Herpes-Type Virus Recovery from "Virus-Free" Frog Kidney Tumors* (32809)

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Spontaneous renal adenocarcinomas of *Rana pipiens* exist in two seasonal forms within the same populations of northern United States frogs. Tumors from animals collected in *winter* months display negligible mitotic activity and contain numerous cells characterized by enlarged nuclei with marginated chromatin and Cowdry (1) type A inclusions. A constant association of herpes-type virus with inclusion-containing cells has been demonstrated by electron microscopic studies of these naturally occurring winter tumors (2,3). In contrast, tumors from *summer* frogs are mitotically active and lack viral inclusion-containing cells. These observations have led to the postulation of suggested life cycles for the Lucké frog tumor (4) and for its associated virus (5).

This tumor dichotomy appears to be a temperature-related phenomenon: tumors arising in laboratory-housed frogs, regardless of season, display "summer" morphology when host animals are maintained at 20–26°C, or "winter" morphology when maintained at 5°C (6). The dependence of winter tumor morphology upon low temperature was further substantiated by studies in which tumors with typical "summer" morphology developed "winter" characteristics (including intranuclear inclusions) after the tumor-bearing frogs had been maintained at 4°C for several months (7).

In the present study "virus-free" primary tumors and eye chamber transplants from these tumors were subjected to prolonged low temperature treatment. Herpes-type virus developed both in primary tumors and in transplants, and herpesvirus was recovered from primary tumor.

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Materials and Methods. Tumor tissue to be examined by light microscopy was fixed in Bouin's solution, embedded in paraffin and sectioned at 8 μ. Serial sections were stained with hematoxylin and eosin. Tumor tissue for electron microscopy was fixed in sodium cacodylate-buffered glutaraldehyde, postfixed in osmium and embedded in epon. Thick sections (0.5–1.0 μ) were stained with toluidine blue and examined with the light microscope; thin sections were stained with uranyl acetate and lead citrate and examined with an RCA EMU-3F electron microscope.

Preparations of isolated virus particles were obtained from tumor extracts by differential centrifugation or by equilibrium density gradient centrifugation, placed on celloidin-coated grids and negatively stained with 1% phosphotungstic acid (pH 7.2). Details of these procedures will be published elsewhere.

Autoradiographs of transplants were prepared in the following manner: transplants were removed from eye chambers and placed for 3 hours at 7–8°C in sterile amphibian Ringer's solution containing 50 μC of thymidine-^{3}H (Schwarz BioResearch, Inc., specific activity 6.0 C/mMole). Thick epon sections were mounted on slides which were then dipped in melted L.4 nuclear emulsion (Ilford, Ltd.), diluted 1:1 with distilled water. Slides were incubated at 4°C. for 2 weeks prior to photographic development and then stained with toluidine blue.

Results. Primary tumors. Four "virus-free" tumors developed in laboratory-housed frogs maintained at room temperature, two in adult Vermont frogs (VRA & VRB) and two in Wisconsin frogs (WRC & WRD). Biopsy material was obtained from each tumor and divided into three small portions; two of these portions were used for light and electron microscopy; the third portions of VRB, WRC, and WRD were used for the explant experi-

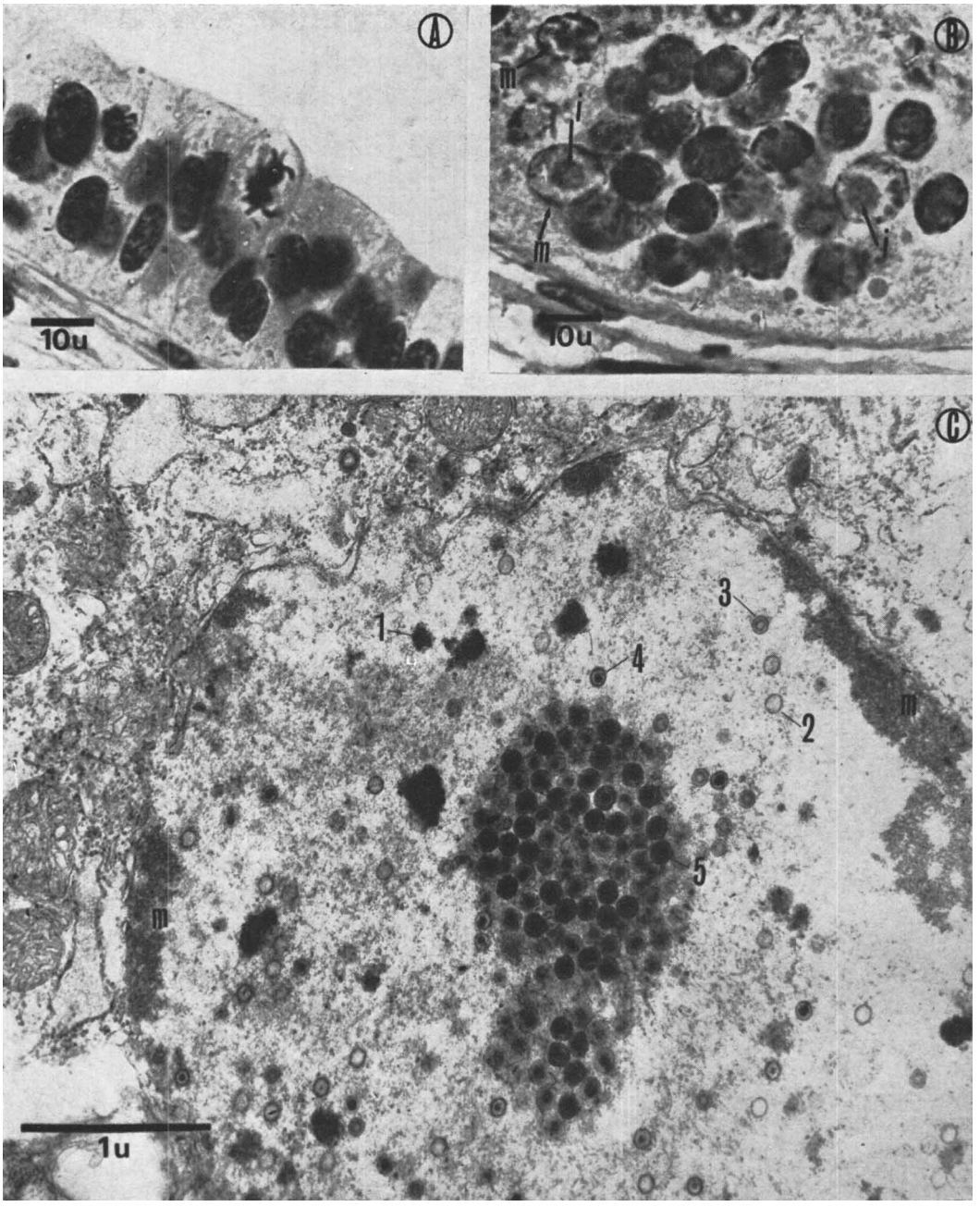

FIG. 1. PRIMARY TUMORS: (A) Light micrograph of primary tumor VRA, from biopsy taken before placing host frog at low temperature. Randomly dispersed chromatin and mitotic figures are apparent. (B) Light micrograph of same tumor (VRA) after 16 weeks at low temperature. Note marginated chromatin (m) and intranuclear inclusions (i). (C) Electron micrograph of same tumor (VRA) after 16 weeks cold treatment, illustrating the typical marginated chromatin (m) and an intranuclear cluster of herpes-type virus particles often found in these cells after cold treatment. Also seen are various types of particles which Fawcett (2) suggested may represent different maturational stages of this virus: 1, dense aggregates; 2, single-membraned empty viral protein coats, or capsids; 3, double-membraned empty capsids; 4, single-membraned capsids with enclosed nucleoids; and 5, double-membraned capsids with enclosed nucleoids.

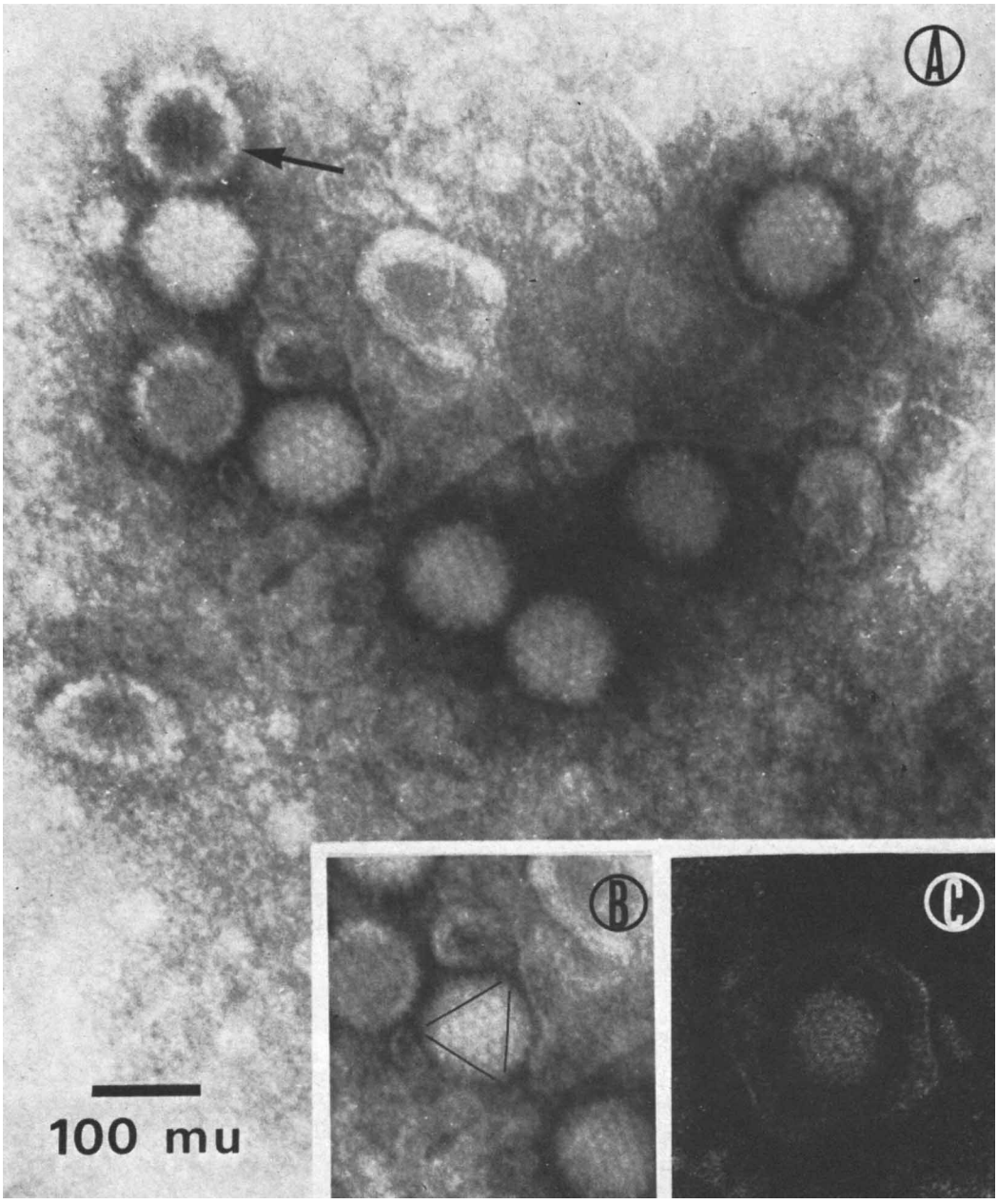

FIG. 2. NEGATIVE-STAINED VIRUS PARTICLES: All photographs are of virus particles recovered from primary tumors by either differential centrifugation (A and B) or equilibrium density gradient centrifugation (C) and stained with phosphotungstic acid. (A) unenveloped virus particles, the majority of which are "full"; arrow indicates an "empty" particle. (B) Virus shown in (A) with triangular facet outlined; the 5 capsomeres along each edge of the facet indicate that this icosahedral virus contains a total of 162 capsomeres, characteristic of herpesviruses. (C) Enveloped virus particle.

ments described below. Exhaustive examination of biopsy tissue from all four tumors with both light and electron microscopy verified the absence of virus (Fig. 1A). After these initial biopsies, the tumor-containing frogs were placed into a low temperature (7–8°C) cabinet. Periodic biopsies revealed no changes after 2, 4, and 6 weeks in two of the tumors, WRC and WRD; but after 16 weeks at low temperature VRA exhibited numerous (approximately 35%) virus-containing cells (Figs. 1B, 1C). The fourth primary tumor, VRB, also demonstrated virus particles when examined after 7 months at low temperature. The viral particles observed in thin sections of low temperature-treated tumors (Fig. 1C) were identical to the various stages of maturation of the herpesvirus previously described in primary tumors of winter frogs (2, 3). Furthermore, isolated virus from a low temperature-treated tumor (VRB) was identical in negatively-stained preparations to virus particles from winter tumors [see Lunger (8) and Fig. 2].

Eye chamber transplants. A frog kidney tumor often develops from several separate foci which eventually fuse to form a "single" tumor. Successive biopsies of a primary tumor could, therefore, actually represent samplings of several separate tumors. Furthermore, repeated biopsy frequently results in premature death of tumor-bearing frogs. In order to overcome these limitations, the remaining third portion of the original biopsy tissue from each of the three primary tumors (VRB, WRC, WRD) was divided into several 1 mm^3 pieces and each single piece was transplanted to an adult frog anterior eye chamber. The eye chamber is a privileged immunological environment which is used for the growth and maintenance of frog kidney tumor (9). Host frogs were housed at laboratory temperatures for 2 weeks,[1] after which the frogs were transferred to the low temperature cabinet (7–8°C). Periodically, frogs were sacrificed and transplants were removed and examined. Transplants of one primary tumor (WRDT) appeared unchanged after 2 weeks at low temperature, but other transplants of the same tumor examined after 26 weeks contained virus particles. Transplants of another primary tumor (WRCT) lacked virus after 6 weeks, but virus was detected 8 weeks later (14 weeks). Virus was present in approximately 5–6% of the cells of transplants from the third primary tumor (VRBT) as early as 11 weeks after the frogs were placed at low temperature. Two weeks later (13 weeks), examination of another transplant of this series indicated that the percentage of virus-containing cells was unchanged.

Thus, although each transplant series was obtained from a different primary tumor and these primary tumors included both Vermont and Wisconsin carcinomas, low temperature treatment resulted in virus induction in all three transplant series. The transplant experiments indicated that virus could be obtained as early as 11 weeks after treatment commenced and that virus was present as late as 26 weeks, when the experiment was terminated. In all cases of virus induction in transplants, the incidence of virus-containing cells varied from 1–6%. The virus particles observed in transplants (Figs. 3A, 3B) were identical to the various stages of maturation of the herpesvirus previously described in primary tumors of winter frogs (2, 3).

After 13 weeks at low temperature, a VRBT transplant was removed from the eye chamber and, prior to fixation, was incubated *in vitro* with thymidine-^3H. Thirteen weeks residence at low temperature was expected to markedly reduce if not completely "turn off" *cellular* DNA synthesis, since winter tumors are characterized by a paucity of mitosis. Light microscopy examination of thick-section autoradiographs did disclose that the majority of the cells (approximately 95%) were unlabeled; the tumor cells that did show nuclear labeling also exhibited nuclear characteristics associated with virus production (Fig. 3C). Electron microscopic examination of adjacent thin sections revealed that *all* labeled cells contained virus (see Fig. 3D). The restriction of labeling to nuclei which contain virus particles indicated that ongoing virus-associated DNA synthesis occurred at this reduced temperature.

[1] When explanted to eye chambers of frogs housed at room temperature, virus-containing tumors lose their virus within 2 weeks (10).

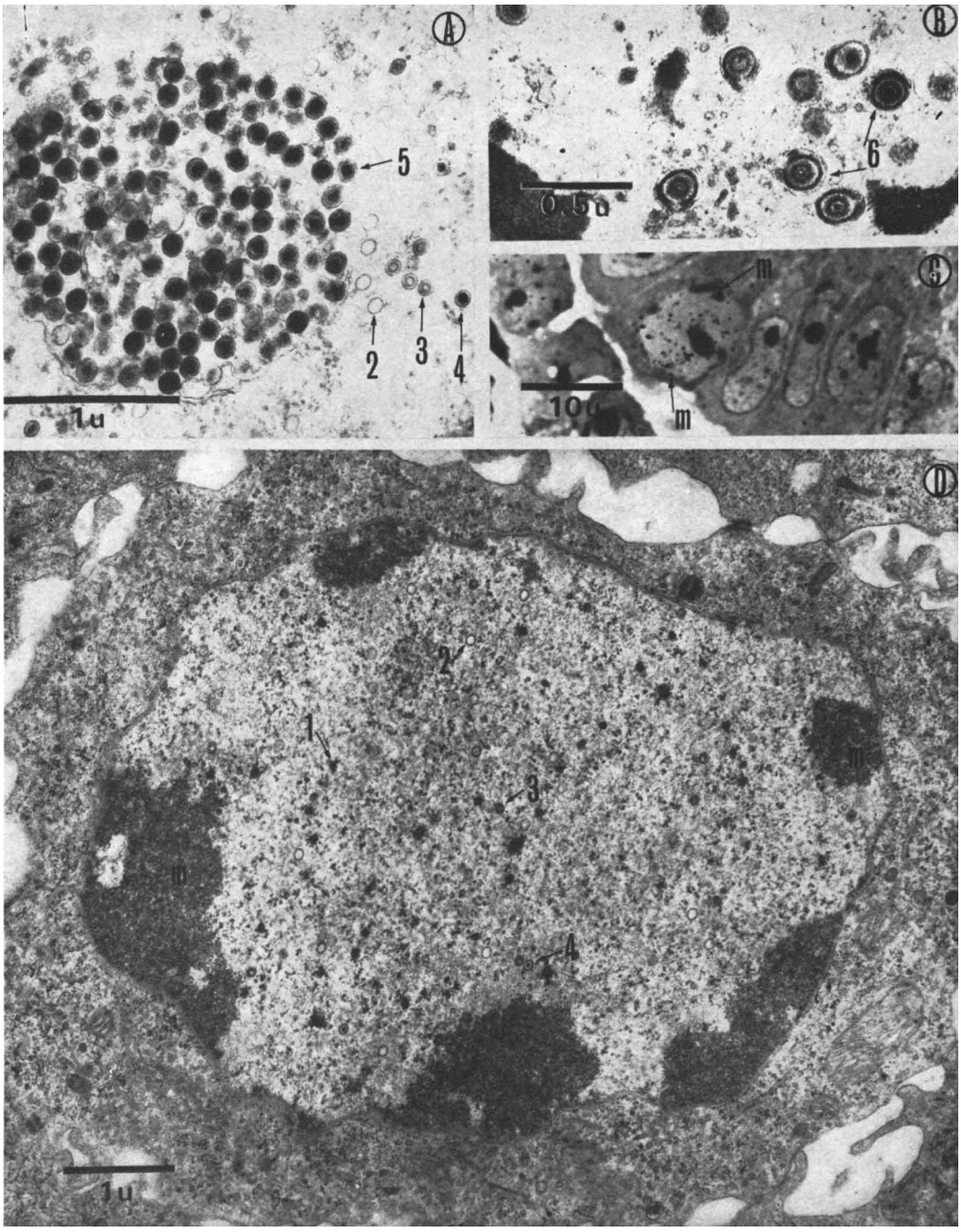

FIG. 3. EYE CHAMBER TRANSPLANTS: (A) Nuclear region of a cell of WRCT after 14 weeks at low temperature showing a membrane-bound sac of virus and other viral particles similar to those described in Fig. 1C. (B) Peripheral cytoplasmic region of cell in same transplant showing another type of virus particle (6), this one with an additional envelope. (C) Autoradiograph of thick epon-embedded section from a VRBT transplant after 13 weeks at low temperature. Silver grains are confined to the single nucleus with marginated chromatin (m). (D) Electron micrograph of a cold-treated transplant depicting the typical ultrastructural appearance of the labeled cell shown in 2C. Also note the marginated chromatin (m) and the various types of virus particles (described in Fig. 1C).

Discussion. Our results indicate that the two types of frog kidney tumor are temperature-related states of the same carcinoma. In populations in which this carcinoma is endemic, frogs undergo marked seasonal changes in body temperature and enter a state of hibernation during the winter. The winter tumor and its associated virus may, therefore, result from low temperature-mediated emergence and synthesis of virus from cells of "virus-free" tumor. In the laboratory, "virus-free" frog tumors and transplants of these tumors, after being subjected to low temperature, become similar to naturally-occurring winter tumors, in cell appearance and in the types and distribution of viral particles.

The herpes-type virus observed in, and isolated from, intranuclear inclusion-containing cells of the frog carcinoma conforms, on the basis of morphology [(8); see Figs. 1–3] and histochemical evidence (11), to the criteria established for classification as a member of the herpesviridae (12). The involvement of this herpesvirus in Lucké tumor etiology is suggested by Tweedell's report of tumor development in metamorphosing frogs which were injected while embryos with subcellular fractions of virus-containing tumors (13). Although no member of this ubiquitous group of viruses has been shown to induce tumors in warm-blooded animals, herpes-like virus particles have been found in nearly all cell lines of Burkitt's lymphoma of children (14). One of these cell lines has recently been reported to induce continuous growth in, and transmit herpes viral antigen to, normal leukocytes in culture (15).

A number of DNA viruses, including polyoma and SV40, are known to induce tumors in homeothermic vertebrates, but it has not been possible to recover virus from these induced tumors (16). However, the persistence of viral genetic information has been demonstrated in several "virus-free" tumors by a variety of indirect techniques, including nucleic acid homology (17, 18) and viral genetic marker rescue studies (19). Direct attempts to induce the vegetative production of virus from these mammalian tumors have either been unsuccessful (20–22), or have not produced consistent results (23). The inability to recover virus has led to two contrasting views of virus–tumor cell interaction: (i) only a small portion of the viral genome persists within the tumor cell (with insufficient information for virus production); or, (ii) a slightly "defective" or masked genome is present in the tumor cell (virus may be recoverable under proper conditions *or* it is irreversibly integrated within the cell and therefore is not recoverable).

Our results with the frog tumor–DNA virus system suggest that a sufficiently complete viral genome is present in some "summer" tumor cells so that, in this cold-blooded system (or perhaps, *because this is a poikilothermic system*), virus is recoverable.

The current inability to grow the herpesvirus of the frog tumor in tissue culture[2] has hampered development of immunological techniques and other sensitive methods of viral detection. The present light and electron microscopic evidence suggests, but does not prove, that viral genetic information exists within the genome of "summer" tumor cells.

However, it is possible that virus, per se, may be maintained in "summer" tumor cells at a level too low to be detected by electron microscopy. It is also possible that during the summer the herpesvirus may reside in frog tissues other than the tumor; reduced temperature might cause the preferential accumulation and reproduction of the virus in winter tumor cells. Experiments are in progress to test these alternatives.

Temperature dependence of tumor type and vegetative virus production should permit utilization of the unique poikilothermic properties of the frog for experimental approaches to mechanisms involved in virus-associated tumorigenesis.

Summary. Virus production was induced by low temperature treatment of the "virus-free" form of the frog renal adenocarcinoma (Lucké tumor). Virus recovered after this low temperature treatment fulfilled the morphological requirements of herpesvirus. Herpesvirus development was also induced in eye chamber transplants from these "virus-free" primary tumors. Results indicate that the two

[2] A different frog virus (24, 25), found occasionally in the cytoplasm (never in the nucleus) of tumor cells and also isolated from normal frog tissues, has been cultured successfully (26).

naturally-occurring seasonal forms of this tumor are temperature-related states of the same tumor and suggest that the "virus-free" tumor contains a relatively complete viral genome in masked or latent form.

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Plaque Titration of Herpes Simplex with Antibody-Free Liquid Overlay* (32810)

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Dilute inocula of herpesvirus produce discrete lesions or plaques in tissue culture monolayers. Discrete plaques persist for a limited time in the presence of liquid culture medium which contains neither specific antibody nor materials such as agar, starch, or methyl cellulose designed to prevent viral spread through the culture medium. Eventually virus spreads from primary plaques to induce multiple areas of secondary infection. Between the development of the maximum number of primary plaques and the appearance of secondary lesions there is an interval when plaque counting in this liquid overlay system is a reliable means of titration.

Utilizing the above principles Farnham (1) and Wheeler (2) described plaque assays for herpesvirus in HeLa cultures overlayed with antibody-free liquid medium. Wheeler performed plaque counts on living, unstained cultures with a microscope at 50× magnification. The method has been reliable and extremely useful but counting at 50× has been tedious and time consuming and counts had to be done at a fixed time after infection

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