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VIRUS MULTIPLICATION IN CULTURE CELLS SYNCHRONIZED BY EXCESS THYMIDINE TREATMENT
II. EFFECT OF VIRAL DNA SYNTHESIS OF SHOPE FIBROMA VIRUS AND COWPOX VIRUS UPON NUCLEAR DNA SYNTHESIS OF SYNCHRONIZED FL CELLS

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SUMMARY Viral and cellular DNA synthesis were examined in synchronized FL cells by double excess thymidine treatment using autoradiography. FL cells were infected at various stages in the cell cycle with Shope fibroma virus or cowpox virus, to compare the effects of an oncogenic poxvirus and a non-oncogenic poxvirus on DNA synthesis in the host cell. The two viruses had similar effects. When viral DNA was synthesized during the G_1 phase, cellular DNA synthesis did not occur. When viral DNA synthesis began in the S phase, cellular DNA synthesis was partially but not completely, inhibited, and proceeded during the period corresponding to the physiological S phase.

INTRODUCTION

The viral DNA synthesis of poxvirus, which occurs in the cytoplasm, is easily distinguishable from nuclear DNA synthesis in cells at a cellular level by autoradiography of ^{3}H-thymidine (Magee 1960; Kato et al. 1960; Cairns 1960). The site of viral DNA synthesis of poxvirus corresponded to the " B " type inclusions of poxvirus which seem to be a good indicator of virus growth at a cellular level (Kato et al., 1960, 1964 and 1965). Kato et al. also noticed that nuclear DNA synthesis of the inclusion bearing cells is generally suppressed. Quantitative analysis of autoradiograms of cells infected with cowpox virus, or oncogenic poxviruses such as Shope fibroma virus and molluscum contagiosum agent, revealed that immediately after viral DNA synthesis begins, nuclear DNA synthesis is suppressed (Kato et al. 1964, 1965 and 1966; Tanigaki and Kato, 1967). Kit et al. (1963) also observed suppression of nuclear DNA synthesis in cells infected with vaccinia virus. Mantani et al. (1968) made more precise studies on this supression of nuclear DNA synthesis in synchronized cultures infected with poxviruses using autoradiography of ^{3}H-thymidine and found

that nuclear DNA synthesis in synchronized cells showing viral DNA synthesis was definitely, but not completely, suppressed and occurred exclusively during the period corresponding to the physiological S phase of these cells. The preceeding paper (Mantani et al., 1970), showed that Shope fibroma virus could multiply in the presence of excess thymidine and this excess thymidine had no direct effect on virus growth. Thus it is possible to keep cells infected with Shope fibroma virus in the early S period by treatment with excess thymidine, and to allow the virus to propagate in the presence of excess thymidine.

Using this method and synchronizing cells infected with Shope fibroma virus, the interactions between cellular DNA synthesis and poxvirus growth, especially the oncogenic poxvirus, Shope fibroma virus were studied.

MATERIALS AND METHODS

1. *Virus*

Cowpox virus (CPV) (LB red strain) and Shope fibroma virus (SFV) (Madison strain) were used. The procedures used to prepar virus materials were described in the previous report (Mantani et al., 1970).

2. *Virus titration*

Plaque assays of CPV and SFV were carried out as described previously (Mantani et al., 1970).

3. *Preparation of cloned cells*

Cloned FL cells was used throughout and were prepared as described previously (Mantani et al., 1968).

4. *Treatment with excess thymidine (E-Tdr) and method of assay in Leighton tubes*

The cloned cells were dispersed in Leighton tubes with cover glasses at the bottom. Eagles'minimum essential medium (MEM) supplemented with 10% calf serum was used as growth medium (GM). After one day, the GM was replaced by medium containing 2.5 mM-Tdr. The cloned FL cells were treated twice at 24 hr intervals with E-Tdr. Treatment with E-Tdr and the method of assay were as described by Mantani et al. (1968).

5. *Virus infection of synchronous cultures in Leighton tubes*

The method of assay of non-infected Leighton cultures was described above. As shown in Fig. 1, another series of Leighton cultures treated with E-Tdr was infected with CPV or SFV at various phases of the cell cycle.

Four experiments (A, B, C, D) were made.

A) FL cells were infected with viruses soon after removal of E-Tdr to allow viral DNA synthesis during the S phase.

B) FL cells were infected with SFV 13 hr after removal of E-Tdr and with CPV 21 hr after its removal to allow viral DNA synthesis during the G_1 phase.

C) FL cells were infected with SFV in the presence of E-Tdr. Seven hr later E-Tdr was removed and synchronization of both infected and noninfected cells started.

D) FL cells were infected with SFV in the presence of E-Tdr. After 7, 17 and 22 hr E-Tdr was removed. Samples were taken at the beginning of the S phase, namely 6 hr after removal of E-Tdr.

A period of 1 hr was allowed for CPV adsorption and of 2 hr for SFV adsorption. After adsorption, cells were washed 3 times with Hanks' solution and incubated with GM (A, B) or with GM containing 2.5 mM E-Tdr (C, D).

GM containing 2.5 mM E-Tdr was removed by washing with Hanks' solution at appropriate times after infection, and replaced by GM. The cover glasses of two Leighton tubes were taken out at appropriate times. One hr before taking out the cover glasses, GM was replaced by prewarmed MEM containing ^{3}H-Tdr. The concentration of the isotope was 2 μc/ml (Specific activity 5 c/mM) and all subsequent procedures were the same as for assay of non-infected synchronous cultures.

6. *Autoradiography*

The procedure used for autoradiography was as described previously (Mantani et al., 1970). The exposure times were 3 days for FL cells infected with CPV and 6 days for those infected with SFV. Giemsa was employed for post-staining to demonstrate "B" type inclusions of poxvirus. The percentage of labeled cells was estimated on at least 500 cells.

RESULTS

1. *Synchronous nuclear DNA synthesis of FL cells*

Synchronous nuclear DNA synthesis was similar to that reported by Bootsma et al. (1964) and Mantani et al. (1968). Pulse labeling with ^{3}H-Tdr showed that after double thymidine treatment, 80–90% of the cells were in the S phase between 4 and 9 hr after removal of E-Tdr (Fig. 9), and that less than 10% of the cells were in the S phase between 16–23 hr after removal of E-Tdr (Fig. 10). The peak of the mitotic index was about 15% 15 hr after removal of E-Tdr (Fig. 11). Twenty six hr after removal of E-Tdr the cells entered the S phase following G_1 although the degree of synchronization was much worse.

The average time of each period of the cell cycle are shown in Fig. 1.

2. *Nuclear DNA synthesis of synchronous FL cells infected with SFV or CPV soon after removal of E-Tdr*

These experiments were designed to compare the nuclear DNA synthesis of synchronous FL cells infected with virus with that of non-infected cells.

One ml of the virus inoculum was introduced into each Leighton tube just after removal of E-Tdr, and the methods shown in Fig. 1 (Expt. A) were followed. At each point, cells were divided into 2 groups. That is, cells showing viral DNA synthesis in cytoplasmic areas which corresponded exclusively to that of the "B" type inclusions of poxvirus, and cells showing both cytoplasmic and nuclear DNA synthesis.

The term "B" type inclusion-bearing cells ("B"-bearing cells) is used here after to represent cells showing cytoplasmic DNA synthesis. The percentage of cells in each group were counted as follows. (1) Percentage of "B"-bearing cells among total cells. (2) percentage of "B"-inclusion bearing cells with labeled nuclei (with more than 10 grains per nucleus). The percentages were calculated

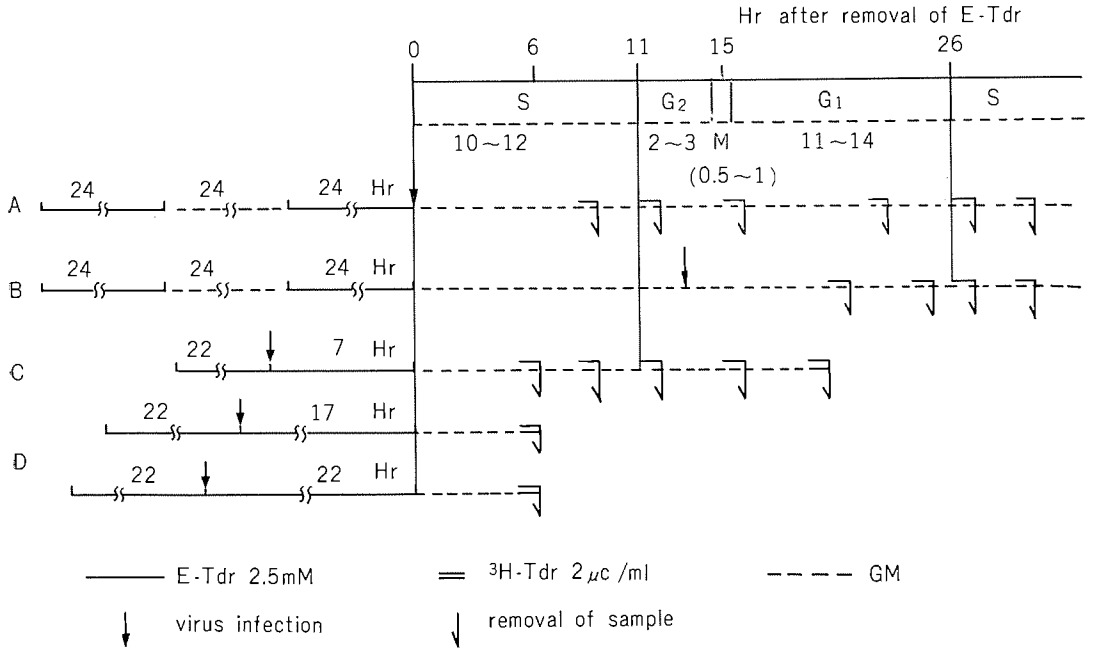


FIGURE 1. *Scheme of experiment on double thymidine treatment and virus infection. Two Leighton tubes were used at each point.*

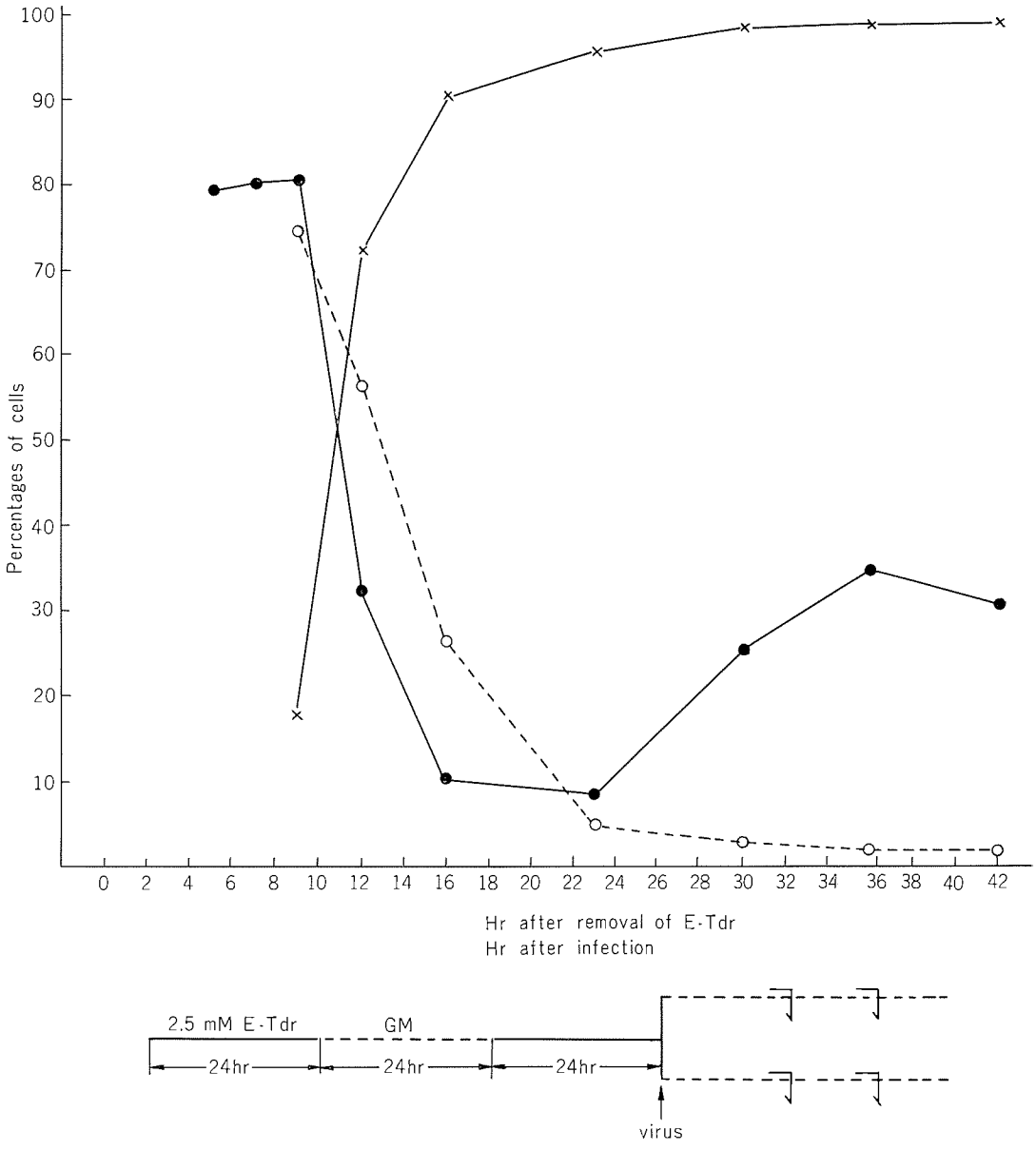


FIGURE 2. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with SFV just after removal of E-Tdr.*

moi 100

●——● *non-infected cells with labeled nuclei*

○------○ *" B "-bearing cells with labeled nuclei*

×——× *" B "-bearing cells*

on more than 500 randomly selected cells.

Generally the number of grains on the nucleus of cells showing cytoplasmic viral DNA synthesis was less than that on the nucleus of non-infected cells through the experiments.

As shown in Fig. 2, the number of " B "-bearing cells increased rapidly after infection and was maximam 14 hr after infection.

The curve of the " B "-bearing cells with labeled nuclei apparently corresponded to that of non-infected cells till 22 hr after removal of E-Tdr. Then non-infected cells gradually entered the second S phase, 24 hr after removal of E-Tdr. But scarcely any " B "-bearing cells with labeled nuclei were observed in the period corresponding to the second S phase of non-infected cells.

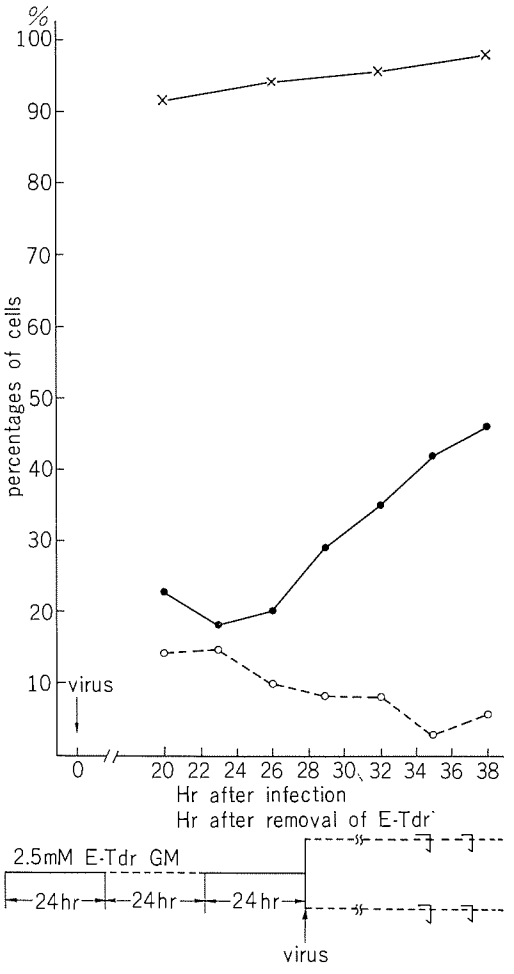


FIGURE 3. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with CPV just after removal of E-Tdr.*
moi 1
●——● *non-infected cells with lebeled nuclei*
○------○ *" B "-bearing cells with lebeled nuclei*
×——× *" B "-bearing cells*

The " B "-bearing cells could not enter the second S phase. This means that when viral DNA synthesis of SFV began after the onset of the first S phase, the infected cells could not enter the second S phase following G_2 and G_1 with respect to cellular DNA synthesis.

Mitotic cells bearing inclusion bodies of SFV were rarely observed throughout the experiments as described in the report of Mantani et al. (1968).

Similar experiments were carried out with CPV. As shown in Fig. 3, non-infected cells, but not infected cells, entered the second S phase 26 hr after removal of E-Tdr.

These results raise the following questions. 1) Does nuclear DNA synthesis of FL cells in which viral DNA synthesis was induced after removal of E-Tdr occur during the period corresponding to the S phase of non-infected cells, as shown with CPV by Mantani et al. (1968)? 2) The curve of the percentage of " B "-bearing cells with labeled nuclei seems to decrease corresponding to that of non-infected cells with labeled nuclei for 23 hr after removal of E-Tdr. Is this due to increased inhibition of cellular DNA synthesis due to the developing inclusion bodies of SFV? 3) Do " B "-bearing cells enter the S phase following G_1, when viral DNA is synthesized during G_1? Experiments were made to answer these questions.

3. *Synchronous nuclear DNA synthesis of FL cells allowing multiplication of SFV for 7 hr in the presence of E-Tdr*

As shown in Fig. 1 (Expt. C) and Fig. 4 (Expt. C), cloned FL cells were infected with SFV (moi 75) after 22 hr treatment with 2.5 mM E-Tdr. After the virus infection, infected

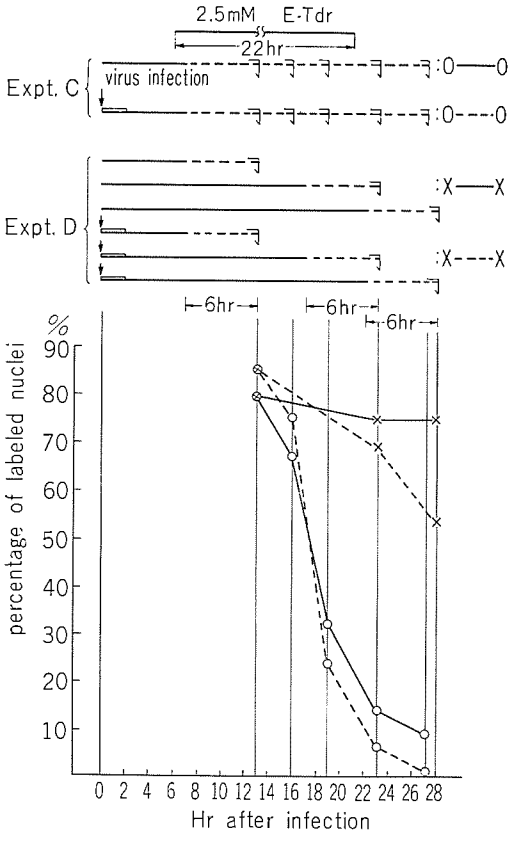


FIGURE 4. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with SFV in the presence of E-Tdr.*

○------○ × ------ × } *" B "-bearing cells with labeled nuclei*

○——○ × ——× } *non-infected cells with labeled nuclei*

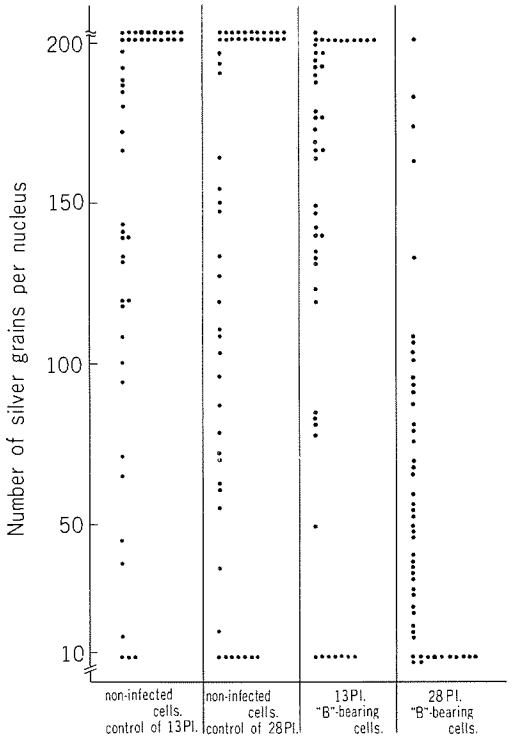


FIGURE 5. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with SFV in the presence of E-Tdr.*

Grain counts were made on 50 randomly selected nuclei of the same samples as those in experiment D in Fig. 4.

cells were again incubated with GM containing 2.5 mM E-Tdr for 7 hr. Then, these cells were washed 3 times with Hanks' solution and incubated in GM only. As a control, non-infected FL cells were similarly treated with E-Tdr. At appropriate times, these cells were pulse labeled with ^{3}H-Tdr for 1 hr and taken out for autoradiography. Curve of a percentages of " B "-bearing cells with labeled nuclei was compared with those of non-infected cells with labeled nuclei.

As shown in Fig. 4 (Expt. C), the lower two curves clearly corresponded with each other. If the correspondence of the curve of the percentage of " B "-bearing cells with labeled nuclei with that of non-infected cells in the S phase in Fig. 2 depended on progressive inhibition of cellular DNA synthesis due to developing inclusion bodies, the curve of " B "-bearing cells with labeled nuclei in Fig. 4 should not correspond with that of non-infected cells. Namely, the curve of " B "-bearing cells with labeled nuclei should begin to decrease 7 hr sooner than that of non-infected cells. The present result shows that nuclear DNA synthesis of cells infected with SFV was inhibited, but proceeded during the period corresponding to the S phase of non-infected cells.

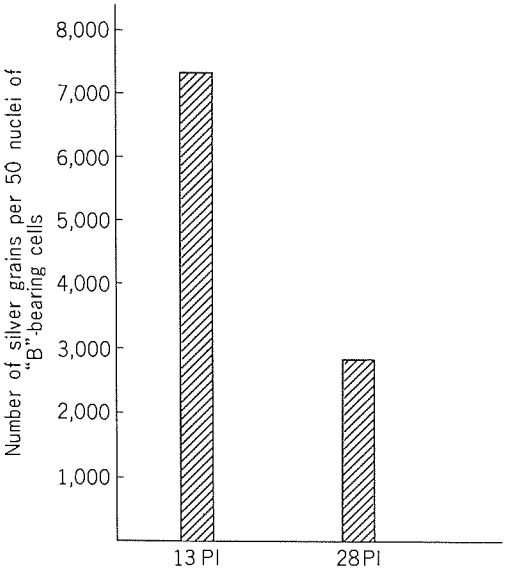


FIGURE 6. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with SFV in the presence of E-Tdr.*

Total number of silver grains per 50 nuclei from samples 13 hr and 28 hr after infection. (*see Fig. 4.*)

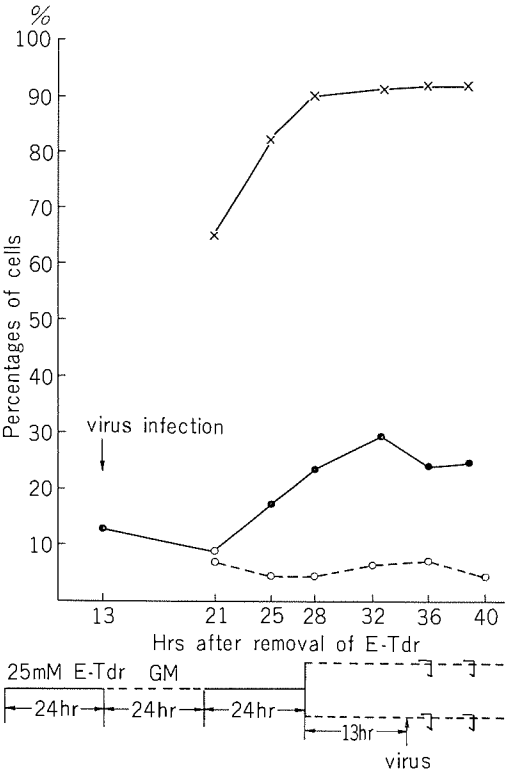


FIGURE 7. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with SFV in the G period.*

moi 100

●——● *non-infected cells with labeled nuclei*
○------○ *" B "-bearing cells with labeled nuclei*
×——× *" B "-bearing cells*

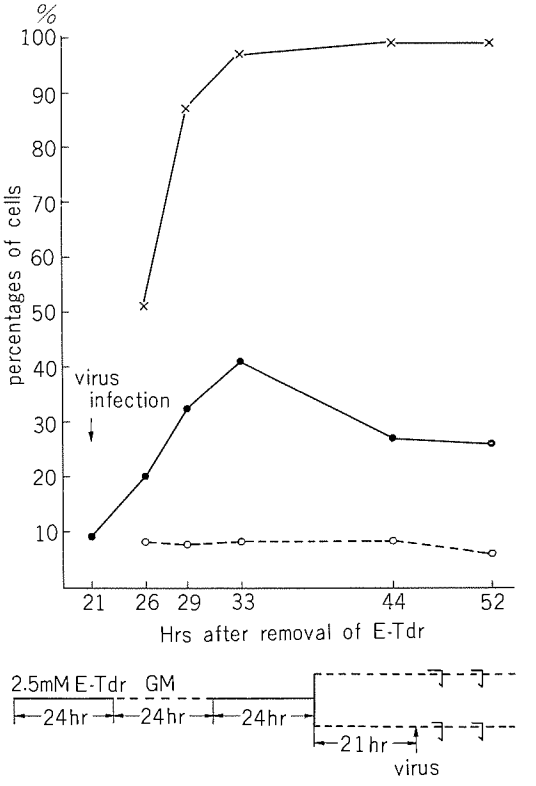


FIGURE 8. *Effect of viral DNA synthesis upon synchronous nuclear DNA synthesis of FL cells infected with CPV in the G period.*

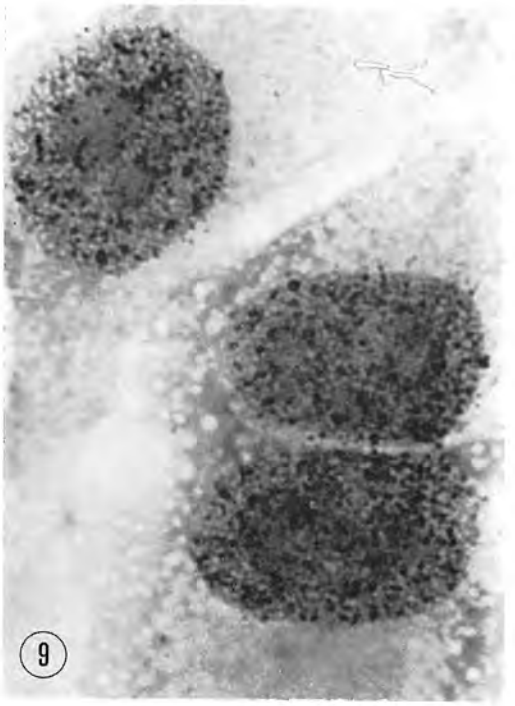
moi 25

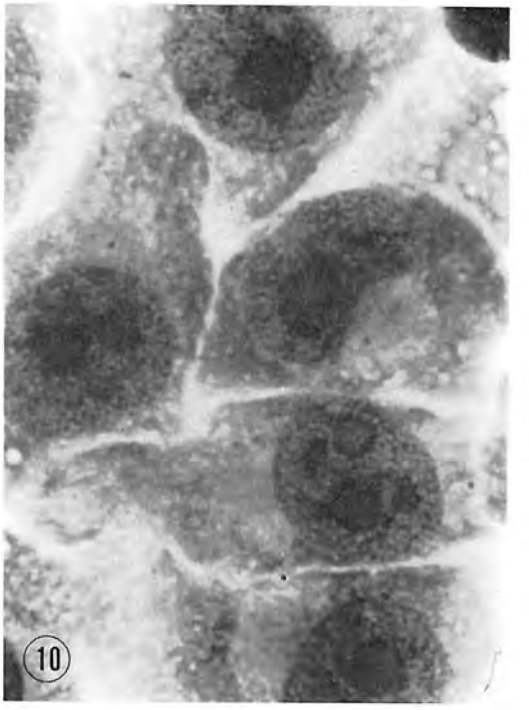
●——● *non-infected cells with labeled nuclei*
○------○ *" B "-bearing cells with labeled nuclei*
×——× *" B "-bearing cells*

4. *Degree of inhibition of nuclear DNA synthesis at the beginning of the S phase caused by SFV infection*

This experiment was designed to study the degree of inhibition of nuclear DNA synthesis caused by SFV infection.

As shown in Fig. 1 (Expt. D) and Fig. 4 (Expt D), FL cells were infected with SFV after 22 hr incubation with 2.5 mM E-Tdr.





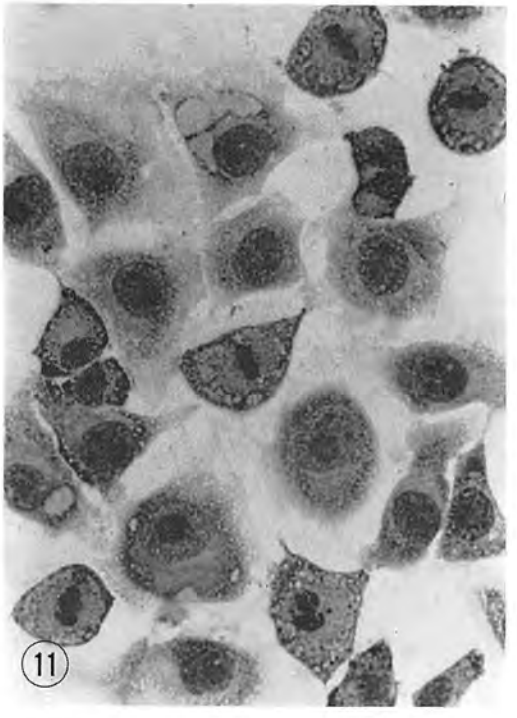


FIGURE 9. *Autoradiogram of non-infected synchronous FL cells. Six hr after removal of excess thymidine. All FL cells have labeled nuclei (S phase).*

FIGURE 10. *Autoradiograms of non-infected synchronous FL cells 23 hr after removal of excess thymidine. No nuclei are labeled with ^{3}H-thymidine (G phase).*

FIGURE 11. *Autoradiogram of non-infected synchronous FL cells 15 hr after removal of excess thymidine. Mitotic figures at various stages are seen.*

▶

FIGURES 12, 13, 14 and 15. *Autoradiogram of synchronous FL cells infected with Shope fibroma virus.*

Figure 12: Six hr after removal of E-Tdr and 13 hr after infection.

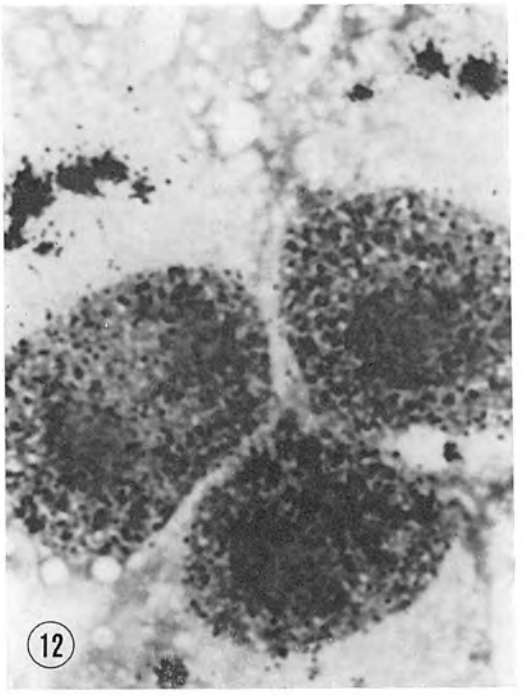
Figure 13: Eight hr after infection and 22 hr after removal of E-Tdr.

" B "-bearing cells without labeled nuclei are seen.

Figure 14: Six hr after removal of E-Tdr and 23 hr after infection.

" B "-bearing cells with labeled nuclei are seen.

Figure 15: Six hr after removal of E-Tdr and 28 hr after infection. Both " B " inclusions and nuclei are slightly labeled.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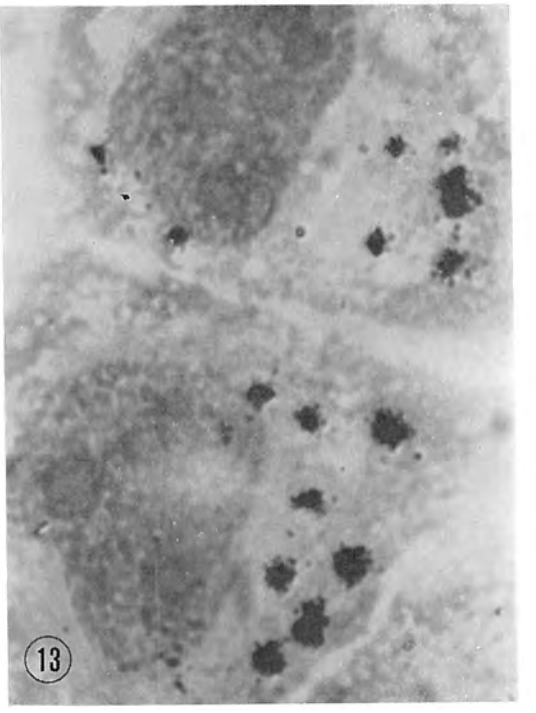
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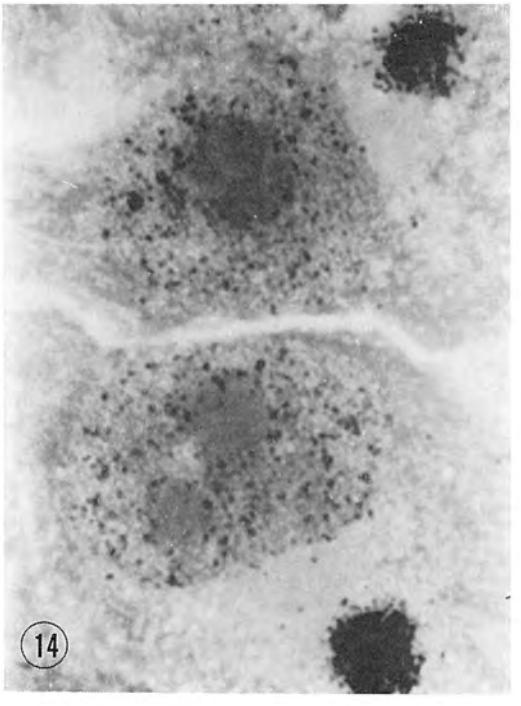
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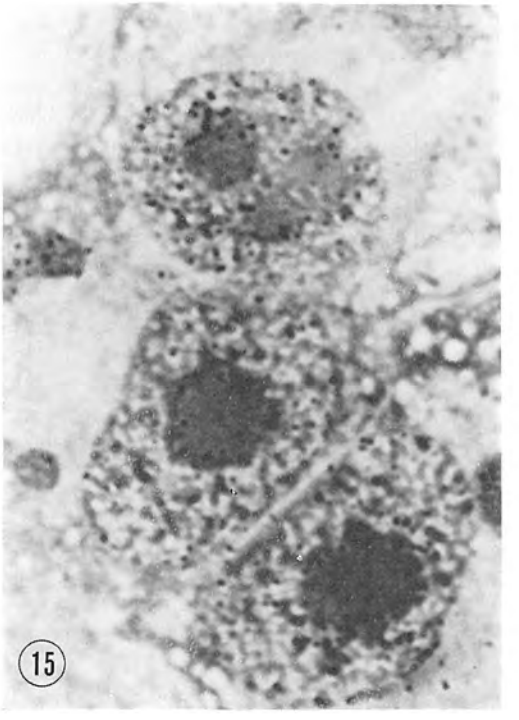
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After the virus infection, the infected cells were again incubated with 2.5 mM E-Tdr. These cells were labeled with ^{3}H-Tdr 6 hr after removal of E-Tdr. These cells were labeled with ^{3}H-Tdr 6 hr after removal of E-Tdr, as shown in Fig. 4 (Expt. D). All the cells were labeled with ^{3}H-Tdr from 6 hr to 7 hr after removal of E-Tdr, that is, when all the cells were at the beginning of the S phase. There were 78%, 92% and 94% of " B "-bearing 13 hr, 23 hr and 27 hr, respectively, after infection. The percentage of non-infected cells with labeled nuclei was 75–80% throughout this epxeriment. The percentage of " B "-bearing cells with labeled nuclei decreased from 85% 13 hr after infection to 53%, 28 hr after infection (Fig. 12, 14 and 15).

The previous report (Mantani et al., 1970) showed that E-Tdr had no direct effect on virus growth. Thus the decrease in the percentage of " B "-bearing cells with labeled nuclei showed the actual degree of inhibition of nuclear DNA synthesis caused by the " B "-inclusion bodies developed in the cytoplasm.

Grain counts were made on these samples, on 50 randomly selected nuclei (Fig. 5). Fig. 6 shows the total number of silver grains in these 50 nuclei.

Figs. 5 and 6, show that nuclear DNA synthesis of " B "-bearing cells in the samples examined 28 hr after infection, was definitely supressed, compared with that 13 hr after infection or that in non-infected cells. The number of grains per 50 nuclei 28 hr after infection was about 1/3 of that 13 hr after infection (Fig. 6), and about 2/3 of the nuclear DNA synthesis was inhibited during the 15 hr period of virus growth from 13 hr to 28 hr after infection. These results also show that the correspondence of the curve of the percentage of " B "-bearing cells with labeled nuclei with that of non-infected cells in the S phase in Figs. 2 and 4 did not depend on progressive inhibition of cellular DNA synthesis due to developing inclusion bodies and that nuclear DNA synthesis of FL cells in which viral DNA synthesis of SFV was induced in the S phase occurred during the period corresponding to the S phase of non-infected cells.

5. *Effect of the onset of viral DNA synthesis during G_1 on cellular DNA synthesis*

An experiment was designed so that viral DNA synthesis of CPV or SFV occurred in the G_1 phase and nuclear DNA synthesis of " B "-bearing cells in the S phase was studied.

As shown in Fig. 1 (Expt. B) and Fig. 7, FL cells were infected with SFV 13 hr after removal of E-Tdr, which corresponded to the time of the G_1 phase. The percentages of " B "-bearing cells increased rapidly from 85% at the end of the G_1 phase to 90% in the middle of phase S. The percentage of non-infected cells with labeled nuclei gradually increased for 21 hr after removal of E-Tdr, being 30% 32 hr after removal of E-Tdr. However, " B "-bearing cells with labeled nuclei did not increase, even at the period corresponding to the S phase in non-infected cells (Fig. 13).

Fig. 8 shows that when CPV-DNA was synthesized during the G_1 phase, cellular DNA synthesis did not occur in the period corresponding to the S phase of non-infected cells.

DISCUSSION

Poxvirus is known to have marked proliferative effects upon cells. In fact most poxviruses can produce pocks as a result of cell proliferation with some cell infiltration. Several viruses of this group, such as the Shope fibroma and Yaba monkey tumor, can produce tumors.

Extensive studies on the relationship between nuclear and cytoplasmic DNA synthesis in cells infected with various poxviruses, have been carried out in our laboratory using autoradiography of ^{3}H-thymidine. The results obtained were briefly as follows. 1. Poxviruses, whether oncogenic or non-oncogenic, multiply in the cytoplasm of infected cells, forming " B " type inclusions which are the

sites of viral DNA synthesis and of viral antigen. 2. Celluar proliferation, whether hyperplasia, benign growth, or formation of a sarcomatous tumor, is always accompanied by active virus multiplication. 3. Nuclear DNA synthesis in cells in which multiplication of any type of poxvirus occurred was a definitely suppressed. Therefore cells producing virus did not multiply, but degenerated. Our studies were focused on the relationship between virus DNA synthesis and nuclear DNA synthesis in poxvirus infection. The effect of viral DNA synthesis of cowpox virus upon nuclear DNA synthesis of infected cells was analyzed using synchronizing FL cells induced by double thymidine treatment (Mantani et al., 1968). Nuclear DNA synthesis in synchronized cells showing viral DNA synthesis was definitely, but not completely, suppressed, and slow nuclear DNA synthesis occurred exclusively during the physiological " S " phase of these cells. As shown in the previous report (Mantani et al., 1970), CPV and SFV multiply well in FL cells in the presence of E-Tdr. This finding made it possible to induce viral DNA synthesis in synchronous cells during a desired cell phase. In the present experiments Shope fibroma virus (SFV) was used as oncogenic virus in addition to the cowpox non-oncogenic virus.

The difference in viral multiplication of SFV and CPV in cultured FL cells were as follows. 1. SFV produced only " B " type inclusions and did not produce "A" type inclusions. 2. The rate of viral multiplication of SFV was slower than that of CPV. 3. " B "-bearing FL cells infected with SFV did not degenerate rapidly and remained on the surface on the culture glass for a longer period than cells infected with CPV. 4. The SFV-FL cell system readily becomes in the state of a "carrier culture " with a low moi, as reported by Kato et al. (1959, 1965), while CPV causes total degeneration of cultured FL cells. However, no differences could be found between the effects of viral DNA synthesis of SFV and CPV upon nuclear DNA synthesis. Thus, when viral DNA synthesis of either virus began before the " S " phase of infected cells, no nuclear DNA synthesis was initiated. As suggested by Ensminger and Tamm (1970) from studies on L cell-RNA virus systems, some protein involved in initiation of DNA synthesis, may be reduced as a result of viral growth.

When viral DNA synthesis of either virus began after nuclear DNA synthesis had been initiated in the infected cells, slow nuclear DNA synthesis occurred exclusively during the physiological " S " phase of the cells, as shown with CPV (Mantani et al. 1968). However, in the following " S " phase in the 2nd cell cycle of the infected cells, no nuclear DNA synthesis was initiated. Hodge and Scharff (1969) found a similar relationship between viral and cellular DNA synthesis in cells infected with adenovirus using biochemical methods. The mechanism of the slow nuclear DNA synthesis is unknown. Recently, Ensminger and Tamm (1970) studied cellular DNA synthesis inhibited by infection with Newcastle Disease virus or Mengo virus using biochemical methods, and showed that high molecular weight cellular DNA is not degraded and nascent DNA chains grow at the normal rate when over all DNA synthesis is inhibited by viral infection. Their interpretation of their results could be applied to explain the slow progress of nuclear DNA synthesis in the " S " phase of infected cells. Therefore, cells infected with either SFV or CPV finally died. Our data support the hypothetical mechanism of oncogenesis of poxvirus, presented by Kato et al. (1963).

Tompkins et al. (1970) reported that rabbit kidney cells infected with SFV (Patuxent strain) did not die, but that 35 to 38 hr after infection, nuclear synthesis of DNA commenced and the cells later divided at a rate similar to that of uninfected cells. From studies at a cellular level this does not seem to occur in our system. In the carrier culture state demonstrated with the FL cell-SFV system and the FL cell-rabbit myxoma virus system, there seems to be a balance between virus producing cells which

will die, and non-infected cells which will multiply at least until they are infected with virus. (Kato et al., 1965).

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