Polyoma Virus Genetic Material in a Virus-Free Polyoma-Induced Tumor

Abstract. *The DNA-agar technique for homology studies was used to investigate the DNA of a polyoma-induced virus-free tumor for the presence of the viral genetic material. Complementary polynucleotide sequences were found between the polyoma DNA and the DNA of several mammalian species. The increased complementarity of the polyoma DNA to the DNA of polyoma-induced tumor reflects an increased frequency of common polynucleotide sequences in the tumor DNA.*

Polyoma virus induces malignant transformation in various rodents and in selected rodent tissue cultures in vitro. Although the virus appears to be directly responsible for the transformation in vivo of normal cells to tumor cells, a significant proportion of gross tumors are free of virus (*1*). Tumors originally containing demonstrable infectious virus frequently lose it on subsequent transplantation. Habel and Silverberg (*1*) and others (*2*) have attempted to induce these virus-free tumors to produce virus both in vivo and in tissue cultures prepared from the tumors. Exposures of the tumors in animals to x-irradiation, cortisone, and starvation, and of tissue cultures to x-irradiation, ultraviolet irradiation, nutritional deficiencies, superinfection with cytolytic and other oncogenic viruses, or growth on a sensitive feeder layer have failed to activate any latent polyoma virus. These virus-free polyoma tumors, however, still contain specific polyoma-induced transplantation and complement-fixing antigens (*3*). Such evidence for the persistence of virus-induced expression of genetic information suggested that appropriately sensitive methods might reveal the continued presence of the viral genome itself.

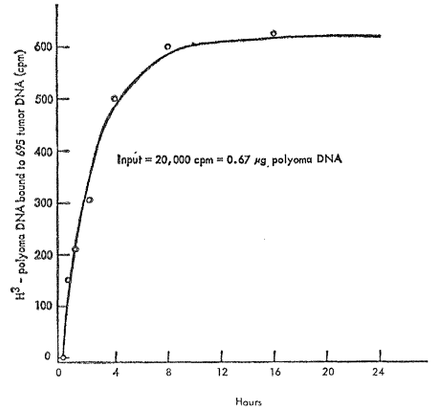

Fig. 1. Rate of reaction of labeled polyoma virus DNA fragments with mouse tumor DNA trapped in agar. The polyoma DNA bound in duplex with the DNA in the agar is plotted as a function of the time of incubation at 60°C. One-half gram of agar gel containing 180 μg of tumor DNA was incubated with 0.500 ml of saline citrate of double the standard concentration, containing the DNA fragments: 0.67 μg of H^3-labeled polyoma DNA (30,000 count/min per μg). CPM: counts per minute.

The highly specific nature of DNA-RNA hybrid formation provides a possible approach to the detection of the polyoma DNA within the virus-free transformed cell. Unsuccessful attempts to detect polyoma DNA in the transformed cells by hybridization with a polyoma messenger RNA (mRNA) synthesized in vitro have been reported (*4*). Efforts to detect a virus specific mRNA in polyoma-transformed cells by hybridization of transformed cell RNA with polyoma DNA have also met with little success. In addition to the formation of DNA-RNA hybrids, a more direct approach depends upon duplex formation between two complementary DNA molecules. The recent demonstration of complementarity between the DNA of the lysogenic bacteriophage lambda and the DNA of the lysogenized bacterial cell (*5*) suggested that the DNA-agar technique (*6*) might provide a suitably sensitive assay procedure for the detection of any homology between the DNA's of the polyoma virus and of the polyoma transformed tumor tissue. We have now demonstrated by applying this procedure that the DNA of polyoma-induced tumors does indeed contain polynucleotide sequences complementary to those in the DNA of the polyoma virus. We also have evidence that the normal DNA of several mammalian species, including man, contains sequences in common with the polyoma DNA.

DNA was extracted from nuclei of a virus-free, polyoma-induced, transplantable mouse tumor in its 46th passage and from tissues of normal animals of the same strain of mice (C57Bl/6JN) by Marmur's method (*7*). The DNA's were denatured and embedded in agar. The quantity of entrapped DNA was measured by dissolving the DNA-agar in hot 5*M* sodium perchlorate (*8*) and measuring the ultraviolet absorbance at 260 mμ.

Polyoma virus was grown in cul-

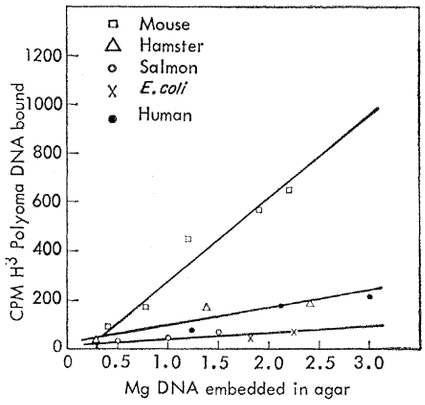

Fig. 2. Linear dependence of the fraction of polyoma DNA fragments that duplexed on the concentration of several normal DNA's embedded in agar. Tritiated (30,000 count/min per μg) DNA (0.67 μg) was incubated with quantities of DNA varying from 0.3 to 3.0 mg for 16 hours at 60°C. The quantity of agar in which the DNA was embedded varied from 0.3 to 1.0 g. The ratio of the total volume to the volume of DNA-agar gel was adjusted to maintain a ratio of 1.

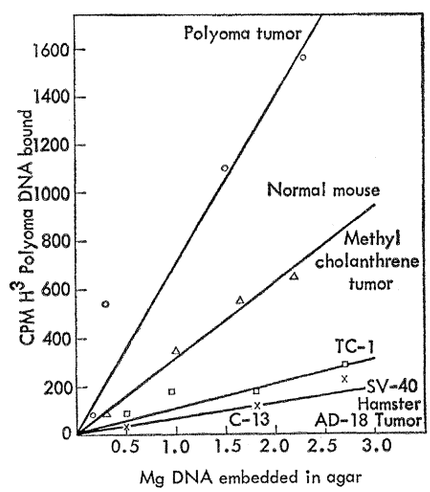

Fig. 3. Comparison of the linear dependence of the fraction of polyoma DNA fragments duplexed with DNA's from polyoma tumor, normal tissues, other virally induced tumors, and a tumor not induced by virus. Conditions of incubation and duplex formation are identical with those described in Fig. 2. The actual results are plotted for the cultured cells of the mouse polyoma tumor, normal mouse, and hamster (TC-1 and C-13). The results with mouse tumor induced by methylcholanthrene corresponded so closely with those for normal mouse that they are represented by a single line; correspondence of results on SV-40 and adenovirus 18 tumors with those on C-13 cells was similar.

tured suckling mouse kidney cells (*9*) in the presence of tritiated thymidine with high specific radioactivity. When the cytopathogenic effect was complete, cells and supernatant fluid were harvested; the crude virus suspension was concentrated by ultracentrifugation, treated with deoxyribonuclease at 20 μg/ml, and purified by two banding centrifugations in CsCl gradients. After the virus had been passed through a Sephadex G-100 column to remove low-molecular-weight compounds, electron microscopy revealed physical homogeneity of the virus particles. Virus nucleic acid was prepared by the procedure described by Borenfreund *et al.* (*10*). This viral DNA was fragmented by shearing it in a French pressure cell to a molecular weight of 5×10^5. These fragments were denatured by heating to 100°C to provide separated single-stranded fragments of DNA which are able to diffuse freely through the gel structure of the agar because of their relatively small size. Portions of the viral DNA solution were stored at −20°C. Immediately before each experiment, a portion was heated again to assure single-strandedness of the DNA, which is known to undergo rapid renaturation (*11*).

The radioactive, single-stranded, polynucleotide fragments of polyoma virus DNA were incubated with various DNA-agar preparations from cells, as previously described (*12*), and the amounts of hybridizable nucleic acid were determined. Figure 1 illustrates the time course of hybrid formation in the reaction of the polyoma DNA fragments with the DNA from a virus-free polyoma-induced tumor; the reaction progresses slowly and smoothly as would be expected from the complementary association of polynucleotide sequences, and is essentially complete in 8 hours. The observed time course for the reaction resembled rates reported for the complementary association of a variety of other interacting DNA species.

Figure 2 illustrates the extent of duplex formation between labeled polyoma DNA (0.67 μg, 20,000 count/min) and the DNA of several normal species. Although the DNA samples examined are few, there are significant reactions between the fragments of the polyoma virus DNA and the mammalian DNA's. There is no reaction with the DNA of bacteria, and little or none with that of salmon. The presence in polyoma of polynucleotide sequences complementary to sequences in the normal DNA's of mice, hamsters, and man suggests that the complementarity is directed toward some of the polynucleotide sequences common to these higher organisms (*13*).

When the binding of polyoma virus DNA to similar concentrations of embedded normal and polyoma mouse tumor DNA is compared (Fig. 3), there is a significant increase in the binding to the tumor material. Similar experiments were carried out with DNA from a continuously cultured cell line (C-13) from normal hamster tissue and the DNA from a polyoma-transformed cell line (TC-1) derived from it (*14*). Although the results were less striking than those with the mouse tumor, there were consistent differences in the binding of the polyoma DNA to that of the normal and transformed hamster culture cell. That this increased binding is a specific property of DNA of the polyoma-induced transformed cell rather than a general property of tumor DNA is evidenced by its absence from DNA's of tumors caused by methylcholanthrene in the mouse and of tumors induced by SV-40 or adenovirus 18 in the hamster. Although the karyotype of these tumors was not determined, other studies indicate that many virus-induced tumors are randomly heteroploid (*15*). The absence of increased polynucleotide binding with DNA's other than those from polyoma tumors indicates that the binding is not related to a specifically altered karyotype of the tumor cell.

Although care was taken to avoid

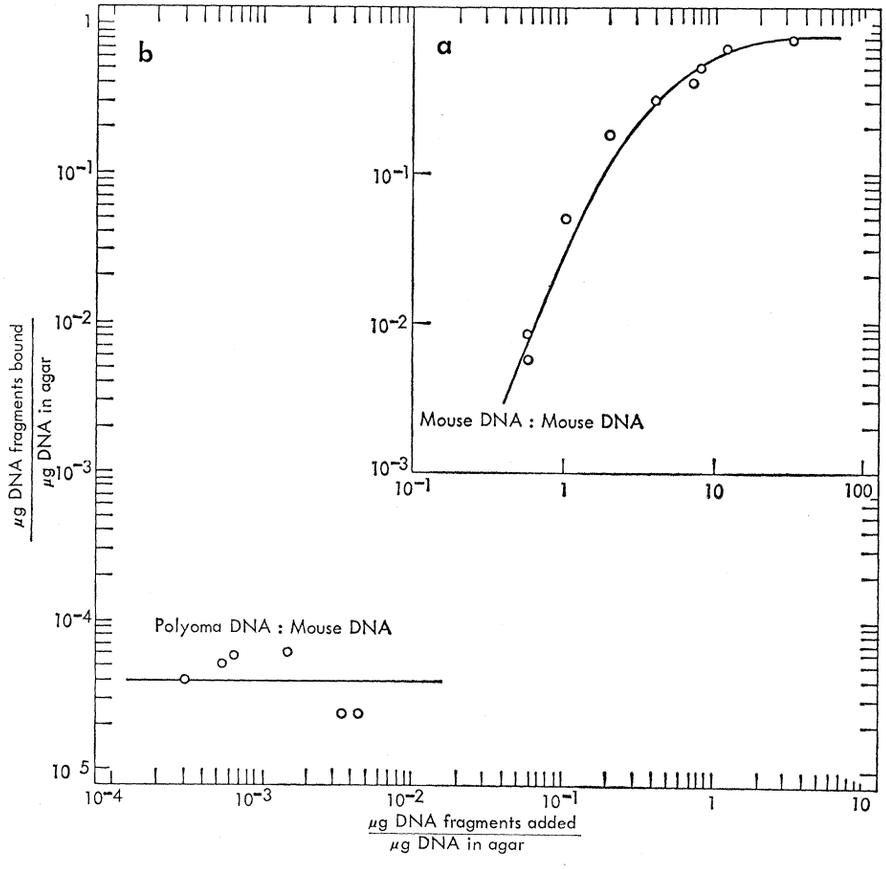

Fig. 4. *a*, Dependence of the ratio of mouse DNA fragments bound to the amount of entrapped DNA on the ratio of the amount of DNA fragments added to the amount of entrapped DNA. The slope of the curve approaches 0 only when the ratio of mouse DNA fragments added to the embedded DNA approaches 10. The fraction of DNA fragments bound was determined when various quantities of P^{32}-labeled (200,000 count/min per μg) DNA fragments from mouse L cells were incubated with 0.5 g of agar containing 200 μg of mouse DNA. *b*, Lack of dependence of the ratio of polyoma DNA fragments bound to the embedded DNA on the ratio of the polyoma DNA fragments added to the embedded DNA. At ratios of polyoma DNA fragments to the embedded DNA as low as 10^{-4}, the slope remains 0. Conditions of incubation and quantities of DNA used are described in Figs. 2 and 3.

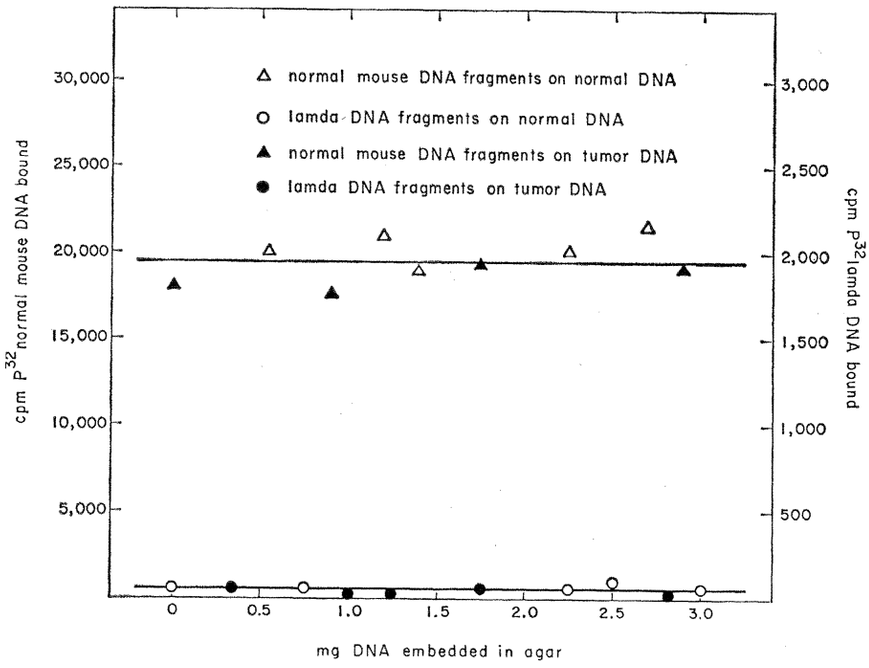

Fig. 5. Lack of dependence of the fraction of combined fragments of normal mouse DNA on the concentration of embedded normal or tumor mouse DNA, and lack of evidence for nonspecific binding of bacteriophage-λ DNA to the embedded DNA's. In this experiment, 0.67 μg of P^{32}–normal mouse DNA fragments (50,000 count/min per μg), or 3.0 μg of P^{32}–bacteriophage-λ DNA (7,500 count/min per μg), was incubated with the DNA-agar preparations under the conditions previously described.

contamination with host DNA in preparing the virus DNA, it may still be argued that the complementarity observed resulted from small quantities of contaminating DNA from the host cell. At any concentration of DNA in the agar, the number of sites available to accommodate the radioactive input fragments is limited. The greater the quantity of DNA embedded in the agar, therefore, the greater the quantity of labeled DNA bound. At the concentrations of labeled and embedded DNA used in these experiments, the fraction of fragments bound would be very much increased if the labeled DNA were random segments of host material; that is, more than one-third would be bound (*16*), whereas in fact less than one-thirtieth was bound. The expression of this relation of binding as a function of the quantity of labeled DNA fragments to the quantity of DNA embedded in the agar is shown in Fig. 4*a* for a mouse–mouse DNA reaction, and in Fig. 4*b* for the tumor DNA–polyoma DNA reaction illustrated in Fig. 3. In the authentic host DNA interactions (Fig. 4*a*), saturation of the available sites does not occur before an input ratio (abscissa) of approximately 10 is reached; while at every input ratio for the experiments of Fig. 3 (even as low as 10^{-4}) saturation has already occurred.

The binding of labeled fragments of normal mouse DNA to both normal and tumor DNA was tested directly: 0.67 μg of mouse fragments was added to the same DNA-agar preparations used in the previous experiments. In all these experiments with both the normal and tumor DNA, approximately 70 percent of the mouse input fragments were bound to the DNA in the agar. The zero slope and the identity of the points obtained in the authentic host interaction (Fig. 5) represent a very different situation from that obtained in the interaction of the polyoma with the same DNA-agar preparations.

To demonstrate further the specificity of the duplex formation, experiments were carried out in which fragmented DNA from bacteriophage lambda was added to similar quantities of the normal mouse and tumor DNA's. When the quantities of DNA (0.67 μg) from bacteriophage lambda and from polyoma were the same, no significant binding was observed. The binding was essentially identical with that between DNA from *Escherichia coli* and the DNA-agar preparation from the normal mouse. When the DNA from bacteriophage lambda was tested with *E. coli* DNA embedded in the agar, 30 percent of the fragments of bacteriophage lambda DNA formed stable duplexes.

A more subtle argument may propose that during virus production the viral DNA acquires a specific segment of normal host DNA which is responsible for the observed binding. To account for the increased binding in the experiment with tumor DNA, the unlikely requirement would be imposed that the specific complementary region in the tumor DNA be multiplied several times compared with other polynucleotide sequences in randomly heteroploid cells.

In conclusion, the results are due to the specific binding of polyoma DNA fragments with complementary regions of mammalian DNA's; the increased binding with polyoma-induced tumor DNA reflects an increased frequency of complementary regions in the tumor DNA. This increase may be due to viral genetic material intimately associated with the cell genome as the result of oncogenic transformation. This technique, therefore, may provide a method of determining the presence of the associated genome of a particular virus in a tumor ostensibly virus-free.

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16. Calculation based on the observation of Hoyer *et al.* (*8*) as well as our additional experiments (Fig. 5).

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Endogenous Pyrogen Release from Rabbit Blood Cells Incubated in vitro with Parainfluenza Virus

Abstract. *Rabbit blood cells incubated in vitro with purified parainfluenza-5 virus (DA strain) released a rapidly acting pyrogen. Spleen and lymph node cells were inactive. The pyrogen resembled in behavior a pyrogen extracted from granulocytic exudates. Similar cells in the blood are believed to be activated by virus in vivo to produce the circulating endogenous pyrogen that mediates virus-induced fever.*

The mechanism by which viruses induce fever is obscure. After intravenous inoculation in rabbits, myxoviruses produce fever indirectly by releasing a circulating pyrogen of endogenous origin (*1*). This pyrogen was presumed to act directly on thermoregulatory centers of the brain. Unlike virus, which caused fever only after 45 to 60 minutes of latency, the pyrogen appearing in the serums of febrile donor animals injected with virus produced immediate transient fevers when transfused into recipient rabbits. Injection of Coxsackie B-1 virus into rabbits has produced similar results (*2*).

There is now considerable evidence that circulating granulocytes, though containing little or no preformed pyrogen, are capable of generating large amounts of pyrogen after contact with specific stimuli (*3*). Two such stimuli, the endotoxins of gram-negative bacteria (*4*) and certain particulate materials that are phagocytized (*5*), liberate pyrogen from granulocytes in vitro. Similar events in vivo are believed to account for fevers caused by intravenous injection of endotoxins (*6*) or various particulate substances (*7*).

Parainfluenza virus was added to rabbit blood cells in vitro to determine whether it would activate these cells to release endogenous pyrogen. In correlated studies, virus was also incubated with mononuclear cells from the spleens and mesenteric lymph nodes of the same animals, because homogenates of spleen and other normal tissues contain a substance or substances with similar pyrogenic properties (*8*).

The DA strain of parainfluenza-5 virus (*9*) was grown in primary monolayer cultures of rhesus monkey kidney and in replicating cultures of rhesus (LLC) and African green monkey kidneys (BSC-1). Culture fluid containing virus was harvested 7 to 10 days after infection and clarified by centrifugation and filtration. The supernatant fluid was then centrifuged in a Spinco model L ultracentrifuge at 34,000*g* for 45 minutes; the pellet was resuspended in saline. An equal amount of 12.5 percent barium sulfate was added, the mixture was kept overnight at 4°C, and the barium plus the adsorbed virus was washed twice in distilled water. Virus was eluted from the barium into citrated saline and the barium was removed by centrifugation. Under pyrogen-free conditions, the virus was then washed 2 or 3 times in saline by centrifugation at 34,000*g* until the supernatant fluid was free of pyrogenic activity when tested in rabbits. Antibiotics were added and cultures made to insure bacterial sterility of all material for injection. Virus preparations showed a single sharp band of hemagglutinin and protein when centrifuged in a potassium tartrate equilibrium density gradient and presented a homogeneous array of particles under electron microscopy (*10*). Procedures used for assay of fever-inducing materials in rabbits have been described (*1*).

Figure 1*a* depicts the mean fevers produced by two different virus samples injected intravenously into two groups of two rabbits each. Duration of the latent period between injection of the virus and onset of fever was inversely related to the dose of virus: more than 30 minutes for the larger dose and extending to more than 75 minutes with the smaller dose.

To determine whether virus could activate blood cells, purified virus was added to heparinized rabbit blood cells that had been washed once and resuspended in saline. The mixture was incubated in vitro at 37°C for 4 hours, and the cells were removed by centrifugation. The supernatant fluid was then injected intravenously into rabbits. The results (Fig. 1*b*) show that samples of supernatant fluid from the virus-blood cell mixture produced immediate monophasic fevers which reached peaks after 45 to 60 minutes and subsided rapidly. The configuration of the febrile responses closely resembled that induced by leucocyte pyrogen obtained from rabbit blood cells incubated with purified endotoxin derived from *Proteus vulgaris* (*8*). Samples of supernatant fluid from similarly incubated mixtures of virus and rabbit spleen or lymph node cells failed to produce immediate fevers.

These results indicate that "purified" DA virus is capable, like endotoxin, of activating cells in the blood to release endogenous pyrogen in vitro. With endotoxins, there is good evidence that the cell releasing pyrogen is the granulocyte. Normal red blood cells and platelets have not been shown to contain a pyrogen, and incubation of virus with mononuclear cells from two different sources—spleen and lymph nodes—fails to release an endogenous pyrogen. It seems likely, therefore, that the granulocyte is also the responsible cell in fevers induced by the intravenous inoculation of DA virus. The results were similar when tuberculin was incubated in vitro with blood cells of rabbits sensitized with

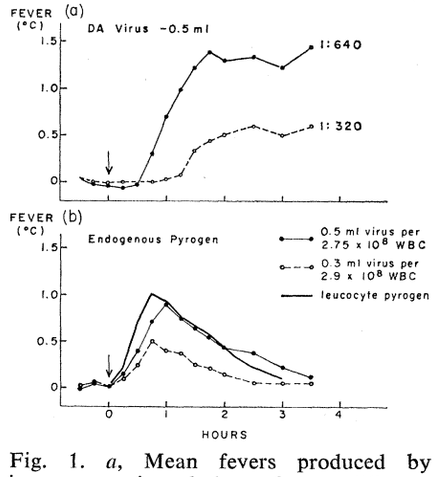

Fig. 1. *a*, Mean fevers produced by intravenous inoculation of two different doses of virus (represented by hemagglutinin titers). *b*, Mean fevers produced by saline supernatants of blood cells incubated with virus at the two concentrations shown. For comparison, responses of five rabbits to leucocyte pyrogen (obtained from 8 to 16 × 10^7 blood leucocytes incubated with 0.003 μg of *Proteus vulgaris* endotoxin) are shown. Figures given for endogenous pyrogen represent individual dosages. Each curve represents the mean response of two or three rabbits.