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PRELIMINARY REPORT

# ISOLATION OF TEMPERATURE-SENSITIVE CONDITIONAL LETHAL MUTANTS OF AN AVIAN ADENOVIRUS (CELO), AND LOCALIZATION OF VIRAL ANTIGEN IN CELLS INFECTED WITH THEM

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Chicken embryo lethal orphan (CELO) virus was first described by Yates and Fry (1957). Several characters of this virus are quite similar to those of mammalian adnoviruses (Petek et al., 1963a; Petek et al., 1963b; Dutta and Pomeroy, 1963). From studies on adenovirus DNA (Green et al., 1967), the viral chromosome seems to consist of 30–50 genes. This virus is readily propagated in primary chicken kidney cell (CKC) cultures so I select it as a model system to analyze the replication and oncogenesis of adenovirus using temperature-sensitive conditional lethal (*ts*) mutants, which replicate well at the non-restrictive temperature of 31 C but scarcely at all at the restrictive temperature of 40 C. This paper reports the isolation of these *ts* mutants and the localization of viral antigen in infected cells under restrictive and non-restrictive conditions.

Virus propagation and plaque formation were done in CKC cultures as originally described by Kawamura et al. (1963). The Ote strain of CELO virus, isolated and identified by the cross neutralization test with Phelp's strain of Yates (Kawamura et al., 1963), was kindly supplied by Dr. H. Kawamura. A26, a clone of the Ote strain was obtained by five repeated single plaque isolations in CKC cultures, and was used as the original wild type strain in the present study. Before treatment with mutagen A26 was propagated in CKC at 40 C to dilute out possible spontaneous *ts* mutants in the viral population. The A26 stock thus obtained (infectious titer, $2\times10^9$ PFU/ml) was exposed to hydroxylamine, as described by Freese and Freese (1965). Then, the virus was seeded into CKC cultures after appropriate dilution, and incubated at 31 C for plaque formation. Clones were isolated from well separated plaques and their growth at 40 C and 31 C was examined. About 1,800 clones were examined and 49 *ts* mutants were picked up. Preliminary results indicated that they did not form plaques when $10^3$–$10^5$ PFU were inoculated onto *ca* $10^6$ cells and incubated at 40 C (Table 1). The ratio of infectious

TABLE 1.

| | *Efficiency of Plating at 40 C to at 31 C* |
|---|---|
| *ts* 1 | $<4.3\times15^{-5}$ |
| *ts* 2 | $<1.3\times10^{-5}$ |
| *ts* 3 | $<1.3\times10^{-4}$ |
| *ts* 4 | $<3.8\times10^{-5}$ |
| *ts* 5 | $<2.0\times10^{-4}$ |
| *tt* 8 | $<1.1\times10^{-4}$ |
| *ts* 10 | $<8.2\times10^{-5}$ |
| *ts* 11 | $<2.5\times10^{-6}$ |
| A 26 | $7.0\times10^{-1}$ |

TABLE 2.

*Infectious Virus Yields at 40 C and 31 C*

| mutant | 40C | 31C | Ratio : 31C/40C |
|---|---|---|---|
| *ts* 1 | $1.0\times10^{4}$ | $4.9\times10^{7}$ | $4.9\times10^{3}$ |
| *ts* 2 | $2.1\times10^{3}$ | $7.0\times10^{7}$ | $3.3\times10^{4}$ |
| *ts* 3 | $2.0\times10^{4}$ | $4.2\times10^{7}$ | $2.1\times10^{3}$ |
| *tt* 4 | $5.2\times10^{3}$ | $7.1\times10^{7}$ | $1.4\times10^{4}$ |
| *ts* 5 | $2.3\times10^{3}$ | $4.7\times10^{7}$ | $2.0\times10^{4}$ |
| *ts* 8 | $2.0\times10^{4}$ | $5.2\times10^{7}$ | $2.6\times10^{3}$ |
| *ts* 10 | $1.7\times10^{3}$ | $1.0\times10^{8}$ | $5.9\times10^{4}$ |
| *ts* 11 | $7.1\times10^{2}$ | $1.6\times10^{8}$ | $2.3\times10^{5}$ |
| A26 | $5.4\times10^{8}$ | $3.4\times10^{7}$ | $6.3\times10^{-2}$ |

*CKC cultures were infected by adding 10–20 PFU/cell and incubated at 40 C for 2 hr for adsorption. Then, cultures were washed once with fresh medium and incubated at 40 C or 31 C. Cultures incubated at 40 C were harvested 34 hr after infection, and cultures incubated at 31 C were harvested when the cytopathic change of infected cells was nearly complete*

virus yield after one growth cycle of *ts* mutants at 31 C to that at 40 C was $10^{3}$–$10^{5}$ (Table 2).

Immunofluorescent studies on the viral antigen were done as follows. Partially purified virus was obtained from a lysate of CKC infected with A26 by treatment with fluorocarbon and centrifugation at $70{,}000\times g$ for 90 min. The virus was emulsified with complete Freund's adjuvant and antiserum was prepared by infecting it intramuscularly into a rabbit. $\gamma$-Globulin conjugated with fluorescein isothiocyanate was prepared from the serum as described by Shimojo et al. (1967). Coverslip cultures of CKC infected with one of the *ts* mutants (input multiplicity 10–20 PFU/cell) were fixed with acetone at −20 C for 15 min and allowed to react with the conjugated $\gamma$-globulin for 1 hr. The wild type strain, A26, accumulated viral antigen

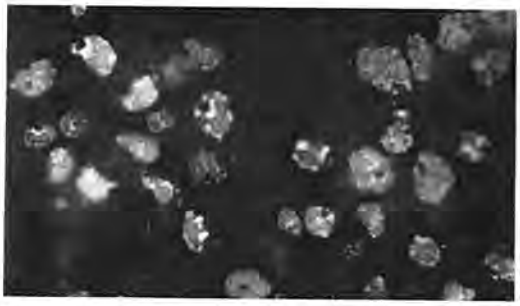

FIGURE 1. *Nuclear accumulation of viral antigen in infected with the wild type strain, A26, after 18 hr incubation at 40 C.*

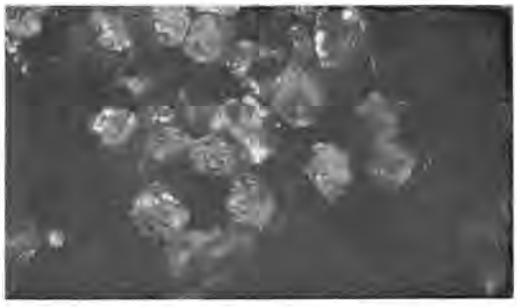

FIGURE 2. *Nuclear accumulation of viral antigen in cells infected with ts8 after 68 hr incubation at 31 C.*

TABLE 3. *Classification of ts mutants on the basis of the localization of viral antigen in infected cells at the restrictive temperature.*

| Type of mutant | Location of viral antigen | Strain number of *ts* mutant | Total |
|---|---|---|---|
| N type | nucleus | *ts* 3, 7, 9, 12, 16, 18, 24, 25, 27, 31, 32, 33, 34, 36, 38, 40, 41, 42, 43, 45. | 20 |
| C type | cytoplasm | *ts* 5, 8, 10, 13, 19, 28, 29, 30, 49. | 9 |
| FA$^{-}$ type | neither in cytoplasm nor nucleus | *ts* 4, 6, 11, 14, 15, 17, 22, 44. | 8 |

in the nucleus at either 40 C or 31 C (Fig. 1). At the non-restrictive temperature of 31C, all the *ts* mutants accumulated viral antigen in the nucleus (Fig. 2), although some *ts* matants retained some antigen in the cytoplasm during an early stage of incubation. As summarized in Table 3, *ts* mutants can be classified into 3

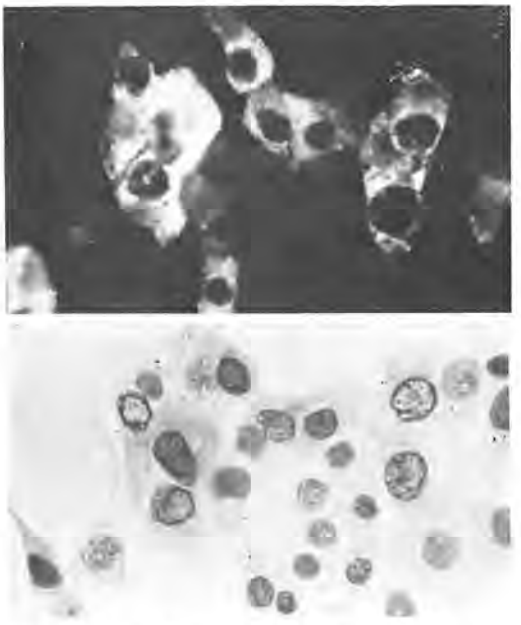

FIGURE 3. *a) Cytoplasmic retention of viral antigen in cells infected with ts8 after 25 hr incubation at 40 C.*
*b) The area shown in a) was observed after Giemsa's staining.*

groups on the basis of the localization of viral antigen in infected cells at the restrictive temperature of 40 C. The first group of *ts* mutants (N type) accumulated the antigen in the nucleus like the wild type strain. The second group (C type) retained viral antigen in the cytoplasm but not in the nucleus even after prolonged incubation (Fig. 3 and 4). By staining immunofluorescent samples with Giemsa's solution it was found that with *ts* 8 cells in which the cytoplasm showed fluorescence (Fig. 3a) had inclusion-like structures in the nucleus (Fig. 3b). However, this staining procedure does not allow such clear elucidation of intranuclear inclusions as hematoxylin-eosin staining after Carnoy's fixation. The third group ($FA^-$ type) produced no, or only slight antigen even after prolonged incubation at

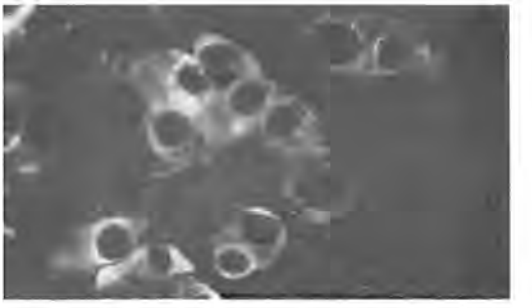

FIGURE 4. *Cytoplasmic retention of viral antigen in cells infected with ts10 after 31 hr incubation at 40C.*

40 C.

When cytosine arabinoside, an inhibitor of DNA synthesis (10 or 40 $\mu$g/ml) was added at the time of virus inoculation, neither cells infected with A26 nor those infected with *ts* 8 showed viral antigen detectable by antibody-staining. This suggests that the antigen observed with the present antibody represented viral structural proteins produced after synthesis of viral DNA.

The above immunofluorescent study demonstrated an unexpected new class of *ts* mutants designated as the C type. At the restrictive temperature they seem to be defective in transportation from the cytoplasm to the nucleus of viral structural protein(s), claimed to be synthesized on cytoplasmic polysomes in a human adenovirus-KB cell system (Thomas and Green, 1966; Velicer and Ginsberg, 1968).

A more detailed report of this work, including results on complementation between *ts* mutants and on temperature-shift will appear elsewhere.

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