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The Presence of the Lucké Herpesvirus Genome in Induced Tadpole Tumors and Its Oncogenicity: Koch–Henle Postulates Fulfilled*

(frog-kidney tumor/frog herpesvirus/frog embryo)

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ABSTRACT **Herpesvirus extracted from a naturally occurring frog renal carcinoma (Lucké tumor) induced virus-free Lucké tumors in developing frogs. Herpesvirus recovered from an induced tumor after incubation at low temperature of tumor fragments cultured *in vitro* was oncogenic when injected into developing frog embryos. With the exception of the "pure culture" requirement, this experiment fulfills Koch–Henle postulates for the identification of the causative agent of the Lucké tumor.**

The renal adenocarcinoma (Lucké tumor) of the leopard frog *Rana pipiens* occurs with high frequency in a wild heterogeneous animal population. Tumor cells of frogs maintained at 4°–9°, whether in nature or in the laboratory, contain intranuclear inclusion bodies (Cowdry type A) and herpesvirus (Lucké herpesvirus, LHV) (1–3). In contrast, neither inclusions nor LHV is found in tumor cells of frogs captured in the warm months of the year (usually summer) or held in the laboratory at 20°–25° (1–3). However, transcripts of LHV DNA are present in virion-free tumors (4). Exposure to low temperature (4°–9°) of (*i*) virus-free, tumor-bearing frogs (5), (*ii*) normal frogs bearing anterior eye-chamber transplants of virus-free tumor tissue (6), or (*iii*) fragments of virus-free tumor cultured *in vitro* (7) induces complete expression of the viral genome and virus replication. Thus, virus replication is dependent on low temperature; the molecular relationship between host and viral genome in virion-free tumor cells is, however, unknown.

Lucké tumors can be induced in developing frog embryos by cell-free tumor extracts containing LHV (8, 9) or by ascitic fluid containing the virus (10). When froglets bearing virus-free induced tumors are placed at 9°, intranuclear inclusions are found in tumor cells after 4 weeks (11); although not examined, such inclusions likely contain herpesvirus (9).

Spontaneous tumor formation in developing frog embryos and laboratory-reared froglets has never been reported, and tumors cannot be induced by normal frog-kidney extracts or by extracts of virus-free tumors (8).

The available evidence supports a herpesvirus etiology of the Lucké tumor. However, the failure to find an *in vitro* cell system, susceptible to LHV, that could provide "pure" culture of LHV and the association of other viruses with Lucké tumors (12) indicate a need to more firmly establish the relationship of LHV to tumor formation. Some years ago Rivers (13) suggested that proof of the specific relationship of a virus to disease cannot follow blind adherence to Koch–Henle postulates. This is particularly true of virus-induced neoplasia. Nevertheless, the fulfillment of classical Koch–Henle postulates can aid in identifying the causal agent of a tumor. The following experiment, using the information on the Lucké tumor summarized above, was designed to meet the requirements of Koch–Henle postulates for establishing the causative agent of the Lucké tumor.

METHODS

A tumor-bearing male *Rana pipiens* received from Hazen Co., Vermont, during January of 1972 was killed, and a bilateral tumor weighing 3.9 g, comprising about 14% of the body weight of the frog, was removed. Light and electron microscopic examination revealed a typical Lucké tumor with 90% of the tumor cells containing intranuclear inclusions and herpesvirus. Virus was extracted from the tumor according to the method of Tweedell (8). The tumor was homogenized (5 min, 4°) with a Teflon–glass homogenizer in 20 mM phosphate buffer, pH 7.2, containing 0.24 M sucrose (sucrose–phosphate buffered saline), and the cytoplasmic supernatant fluid was separated from the nuclear pellet (P_1) by centrifugation at 800 × *g* (10 min, 4°). The nuclear pellet was resuspended and the same procedure was repeated twice more. The combined supernatant fluids (S_1) were centrifuged at 5090 × *g* (15 min, 4°), and the resulting supernatant fluid (S_2) was recentrifuged at 32,000 × *g* (1 hr, 4°); the pellet (P_3) was resuspended in cold distilled water and skimmed milk (1:1) and was used as inoculum within 24 hr of preparation. Electron microscopic examination revealed all morphological forms of herpesvirus, including full and empty, enveloped and unenveloped particles.

Tailbud embryos (S-17) originating in the laboratory from eggs obtained by induced ovulation of adult *Rana pipiens* were used in tests for oncogenic activity (8). One hundred embryos were inoculated in the nephrogenic ridge with 0.2–0.4 μl of the material under test; 50 control animals were similarly inoculated with distilled water–skimmed milk. All animals were reared at 20°–22°.

Fragments of tumors were cultured *in vitro* by cutting the tissue into 3-mm³ pieces and placing them on small triangular grids (Falcon) in 60-mm plastic petri dishes. Five milliliters of Leibovitz (L-15) medium adjusted to amphibian osmolarity and containing 10% fetal-bovine serum were added; fresh medium changes were made weekly.

Methods used in preparation of tissue (thin section) and

Abbreviation: LHV, Lucké herpesvirus.

* This is paper XV in a series on "Viruses and Renal Carcinoma of *Rana pipiens*." The preceding paper was XIV (ref. 26).

TABLE 1. *Appearance of herpesvirus in induced tumor fragments cultured in vitro at 7.5° or 22°*

Incubation (Days)	Incubation temperature (°C)	Presence of intranuclear inclusions*		Presence of herpesvirus†	
		No. of positive cells/ Total no. of cells examined	% Positive	No. of positive cells/ Total no. of cells examined	% Positive
0	22	0/1000	<0.1	0/1000	<0.10
50	22	0/600	<0.17	0/600	<0.17
	7.5	29/600	4.8	27/600	4.5
70	22	0/600	<0.17	0/600	<0.17
	7.5	180/600	30.0	238/600	39.4

* Presence of intranuclear inclusions was determined by hematoxylin-eosin staining.

† Presence of herpesvirus was determined by electron microscopic examination of thin sections of tumor fragments.

virus (negative stain) for electron microscopy are fully described elsewhere (14, 15).

RESULTS

Sixty-two percent of the surviving tadpoles (27 of 44) receiving the P_3 tumor fraction developed typical, virus-free Lucké tumors of the pronephros and/or mesonephros within 3–8 months after inoculation. None of the surviving control embryos (0 of 37) developed tumors during this period.

To determine whether the LHV genome was present in induced tumors and could be made to replicate *in vitro* by treatment at low temperature, fragments of an induced pronephric tumor (12 × 12 × 9 mm, 8 months after inoculation) were placed in culture. The cultures were divided into two groups; one group was incubated at 22° and the other at 7.5°. Tissue samples were taken periodically and examined by light and electron microscopy for the presence of intranuclear inclusions (hematoxylin and eosin stains) and herpesvirus.

The results of this experiment are shown in Table 1. Typical intranuclear inclusions (Fig. 1, *right*) and herpesvirus (Fig. 2) were detected in the cells of tumor fragments maintained at 7.5°. By day 50, the frequency of cells containing herpesvirus was 4.5% in good agreement with the frequency of cells containing inclusions (4.8%). By day 70, at 7.5°, the percentage of inclusion-bearing tumor cells reached 30% and those containing virus 39.4%. The inclusion-bearing cells were frequently found in aggregates throughout the tumor fragments, as is often observed in naturally occurring tumors, suggesting both induction and cell-to-cell spread of virus infection. This was particularly evident in the samples of day 50. No intranuclear inclusions (Fig. 1, *left*) or virus were seen in tumor fragments incubated at 22°, confirming the low-temperature dependence of virus replication.

These results clearly demonstrated the full expression of the LHV genome in induced tumors under *in vitro* conditions in the absence of possible host contributing factors. However, Koch–Henle's third postulate requires that the recovered microorganism must be shown to be the same as that inocu-

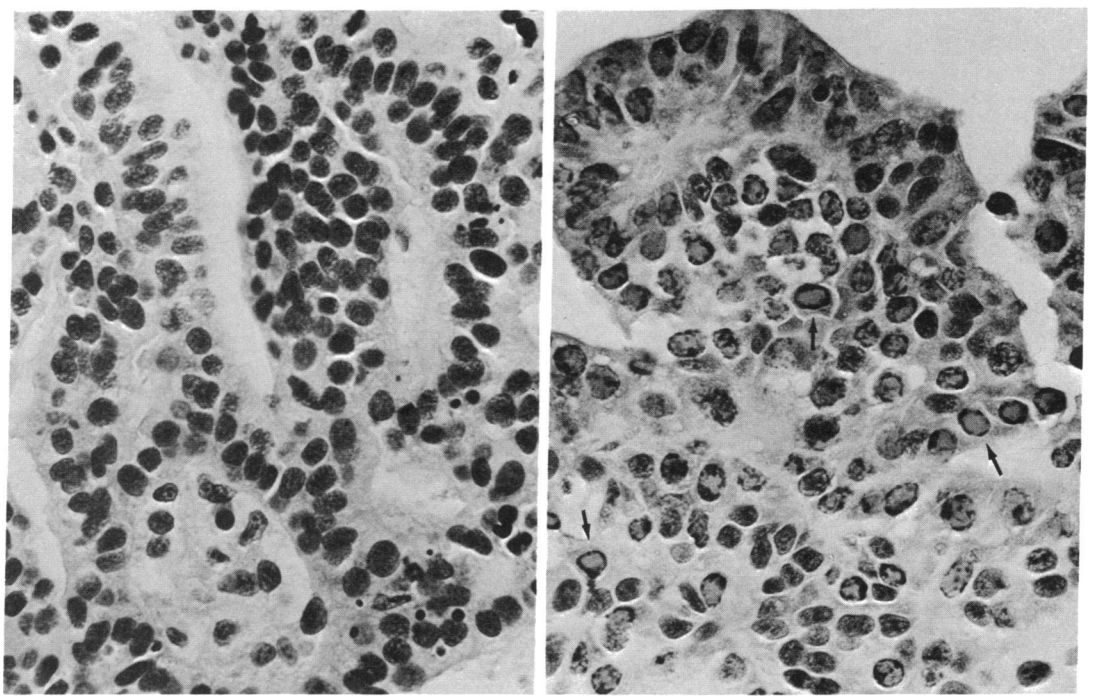

FIG. 1. Histologic appearance of cells from fragments cultured *in vitro* of an LHV-induced Lucké tumor. Hematoxylin and eosin stains. ×410. *Left:* Fragment cultured at 22°. *Right:* Fragment cultured at 7.5°. Examples of cells containing intranuclear inclusions are indicated by *arrows*.

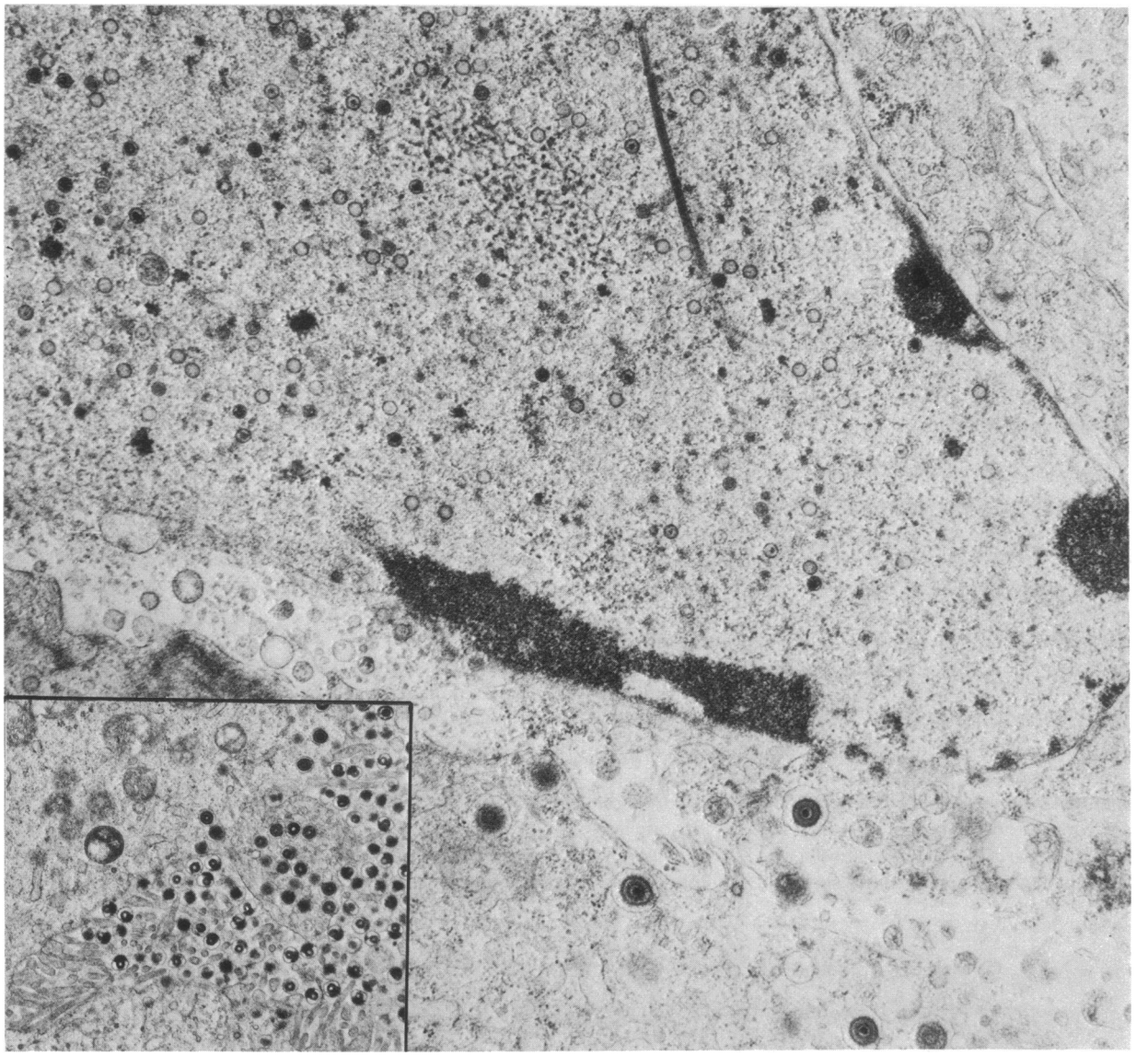

FIG. 2. Electron micrograph of an induced Lucké tumor fragment culture *in vitro* at 7.5°. The nucleus contains numerous unenveloped viral particles approximately 100 nm in diameter. A tubular structure, frequently observed in Lucké tumor cells, is also present in the nucleus. Enveloped viral particles approximately 180 nm in diameter are seen in vacuoles in the cell cytoplasm. ×21,600. (*Insert*) Large numbers of extracellular enveloped virions among villous processes of the cell. ×12,600.

lated. The criterion we used to satisfy this requirement was the demonstration that the virus recovered from the tumor fragments was oncogenic. To this end a group of tailbud embryos was inoculated with virus extracted from tumor fragments maintained at 7.5°. Because of the small amount of tissue available, the following extraction procedure was used. One hundred milligrams of tissue fragments were homogenized in 0.5 ml of sucrose–phosphate–buffered saline at 4°, debris was removed by centrifugation at 600 × *g* (10 min, 4°), and the supernatant fluid was used as inoculum. Electron microscopic examination of a large number of fields revealed only an occasional herpesvirus particle.

TABLE 2. *Appearance of tumors in Rana pipiens embryos inoculated with extracts of tumor fragments cultured in vitro*

Inoculum	No. of embryos inoculated	No. of tumors/ No. of survivors*	Percent
Extract of 7.5° tumor tissue	100	44/68	64.7
Extract of 22° tumor tissue	100	0/56	<1.8
Sucrose–phosphate-buffered saline	50	0/28	<3.6

* All survivors reached metamorphosis (70–80 days).

A second group of embryos was inoculated with an identically prepared extract obtained from tumor fragments maintained at 22°. Additionally, a group of embryos was inoculated with sucrose–phosphate-buffered saline alone. All animals were reared at 22°.

Tumors were first detected at 4 months; after 5.5 months all surviving animals were killed and examined for tumors. The

results are shown in Table 2. Typical Lucké tumors were found at high frequency (65%) in the animals receiving the LHV-containing extract of 7.5° tumor fragments; none occurred in animals receiving extracts of tissue incubated at 22° or sucrose–phosphate-buffered saline alone. The tumors, both pronephric and mesonephric, were free of intranuclear inclusions. Thus, the herpesvirus present in induced tumors retained its oncogenic activity and was, with little doubt, the same agent as originally isolated from the wild tumor.

Fig. 3 schematically illustrates the entire experiment, starting with the original tumor-bearing frog.

DISCUSSION

As noted earlier, LHV has not been isolated and propagated *in vitro* to fulfill the need for obtaining clonally-derived virus. Thus, the role of other viruses in the inocula, not detected by electron microscopy, must be considered. This is particularly important since the extract of 7.5° tumor fragments was poor in herpesvirus, as determined by electron microscopy, but rich in oncogenic activity. The polyhedral cytoplasmic deoxyribovirus (e.g., frog virus 3) (16) can be ruled out since it is highly lethal for frog embryos and surviving animals do not develop tumors (17). Furthermore, the naturally occurring tumor used initially in our experiment was free of polyhedral cytoplasmic deoxyribovirus, as determined by tissue-culture isolation tests. Neither the papova-like virus isolated from Lucké tumors (18, 19) nor an adenovirus isolated from a frog-kidney granuloma (20) is oncogenic when inoculated into frog embryos. Since tumorigenic activity of LHV-containing tumor extracts is inactivated by ether (Naegele and Granoff, unpublished), neither the frog papova-like virus nor adenovirus can be solely causative of the tumor since they are resistant to ether. It does not, however, eliminate a possible, but remote, role as helper virus for either of these two agents.

The remaining virus isolated from tumor-bearing frogs is the herpesvirus, frog virus 4 (5, 15). This virus, completely distinct from LHV (21), has not induced tumors in developing frog embryos (5, 18); it multiplies and produces a lytic infection at 25°. Since it is not seen in tumor cells at 20–25°, it is unlikely that it is causally related to the Lucké tumor.

We believe we have excluded the above viruses isolated from frogs as being directly involved in Lucké tumor formation. The possibility of other, as yet unrecognized, agents, acting either independently or together with LHV in Lucké tumor induction cannot, however, be ruled out. One such agent might be a C-type virus activated by LHV. We have failed to detect C-type virus in LHV-free and LHV-containing tumors by electron microscopy nor have we been able to activate C-type virus by treatment with BrdU of primary Lucké tumor cells cultured *in vitro*.

Our results, therefore, clearly demonstrate a dominant, if not exclusive, role of a herpesvirus, LHV, in Lucké tumor production. Particularly supportive of this conclusion is the isolation of LHV from induced tumors and the oncogenic competency of the isolated virus in its natural host.

Herpes simplex virus types 1 and 2 and cytomegalovirus, after inactivation by ultraviolet light, can transform cells *in vitro* which produce tumors when transplanted to newborn hamsters (22–24). In this experimental situation, permissive cells for lytic infection require inactivation of the virus for cell transformation. If these herpesviruses are oncogenic in man, then, perhaps, naturally occurring inactivated herpesvirus behaves similarly; i.e., active virus results in a productive infection and cell death while infection with an inactivated particle may result in malignant transformation. In contrast, in the Lucké tumor system, temperature affects permissiveness of cells so that at 20°–22° the target cells *in vivo* (most likely pronephros) are nonpermissive for virus replication but permissive for malignant transformation. At lower temperature, the transformed cell becomes permissive for virus replication and cell death follows. The viral genome can, therefore, be kept intact under both conditions. A somewhat analogous situation with herpes simplex virus type 2 has been described where raising the incubation temperature of the virus-infected human embryonic cell cultures from 37° to 42° inhibits lytic infection. The resulting abortively infected cells acquire properties of transformed cells (25).

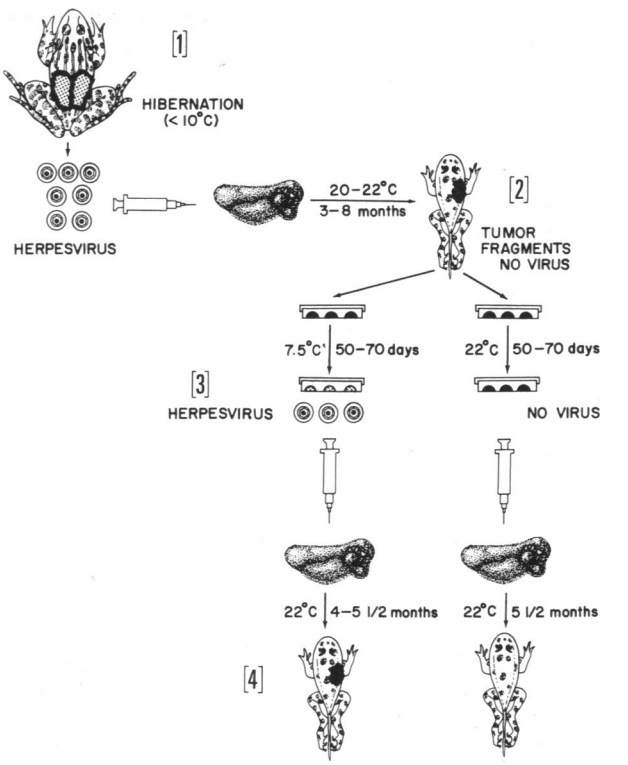

FIG. 3. Schematic representation of the Lucké tumor experiment fulfilling Koch–Henle postulates. [*1*] Association of the agent with the disease. [*2*] The agent must induce the same disease in a susceptible host. [*3*] The agent must be isolated from the induced disease. [*4*] The isolated agent must be identified as the same agent originally associated with the disease.

Mechanisms involved in the temperature-virus-host cell interaction of the Lucké tumor system are presently unknown. Clearly, propagation of LHV in a susceptible *in vitro* cell system will be needed before insight is gained into the mechanism of LHV oncogenesis and the natural history of tumor evolution.

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