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Genetics

Isolation and characterization of a temperature-sensitive mutant of avian myeloblastosis virus

(myeloid leukemia/mutagenesis)

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ABSTRACT **A temperature-sensitive (*ts*) mutant, GA 907/7, was isolated after mutagen treatment of avian myeloblastosis virus. When bone marrow cells or secondary yolk sac macrophages were infected with GA 907/7, the expression of transformation was greatly reduced at 41°C. The results of temperature-shift experiments suggest that in GA 907/7 the putative v-*myb* gene product is functional only at 35.5°C. Moreover, when *ts*-induced transformed cells were shifted to 41°C, a partial morphological conversion to macrophage-like cells was obtained, while the majority of the cells underwent senescence and lysis. No leukemia was obtained when GA 907/7 was injected in 1-day-old chickens. Finally, a continuous cell line releasing genetically stable mutant virus was obtained after transformation of secondary yolk sac cells.**

The oncogenic and transforming properties of avian myeloblastosis virus (AMV) have been thoroughly examined (1, 2) and have shown that AMV is responsible for inducing a relatively rapid acute myeloid leukemia in infected chickens. No oncogenic response other than leukemia has ever been obtained with this virus. However, survivors may eventually succumb much later with liver lymphomas or nephroblastomas caused by the presence of two nontransforming associated viruses in the viral stock (3). Previous studies had shown that AMV is defective in its replication and, therefore, depends for this function on two antigenically distinct helper viruses labeled MAV1 and MAV2, respectively (4). *In vitro* studies demonstrated that AMV and E26 virus (5–7), unlike other avian defective leukemia viruses, are capable of transforming only cells derived from hemopoietic tissues. The AMV cells from leukemic birds or from cells transformed *in vitro* express markers typical of the myeloid lineage (8).

The oncogenic potential of AMV has been tentatively ascribed to a genetic locus (v-*myb*) situated at the 3′ end of the AMV genome (9, 10). A detailed description of this region has been reported (11, 12), but the protein(s) encoded by this region has yet to be identified. It is suggested that a spliced mRNA found in all AMV-transformed cells may be responsible for the expression of the v-*myb* (9, 13).

In recent years, successful isolation and characterization of temperature-sensitive (*ts*) mutants from avian nondefective sarcoma tumor viruses have been instrumental in defining the processes of transformation by these viruses (14, 15).

These findings have shown that the product of a viral gene was directly responsible for cellular transformation (14–17). Moreover, some of the *ts* mutants provided the evidence that the product of the viral transforming gene has a particular enzyme activity such as a protein kinase (18–21).

Attempts to isolate temperature-sensitive mutants from defective leukemia viruses have been successful, and a *ts* mutant of avian erythroblastosis virus has been characterized (22). The present study reports the isolation of a *ts* mutant of AMV and its transforming pattern in chicken hemopoietic cells.

MATERIAL AND METHODS

Viruses. The origin of AMV subgroup C (also referred to as *wt* AMV) used in this study was a pseudotype of AMV obtained by superinfecting AMV nonproducer cells with a subgroup C (RAV7) helper virus (2). The virus derived from the challenged cells had an average transforming titer of 1×10^5 focus-forming units/ml by yolk sac macrophage assay and of 2×10^4 colony-forming units/ml by infecting bone marrow cells derived from tibias of 2- to 5-day-old chicks (4).

Mutagen Treatment of Virus. The strategy used to isolate AMV *ts* mutants was as follows. A continuous line of producer-transformed myeloblasts releasing a high titer of AMV subgroup C was treated with 100 μg of 5-azacytidine (Calbiochem) per ml overnight at 35.5°C. The mutagen was added to $\approx 10 \times 10^6$ myeloblasts per ml in suspension in 60-mm Falcon plastic dishes. After 18 hr at 35.5°C, the myeloblasts were removed by low-speed centrifugation, and the supernatant fluid was centrifuged at 70,000 × *g* for 30 min to pellet the virus. The pellet resuspended in half of the initial volume was used to infect fresh bone marrow cells and was seeded at different cell densities in 35-mm dishes in methylcellulose-containing medium. After 3–4 weeks of incubation at 35.5°C, myeloblast colonies were isolated with a micro Pasteur pipette and transferred into the 15-mm wells of a multidose tissue culture tray (Linbro). Each well contained 0.5 ml of growth medium to which helper virus RAV7 was added. Cells were propagated at 35.5°C until at least 10^6 cells were obtained from each colony.

The helper virus was added to ensure that every isolated clone produced infectious virus because, from previous attempts, the chance of isolating nonproducer clones in these conditions was high. Supernatant fluids were harvested, filtered, and individually tested for transforming activity by infecting dishes containing secondary yolk sac macrophage cultures. Half of the dishes were kept at 35.5°C and the other half, at 41°C. From the >600 clones screened, one mutant was found to produce a relatively low number of colonies at nonpermissive temperatures. This mutant, following the suggested nomenclature for retrovirus mutants (23), was designated GA 907/7 (GA for Gainesville). It was recloned by bone marrow colony assay four times. Each time the results were identical to the original finding—i.e., a strong reduction in the number of transformed colonies at 41°C.

RESULTS

Transforming Properties of GA 907/7. The transforming activity of GA 907/7 was compared with that of *wt* AMV at per-

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Abbreviations: AMV, avian myeloblastosis virus; *ts* mutant, temperature-sensitive mutant.

missive (35.5°C) and at nonpermissive (41°C) temperatures by simultaneously infecting bone marrow cells for colony formation and yolk sac macrophages for focus formation. A reduction to 1/50 in the number of foci was obtained at nonpermissive temperatures with the macrophage assay. A 2- to 3-fold increase in the number of foci was obtained with the wild type at 41°C; this behavior was observed consistently in all of our experiments with wild-type AMV maintained at this temperature. The fact that 41°C is the natural body temperature of the chicken may account for this increase. The transforming assay in yolk sac macrophages was usually scored after 3 weeks at 35.5°C, whereas the corresponding test at 41°C was scored after 12 days. Macrophage cultures under these conditions (i.e., under agar and at 41°C) usually deteriorated after 2 weeks. Metabolic activities for these adherent cells were all accelerated; therefore, senescence of these cells occurred more rapidly at 41°C.

Similarly, when bone marrow cells were infected with GA 907/7, a significant reduction in the number of colonies was observed (Table 1). Unlike the yolk sac assay, the bone marrow colonies could be scored at both temperatures after 3–4 weeks. At 41°C the deterioration described earlier for the macrophages was not observed.

Nature of the Lesion. Temperature-shift experiments were performed to determine the nature of the lesion. Yolk sac cultures were infected with GA 907/7 or *wt* AMV, overlaid with agar, and incubated at 35.5°C or 41°C, respectively. Some of the plates were shifted to the alternate temperature at 30, 48, and 72 hr after infection. The results (Tables 2 and 3) suggest that the lesion may involve a particular step necessary to maintain the transformed state. The data on the up-shift (Table 2) showed convincingly that the product of v-*myb* was not functional at the nonpermissive temperature. Moreover, the down-shift experiments (Table 3) indicate that once the cultures were shifted to the permissive temperature, the product became functional. The reduction in the number of foci obtained at 35.5°C after a 72-hr incubation at 41°C was consistent with the observation that during this time ≈50% of the infected cells remained viable. On the basis of these results, we suggest that GA 907/7 expresses characteristics of a maintenance mutant.

Virus Replication from *ts*-Transformed Cells. Virus production was tested from transformed cells derived from two different sources of hemopoietic tissues—namely, (*i*) cells derived from one colony obtained after infection of bone marrow cells with GA 907/7 and (*ii*) cell line 950/2A (described in a subsequent paragraph) derived from secondary yolk sac macrophages infected with the same *ts* mutant. AMV myeloblasts releasing *wt* virus were used as controls. In the case of line 950/2A, virus replication was tested as follows: cultures were kept at 35.5°C and 41°C for 4 days. Every 24 hr, viable cells were determined by trypan blue exclusion; supernatant fluids were harvested by centrifugation, and cells were resuspended in fresh medium. Although cell growth increased at both temperatures during the first 24 hr, an arrest in cell replication followed by cell lysis occurred thereafter. Filtered infectious fluids were tested for transforming activity by conventional yolk sac assay at 35.5°C (4). The results indicated that virus production occurred at both temperatures (Table 4). The reduction in the virus titer obtained from line 950/2A after 4 days of incubation at 41°C reflected the fact that under this condition only half of the original cells remained viable. Comparative results were obtained with transformed cells derived from the bone marrow colony. A distinct pattern occurred at 41°C after 72 hr of incubation: in addition to cell death, 10–30% of the cells had reverted to macrophages and had become attached (see next paragraph). At 35.5°C no macrophage reversion was observed, and transformed cells remained in suspension (data not shown).

Phenotypic Changes of Transformed Cells. Bone marrow cells from tibias of 2–5-day-old chicks were infected with GA 907/7, seeded into semisolid growth medium for colony formation, and maintained at 35.5°C. Colonies were generally obtained between 3 and 4 weeks. Colonies were individually picked and propagated in Linbro wells containing growth medium. Progeny from several colonies were tested for phenotypic changes at the nonpermissive temperature. A dramatic sequence of morphological changes occurred between 48 and 96 hr leading to a partial reversion (10–30%) of cells expressing markers of mature macrophages (i.e., adhesion of cells to the bottom of the dish, accompanied by positive immunophagocytosis of opsonized sheep erythrocytes (8) (Fig. 1 *Left* and *Right*). The rest of the cell population incurred senescence and death. Wild-type transformed cells did not show such morphological changes at 41°C and remained viable and nonadherent. Some of these *ts*-colony-derived cells were further tested for colony formation at both temperatures, and again a dramatic difference in the phenotype of the colonies was observed. Colonies at 41°C displayed a very loose and sparse morphology consisting of macrophage-like cells confirmed by staining of Cytofuge preparations (Fig. 2 *Left*). Such colonies were indeed similar to those obtained with normal hemopoietic cells (24). Conversely, colonies obtained at

Table 1. Colony formation by GA 907/7 in soft agar*

Virus	Virus dilution	No. of colonies at 35.5°C	No. of colonies at 41°C
GA 907/7	Undiluted	543	10
Wild type	1:10	190	230

* Infected bone marrow cells were seeded at a density of 5.0×10^5 cells per 35-mm dish. Colonies were scored after 3–4 weeks at both temperatures.

Table 2. Influence of temperature shift from 35.5°C to 41°C on focus formation in yolk sac macrophages

Incubation at 35.5°C prior to up-shift, hr	No. of foci in culture* GA 907/7†	No. of foci in culture* Wild type‡
0	13	380
30	31 (2.38)	290 (0.76)
48	38 (2.92)	405 (1.07)
72	49 (3.76)	401 (1.06)

* Compared with 638 foci obtained in cultures maintained at 35.5°C during the entire length of the experiment. Numbers in parentheses are ratios of the no. of foci in culture undergoing temperature up-shift to the number of foci in cultures maintained at 41°C.
† Cultures infected with 0.1 ml of undiluted virus.
‡ Cultures infected with 0.1 ml of 1:100 dilution of virus.

Table 3. Influence of temperature shifts from 41°C to 35.5°C on focus formation in yolk sac macrophages

Incubation at 41°C prior to down-shift, hr	No. of foci in cultures* GA 907/7†	No. of foci in cultures* Wild type‡
0	638	169
30	586 (0.90)	171 (1.00)
48	403 (0.63)	137 (0.80)
72	346 (0.54)	159 (0.94)

* Numbers in parentheses are the ratios of the number of foci in cultures undergoing temperature down-shift to the number of foci in cultures maintained at 35.5°C.
† Cultures infected with 0.1 ml of undiluted virus.
‡ Cultures infected with 0.1 ml of 1:100 dilution of virus.

Table 4. Virus replication in line 950/2A and in wild-type myeloblasts at permissive and nonpermissive temperatures

Source of transformed cells	Incubations							
	24-hr		48-hr		72-hr		96-hr	
	35.5°C	41°C	35.5°C	41°C	35.5°C	41°C	35.5°C	41°C
Line 950/2A								
No. of cells per ml*	3.0×10^6	3.4×10^6	3.0×10^6	1.6×10^6	4.5×10^6	1.8×10^6	4.5×10^6	1.0×10^6
Virus titer per ml†	6.6×10^4	8.5×10^4	7.5×10^4	7.1×10^4	1.1×10^5	6.2×10^4	1.1×10^5	2.5×10^4
Wild type myeloblasts								
No. of cells per ml*	2.5×10^6	8.0×10^6	3.4×10^6	7.0×10^6	4.2×10^6	9.0×10^6	3.4×10^6	1.3×10^7
Virus titer per ml†	2.5×10^4	1.2×10^5	2.5×10^4	1.1×10^5	3.2×10^4	1.5×10^5	2.8×10^4	2.0×10^5

* Dishes were initially seeded at a cell concentration of 2.0×10^6 cells per ml; viable cells were determined by trypan blue exclusion.
† Determined by standard transformation assay on secondary yolk sac macrophages (4).

35.5°C displayed a phenotype indistinguishable from that obtained with wild type (Fig. 2 *Right*).

GA 907/7 Lesion Resides in the AMV Genome. In order to determine whether the lesion affected the transforming component of the mutant rather than the helper, bone marrow cells were infected with GA 907/7 at low multiplicity of infection in an attempt to obtain nonproducer-transformed colonies (5, 25). Few colonies were obtained at 35.5°C. Of seven colonies picked, two proliferated extensively. Supernatant fluids were assayed for transformation and reverse transcriptase and found to be negative for virus production. These nonproducer cells were then tested for colony formation at both permissive and nonpermissive temperatures. Discrete compact colonies were obtained at 35.5°C, whereas the characteristic dispersed colony appeared at 41°C. These data paralleled those described above with the producer cells. This indicates that a function of the putative v-*myb* gene is affected excluding any involvement of the helper virus.

Isolation of Cell Line Synthesizing *ts* Virus. In order to obtain a constant source of *ts* virus for biochemical and biological studies, we attempted to establish a continuous line of transformed myeloblasts releasing the mutated virus. Spafas yolk sac macrophage cultures were infected with GA 907/7 and maintained in liquid condition at the permissive temperature until a fully transformed culture was obtained. This occurred after 4 weeks, and subcultures of the transformed cells led to the es-

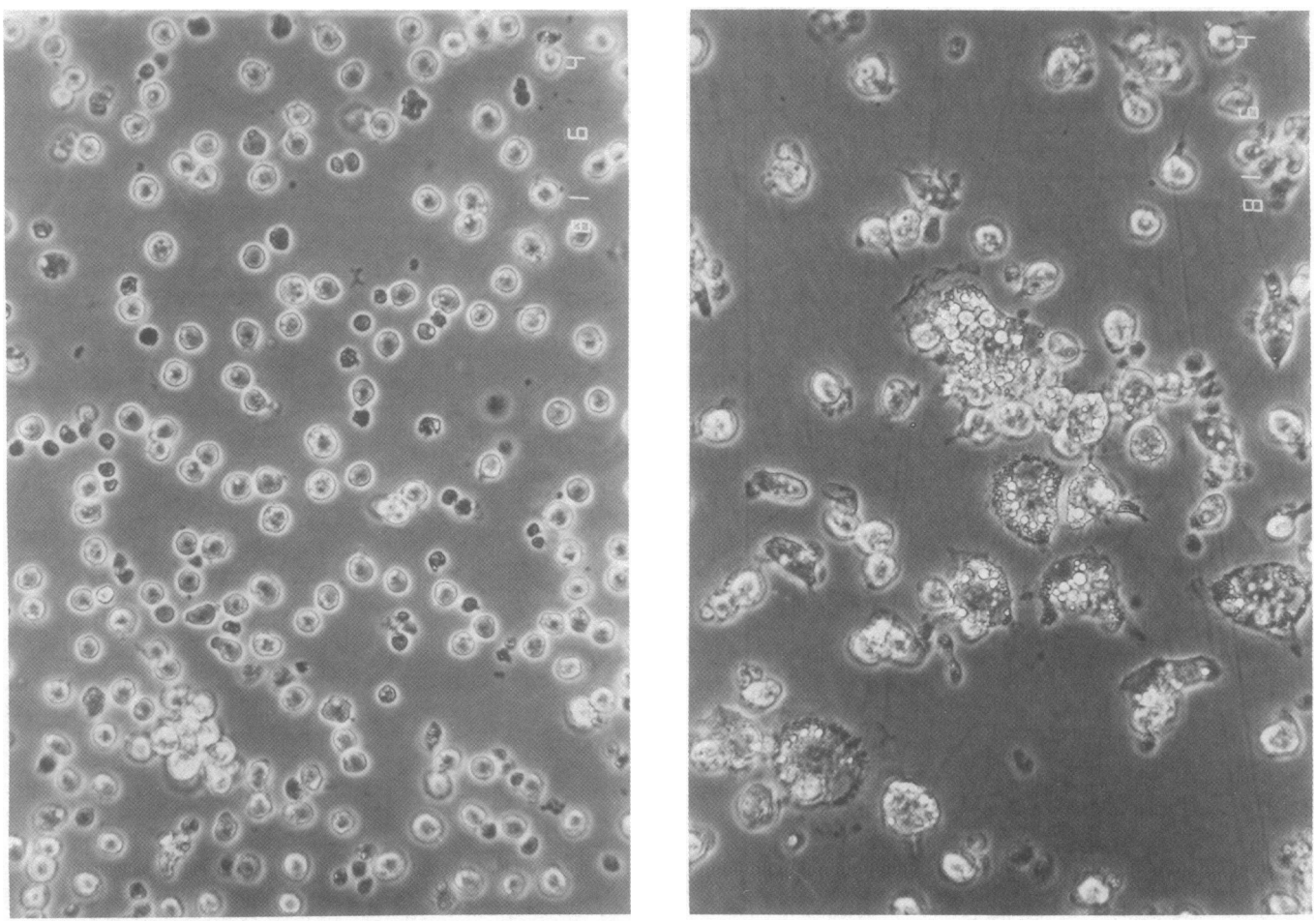

FIG. 1. (*Left*) Bone marrow cells originally transformed by GA 907/7 and kept at 35.5°C in liquid growth medium. Cells remain nonadherent as long as they are maintained at the permissive temperature. (×120.) (*Right*) Same cells as in *A* but shifted to 41°C. Morphological changes can be seen leading to adherent macrophage-like cells. (×120.)

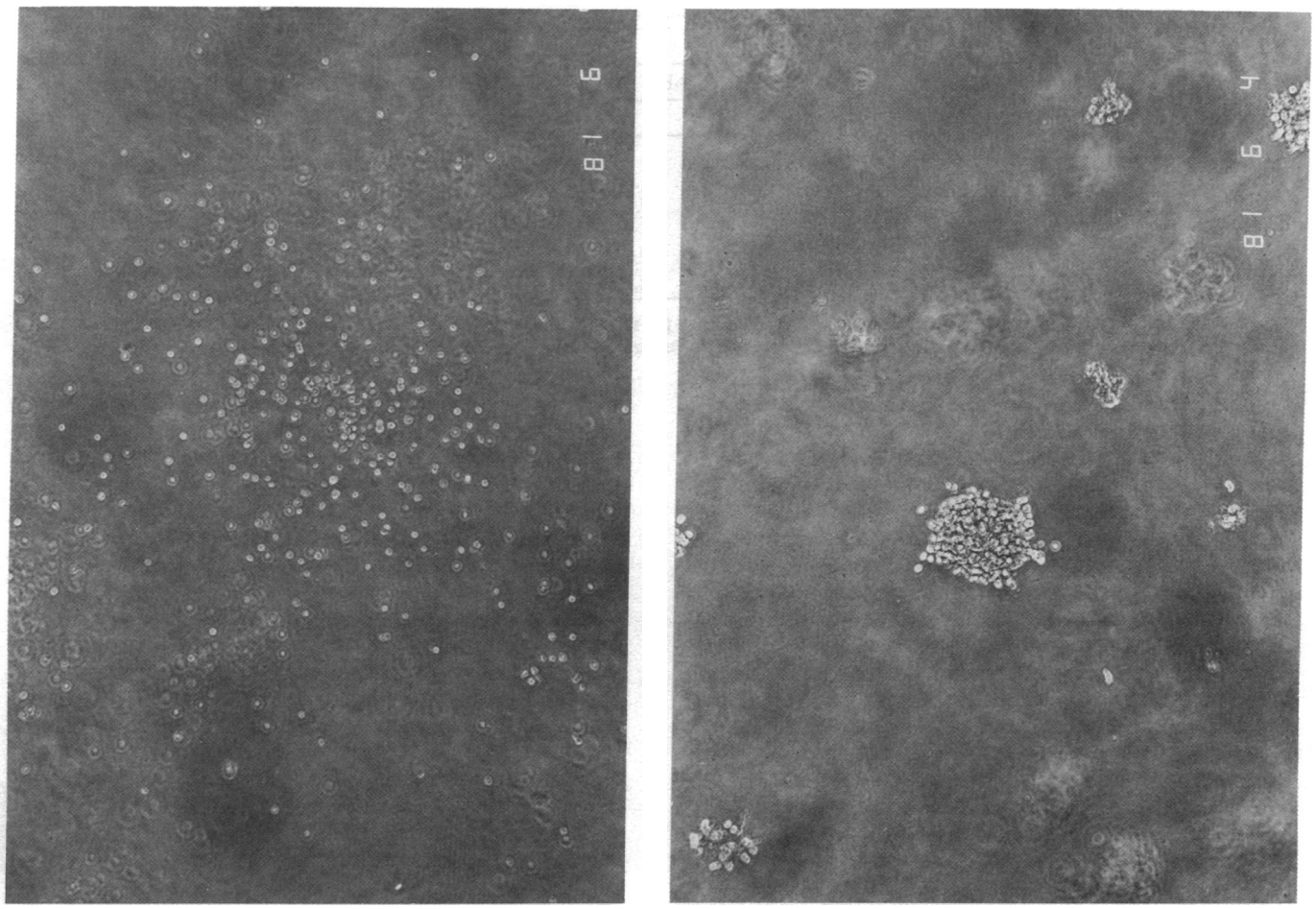

FIG. 2. (*Left*) Cells derived from a *ts* colony seeded in semisolid medium and incubated at 41°C. Proliferation of macrophage-like cells with a lack of colony formation. (×30.) (*Right*) Gross morphology of a typical *ts* colony obtained at 35.5°C is similar to *wt* colony morphology. (×30.)

tablishment of a permanent line, labeled 950/2A. The virus production in mutant-infected cells was tested at various intervals, and the genetic stability of the viral population was confirmed.

When line 950/2A underwent a shift in temperature from 35.5°C to 41°C, no difference in the number of cells was observed in the first 24 hr as compared with cells maintained at 35.5°C. Viable cells in suspension decreased steadily thereafter, with only one-third surviving after 96 hr. Unlike the previously described bone-marrow-derived transformed cells, only a few 950/2A cells did attach, reverting to their initial secondary macrophage state. This reduction of viable cells coincided with the reduction in the amount of virus released after 4 days in culture at 41°C (Table 4).

Leukemogenicity of GA 907/7. No leukemia was observed in any birds injected with GA 907/7, compared with the relatively high incidence of leukemia obtained when an equal titer of wild-type AMV(C) was used (Table 5). Blood smears were obtained twice a week, and all birds were observed for at least 8 weeks. Birds injected with the wild type usually died quite rapidly after the onset of disease was detected. At the end of the experiment, all of the surviving birds were autopsied and checked only macroscopically for possible lesions, none of which were observed.

Table 5. *In vivo* assay of GA 907/7

Virus used	Chicken strain	Leukemia incidence*
wt AMV(C)	SPAFAS	42/87
wt AMV(C)	Line 6	18/53
GA 907/7	SPAFAS	0/38
GA 907/7	Line 6	0/19

* One-day-old birds injected intravenously with 0.1 ml of undiluted virus—number infected/total number injected.

DISCUSSION

This paper describes isolation of a *ts* mutant from mutagen-treated AMV. The results obtained and presented here indicate that GA 907/7 shows a drastic inability to transform either bone marrow cells or yolk sac macrophages at the nonpermissive temperature of 41°C.

The experiments involving temperature down- and up-shift have provided data strongly indicating that this *ts* mutant has properties that affect a function required for the maintenance of the transformed state. The reduction obtained in the down-shift experiments may be explained by the fact that part of the infected cells are lysed during the incubation period at 41°C (72 hr), resulting in ≈50% survival of cells in which the alleged v-*myb* product can become functional after the cells were shifted at the permissive temperature. The survival pattern observed when transformed cells were shifted at nonpermissive temperature was different when yolk sac or bone marrow-derived cells were examined. This finding is consistent with the proposed model for cell transformation with AMV (26), which invokes a

mechanism of partial dedifferentiation when terminally differentiated yolk sac macrophages are infected and transformed with AMV. Hence, the shift to the nonpermissive temperature causes a rapid return to their original differentiated state, followed by their inherent limited life-span. Conversely, in the bone marrow, it is likely that a more immature myeloid precursor is infected and transformed; therefore, more sequential steps are required to reach terminal differentiation and death.

We were able to immortalize a transformed cell line (line 950/2A) after infection of yolk sac macrophages with GA 907/7. This line produces high titers of transforming virus (Table 4), which seems to be genetically stable.

Finally, leukemia was not obtained when two strains of chicken maintained in this laboratory were injected with GA 907/7. Cell line 950/2A may be used as a powerful tool for the identification of the putative transforming protein that has been recently postulated (11, 12).

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