THE MECHANISM OF INTERNUCLEAR TRANSMISSION OF
SV40-INDUCED COMPLEMENT FIXATION ANTIGEN IN
HETEROKARYOCYTES*

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During the investigation of the action of SV40 virus by means of fusion of
SV40-transformed cells with virus-susceptible monkey kidney cells,1 it became
apparent that SV40-induced complement fixation antigen (ICFA)2 was trans-
mitted to the nuclei of the nontransformed cells whether or not fusion between the
two systems resulted in the isolation of infectious virus.1, 2 This was not sur-
prising, since the mechanism of ICFA synthesis apparently is unrelated to
the synthesis of viral protein and infectious virus, and the antigen is found in many
non-virus-yielding transformed cells. As a corollary of these observations, an
investigation of transmission of ICFA in heterokaryocytes to nuclei originating
from cells resistant to SV40 infection became of interest. Further, a study of the
mechanism of the transmission of ICFA was instituted to elucidate the process of
its intracellular synthesis.

Materials and Methods.—Tissue cultures: Table 1 lists cell types used for fusion exper-
iments. The origin and history of SV40-transformed human, monkey, and hamster cell
lines were described previously.1 ICFA (T-antigen)2 was present in cell nuclei of all
these lines, referred to as “donor” lines. The “recipient” cells described in Table 1 were
characterized by the absence of ICFA in their nuclei. This was confirmed repeatedly
during each fusion experiment.

All cells were grown in double-strength Eagle’s basal medium in Earle’s balanced salt
solution with 10% fetal calf serum and aureomycin (50 μg/ml).

Technique of cell fusion: The fusion technique was essentially similar to the one de-
scribed previously.1 Recipient cells were labeled with 0.2 μc/ml thymidine methyl-H3
(specific activity 14.5 c/mM) for 4 days prior to fusion. Metabolic inhibitors, FUdR
(Hoffman LaRoche, Nutley, N. J.), actinomycin (Lyovac Cosmogen, Merck Sharp and
Dohme, Rahway, N. J.), and cycloheximide (Actidione, Upjohn, Kalamazoo, Mich.),
were added at the time of fusion, and the fused cells were then grown in inhibitor-con-
taining medium.

SV40 ICFA determination and autoradiographic techniques: The presence of ICFA
was detected according to the previously described technique.1 At least 1000 nuclei
were counted under the fluorescent microscope, and nucleograms were constructed, show-
ing the percentage of the total cells counted containing various proportions of fluorescent
and nonfluorescent nuclei. The percentage of multinucleated cells containing only
ICFA-positive nuclei was determined and used as an index of transmission of ICFA
(see Figs. 1, 3, 4, and 5). In some experiments we determined the origin of the nuclei in
multinucleated fluorescent cells by subsequent autoradiography of the same cells, as
previously described.1

Results.—Appearance of ICFA in the nuclei of recipient cells after fusion with
transformed ICFA-containing donor cells: The results in Table 1 indicate that
ICFA always appeared in the nuclei of the recipient cells after fusion with SV40-
transformed cells. Transmission of ICFA took place regardless of the origin of the
donor and recipient cells and regardless of whether the transformed cell lines
were capable of producing infectious SV40 spontaneously or after fusion with
susceptible cells. ICFA was also transmitted from F5-1 and from three GMK EVa clones from which infectious virus has never been recovered.

Transmission of ICFA was also unrelated to the susceptibility of the recipient cell types to SV40 infection, since ICFA was transmitted not only to African green monkey kidney (AGMK) and CV-1 cells, which are susceptible to infection, but also to mule^4 and hamster embryo cells, both of which are much more resistant to infection than AGMK and CV-1 cells.

Figure 1 presents results of ICFA transmission obtained after the fusion of

![Graph](image)

**Fig. 1.—Transmission of SV40 ICFA after fusion of F5-1 with AGMK cells.**

Cells containing only ICFA (+) nuclei, ——; cells containing only ICFA (−) nuclei, . . . . ; cells containing both ICFA (+) and ICFA (−) nuclei, ———.

F5-1 cells with AGMK cells. The percentage of multinucleated cells containing only ICFA-positive nuclei rose rapidly between three and nine hours after fusion, with a concomitant decrease in the percentage of multinucleated cells containing both ICFA-positive and ICFA-negative nuclei. The percentage of multinucleated cells containing only ICFA-negative nuclei remained essentially unchanged throughout the observation period, indicating that these cells did not contribute to the increase in cells containing only ICFA-positive nuclei. It appears that transmission of ICFA from the donor to the recipient nucleus occurs shortly after fusion and is virtually complete by 25 hours, when less than 7 per cent of the multinucleated cells contain both ICFA(+) and ICFA(−) nuclei.

Autoradiographic studies showed that 24 hours after fusion of various types of donor and recipient cells, approximately 30–50 per cent of the multinucleated cells are true heterokaryocytes.

Figure 2 shows the same cells after ICFA staining (A, C) and autoradiography (B, D). In the cell in Figure 2A, two of the five ICFA-positive nuclei are labeled (B) and are from the recipient cells. In Figure 2C, one of the six ICFA-positive nuclei on the lower left is heavily labeled (D) and is clearly a nucleus from a recipient cell.

**Effect of metabolic inhibitors on transfer of ICFA:** Since synthesis of ICFA in
the nuclei of the donor cells must be directed in the course of numerous passages by some part of the viral genome dividing in synchrony with the cells, transmission of ICFA in heterokaryocytes may be mediated by the passage of this viral genome from the donor to the recipient nucleus. Transmission may also occur through the passive transfer of already synthesized antigen or by transfer of mRNA to the recipient nuclei, followed by synthesis of ICFA. ICFA may also be synthesized in the cytoplasm of the heterokaryocytes and then taken up by the recipient nucleus without passage of the mRNA from the donor to the recipient nucleus. In order to investigate these possibilities, experiments with metabolic inhibitors were initiated.

In preliminary investigations, transmission of ICFA was not affected by the addition of $10^{-6} M$ 5-fluoro-2'-deoxyuridine (FUdR) to F5-1 cells at the time of fusion with HE or AGMK cells. Further experiments indicated that the fusion of W98VaE and AGMK cells was not affected by $10^{-5} M$ or $10^{-4} M$ FUdR with $10^{-5} M$ thymidine and that the percentage of multinucleated cells containing only ICFA-positive nuclei was the same at 48 hours after fusion

<table>
<thead>
<tr>
<th>Donors</th>
<th>Isolation of Infectious SV40</th>
<th>After fusion with AGMK</th>
<th>Transmission of ICFA to “recipient” cell nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species of origin</td>
<td>Spontaneously or by cocultivation</td>
<td>virus</td>
<td></td>
</tr>
<tr>
<td>Cell line</td>
<td></td>
<td></td>
<td>Recipients</td>
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<tr>
<td>W126V2a</td>
<td>Man</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>W18V2a</td>
<td>Man</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W98VaE</td>
<td>Man</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMK Eva</td>
<td>None</td>
<td>+</td>
<td>AGMK +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl. 5-1</td>
<td>Green monkey</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Cl. 2A-1</td>
<td>Green monkey</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cl. 2B-1</td>
<td>Green monkey</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cl. 8-1</td>
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<tr>
<td>GMK LL&quot;E-46&quot;</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
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<tr>
<td>SV-3T3-101‡</td>
<td>Mouse</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

AGMK, primary cultures of African green monkey kidney; CV-1, an established AGMK line; Mule, mule fibroblast culture; KB, cell line originating from human tumor; HE, secondary culture of hamster embryo fibroblasts.

* With SV40-susceptible green monkey kidney cells.
† SV40-induced hamster tumor cultivated for numerous generations in tissue culture.
‡ A clone obtained from Dr. Howard Green, Dept. of Pathology, New York University School of Medicine.
Fig. 2.—Heterokaryocytes between W98VaE and AGMK cells. SV40 ICFA fluorescent staining and autoradiograms on the same cells, as described in text.

(A) Positive fluorescence for SV40 ICFA in all nuclei.

(B) Autoradiogram of the same heterokaryocyte shows two labeled nuclei (arrows in A).

(C) Positive fluorescence for SV40 ICFA in cell nuclei.

(D) Autoradiogram of the same heterokaryocyte shows one (arrow in C) heavily labeled (AGMK) nucleus.

(E, F) Treated with cycloheximide for 48 hr after fusion. (E) One of six nuclei with positive fluorescence for SV40 ICFA. (F) Autoradiogram of the same heterokaryocyte shows five labeled AGMK nuclei which did not fluoresce in (E).
regardless of the presence or absence of FUdR. This indicates that replication of DNA is not necessary for internuclear transmission of ICFA.

To investigate the possibility that previously synthesized antigen is passively transferred from one nucleus to another in a heterokaryocyte, fusion was performed in the presence of 30 μg/ml of cycloheximide, a protein synthesis inhibitor. In this experiment, F5-1 and AGMK cells were fused in the presence of 30 μg/ml of cycloheximide; the inhibitor was then left in the medium at the same concentration for 26 hours of incubation. In another set of cultures, the cells were fused in the absence of the inhibitor; cycloheximide was then added, either immediately after the cells were seeded on Petri dishes, or five hours later. One set of cells from the same fusion was kept as control without cycloheximide.

The results of these experiments are shown in Figure 3. It can be observed that cycloheximide added at the time of fusion or immediately after fusion effectively blocked transmission of ICFA. The percentage of multinucleated cells containing ICFA-positive and ICFA-negative nuclei did not change essentially in the presence of cycloheximide, but decreased, in the control cultures, from 56 per cent at 3 hours after fusion to 3 per cent at 26 hours after fusion. In contrast, in the control cultures, the percentage of multinucleated cells containing only ICFA-positive nuclei increased after fusion from 10 to 65 per cent within 26 hours; in the

![Figure 3](image-url)

**Figure 3.** The effect on transmission of SV40 ICFA of cycloheximide added at time of fusion and at 0 and 5 hr after fusion of F 5-1 and AGMK cells.

Control, 
Cycloheximide added at time of fusion, 
Cycloheximide added at 0 hr of fusion, 
Cycloheximide added at 5 hr after fusion,
presence of cycloheximide the percentage of multinucleated cells with only ICFA (+) nuclei remained the same even 26 hours after fusion. No difference was observed in the percentage of multinucleated cells containing only ICFA-negative nuclei whether they were or were not treated with cycloheximide. Addition of the inhibitor five hours after fusion, when internuclear transmission of ICFA had already begun (see above), resulted in the inhibition of ICFA transmission.

Figures 2E and F show a representative heterokaryocyte after treatment for 48 hours with cycloheximide. It can be noted that, in contrast to Figure 2A, B, C, and D, the labeled "recipient" cells' nuclei do not show ICFA-fluorescence.

Inhibition of ICFA transmission by cycloheximide seemed to indicate that protein synthesis may be necessary for ICFA transmission.

In order to investigate the role of mRNA in ICFA transmission, F5-1 cells and HE cells were fused in the presence of actinomycin D (Fig. 4). Because of the cytotoxic effect of the inhibitor, observations of the culture could not be extended beyond 26 hours after fusion. The results shown in Figure 4 indicate that at 26 hours after fusion, in the absence of the inhibitor, 78 per cent of the multinucleated cells contained only ICFA-positive nuclei, whereas the corresponding figure obtained for cells exposed to actinomycin D (0.1, 0.5, and 1.0 μg/ml) was below 5 per cent.

A similar suppressive effect of actinomycin D on transmission of ICFA was observed in heterokaryon cultures obtained by fusion of W126Va4 or GMK-EVα cells with AGMK cells. These results indicate that actinomycin effectively blocks internuclear transmission of ICFA, regardless of species origin of the transformed cell line.

Since, in these experiments, actinomycin was added to the cultures at the time of fusion, we may conclude that inhibition of mRNA synthesis occurred at the
level of the donor nucleus. However, it was still impossible to distinguish whether synthesis of ICFA took place in the cytoplasm of the heterokaryocyte under the direction of mRNA from the donor nucleus, or in the recipient nucleus after internuclear passage of mRNA. In order to test these two possibilities, an experiment was designed in which cycloheximide was removed from the fused cultures after a short period (5 or 9 hr) (Fig. 5). After removal of the medium with the inhibitor and washing of the cultures, normal medium was added, and the cells were examined for internuclear ICFA transmission at various time intervals. If, during the period of inhibition of protein synthesis, passage of mRNA had occurred to the recipient nucleus, resumption of ICFA synthesis following removal of inhibitor would rather rapidly occur. This, as shown in Figure 5, was not the case, since the appearance of ICFA in the recipient nuclei in heterokaryocytes was gradual. Thus, either we are dealing with an unstable messenger, or ICFA is being produced in the cytoplasm of the heterokaryocytes and the finished product is slowly taken up by the recipient nucleus.

Discussion.—Analysis of the effect of metabolic inhibitors on transmission of ICFA which takes place in heterokaryocytes produced through fusion of ICFA-positive SV40-transformed donor cells with ICFA-negative recipient cells indicates that replication of the gene directing ICFA synthesis is not necessary for ICFA transmission from the donor to the recipient nucleus. It is also highly improbable that a previously synthesized antigen is passively transferred from one nucleus to another since, in this case, the presence of cycloheximide would not have inhibited transfer. Our results showing the effective blocking of internuclear ICFA transmission by actinomycin D are similar to the results obtained by

**Fig. 5.**—Effect of removal of cycloheximide on the transmission of SV40 ICFA after fusion of W98 VaE with AGMK cells.
others\(^6\) that actinomycin definitely inhibits ICFA formation after exposure of AGMK cells to SV40 virus.

The site of synthesis of ICFA protein is a matter of conjecture as long as it is not known whether synthesis of any protein of this type may take place in the nucleus. Although ICFA is localized by means of ferritin-conjugated antibody only in the nucleus of an SV40-infected cell, and not in its cytoplasm,\(^5\) \(^7\) the higher concentration of the antigen in the nucleus may account for its visibility; a lower concentration in the cytoplasm would not be visible by this type of observation. ICFA apparently does not represent a "shuttle cytonucleoprotein" similar to the one described by Byers \textit{et al.},\(^8\) since it cannot transfer by simple diffusion as a preformed product. It is most probable that ICFA, similarly to the adenovirus 2-coded protein,\(^9\) is synthesized in the cytoplasm of a heterokaryon under the direction of mRNA from the nucleus of the transformed donor cell; the finished product is then "taken up" by the recipient nuclei. This suggests that the absence of viral coat protein in these nuclei cannot be ascribed to their inability to take up proteins synthesized outside the nucleus. Within this context it is doubtful that presence of ICFA in the nucleus may \textit{per se} be considered as a hallmark for the transformation of the cells.

**Summary.**—Fusion of transformed cells with cells of various origins results in the transmission of ICFA to the recipient nuclei. This transmission is insensitive to FUdR, indicating that DNA synthesis is not necessary. Actinomycin D inhibits ICFA transmission, indicating the necessity of new mRNA synthesis for transmission. Cycloheximide also inhibits the transmission of ICFA, indicating that protein synthesis is necessary for transmission; simple diffusion does not account for the observed internuclear protein migration.

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