Isolation and Characterization of Temperature-sensitive Mutants of Abelson Murine Leukaemia Virus that Exhibit Dissociation among Morphological Transformation, Soft Agar Colony-forming Ability and Tyrosine Kinase Activity

By Y. OKA, 1 H. SUGIYAMA, 1 S. TSUKADA, 1 Y. SHIMIZU, 1 H. INOUE, 2 A. HAKURA 2 and S. KISHIMOTO 1

1 The Third Department of Internal Medicine, Osaka University Medical School, Fukushima-ku, Osaka 553 and 2 Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

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SUMMARY

Seven temperature-sensitive (ts) mutants of Abelson murine leukaemia virus (A-MuLV) were isolated on the basis of the temperature dependence of their soft agar colony-forming ability. These seven ts mutants exhibited similar characteristics and were not ts for morphological transformation and autophosphorylation of P120\(^{PS\rightarrow abl}\) protein. The dissociation of the properties of morphology, soft agar colony formation and tyrosine kinase activity might suggest that the v-\(abl\) product has more than one primary intracellular target.

Abelson murine leukaemia virus (A-MuLV) is a replication-defective retrovirus capable of transforming lymphoid and fibroblast cells in vitro (Rosenberg et al., 1975). It predominantly induces lymphosarcomas of the B cell lineage in vivo (Abelson & Rabstein, 1970; Sugiyama et al., 1982). The product of the v-\(abl\) oncogene of A-MuLV has tyrosine kinase activity that is thought to be responsible for oncogenesis by A-MuLV (Witte et al., 1980). Exactly how the v-\(abl\) oncogene product transforms the cell, however, is still unknown, and to elucidate the mechanism of transformation by the v-\(abl\) oncogene, temperature-sensitive (ts) mutants of A-MuLV would be very useful. We therefore attempted and succeeded in the isolation of ts mutants of A-MuLV. In the present study, we describe the isolation and characterization of seven such mutants, all of which are ts for soft agar colony-forming ability (anchorage independence), but not for morphological transformation and tyrosine kinase activity.

To mutagenize A-MuLV, ANN-1 cells (Scher & Siegler, 1975) were superinfected with HIX virus (Fischinger et al., 1975) and cultured in the presence of 5 to 15 \(\mu\)g/ml of 5-azacytidine (5-AC; Sigma) at 37 °C for 12 h. The culture supernatant was collected and used as the mutagenized A-MuLV stock. NIH 3T3 cells (2 \(\times\) 10^4 to 4 \(\times\) 10^4/60 mm dish) were infected with 5-AC-treated virus stock at an m.o.i. of 0.01 to 0.1. After overnight culture at 37 °C, the temperature was shifted down to 35 °C. Ten to 14 days after infection, the cells were trypsinized, and 5 \(\times\) 10^4 cells were seeded per 60 mm dish with Earle's modification of Eagle's minimal essential medium (E-MEM) containing 5% foetal calf serum and 1.7% methylcellulose and again cultured at 35 °C. About 2 weeks later, when several hundred visible colonies were detected in the semisolid medium, the culture temperature was shifted up to 39 °C for 2 days. Then, 10 to 20 \(\mu\)g/ml of 5-fluoro-2'-deoxyuridine (FdUrd; Sigma) and 125 \(\mu\)g/ml of uridine (Sigma) were added to the culture dishes, and the culture was continued at 39 °C for a further 2 days. The cells were collected by centrifugation, and then washed and transferred to liquid culture medium (E-MEM with 5% foetal calf serum) at 35 °C. These experiments were performed with 45 dishes using different virus stocks individually prepared by independent
Infection by 5-AC-mutagenized virus stock

Liquid culture

37 °C

NIH 3T3

Transfer of cells to semisolid medium

Appearance of visible colonies

35 °C

Transfer of cells to liquid culture

Culture in semisolid medium

Cloning of surviving cells

ts growth assay in soft agar

ts mutant-infected NIH 3T3 cell clones

12 h 10-14 days about 2 weeks 2 days 2 days 2-3 weeks

Fig. 1. Strategy for the isolation of ts mutant-infected NIH 3T3 cells.

Fig. 2. Temperature-sensitive soft agar colony formation. NIH 3T3 cells (1 x 10^4 cells/60 mm dish) transformed with tsOS-170 were seeded in 0.33% soft agar and cultured at the permissive (35 °C) or non-permissive (39 °C) temperatures for 2 to 3 weeks.

mutagenizations of A-MuLV. After 2 to 3 weeks, generally only one colony of surviving cells was detected per dish. Surviving cells were cloned in semisolid medium and only one clone per dish was selected. To check the temperature sensitivity of each clone for soft agar colony formation, 3 x 10^3 cells were seeded in 3 ml of 0.33% soft agar medium over 4 ml of 0.5% soft agar medium in a 60 mm dish and incubated at permissive (35 °C) or non-permissive (39 °C) temperatures. After 2 to 3 weeks, the number of colonies was scored. When the ratio of the number of colonies at the non-permissive temperature to the number of colonies at the permissive temperature was <0.1, the clone was considered to be ts mutant-infected. Fig. 1 schematically represents our strategy for the isolation of ts mutant-infected NIH 3T3 cells. Of 20 surviving cell clones, seven exhibited temperature sensitivity for soft agar colony formation. All seven cell clones were independently isolated from different cultures, infected with virus stocks prepared from independent mutagenization experiments.

To confirm that the temperature sensitivity of the seven cell clones was caused by infection with ts mutants of A-MuLV, NIH 3T3 cells were infected with the culture supernatant from the seven cell clones, seeded into soft agar medium, and cultured at 35 °C or 39 °C for 2 to 3 weeks. As expected, the soft agar colony formation of the infected NIH 3T3 cells exhibited temperature sensitivity, although the number of the colonies formed in soft agar was small because of the low titre of A-MuLV (10^2 to 10^3 f.f.u./ml) in the culture supernatants (Fig. 2 and Table 1). Therefore, it was confirmed that all seven clones with ts growth in soft agar released ts virus.
The morphology of NIH 3T3 cells transformed by the seven ts mutants of A-MuLV was rounded and refractile at the permissive temperature (35 °C) and did not change when the transformants were shifted to the non-permissive temperature (39 °C) (Fig. 3).

Tyrosine kinase activity was assayed in cells infected by the ts mutants. Cells were cultured for 5 to 10 days at the permissive or non-permissive temperature and lysed in RIPA buffer (50 mM-Tris-HCl pH 8.2, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.15 M-NaCl, 20 mM-EDTA, 100 kallikrein-inhibiting units/ml Trasylol) and clarified by ultracentrifugation (8500 g, 30 min). Cell lysates (100 μg of protein) were immunoprecipitated with anti-gag antibodies and the immune complexes bound to Protein A-Sepharose CL-4B beads were assayed for tyrosine kinase activity as previously described (Maeda et al., 1987). The reaction was initiated by the addition of 5 μCi of [γ-32P]ATP. After incubation at 30 °C for 30 min, the reaction was terminated and the samples were analysed on 10% SDS–polyacrylamide gels. As shown in Fig. 4, no significant difference in tyrosine kinase activity at the permissive and non-permissive temperature could be detected. Furthermore, we determined the amount of P120 gag-abl by immunoprecipitation (Maeda et al., 1987). Cells were cultured for about 10 days at the permissive or non-permissive temperature, and then 10^6 cells were washed with phosphate-buffered saline (PBS) and starved for 3 h in methionine-free medium. Then, after washing once, the cells were incubated with 250 μCi of [35S]methionine for 4 h at the permissive or non-permissive temperature. The labelled cells were washed with PBS and lysed in RIPA buffer. After centrifugation at 10000 r.p.m. for 30 min, the supernatants (6 × 10^6 c.p.m.) were immunoprecipitated with anti-gag antibodies, and the immune complexes were adsorbed on Protein A-Sepharose beads by mixing for 30 min at 4 °C. The beads were washed intensively and suspended in sample buffer (2% SDS, 0.0625 M-Tris–HCl pH 6.8, 10% glycerol, 5% mercaptoethanol, 0.02% bromophenol blue). After boiling for 2 min, the supernatants were applied to 10% polyacrylamide gels. No significant difference in the amount of P120^gag-abl at the permissive or non-permissive temperature could be detected (Fig. 5). Therefore, no significant differences existed in tyrosine kinase activity or the amount of P120^gag-abl at the permissive or non-permissive temperature regardless of the remarkable reduction of soft agar colony formation ability at the non-permissive temperature. Thus, the results showed dissociation of the properties of morphology, soft agar colony formation, and tyrosine kinase activity.
Fig. 3. Morphology of NIH 3T3 cells transformed with tsOS-170 and wild-type (wt) virus at the permissive (35 °C) or non-permissive (39 °C) temperatures. Uninfected cells are shown as a control. Bar marker represents 100 μm.

Fig. 4. Temperature-independent autophosphorylating activity of NIH 3T3 cells transformed with ts mutants. No autophosphorylated P120 protein was immunoprecipitated with normal rabbit serum.

Our ts mutants exhibited temperature dependence in soft agar colony formation, but not in morphology or tyrosine kinase activity. On the other hand, ts mutants isolated by Kipreos et al. (1987) were ts for these three properties. The ts mutants isolated by Engelman & Rosenberg
Fig. 5. Temperature-independent P120$^{gag-\text{abl}}$ production. No P120$^{gag-\text{abl}}$ was immunoprecipitated from wild-type A-MuLV-infected NIH 3T3 cells with normal rabbit serum (NRS).

(1987) and Takemori et al. (1987) were ts in their morphology and tyrosine kinase activity, but soft agar colony formation was not examined. Therefore, our ts mutants appear different from previously isolated ts mutants. Molecular cloning and DNA sequencing of the seven ts mutants should reveal interesting differences at the gene level. The results suggest that the v-\text{abl} product may have more than one primary intracellular target, as with pp60$^\text{src}$ (Becker et al., 1977; Weber & Friis, 1979; Anderson et al., 1981).

Some ts mutants of Rous sarcoma virus (RSV) showed reductions in focus formation and soft agar colony formation regardless of the expression of tyrosine kinase activity at non-permissive temperatures (Garber et al., 1983; Stoker et al., 1984). It has been shown that lipid association is temperature-sensitive in cells infected with RSV mutant ts NY68, whose pp60$^\text{src}$ membrane association is ts (Garber et al., 1983). The biological characteristics of our ts mutants might be explained by analogy to ts NY68: they might be ts in the interaction responsible for soft agar colony formation between viral tyrosine kinase and substrate(s) for tyrosine kinase in the cell.

In the present study, we used ts growth of transformed fibroblasts in a semisolid medium as the selection marker because anchorage-independent growth had the strongest correlation with tumorigenicity. Furthermore, our procedure makes it possible to check efficiently the temperature sensitivity of growth in a semisolid medium for numerous mutagenized A-MuLV. We isolated seven ts mutants from approximately $10^4$ c.f.u. (probably corresponding to $10^5$ to $10^6$ f.f.u.) of mutagenized A-MuLV. As it is difficult to check a large number of mutagenized A-MuLV for ts focus formation or their effects on B cell differentiation, our procedure is useful to isolate ts mutants from a large amount of mutagenized virus, and could be applied to isolate ts mutants of other transforming viruses.

It is surprising that our seven ts mutants showed similar characteristics although they had been isolated from different virus stocks individually prepared by independent mutagenization events. Four immature B cell transformants independently transformed by tsOS-41 did not exhibit temperature sensitivity in growth and differentiation (as measured by an increase in intracytoplasmic $\mu$-positive cells), whereas three immature B cell transformants independently transformed by tsOS-59 did show temperature sensitivity in growth and differentiation (data not shown). These findings strongly indicate that our seven ts mutants are similar but different. Molecular cloning and DNA sequencing of the seven ts mutants is currently under way. The fact that our ts mutants are similar in phenotype even though isolated independently suggests inherent pressure(s) for preferential selection of such ts mutants.

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Short communication

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