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TUMOR PRODUCING CAPACITY OF TEMPERATURE SENSITIVE MUTANTS OF AVIAN SARCOMA VIRUSES IN CHICKS

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SUMMARY When temperature sensitive (*ts*) mutants were inoculated into the wing webs of chicks, the incidence of tumors was low. However, when the inoculated chicks were exposed to unfavourable conditions during the first week of infection, which lowered the temperature of the inoculated site, tumors appeared at high frequencies and grew rapidly. These results suggested that mutants which possess *ts* characters of cell transformation in vitro also have a tumor producing capacity in vivo which is affected by temperature. Some, though not all, of the tumors produced in vivo by *ts* mutants contained back mutants. Thus some of the tumors produced in vivo by *ts* mutants were due to back mutation of viruses. Some may also be partly due to " leaky " infection, since the incidence of tumors varied with the virus dose.

INTRODUCTION

Many *ts* mutants for cell transformation have been isolated from avian sarcoma viruses (Toyoshima and Vogt, 1969; Martin, 1970; Kawai and Hanafusa, 1971; Biquard and Vigier, 1972; Bader, 1972; Wyke and Linial, 1973). The characteristics of the transformed cells induced by most of these *ts* mutants can be reversibly regulated between the transformed and non-transformed states by reciprocal shift between the permissive and non-permissive temperature. These mutants could be expected not to produce tumors in vivo, since the rectal temperature of chicks is about 42 C. However, three mutants have been reported to produce some tumors in chicks, although their tumor producing capacity was lower than that of wild type viruses (Friis, Toyoshima and Vogt, 1971; Kawai and Hanafusa, 1971). In the present study, tumor production by five *ts* mutants with different characters were tested to examine the relation between temperature sensitivity of cell transformation in vitro and the tumor producing capacity in vivo.

MATERIALS AND METHODS

1. *Viruses*

B77-*ts*X334-C (*ts* 334) and B77-*ts*X336-C (*ts* 336)

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were formerly referred to as *ts* 75 and *ts* 149, respectively (Toyoshima and Vogt, 1969). B77-*ts*O260-C (*ts* 260) was isolated from B77 after UV-irradiation. SR-*ts*O122-D (*ts* 122) was isolated from SR-RSV D after treatment with BUDR for 24 hr after infection and SR-*ts*O538-A (*ts* 538) was isolated from SR-RSV A after treatment with 5-azacytidine. The cell transforming abilities of all three newly isolated mutants are temperature sensitive but these mutants replicate better at 41 C than at 36 C. All wild type viruses were gifts from Dr. P. K. Vogt.

2. *Chicks and eggs*

One-day-old chicks and fertilized hen eggs were kindly provided by the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University. Both chicks and eggs were free of known leukosis viruses, but harbored group specific antigen of avian RNA tumor viruses and cell associated helper factor.

3. *Culture and focus assay*

Culture and focus assay were done essentially as described by Vogt (1969) with some modification (Owada and Toyoshima, 1973). The permissive and nonpermissive temperatures were 36 C and 41 C, respectively.

4. *Neutralization test*

Chicks were bled by heart puncture 40 days after inoculation and neutralizing antibody in the sera were tested by their inhibition of focus formation (Ishizaki and Vogt, 1966). None of the chicks possessed neutralizing antibody to uninoculated subgroup viruses at a dilution of 1 : 10.

5. *Test of viruses recovered from tumors*

The supernatant of 10% emulsions of tumors was assayed at both 36 C and 41 C to test the temperature sensitivity of virus in the tumors. If necessary, the test of temperature sensitivity for cell transformation was repeated with virus recovered from foci appearing at 41 C.

RESULTS

1. *Tumor formation in chicks kept under 2 different conditions*

In experiment A, 10 to 15 one-day-old chicks (body temperature: 39–40 C) in each group were inoculated subcutaneously into the wing webs with 0.1 ml of one of the viruses. The chicks in each group were kept at 30 C in separate cages without a chick-bulb-heater and were moistened during the first week of infection. The temperature of the wing webs was lowered 1–3 C under these conditions because of the poor regulation of body temperature of new born chicks. Ten to fifty per cent of the chicks died without tumors during the first week and these were omitted from calculations of tumor incidence.

In experiment B, 10 to 15 ten-day-old chicks were inoculated by the same route and were kept in dry cages at 30 C without a heater. No death was recorded during the first week of infection.

Wild type B77 produced tumors in high incidence in both experiment A and B, although some chicks recovered from these tumors in experiment B (Figs. 1A and 1B). Wild type SR-RSV D and SR-RSV A were essentially similar to B77 in tumor production.

ts 334 produced tumors with high efficiency in experiment A when 2.7×10^3 or more FFU was inoculated (Fig. 2A). Inoculation with 2.7×10^2 FFU of the same mutant resulted in 40 per cent incidence of tumor. All tumors increased progressively in size and killed some chicks within 1 month. In experiment B, the incidence of tumors induced by *ts* 334 was low, even with 2.5×10^4 FFU of the virus and tumors were less progressive than those in experiment A (Fig. 2B).

ts 336 showed a similar tendency to *ts* 334 in both experiment A and B (Figs. 3A and 3B). However, the difference of the incidences of tumors in experiment A and B was more pronounced than with *ts* 334 and in experiment B, only 20 per cent of the chicks developed progressive tumors with an inoculum of *ts* 336 as large as 2.5×10^5 FFU.

ts 260, which had the replicating character of the wild type, induced similar incidences of tumors in experiments A and B. However, progression of the tumor was much less in experiment B than in experiment A (Figs. 4A

and 4B).

ts 122 produced one tumor in experiment A when 1×10^3 FFU was inoculated and *ts* 538 did not produce any tumor in experiment B when 1.3×10^2 FFU was inoculated.

2. *Subcutaneous and intramuscular inoculations of cells transformed by ts 122*

Chick embryo fibroblast cells transformed in vitro by wild type viruses produced tumors rapidly in chicks. Cells transformed by *ts* mutants produced tumors in relatively high frequency (Friis et al., 1971; Toyoshima, 1972). The tumor producing ability in vivo of cells transformed by *ts* 122 was tested by subcutaneous and intramuscular inoculations (Figs. 5A and 5B). The latter route was used to minimize the leak of the temperature sen-

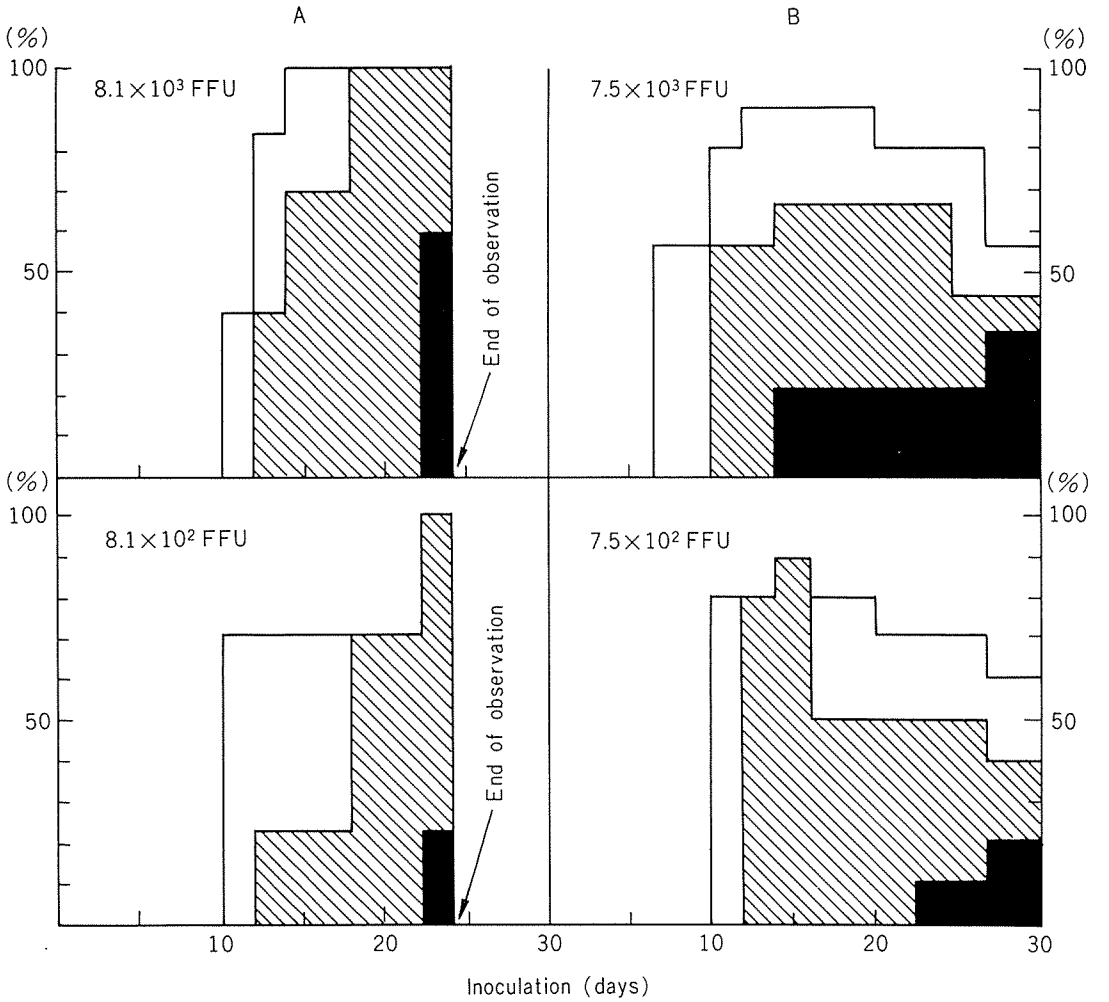

FIGURE 1. *Tumor production by wild type B77, inoculated subcutaneously into the wing webs of chicks. A: One-day-old chicks were inoculated and exposed to unfavourable conditions (see text) during the first week. B: Ten-day-old chicks were inoculated and kept at 30 C. Focus forming units were determined at 36 C on C/E chick embryo fibroblast cells and virus titers indicate amount inoculated per chick.*
□, *nodule (less than 1 mm diameter);* ▒, *tumor;* ■, *death with tumor.*

sitive function for cell transformation, since the temperature under the wing web was about 1 C lower than the rectal temperature, even the wing was tightly closed. Tumors appeared much earlier and were more progressive after subcutaneous inoculation (group A) than after intramuscular inoculation (group B).

3. *Antibody response of chicks in experiment B*

Chicks were bled 40 days after inoculation in experiment B and their neutralizing antibodies were examined. Table 1 shows the antibody responses of chicks inoculated with 3 different type mutants of B77. The antibody response seemed to be affected by the inoculum size. However, it was not correlated directly with the presence or absence of tumors or with the replicating ability of the mutants in vitro at the nonpermissive temperature.

4. *Characters of the viruses recovered from tumors*

A certain proportion of viruses recovered from tumors induced by *ts* mutants contained

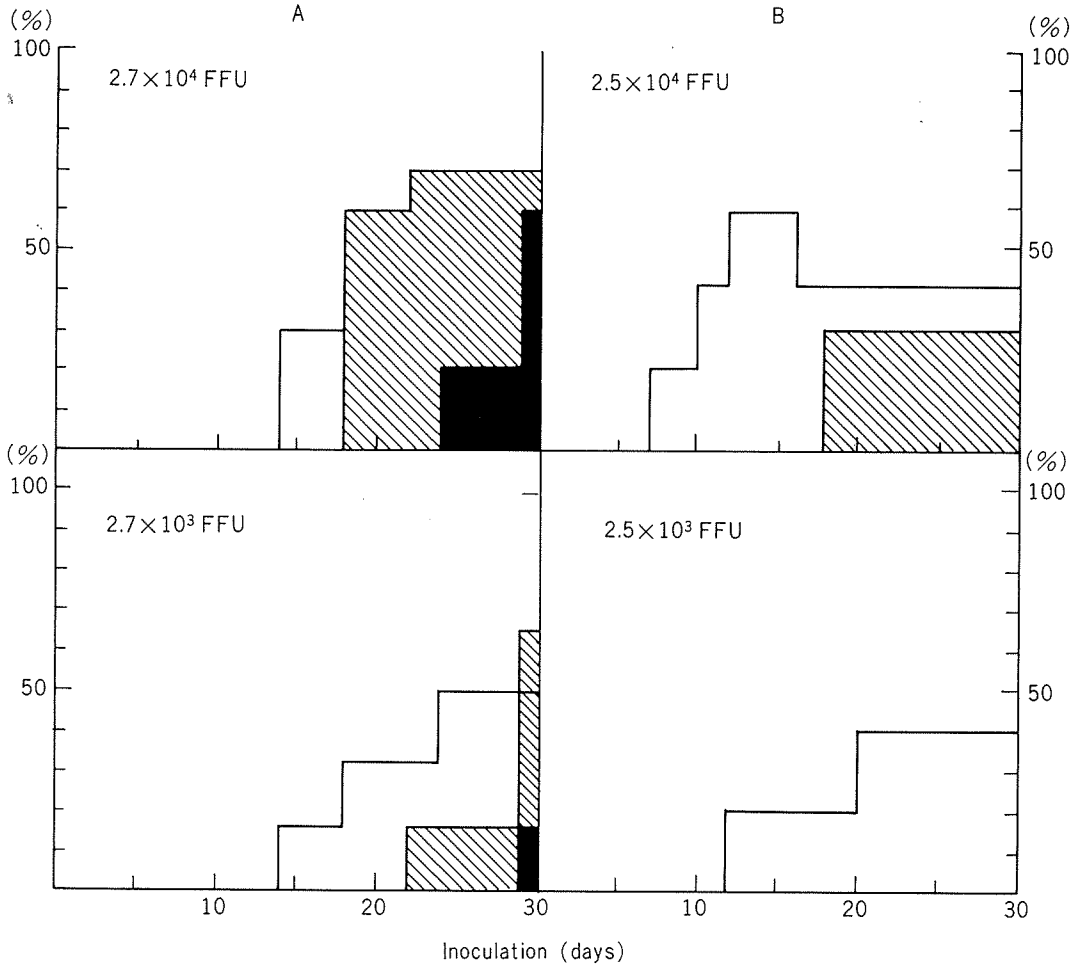

FIGURE 2. *Tumor production by ts 334 inoculated subctaneously into wing webs of chicks. A: One-day-old chicks. B: Ten-day-old chicks. Treatments and symbols are the same as those in Fig. 1.*

TABLE 1. *Antibody response of chicks 40 days after inoculation of ts mutants of B77*

Tumor	Virus inoculated[a]				
	ts 334		*ts* 336		*ts* 260
	2.5×10^4	2.5×10^3	2.5×10^5	2.5×10^4	7.5×10^2
+	3/3	2/5	4/4	2/2	1/5
−	2/4	1/4	4/4	5/6	2/6

[a] Virus titers are expressed by FFU/inoculum.
Numerators: numbers of chicks with neutralizing antibody titers of 1 : 10 or more.
Denominators: numbers of chicks tested.

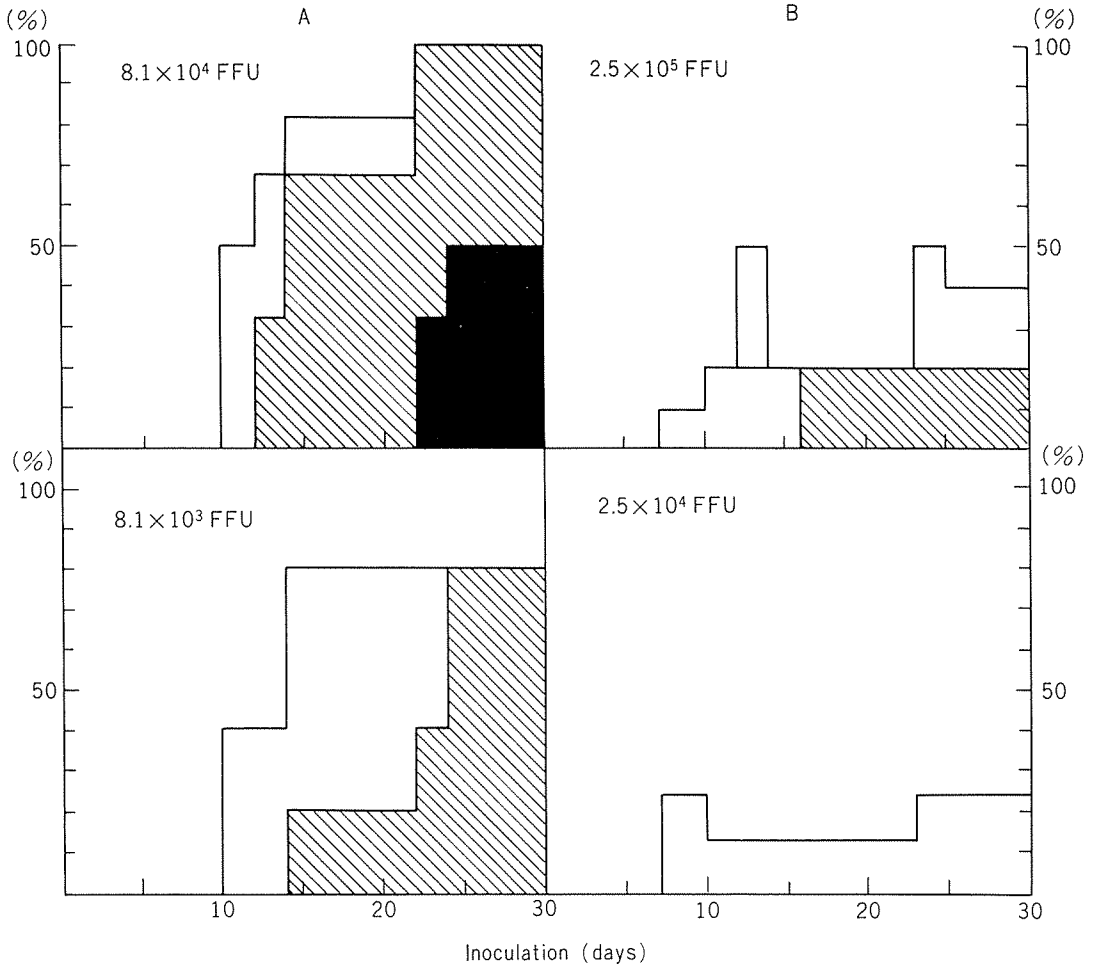

FIGURE 3. *Tumor production by ts 336 inoculated subcutaneously into the wing webs of chicks. A: One-day-old chicks. B: Ten-day-old chicks. Treatments and symbols are the same as those in Fig. 1.*

back mutants which could transform chick embryo fibroblast cells at the nonpermissive temperature (Table 2). Thus part of the tumor formation by *ts* mutants seemed to be caused by back mutation of the *ts* character. However, back mutants could not be detected in more than half the tumors induced by *ts* 334, *ts* 336 or *ts* 260. These tumors might be

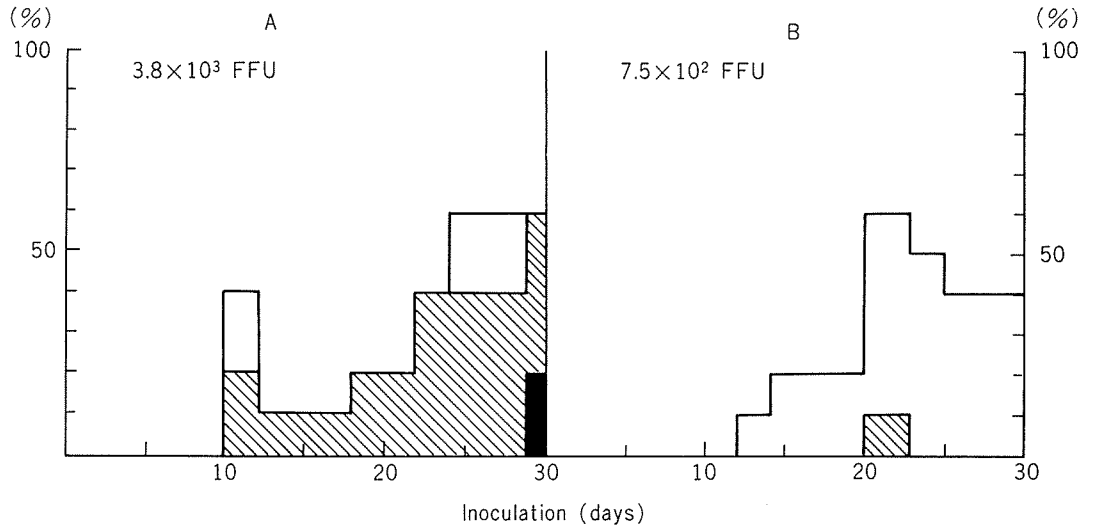

FIGURE 4. *Tumor production by ts 260 inoculated subcutaneously into wing webs of chicks. A: One-day-old chicks. B: Ten-day-old chicks. Treatment and symbols are the same as those in Fig. 1.*

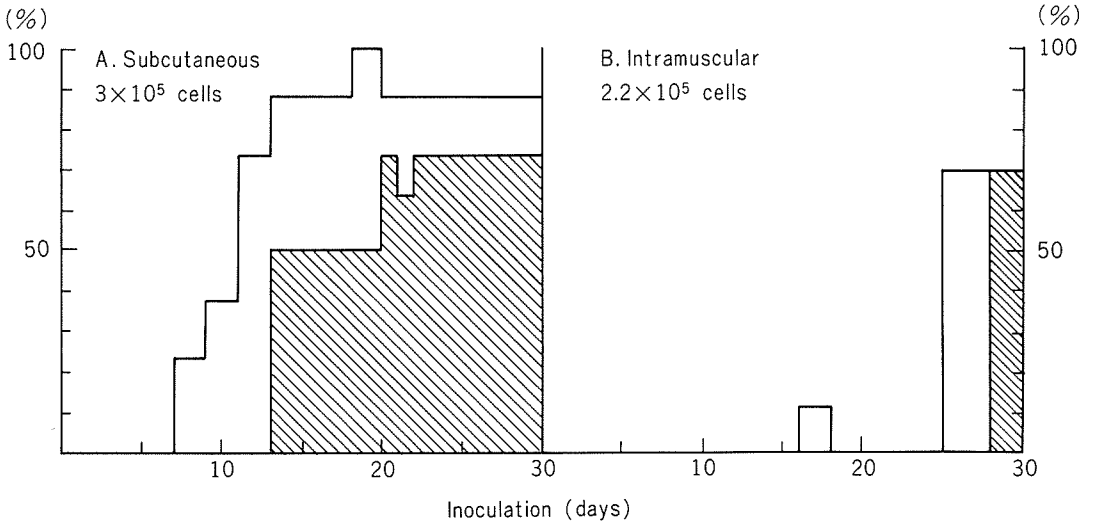

FIGURE 5. *Tumor production by the cells transformed in vitro by ts 122. Ten-day-old chicks were inoculated with transformed cells subcutaneously (A) or intramuscularly (B) into wing webs and were kept at 30 C without chick bulb-heater.*
□, *nodule (less than 1 mm diameter);* ▒, *tumor.*

TABLE 2. *Test of temperature sensitivities of viruses recovered from tumors*[a]

	Tumors produced by			
	ts 334	*ts* 336	*ts* 260	*ts* 122
No. tested	10	3	10	9
Virus (−)[b]	2	0	0	2
Back mutant (+)[c]	2	0	3	6

[a] Tumors were removed 40 days after inoculation and viruses in 10% emulsions of tumors were tested at 36 C and 41 C.
[b] Virus was not recovered from the tumor at either 36 C or 41 C.
[c] Virus which could produce foci at 41 C.

caused by "leaky" infection of *ts* mutants. Tumors produced by *ts* 122-transformed cells contained a high proportion of back mutants and all the tumors which appeared after intramuscular inoculation of the cells contained back mutants.

DISCUSSION

Many *ts* mutants of avian RNA tumor viruses have been reported to have cell transforming ability only at the permissive temperature (Toyoshima and Vogt, 1969; Martin, 1970; Kawai and Hanafusa, 1971; Biquard and Vigier, 1972; Bader, 1972; Wyke and Linial, 1973), but little is known about the relation between the temperature sensitivity of these mutants in vitro and their tumor forming capacity in vivo. Three mutants have been reported which showed a higher tumor producing capacity than that expected from their *ts* characters, since the rectal temperature of chicks is 42 C (Friis et al., 1971; Kawai and Hanafusa, 1971; Toyoshima, 1972).

To elucidate this problem, tumor formation in vivo of *ts* mutants was tested under various conditions. Tumors appeared at higher frequencies and grew faster in chicks exposed to unfavourable conditions than in chicks kept under normal conditions. These unfavourable conditions killed a certain proportion of the chicks during the first week, suggesting that under these conditions the body temperature of the new born chicks decreased. Under normal conditions, the frequency and progression of the tumors were considerably less in chicks inoculated with *ts* mutants than those inoculated with wild type viruses. Tumors rarely developed when a dose of less than 10^2 FFU of the mutants was inoculated. However, inoculation with 10^3 FFU or more of the mutants resulted in formation of some tumors. Some of these tumors might have been produced by back mutants, but most of those produced by *ts* 334 and *ts* 336 might be explained by "leaky" infection.

The results suggested that *ts* mutants for cells transformation in vitro also had a temperature-dependent character for tumor production in vivo. This suggestion was supported by results on tumor production by *ts* 122-transformed cells. Chick fibroblast cells transformed by *ts* 122 rarely formed colonies in soft agar at 41 C. However, even these cells produced nodules as early as 7 days after inoculation and induced tumors in high incidence when inoculated subcutaneously into the wing webs of chicks. When inoculated intramusculary, these cells produced tumors late in the observation period. The incubation period was much shorter after subcutaneous inoculation than after intramuscular inoculation. This difference might be caused by leak of the *ts* function in the former. Once tumors had appeared after intramuscular inoculation, they grew rapidly. All virus samples recovered from these tumors contained back mutants. The chance of back mutation might increase

with a prolonged observation period, since *ts* 122 could replicate at the nonpermissive temperature. These back mutated viruses might produce tumors late in the observation period.

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