Experimental Alteration of the Oncogenicity of Frog Tumor Cell–Viral Fractions[1] (36651)

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Inoculation of *Rana pipiens* embryos (1), or exposure of oocytes during ovulation (2) to cytoplasmic renal tumor cell fractions containing a herpes-type virus (HTV) or purified enveloped HTV (3–5), produce pronephric or mesonephric renal adenocarcinomas as the hosts reach metamorphosis. However, three separate virions have been isolated from normal kidney or renal tumor cells and cultivated *in vitro*, (6, 7) the nuclear HTV, a cytoplasmic DNA polyhedral virus (PCV), and a papova type virus (PTV); all have failed to induce tumors after embryo injection (6, 8, 9).

Early electron microscopic observations (9) and subsequent studies (11, 12) established the association of HTV with the nuclear inclusion body (NIB) phase of the tumor. Nuclear inclusion bodies and HTV are directly related to environmental temperature (13–16), and their formation can be induced in "virus-free" tumors both *in vivo* (17, 18), or *in vitro* (19, 20), by low-temperature manipulation.

The oncogenicity of mitochondrial (P_2) tumor fractions is correlated with the presence of NIB and with membrane-enclosed sacs of nucleated HTV (1, 5) that are seen within the nucleus as well (3, 21–23). As further variables of oncogenicity, an analysis was made of nuclear vs cytoplasmic tumor fractions, the effects of physical-chemical changes on the cell virus fractions and the result of postinjection temperature shock on tumor induction.

Materials and Methods. Tumor sources. Five spontaneous renal adenocarcinomas were obtained from Vermont *Rana pipiens* and one from a Wisconsin *Rana pipiens*. Each renal tumor was selected after biopsy for its oncogenic activity by the presence of intranuclear inclusion bodies (Cowdry, Type A) seen in frozen sections. All tumor animals had been maintained at 9° for 2–4 months. Tumors were unilateral (two) or bilateral (four), (two had metastases to the liver, lung, pancreas or intestine) weighed from 1.7 to 6.2 g (average, 4.4 g) and were equally derived from ♂ and ♀ adults.

Preparation of tumor fractions. Each tumor was homogenized with a teflon-glass homogenizer in 0.24 *M* sucrose and 0.02 *M* phosphate buffer, pH 7.2 for 100 strokes at 265–465 rpm. Iced conditions were used throughout. The cytoplasmic supernatant was separated from the nuclear pellet, P_1 by centrifugation at 800*g* for 10 min. In two tumors (RT 174, 167) the recovered supernatant, S_1 was decanted into two equal aliquots. One portion was treated with kinematic sonication (7300 cps, 22,000 rpm) for 1 min; the other aliquot was untreated. From each aliquot a mitochondrial–viral fraction, P_2, was derived as described previously (1). The remaining sonicated supernatant, S_{2s}, was then centrifuged at 160,000*g* for 5 hr to yield a sonicated microsomal–viral fraction, P_{3s}. The other source tumors were handled similarly except that sonication was omitted.

The original pellet, P_1 (from the tumor homogenate) was resuspended in sucrose buffer, washed twice and repelleted and sonicated at 7300 cps for 3 min, rupturing 98% of the tumor cell nuclei. The suspension was recentrifuged at 800*g* for 10 min to yield a sonicated, nuclear pellet, P_{N1s}. The nuclear supernatant SN1 was then centrifuged at

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5000*g* to yield a precipitate, P_{N2s} containing subnuclear particles comparable in density to the cytoplasmic P_2 fraction.

Host animals and infection. Frog embryos were laboratory reared from eggs produced by induced ovulation in *Rana pipiens* adults obtained from Vermont. Rearing of embryos with the exception of temperature, was described previously (1). Tumor cell fractions (0.2 μl) were injected into the nephrogenic ridge from a 10 μl syringe.

Results. Cytoplasmic P_2 fractions. As a measure of tumor potency for the present experiments a mitochondrial–viral HTV (P_2) precipitate was derived from the supernatants of nuclear inclusion body renal tumors and injected into the nephrogenic ridge of tailbud embryos. Previous injection of similar cytoplasmic fractions of renal tumors into *Rana pipiens* embryos had indicated that from 21 to 93% of the injected embryos would develop renal tumors (1). The diluted (10^{-1}) mitochondrial–HTV pellet was injected into the nephrogenic ridge of Shumway (25) stage 17 (tailbud) *Rana pipiens* Vermont embryos and stock controls were also maintained (Table I). In the injected group 48 out of 60 animals survived. Between 40 and 53% of the injected embryos developed renal tumors at the time of metamorphosis. The first tumor was detected in a premetamorphic animal 2.5 months after injection but most of the renal tumors appeared after completion of metamorphosis. Renal tumors either developed in the pronephros before its regression or in the definitive mesonephros but often both kidney primordia developed tumors.

Effect of sonication. After homogenization of donor tumor, RT 167, a portion of the cytoplasmic supernatant was removed and sonicated. Following centrifugation to remove the mitochondria, a cytoplasmic microsomal–viral fraction (P_{3s}) was derived from the remaining supernatant. Electron microscopic examination of the pellet, based on random sections, indicated some single-membrane HTV particles, a few double-membraned particles and only occasional nucleated HTV particles. Other viral particles were not obvious but could not be excluded. The nephric ridge of *Rana pipiens* Vermont tailbud embryos was injected with 0.2 μl of the undiluted fraction. An equal number of controls were established. Renal carcinomas began to appear in injected premetamorphosing larvae but most of them appeared in postmetamorphic animals. The tumors were mitotically active, nonnuclear inclusion adenocarcinomas. Comparison of nonsonicated P_2 cytoplasmic fractions of RT 167 with the sonicated P_3 cytoplasmic fractions showed only a 17% reduction in induced tumors resulting from the sonicated fraction (Table I). This was not considered significant since previous experiments without sonication showed the P_2 fractions had a greater oncogenic potential than the P_3 fractions (1). No tumors were found in the control animals.

Nuclear (PN_2) fractions. The original tumor cell pellet, P_1 from the source tumor RT 174 was then submitted to further fractionation after the mitochondrial–HTV supernatant was removed. Examination of the pellet by phase-contrast microscopy revealed intact nuclei plus cell debris: Stroma, frag-

TABLE I. Comparison of Sonicated Nuclear vs Cytoplasmic Tumor Cell Fractions on the Induction of Renal Carcinomas in Frog Embryos.

Tumor fraction	Source tumor	Number injected	Number survivors	Tumors induced
Cytoplasmic				
P_2	167	36	26	14
P_2	174	24	22	9
P_{3s} (sonicated)	167	36	33	11
Controls		36	33	0
Nuclear				
P_{N2s} (sonicated)	174	20	16	10
Controls (uninjected)		(30)	20	0

mented membranes, and microscopic cell particulates. This pellet was washed, repelleted, then resuspended in buffer and sonicated to rupture the nuclei. After sonication all nuclei, membranes, and clumps of debris were ruptured or dispersed. Following recentrifugation at the original speed, the new pellet, P_{N1s} was discarded. The remaining supernatant was removed and centrifuged at 5090*g* to produce a pellet of sonicated nucleoplasmic particles, P_{N2s} of similar sedimentation rate to the cytoplasmic–mitochondrial fraction. The latter precipitate contained nuclear fragments of nucleoli, clumps of chromatin, inclusion bodies, and probably some previously bound cytoplasmic particulates that were not removed in the original supernatant. Electron microscopic examination of the pellet revealed single and double-layered virions plus some enveloped, nuclated HTV viruses. The presence of other viruses was not excluded, however.

The undiluted P_{N2s} suspension (0.2 μl) was injected into the left nephrogenic ridge of tailbud embryos obtained from the same stock as before. The sonicated nuclear fraction of RT 174 was just as effective in the induction of renal tumors as the corresponding cytoplasmic fraction of the same tumor. Renal tumors developed in 62% of the survivors. Of 16 survivors injected with the nuclear fraction (PN_{2s}), 10 developed tumors compared to 9 out of 22 injected with the cytoplasmic P_2 fraction of the same tumor and 14 out of 26 from a different tumor. The first tumor was detected 14 weeks after injection. A few tumors were found in premetamorphosing larvae but two-thirds were discovered in young metamorphosed adults. Incidence of renal carcinomas was equivalent in males and females, and tumors developed in the pronephroi, the mesonephroi, or both types of kidneys. The gross appearance and histopathology were indistinguishable from previous induced tumors; the mitotic index was high and nuclear inclusion bodies were absent. No tumors were found in any of the control animals over a 6 month period. It was concluded that sonication alone for periods of 1–3 min did not reduce the oncogenicity of the viral–cell fractions from nuclear inclusion body tumors. In fact, sonication appears to enhance tumor production from nuclear fractions.

Enzyme pretreatment of tumor fractions. The viral–mitochondrial (P_2) fraction of a nuclear inclusion body tumor, RT 187, was diluted to 10^{-1} in sterile 0.05 *M* Tris buffer, pH 7.2 and stored in ice. One aliquot was treated in suspension with 200 mg % of a broad spectrum protease, $\overline{V}$ (Sigma), containing 250 μg Streptomycin and 2500 units of Penicillin for 4 hr at 4°. The suspension was repelleted twice at 5090*g*, washed twice with sterile Tris buffer and resuspended as concentrated P_2 or diluted to 10^{-1} in the same buffer. After enzyme treatment, the fraction was injected into the nephrogenic ridge of S 17 *Rana pipiens* embryos. As a control of tumor oncogenicity, nonenzyme-treated aliquots of the P_2 10^{-1} fraction that had been stored in ice for 3 days were injected into embryos. All embryos were kept at 9° for 2 weeks, then brought to 23°. Larvae began metamorphosis after 3 months.

At metamorphosis pronephric and mesonephric renal tumors began to appear in the embryos injected with the untreated P_2 tumor fractions. Out of 37 survivors reaching metamorphosis, 46% of the embryos developed renal tumors (Table II). Of the 39 embryos surviving inoculation with the enzyme-treated P_2 fractions, none formed renal tumors. Those receiving the enzyme-treated fraction had fewer survivors but they were still refractory after 5 months when the experiment was terminated. Enzyme treatment appeared to completely abrogate the oncogenicity of the tumor fractions.

Effect of lyophilization. Another measure of the stability of the oncogenic virus in tumor cell fractions was obtained by lyophilization of a freshly prepared P_2 tumor fraction after dilution to 10^{-1}. Six days after freezing, the lyophilizate was reconstituted to the original volume with 0.05 *M* Tris buffer. Stage ten embryos were then injected with the reconstituted tumor fraction. A second group of the same embryos were injected with 0.2 μl of a 10^{-1} P_2 fraction of the same tumor that had been stored in ice for 6 days. A third group of controls were noninjected.

TABLE II. Effects of the Enzyme Protease or Lyophilization on the Tumor-Inducing Capacity of Tumor Mitochondrial–Virus Fractions in Frog Embryos.

Tumor fraction	Treatment	Source tumor	Number injected	Survivors (1 month)	Renal tumors induced
P_2 10–1	Untreated	187	48	41	18
P_2 10–1	Enzyme treated	187	36	24	0
P_2 concen.	Enzyme treated	187	24	15	0
P_2 10–1	Untreated	186	36	31	19
P_2 10–1	Lyophilized	186	48	43	0
Controls	(Uninjected)		(36)	30	0

All embryos were kept at 9° until stage 20, then gradually brought to 23°.

Larvae began metamorphosis in all three groups on schedule. The embryos injected with the stored P_2 tumor fractions of RT 186 developed renal tumors before, during, and after metamorphosis in 61% of the survivors (31 at 30 days); the first tumor was detected in a prometamorphic animal at 2.5 months (Table II). In the group injected with the lyophilized P_2 tumor fraction, none of the survivors (43/48) developed any signs of malignancy up to a period of 5 months. The controls were also negative.

Postinjection heat shock. A group of stage 18 *Rana pipiens* embryos (Vermont) were inoculated with 10^{-1} dilutions of the P_2 tumor-fraction RT 199 in the nephrogenic ridge and then placed at 12°. Sixteen days after injection they had reached stage 21 (12°) and two-thirds of the inoculated embryos were warmed 18° by gradually raising the temperature of the water until it reached 30°. They were held at 30° for 1 hr and then transferred back to normal rearing temperature water of 23°.

Of 41 heat treated embryos, 19 did not survive the heat shock and 5 more died within the week. The remaining larvae survived a month or more until metamophosis. None of these animals developed renal tumors (Table III). Out of 14 nonheat-treated larvae reaching metamorphosis, four developed renal tumors of the pronephros indicating a moderate oncogenicity of the tumor fraction.

Hypothermia. Although heat apparently destroyed the activity of the oncogenic virus, latent application of cold after injection had the opposite effect. A group of embryos (S 18) were injected with 10^{-1} dilutions of the mitochondrial fraction from RT 200 and thereafter they were maintained at 12° for 2 weeks. At this time the embryos had developed to stage 23 when they were gradually brought to 23°. After 2 months noninclusion renal tumors began to appear in prometamorphic animals (Table III). At 3 months the remaining animals were transferred to 9°. Animals with induced renal tumors were still harvested in the period immediately following; four such tumors were histologically in the "summer" noninclusion body phase. Later all animals held at 9° that developed renal tumors, formed renal adenocarcinomas

TABLE III. Postinoculation Temperature Shock on Embryos Injected with Oncogenic Mitochondrial–Virus Fractions.

Tumor fraction	Source tumor	Number injected	Number survivors	Treatment	Tumors induced
P_2 10–1	199	16	14	None	4
P_2 10–1	199	41	22	Heat (30°)	0
P_2 10–1	200	32	24	None	6[a]/24 (noninclusion)
				Cold (9°)	5/18 (nuclear inclusion)

[a] Includes four animals autopsied in the first 9 days after cold treatment.

rich in intranculear inclusion bodies. The earliest of these occurred after 4 weeks exposure to 9° and the last was autopsied 4 months after exposure. A total of five inclusion tumors were recovered. Although all of the animals were derived from the same clutch and all were inoculated with the same cell–virus fractions, the histopathology of the "winter" tumors was directly affected by the cold regimen.

Discussion. The experiments reported here further support the contention that oncogenicity of subcellular fractions from the Lucké renal adenocarcinoma is related to the presence of a nuclear Herpes-type virus. The enveloped form of HTV usually occurs singly but often is seen in sac-like vesicles in the cytoplasm (18, 22). Similar sacs of HTV are found in the oncogenic mitochondrial–viral tumor cell fractions (1). When such fractions are purified by rate-zonal centrifugation, the only oncogenic band is that with tightly enveloped, nucleated Herpes virus, that characteristic of the vesicles seen in the cytoplasm (5). These vesicles of viruses may form in the nucleus (18) and are also seen in purified fractions from the nucleus (5). The presence of HTV in intranuclear inclusion body tumors (3) has already been noted. In an electron microscopic survey HTV were seen in 91% of renal inclusion carcinomas examined while the occurrence of the cytoplasmic virus was noted only 9% of the time (21). Similarly, the first study linking the presence of virus in frog tumor cells (9) noted the absence of HTV in frog renal tumors cells from nonnuclear inclusion tumors. It is significant then that the oncogenicity of nuclear fractions possessing similar virus-filled sacs reported here, is equal to that of the cytoplasmic fraction from the same tumor. While contamination of the nuclear viral fraction could not be ruled out, the method of preparation with multiple washings should cause a potential decrease in concentration of cytoplasmic-derived virus to at least 10^{-3}. Such a decrease should have lowered the incidence of tumors produced as shown in previous experiments (1), but rather the incidence of nuclear-derived tumors rose 22%. The former observations and the current results which show that injection of subnuclear fractions of intranuclear inclusion tumors into embryos is just as or more effective in the induction of renal tumors imply that a nuclear Herpes virus rather than a cytoplasmic, polyhedral DNA virion is involved.

Preparations of the tumor cell–virus milieu, whether cytoplasmic or nuclear, were found to be stable to mild sonication of up to 3 min exposure, sufficient to rupture nuclei and mitochondria.

The lability of the oncogenic virus was clearly shown when similar cell–viral fractions were exposed to lyophilization or treatment with a broad spectrum enzyme, protease. The sensitivity to the enzyme could be attributed to a destruction of the viral protein coat. Exposure to pronase has been used to extract DNA from tumor cells with no reduction in infectivity (24) although the concentration used here was 10 times that normally employed.

Since viral replication, as reflected in the formation of nuclear inclusions, is controlled by low temperature exposure and ambient temperatures apparently suppress such activity, we surmised that continued viral proliferation might be abrogated by a high temperature shock. By *in situ* exposure to heat we were able to simulate conditions that might occur to the host after natural infection. The temperature chosen (30°) is in the lethal range (25) for embryos of *Rana pipiens* and no doubt accounts for the larger number of deaths just after exposure. Within the period of observation of the infected animals, the high temperature shock either inhibited or permanently destroyed the virus inoculation.

Cold development of NIB has been repeatedly demonstrated but it is significant here since induced tumors resulting from embryo *injections* are usually active, mitotic, noninclusion carcinomas. The development of NIB in tumors of the injected hosts after prolonged cold exposure indicates that HTV must be latent in the tumors. Thus, the HTV-derived inclusions, indicative of viral replication, are indistinguishable from those found in the source tumor used for inoculation.

Summary. Renal adenocarcinomas can be induced in *Rana pipiens* with equal success after injection of virus containing cell fractions derived from either the cytoplasmic or nuclear portion of renal tumor cells with intranuclear inclusions. The latter result supports the contention that a nuclear herpes-type virus (HTV) is implicated in tumor induction. Brief periods of sonication do not alter the oncogenicity of either cell fraction. Pretreatment of the viral cell fractions with a proteolytic enzyme or lyophilization of the fractions prior to injection destroys all oncogenic action. Postinjection heat shock of the injected embryos also prevent the formation of renal tumors. Postinjection cold treatment of induced tumors results in formation of intranculear inclusions, an index of viral replication.

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