Retention of Viral Antigen in the Cytoplasm of Cells Infected with Temperature-Sensitive Mutants of an Avian Adenovirus

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Abstract. Immunofluorescent studies were made on chicken kidney cell cultures infected with various temperature-sensitive (ts) mutants of an avian adenovirus, chicken embryo lethal orphan (CELO), which grow well at the nonrestrictive temperature of 31°, but fail to grow at the restrictive temperature of 40°. At 40° some mutants (ts 5, 8, and 10) accumulated viral antigen in the cytoplasm, but scarcely at all in the nucleus. However, at 31° they accumulated the antigen in the nucleus like the wild-type strain at either 31° or 40°. These mutants seemed to complement each other in nuclear accumulation of antigen at 40°. In cells infected with ts 8, the antigen remaining in the cytoplasm during incubation at 40° was partially transferred to the nucleus even in the presence of an inhibitor of protein synthesis when the temperature was lowered to 31°. The results suggest that viral antigen produced in the cytoplasm is transported to the nucleus and that these ts mutants are defective in this process at 40°.

There are apparently two other types of ts mutant: one type (ts 3, 7, and 12), like the wild-type strain, showing nuclear accumulation of antigen at 40° and the other (ts 6, 11, and 17) showing no, or only slight, formation of the antigen at 40°.

Introduction. Studies by immunofluorescence microscopy1–6 and electron microscopy using the immunoferitin technique7, 8 have shown unequivocally that the nucleus of adenovirus-infected cells is the site where a detectable amount of viral structural antigen accumulates and virus particles assemble. However, Thomas and Green9 recently demonstrated in KB cells infected with adenovirus type 2 that the cytoplasmic polyribosome fraction contained much of a messenger type of RNA, hybridizable with denatured type 2 adenovirus DNA and that this fraction actively incorporated labeled amino acids. Using an immune coprecipitation technique with KB cells infected with type 5 adenovirus, Velicer and Ginsberg10 confirmed that cytoplasmic polyribosomes are the site of synthesis of viral structural proteins. They also suggested from autoradiographic studies that after synthesis viral proteins are rapidly transported to the nucleus.

In the present work I used temperature-sensitive (ts) mutants of an avian adenovirus, chicken embryo lethal orphan (CELO), which replicated well at the nonrestrictive temperature of 31° but scarcely at all at the restrictive temperature of 40°. Immunofluorescent studies on these suggest that at the restrictive
temperature one group of ts mutants is defective in the transportation of viral antigen from the cytoplasm to the nucleus, and seem to confirm the conclusion of Velieer and Ginsberg\textsuperscript{18} from their autoradiographic studies.

**Materials and Methods.** Virus: CELO virus, an avian adenovirus, was first described by Yates and Fry.\textsuperscript{11} Several characteristics of this virus,\textsuperscript{12–17} including its helper function in replication of adeno-associated viruses,\textsuperscript{18} are quite similar to those of mammalian adenoviruses. The Ote strain was isolated by Kawamura et al.\textsuperscript{19} from a chicken in Japan and identified by the cross-neutralization test with Phelp's strain of Yates. A26, a clone of the Ote strain, was obtained by repeating single-plaque isolation five times in monolayer cultures of chicken kidney cells following the method of Kawamura et al.\textsuperscript{19} and was used as the original wild-type strain in the present study. The ts mutants used here were independently isolated from A26 stock exposed to hydroxylamine as described by Freese and Freese.\textsuperscript{20} These mutants and A26 were neutralized in the same manner by the antiserum described below. The yields of infectious virus after infections with these ts mutants at 40° were 10⁻²–10⁻⁵ of that of A26 at the same temperature. The isolation and preliminary genetic characterization will be published in detail elsewhere.

**Cell culture and virus inoculation:** Monolayer culture of the primary chicken kidney cell was done as originally devised by Kawamura et al.\textsuperscript{21} The growth medium was YLE (Earle's balanced salt solution supplemented with 0.5% lactalbumin hydrolysate and 0.1% yeast extract) supplemented with 5% bovine serum. Unless otherwise stated, the culture was washed with YLE, seeded with virus (at an input multiplicity of 10 plaque-forming units per cell), and incubated at 37° for 90 min to allow for adsorption of virus. After adsorption, maintenance medium (YLE containing 2.5% bovine serum) was added and the cultures were immediately incubated at the temperature indicated in the results. The first 90-min period of incubation at 37° to allow adsorption of virus is not included in the incubation times reported in later sections. In the experiment with cytosine arabinoside, LE (Earle's balanced salt solution supplemented with 0.5% lactalbumin hydrolysate) without added serum was used as maintenance medium.

**Preparation of antiserum:** A lysate of cells infected with A26 was treated twice with fluorocarbon (Daifron S3) and the aqueous fraction was centrifuged at 70,000 g for 90 min. The resulting pellet was suspended in a small volume of 0.01 M Tris-HCl buffer, pH 8, and emulsified with Freund's complete adjuvant. At 10-day intervals, a rabbit was injected intramuscularly with 0.6 ml of the emulsion. Ten days after the third injection, the animal was exsanguinated to obtain immune serum. The serum was inactivated by heating at 56° for 30 min. Its neutralization titer by the 50% plaque-reduction method was higher than 1:10,000.

**Immunofluorescent staining:** The direct method was used. Fluorescein-conjugated γ-globulin was prepared from the above antiserum as described by Shimojo et al.\textsuperscript{5} The cultures of infected cells on cover slips were fixed by treatment with acetone at -20° for 5 min, and allowed to react with conjugated globulin (staining titer, 4 units) at 37° for 1 hr. In some cases part of the sample was fixed by treatment with acetone at room temperature for 15 min and this procedure gave similar results.

**Results.** Two series of cultures on cover slips each inoculated with one of the ts mutants or the wild-type strain were incubated at the restrictive temperature of 40° and the nonrestrictive temperature of 31°, respectively. At intervals, duplicated cultures in the two series were sampled, and each coverslip was divided into three pieces. One piece was used for immunofluorescent staining and the others were fixed with Carnoy's fluid and stained with hematoxylin-eosin and by the Feulgen reaction, respectively.

Results of immunofluorescent staining for viral antigen are summarized in Table 1. Cells infected with the wild-type strain (A26) and with ts 8 were followed sequentially. At 40° where one growth cycle of wild-type strains of
Table 1. Location of viral antigen in chicken kidney cells infected with the wild-type strain or ts mutants.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>5</th>
<th>8</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>A26</td>
<td>no</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>ts 8</td>
<td>no</td>
<td>C*</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>ts 5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>C</td>
<td>C</td>
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<tr>
<td>ts 10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

Symbols: N, cells carrying antigen exclusively in the nucleus; C, cells with antigen exclusively in the cytoplasm; CN, cells with antigen in both the cytoplasm and nucleus; no, cells with no detectable antigen in either cytoplasm or nucleus; —, not tested.

* At this time of sampling, less than 10% of the cells showed fluorescence, while later the percentage increased to ca. 50% or more.

CELO virus is known to require 20–25 hours, which is two-thirds of that at 37°,²² cells infected with A26 began to show nuclear fluorescence after 8 hours incubation, and more than 50 per cent of the cells on the cover slip showed nuclear fluorescence after 12 hours (Fig. 1a). However, cells infected with ts 8 at 40° showed rather diffuse homogeneous fluorescence from the time of its first detection to a late stage of incubation in the cytoplasm, as shown in Figure 1b. In the center of each cell there was a round dark area negatively outlined by the cytoplasmic fluorescence. These central dark areas were found to correspond to the nuclei of the cells by staining the same samples with Giemsa stain after fluorescence microscopy (Fig. 1c). Moreover, cells in which the cytoplasm stained with fluorescent antibody had a nucleus showing higher affinity for Giemsa stain or showing inclusion-like structures. Unfortunately this staining procedure does not allow such clear elucidation of intranuclear inclusions as hematoxylin-eosin staining after Carnoy’s fixation. However, these observations indicate that cells infected with ts 8 at 40° retain the viral antigen in the cytoplasm but scarcely accumulate it at all in the nucleus. At 31° where one growth cycle of A26 requires about 3 times as long as that at 40°,²² cells infected with either A26 or ts 8 accumulated antigen in the nuclei and did not retain it in the cytoplasm. After five-hour incubation at 40° and 12 hours at 31° cells infected with A26 or ts 8 did not show any specific staining like uninfected control cultures. The T or P antigen in cells infected with human adenoviruses is reported to give characteristic flecks and dots of fluorescence in the cytoplasm,³–⁶ but I did not observe fluorescence of this type. Cells infected with ts 5 or ts 10 at 31° or 40° had essentially the same fluorescent appearance as cells infected with ts 8 (Table 1 and Fig. 1d), although with ts 5 or ts 10 nuclear accumulation of viral antigen was slow at 31°. Cells infected with ts 5 accumulated antigen in both the cytoplasm and nucleus (CN type*) and those with ts 10 showed both C- and CN-type fluorescence after 48-hour incubation at 31°, but later almost all cells with fluorescence became N type. This suggests that control of nuclear accumulation of viral antigen is partially impaired, even at 31°, in cells infected with these mutants.
FIG. 1.—Chicken kidney cell culture infected with (a) A26, the wild-type strain of CELO virus, sampled after 12-hr incubation; (b), (c) ts 8, after 24 hr; (d) ts 5, after 24 hr; (e) ts 8 together with ts 10, after 32-hr incubation. The incubation temperature was 40°C; (a), (b), (d), and (e), show immunofluorescent staining of viral antigen, and (c) shows Giemsa staining of the area shown in (b).

Hematoxylin-eosin staining and the Feulgen reaction demonstrated intranuclear inclusions with high DNA content in cells infected with the three mutants as well as the wild-type strain at both 31°C and 40°C. The number of cells showing these changes in nuclei seemed to correspond with the number of fluorescent cells.

**Possible complementation between ts mutants:** Mutants ts 8 and ts 10 were simultaneously inoculated in a cover-slip culture each at an input multiplicity of 10 plaque-forming units per cell and incubated at 40°C for 32 hours. After fluorescent antibody staining, it was roughly estimated that there were equal numbers of CN- and C-type cells (Fig. 1e). When the input multiplicity of each
mutant was 20, more than 80 per cent of the fluorescent cells were CN and N type. However, scarcely any CN- or N-type cells were observed under similar conditions on infection with either mutant singly. Similar results were obtained on double infection with two other pairs of mutants (ts 5 and ts 8, ts 5 and ts 10).

Inhibition of antigen production by cytosine arabinoside: Cytosine arabinoside (10 or 40 \( \gamma \)/ml) was added to coverslip cultures at the beginning of infection with A26 or ts 8 at 40°. Twenty hours later neither culture showed fluorescent cells, whereas infected cultures without the drug showed numerous fluorescent cells, as cited in Table 1. Hematoxylin-eosin staining showed that cells infected in the presence of the drug carry one or more eosinophilic inclusion in the nucleus. These inclusions were not stained by the Feulgen reaction. The number of nuclei with eosinophilic inclusions was equal to the number of nuclei with characteristic advanced type inclusions\(^8\) in infected cultures without the drug.

Effect of shift down of temperature: Cells inoculated with ts 8 were incubated at 40° for 21 hours and then at 31° for 10 hours. To inhibit de novo protein synthesis during incubation at 31°, 10 or 40 \( \gamma \)/ml of cycloheximide was added one hour before lowering the temperature. After incubation, about equal numbers of infected cells showed CN-type and C-type fluorescence. However, the infected cells not transferred to 31° but kept at 40° for the same duration showed only C-type fluorescence. The results indicate that about 50 per cent of C-type cells at 40° became CN type even in the absence of de novo protein synthesis when the temperature was lowered to 31°. To confirm the effect of cycloheximide as an inhibitor of protein synthesis, the above concentrations of the drug were added to cultures infected with ts 8 after five hours incubation at 40°. In its presence there was neither production of a detectable amount of antigen nor formation of intranuclear inclusions.

Other types of ts mutant: On the basis of immunofluorescent studies, there appeared to be two other types of ts mutant. One type (ts 3, 7, and 12) formed exclusively N-type fluorescence within 24 hrs after incubation at 40°. The other type (ts 6, 11, and 17) gave no, or only slight, fluorescence even after 32 hours incubation at 40°.

Discussion. The present results showed that cells infected with one type of ts mutants (ts 5, 8, and 10) scarcely accumulated any viral antigen in the nucleus but retained it in the cytoplasm at the restrictive temperature of 40°, whereas at the nonrestrictive temperature of 31°, they accumulated it in the nucleus like wild-type strain at either 31° or 40°. The results agree with the conclusion of Velicer and Ginsberg\(^6\) from experiments with a human adenovirus that viral structural proteins synthesized on the cytoplasmic polyribosomes are rapidly transported to the nucleus. Furthermore, the present results with ts mutants suggest that the normal conformation or function of protein(s) coded by the viral gene(s), on which ts mutation 5, 8, and 10 took place, somehow contributes to this process. The experiment on the effect of a temperature shift showed that the viral antigen remaining in the cytoplasm during incubation at 40° was partially transferred to the nucleus even in the absence of de novo protein synthesis when the temperature was lowered to 31°, suggesting that viral protein(s) necessary for antigen transportation was already formed, but was not active at
40°. The above results of the complementation tests suggest that several gene-products work together in nuclear accumulation of antigen.

It has been reported that inhibitors of DNA synthesis do not prevent the production of T antigen or P antigen of adenoviruses, but do prevent the production of capsid antigen.5, 6, 22, 24 The antigen observed in this immunofluorescent study was not produced in the presence of cytosine arabinoside, an inhibitor of DNA synthesis. The result suggests that the antigen observed was viral structural antigen produced after viral DNA synthesis.

The mutants so far examined at the restrictive temperature could be classified into three groups from the resulting immunofluorescence: (1) mutants accumulating viral antigen in the nucleus like the wild-type strain, (2) mutants retaining it in the cytoplasm, and (3) those forming no or only slight detectable antigen.

I am grateful to Dr. H. Kawamura for his generous supply of the Ote strain of CELO virus and technical advice and to Dr. H. Shimojo for his critical reading of the manuscript.

* Abbreviation used: CN type, type of cells accumulating viral antigen in both nucleus and cytoplasm; N type, cells with antigen only in the nucleus; C type, cells with antigen only in the cytoplasm.

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