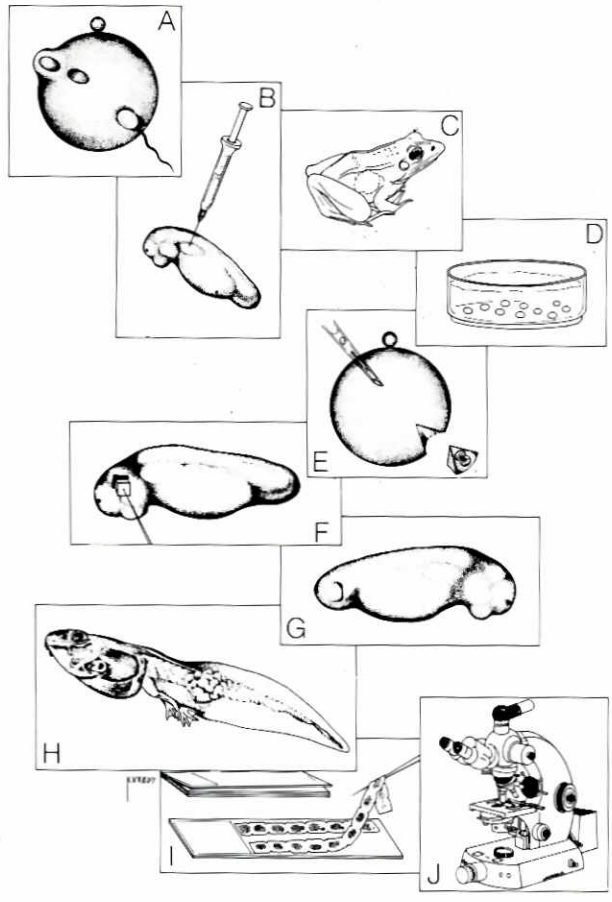

Fig. 2. Rescue of triploid tumor nuclear transplant tissue by allografting to diploid hosts. (A) *Triploidy is induced in freshly inseminated eggs by hydrostatic pressure which inhibits extrusion of the second polar body (1n second polar body + 1n sperm nucleus + 1n mature maternal nucleus= 3n).* **(B)** *Tailbud 3n embryos are injected with a tumor cell homogenate containing Lucké tumor herpesvirus.* **(C)** *Renal adenocarcinomas (dotted line) appear at metamorphosis in animals which were exposed to the virus.* **(D)** *Triploid tumor cells are dissociated and* **(E)** *inserted into previously activated and enucleated ova.* **(F)** *Tissue is harvested from tailbud 3n tumor nuclear transplant embryos (in this case, the eye forming region) and* **(G)** *allografted to diploid normal host embryos.* **(H)** *Forty days later, large triploid grafts appear on the tails of diploid host larvae, the grafts are dissected from the hosts, fixed and sectioned* **(I)** *and histologically examined* **(J)** *(from McKinnell et al., 1991).*

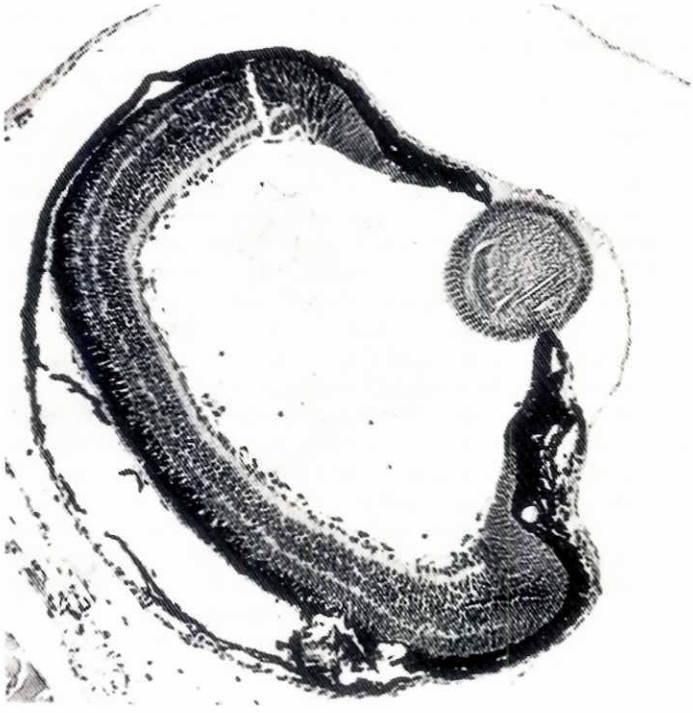

Fig. 3. Section of eye and lens that developed in an allograft of an eye-forming region of a tumor nuclear transplant embryo. *Note normally differentiated retina comparable in histology to the retina of a diploid control about half way to metamorphosis.*

that the development of the recipient ova was attributable to the inserted tumor nucleus and not to improperly enucleated host egg chromosomes. We were aware that triploidy is compatible with normal development in *Rana pipiens* (Briggs, 1947; McKinnell, 1964) and that triploid frogs can be produced easily by exposure of freshly inseminated eggs to hydrostatic pressure (Dasgupta, 1962). Accordingly, we produced triploid embryos which were subsequently exposed to the herpesvirus etiological agent of the Lucké renal carcinoma. Triploid (3n=39) (Fig. 1) renal carcinomas ensued from this treatment (McKinnell and Tweedell, 1970). We reasoned that if we transplanted triploid renal carcinoma nuclei into activated and enucleated ova, we could easily exclude gynogenetic diploid development should it occur. We were gratified that all seven of the postneurulae which developed in the first experiments using triploidy as a nuclear marker were triploid (McKinnell *et al.*, 1969).

While the triploid experiment provided genetic assurance that the donor nuclei were of tumor origin, it did not assure us that epithelial nuclei (i.e., nuclei of tumor parenchymal origin) were providing the genetic information that directed the development of the recipient ova. Obviously tumors are comprised of epithelium, connective tissue and vascular elements. Therefore, we sought to characterize the cells which served as donors in nuclear transfer experiments.

We seek to manipulate nuclear donors minimally. Thus, in our early experiments using primary tumors, or tumors that had been allografted to the anterior eye chamber of adult frog hosts, we exposed the donor tissue to an electrolyte solution deprived of divalent cations (calcium- and magnesium-free Steinberg solution, McKinnell, 1978). We expected that only epithelial cells would dissociate from the tumor fragment under these circumstances. This proved to be true (McKinnell *et al.*, 1976; Seppanen *et al.*, 1984). While ultraviolet fluorescence of dissociated cells stained with acridine orange revealed that most cells were indeed epithelial (the percentage varied in individual experiments from 96.6 to 100%), we continued to seek yet better selective procedures for the cells which would serve as nuclear donors in our transplantation experiments.

D-valine in an L-valine-deprived culture medium has been shown to be permissive for growth of renal epithelium while selectively inhibiting the growth of connective tissue cells (Gilbert and Migeon, 1975, 1977). Further, culture vessels lined with type IV collagen (Kleinman *et al.*, 1982) selectively support the attachment of epithelial cells (Klebe, 1974; Murray *et al.*, 1979; Cameron *et al.*, 1991). We are currently investigating the feasibility of combining the use of D-valine culture medium with growth of tumor cells on a type IV collagen substrate as a means of providing nuclear donors for transplantation experiments (McKinnell *et al.*, 1992). If, as our preliminary data indicate, we find only tumor parenchyma in culture tumor cells, then this procedure in addition to triploidy will provide ample cytological and cytogenetic evidence of the identity of the nuclear donor in our tumor nuclear transplantation experiments.

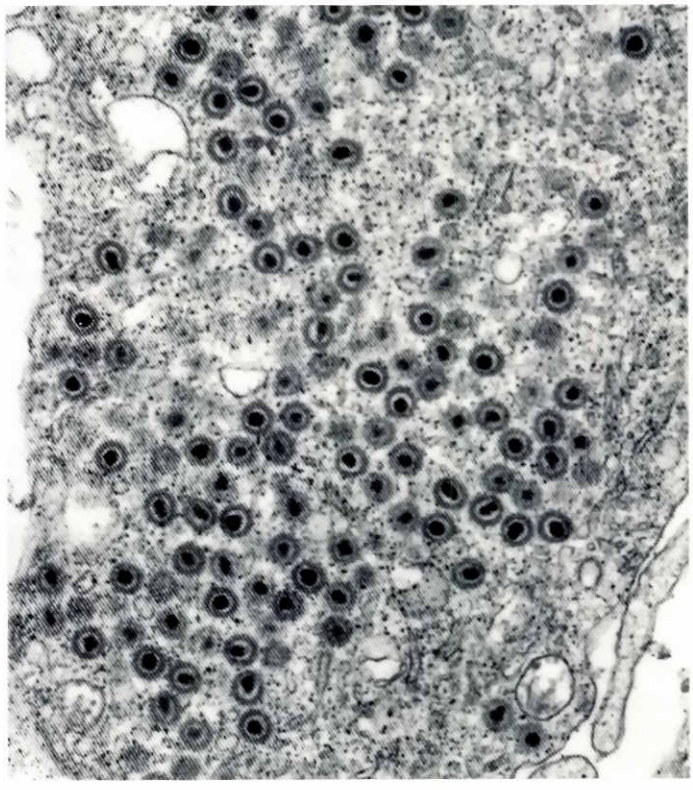

Fig. 4. Lucké tumor herpesviruses induced by culture of renal carcinoma fragments *in vitro* at 7°C *(from McKinnell et al., 1991).*

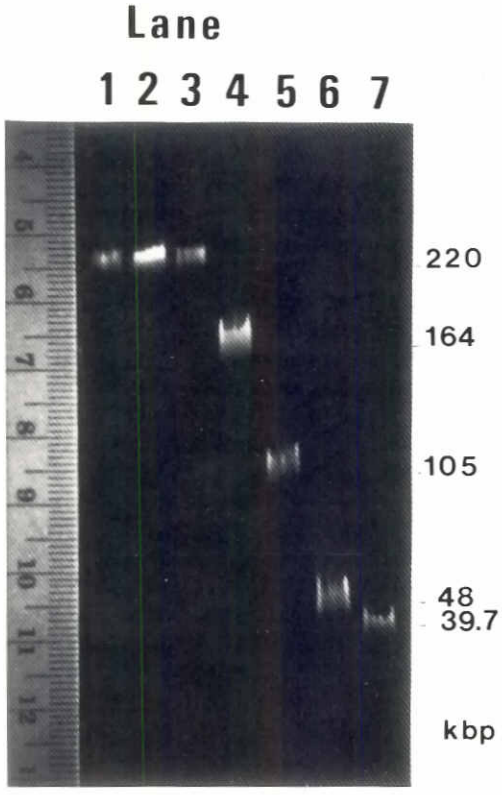

Fig. 5. Field inversion gel electrophoresis of the Lucké tumor herpesvirus DNA on an agarose gel. (Lane 2) *Lucké tumor herpesvirus DNA.* **(Lanes 1 and 3)** *Saccharomyces cereviseae chromosome I DNA (220,000 base pairs).* **(Lane 4)** *T2 DNA (164,000 base pairs).* ***(Lane 5)*** *T5 DNA (105,000 base pairs).* **(Lane 6)** *Lambda DNA (48,000 base pairs).* **(Lane 7)** *T7 DNA (39,700 base pairs).*

Is there latent differentiative potential in a tumor nuclear transplant tadpole?

While very limited development follows the transplantation of maturing and adult cell nuclei using ordinary procedures (McKinnell, 1972; DiBerardino, 1987), DiBerardino and her colleagues have shown in a remarkable series of experiments that significant additional developmental potential can be extracted from the genome of frog erythrocytes and other blood cells by insertion of the genome of these otherwise postmitotic cells into *oocyte* cytoplasm followed by retransfer into mature egg cytoplasm (DiBerardino *et al.*, 1986; DiBerardino and Orr, in press). It should be noted that these oocyte-egg nuclear transfer experiments have resulted in the most extensive development yet obtained from the genome of an adult cell nucleus. Some erythrocyte nuclear transplant larvae fed, developed hind limb anlage and appeared quite normal until the time of their demise. This seemed to be such a promising model system that we began a study of Lucké renal carcinoma nuclei inserted into oocyte cytoplasm followed by retransfer into mature egg cytoplasm. The studies were disappointing because we were unable to obtain any development beyond that reported previously (Carlson and McKinnell, unpublished studies). Nevertheless, the seminal DiBerardino studies prodded us to devise experiments to ascertain whether added developmental potential resided in the genome of tumor nuclear transplant tadpoles.

Experimental embryology has shown that the potentiality of many cell types exceeds the fate of those cells (e.g., see Spemann, 1938) and that tissues with limited developmental potential due to lethal factors can be rescued from their fated early demise by grafting to fully viable hosts (Hadorn, 1936, 1937; Moore, 1947; Beetschen, 1960; Stevens *et al.*, 1977; Stevens, 1978, 1980). We sought to similarly «rescue» tumor nuclear transplant embryonic tissue, fated to die at 10 to 14 days, by allografting fragments of the tissue to normal hosts. Our procedure was to produce triploid renal carcinoma (by injection of Lucké tumor herpesvirus into triploid tadpoles) and use those cells as nuclear donors in conventional nuclear transplantation experiments. When successful nuclear transplant animals reached the embryonic tailbud stage 17 (Shumway, 1940), fragments of this triploid tissue were grafted to the tail mesenchyme of diploid hosts. The grafts were allowed to grow for varying periods of time to permit tissue differentiation. Allografts (of confirmed 3n ploidy) harvested at 40 days contained tissue that had grown and differentiated far in excess of that ever observed in the pre-feeding tumor nuclear transplant tadpoles (Fig. 2) (Lust *et al.*, 1991). The eye serves as an example of the improved growth and differentiation observed in allografts of tumor nuclear transplant embryos. Either no eye primordium (King and McKinnell, 1960) or defective eye primordia (Lust, 1990) developed in conventional tumor nuclear transplant tadpoles. Well differentiated larval eyes, with all retinal layers and normal appearing lenses, were found in the allografts (Fig. 3). This differentiation cannot be distinguished from that observed in allografts of normal diploid or triploid tissue. Further, a diversity of normal cell types, paralleling those which appeared in control grafts, appeared in the tumor nuclear transplant allografts. These tissues were comparable in their histological structure to tissue differentiation that occurs in a free-living 50-day-old larva which is about half way in development from zygote to juvenile frog. Our normal appearing mitotic descendents from neoplastic cells are compatible with the results of pioneer investigators who sought for and found normally differentiated progeny of cancer cells (Kleinsmith and Pierce, 1964; Pierce, 1970, 1972, 1983; Pierce and Wallace, 1971; Pierce and Speers, 1988).

It seems reasonable to ask why the allografts were not permitted to grow longer. Most allografts at Taylor-Kollros (1946) stage IV had been invaded by lymphocytes and eosinophils and were in the process of being rejected by the host larva. We compared the time of immune rejection and the percentage of grafts which elicited the immune response for control and for tumor nuclear transplant tissue and found no difference (Lust *et al.*, submitted for publication). For pragmatic reasons, subsequent grafts were harvested prior to the time that the host could mount an immune response. However, it should be noted here that inasmuch as the grafts of tumor nuclear transplant tadpole tissue and normal diploid and triploid tissue were no different with regard to eliciting an immune rejection response in the host, it seems reasonable to believe that not all the differentiation potential of these cells has been expressed. Another way of stating this is that it is already known that both diploid and triploid embryos have the competence to grow and differentiate into adult frogs, i.e., significant developmental competence was unexpressed in the allografts of the normal tissue. Since the histology of the tumor nuclear transplant allografts was indis-

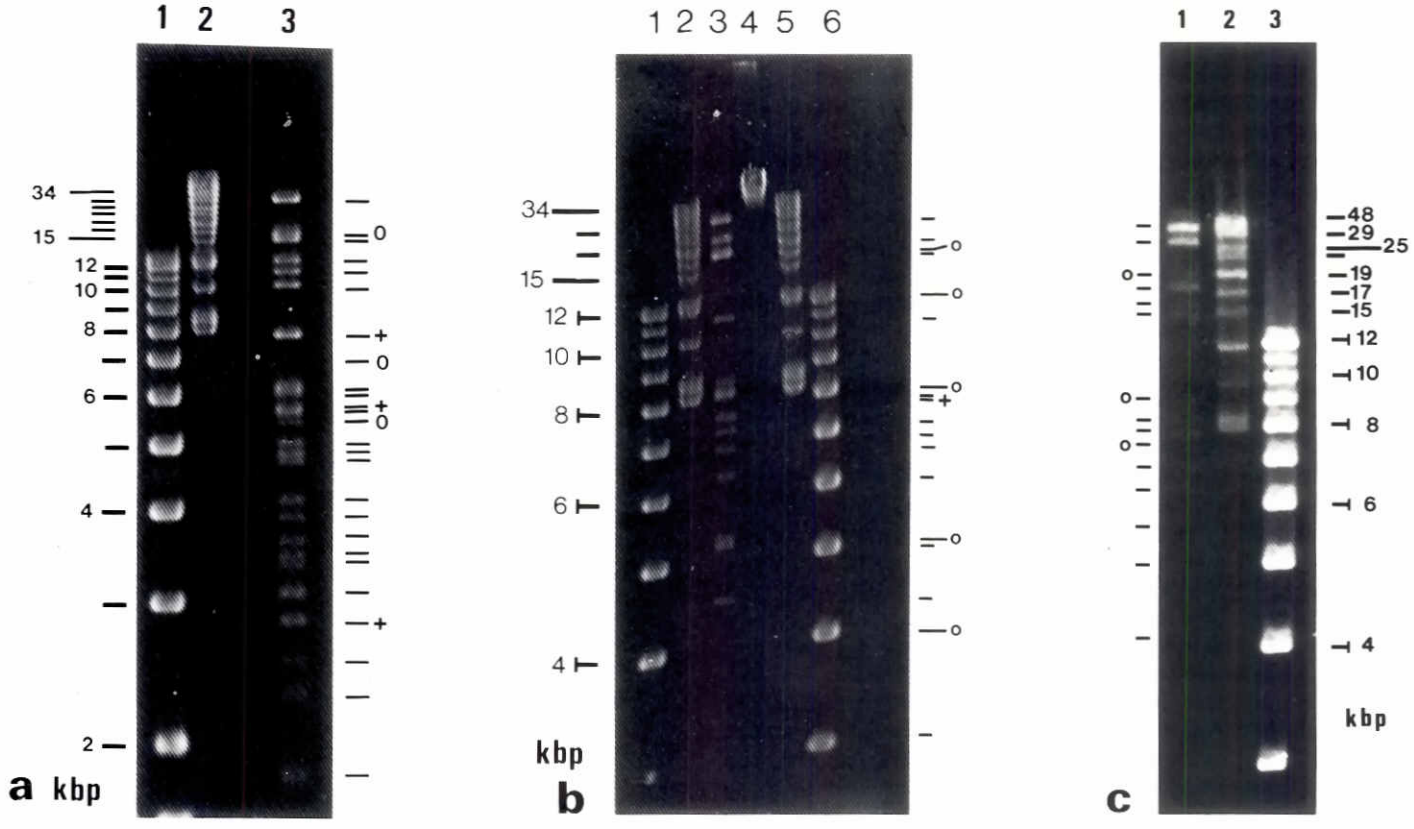

Fig. 6. Restriction fragment analysis of Lucké tumor herpesvirus DNA. (a) *Agarose gel electrophoresis of Lucké tumor herpesvirus/BamHI fragments (Lane 3). Lanes 1 and 2: DNA size markers ranging from 2,000 to 34,000 base pairs. The position of the herpesvirus restriction fragments are indicated by lines on the right. Because of the limited amount of herpesvirus DNA loaded, restriction fragments shorter than 1,800 are not visible on the gel. Fragments that appear to be present in submolar amounts are indicated by a zero next to the position line. Fragments in greater than molar amounts are indicated by a +. The cumulative length of restriction fragments suggests that the size estimation by field inversion gel electrophoresis is likely to be correct.* **(b)** *Agarose gel electrophoresis of Lucké tumor herpesvirus DNA/HindIII restriction fragments (Lane 3). Lanes 1, 2, 5 and 6 are DNA size markers as indicated on the left side of the panel. Approximate lengths of the HindIII fragments vary from 30,000 base pairs at the top to 3,500 base pairs at the bottom. Fragments shorter than 3,500 base pairs are not visible on the gel. Fragments that appear to be present in submolar amounts are indicated by a zero next to the position line. Fragments in greater than molar amounts are indicated by a +.* **(c)** *Agarose gel electrophoresis of Lucké tumor herpesvirus DNA/EcoRI restriction fragments (lane 1). Lanes 2 and 3 are DNA size markers as indicated on the right of the panel. Approximate lengths of EcoRI fragments vary from 35,000 base pairs (top) to 4,200 base pairs (bottom). Fragments shorter than 4,200 base pairs are not visible on the gel. Fragments that appear to be present in submolar amounts are indicated by a zero next to the position line.*

tinguishable from that of normal diploid and triploid control grafts and, further, since allograft rejection of tumor nuclear transplant embryonic tissue was not distinguishable from rejection of diploid and triploid controls, it seems reasonable to suggest that the limitation of differentiation in allografts was ascribable to the immune response of the host and was not due to an intrinsic genomic limitation of the donor tissue.

There are methods of grafting embryonic tissue that are designed to abrogate the allograft rejection reaction of the host embryo. Volpe and Gebhardt (1965) induced immunotolerence by making large grafts of tissue. Co- transplantation of hematopoietic stem cells of the donor with the grafting of specific tissue anlage also will induce immunotolerance (Turpen *et al.*, 1981). We are unable to prognosticate at this time which procedure will enhance tolerance optimally for our experiments.

The Lucké tumor herpesvirus and its role in differentiation of tumor cells

We have demonstrated the pluripotency of the Lucké renal carcinoma genome, and we continue in our efforts to establish whether the genome is totipotent. But, whether the genome is pluripotent or totipotent, we are now in a position to seek resolution of another question that concerns the stability of the differentiated state. Balduin Lucké in his earliest publications asserted that the frog renal carcinoma was probably caused by a herpesvirus (Lucké 1934, 1938). Subsequently, the virus was in fact shown to be the etiological agent of the Lucké tumor (Naegele *et al.*, 1974). All Lucké renal carcinomas contain the etiological agent. In some tumors the agent is in latent form (Zambernard and McKinnell, 1969) while other tumors are characterized by a productive infection with virions

easily detected by electron microscopy (McKinnell and Zambernard, 1968; McKinnell and Ellis, 1972; McKinnell, 1973; McKinnell *et al.*, 1991). But, whether latent or with a productive viral infection, the herpesvirus of the Lucké tumor is invariably associated with the frog malignancy. The presence of the herpesvirus in renal carcinomas of frogs raises an important question in our studies of differentiation. Do the normal appearing cells in the allografts of tumor nuclear transplant tadpole tissue fragments contain latent herpesvirus, or has the process of differentiation resulted in a genome bereft of the herpesvirus? We seek the answer to those questions because it would seem that differentiated cells and stem cells found in mature tissue and containing an oncogenic viral genome might be vulnerable to a subsequent conversion to the malignant condition (because of the presence of that viral genome). We cannot answer those questions at the time of this writing, but we have entered into studies designed to provide answers.

First, to develop modes of viral genome detection, we needed viral DNA. At the time our studies were begun, there was not even agreement on the size of the Lucké herpesvirus genome. Accordingly, we set about to produce the virus *in vitro* by cold treatment (7°C) of tumor fragments explanted to agar plates containing culture medium. Many virions, confirmed by transmission electron microscopy (Fig. 4) were present in the cold treated tissue. The virus-containing tissue was homogenized and viral DNA was prepared from sedimented nucleocapsids. The Lucké tumor herpesvirus DNA was sized by field inversion gel electrophoresis where it was shown to co-migrate with yeast chromosome I (Fig. 5). This establishes the size to be approximately 220,000 base pairs (Sauerbier *et al.*, in press), which is supported by summation of restriction enzyme fragment lengths (Fig. 6). This genome is comparable to that of other slowly proliferating herpesviruses such as human cytomegalovirus DNA at 240,000 base pairs (Stinski, 1990).

The polymerase chain reaction (Innis *et al.*, 1990) is being used in our studies to detect the virus genome in the Lucké tumor (a positive control), in normal embryonic and mature frog tissue, in tumor nuclear transplant tadpoles and in differentiating tissue obtained from allografts of tumor nuclear transplant tadpole tissue (Lust *et al.*, 1991). We are using a partially sequenced Hind III restriction fragment subcloned in plasmid PUC 118 (McKinnell *et al.*, 1991) for PCR amplification. While it is too early to report the outcome of these initial experiments, we are pursuing them because the absence of the viral genome in normally differentiating tumor-derived tissue would imply a correlation between the loss of the viral genome and the loss of the malignant phenotype. However, the continued presence (should it be shown to occur) of the viral genome would demonstrate that normal differentiation of tumor-derived cells can occur despite the presence of the etiological agent and it opens the question as to the long-term stability of normal cells that carry with them significant sequences of an oncogenic virus.

Acknowledgments

This work was supported by Grant 2675 to R.G.M. and Grant 2617 to W.S. from the Council for Tobacco Research-USA, Inc., American Cancer Society, Inc. Grant IN-13-29-28 to W.S. and R.G.M., Grant DCB-900466 from the National Science Foundation to L.A.R-S., NIH/MBRS 5 S06 RR08091-18 to J.W.W. and NIH/DRR/RCMI Grant G12RR 03059-01A1 to C.S.W.

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