Studies on Polyoma Virus DNA Replication in Synchronized C3H2K Cells

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SUMMARY

In G1-arrested cells infected between 1 and 12 h after having been stimulated by fresh serum to progress to S phase, polyoma virus DNA synthesis proceeded in the first half of S phase, and virus and whole cellular DNA accumulated at about the same time. However, in cells infected later than 14 h after serum stimulation, virus DNA synthesis was shifted to the next S phase. Thus, a permissive cell attains competence for polyoma virus DNA replication at a precise moment during an S phase initiated by fresh serum, which can efficiently replace the early virus host DNA stimulation function. When cells were incubated in serum that had lost its capacity to stimulate host DNA synthesis by pre-absorption with growing cells, normal yields of polyoma DNA could nevertheless be observed, which shows that extensive replication of host DNA does not seem to be an obligatory condition for virus DNA replication.

Synthesis of nuclear DNA of eukaryotes is a highly ordered but still poorly understood process (Prescott, 1976). The small and well-defined genomes of some of the DNA viruses, which replicate their DNA during the S phase of their host cells, may be suitable probes to study regulated DNA synthesis in eukaryotes.

Parvovirus H-I a single stranded DNA virus, replicates its DNA at the end of the S phase (Rhode, 1973) and it is known that different populations of eukaryotic DNA replicate at different times during the S phase (Balazs et al. 1973). The cellular double stranded genomes of polyoma and SV40 viruses also replicate during the S phase (Manteuil et al. 1973; Thorne, 1973a, b; Pages et al. 1975). For the latter viruses, however, a detailed study of the relationship between virus and cellular DNA synthesis has not so far been made. Thus, observations recorded for polyoma virus were based on virion formation and not on the synthesis of virus DNA. Furthermore, the use of the metaphase detachment and the double excess thymidine procedure which were used in these experiments did not allow cells arrested in G1 to be obtained in a reproducible way. This goal was achieved in the present work using the permissive C3H2K mouse cell, a continuous cell line derived from a mouse kidney fibroblast culture which, in previous work, has been found to be useful for cell synchronization studies (Yoshikura et al. 1967). These cells have properties similar to those of the 3T3 cell line (Todaro & Green, 1965).

When grown to confluence or when kept in the same medium which became deficient, C3H2K cells were arrested in G1 phase. After renewal of nutrient medium, the cells progressed to S phase and divided in a synchronous way as determined by 3H-thymidine pulse-labelling and cell counts. After a change to fresh medium, incorporation of radioactivity was temporarily diminished in accordance with the work of Cramer & Feinendegen (1966). A synchronized cycle of DNA replication started about 14 h after medium change and maximum DNA synthesis was observed at 24 to 26 h. At this time, about 50% of the cells were found to be in the S phase as detected by autoradiography (not shown), confirming
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Table 1. Time relationship between onset of S phase and virus DNA synthesis*

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<th>Time of infection after medium change (h)</th>
<th>Time lapse between S phase and DNA synthesis † in cultures infected</th>
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* In each experiment, G1 arrested cells were infected before and at different times after medium change and the kinetics of whole cellular and virus DNA synthesis were examined. For pulse labelling of cellular DNA, cells were incubated at different times with 1 μCi/ml 3H-thymidine (10 mCi/mmol) for 1 h and the synthesis was measured by the incorporation of radioactivity in the acid-precipitable fraction. To determine its synthesis, the virus DNA was extracted by the Hirt (1967) method and assayed on mouse cultures for infectivity.

† Values given are the time intervals in hours between the point at which the rate of thymidine incorporation into whole cellular DNA attained 50% of maximal incorporation and the point at which half the yield of virus was produced.

results already described for this cell system (Yoshikura & Hirokawa, 1968). Forty-eight hours after medium change, the cell number had doubled and a second, but usually incomplete, S phase was observed, followed by a progressive loss of synchronization. In non-medium-changed cultures, plateau levels of DNA were observed.

This cell system allowed us to infect cells arrested in G1 at different times after serum stimulation and to study the time relationship of virus and cellular DNA synthesis (Table 1). The length of the S period was determined by measuring radioactivity in the acid-precipitable material derived from synchronized pulse-labelled cells.

Continuous cell labelling was used for a comparative study of virus and cellular DNA synthesis. Whole cell DNA synthesis was again examined as described. Virus DNA synthesis was determined by DNA infectivity titrations using a plaque-purified virus for which 1 infectious unit of virus DNA was found to correspond to about 10⁶ molecules of virus DNA, comparable to earlier findings (Crawford, 1969). Simultaneously, virus DNA synthesis was also determined by isolating virus DNA form I on equilibrium density gradients (Radloff et al. 1967).

Both methods gave almost identical results as far as the kinetics of virus DNA synthesis are concerned. Virus DNA synthesis was found to attain its maximum in the middle of S phase and virus and cellular DNA synthesis proceeded almost synchronously. Virus DNA synthesis was only slightly delayed in cells infected up to 12 h after having been serum-stimulated to progress to S phase, when compared to cells infected immediately after serum stimulation. However, when cells were infected later than 13 h after serum stimulation, when they had already entered S phase, a change to a delayed synthesis occurring in the ensuing S phase was observed in all cases.

These results seem to show that the early virus information needed to initiate virus DNA
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Fig. 1. Whole cellular and virus DNA synthesis in G1 arrested cells infected by polyoma virus and incubated in different media. The method for determining cellular and virus DNA synthesis was described in Table 1. □ - - - □, Virus DNA yield in cultures incubated with fresh medium; ○ - - - ○, virus DNA yield in cultures incubated with medium pre-incubated with growing cells for 3 days; △ - - - △, virus DNA yield in cultures incubated with 100 μM 5'-deoxy-5'-S-isobutyl adenosine/ml serum free medium; ■ - - ■, rate of thymidine incorporation in whole cellular DNA in cultures incubated with fresh medium; ● - - ●, rate of thymidine incorporation in whole cellular DNA in cultures incubated with medium pre-incubated with growing cells for 3 days; ▲ - - ▲, rate of thymidine incorporation in whole cellular DNA in cultures incubated with 100 μM 5'-deoxy-5'-S-isobutyl adenosine/ml serum free medium. Each point represents the mean values from three Petri dishes.

replication must be expressed before a critical period of the S phase is attained (Eckhart, 1974). From the shortening of the latency period observed in cells which were infected 12 h after having been stimulated we can calculate that the expression of this early virus function can occur in less than 9 h, which agrees with the findings that polyoma virus penetrates into the nucleus in about 15 min and that transcription of the early virus genome is completed in about 6 h (Salomon et al. 1977).

The similar yields and kinetics of virus DNA synthesis recorded in cells infected before and 12 h after serum stimulation indicate that serum stimulation factors can efficiently replace the virus host DNA stimulation function which is believed to be an early virus function distinct from that of the TsA needed to initiate virus DNA replication (Eckhart, 1974). With regard to this, one has to consider that the majority of quiescent cells are probably in a similar state of G1 and that, regardless of the type of stimulation, they have to
progress through a defined chemical programme, which cannot be shortened, before cellular DNA synthesis can start (Pardee, 1974). A striking parallel between the stimulatory effects of serum and polyoma virus infection has already been found concerning shifts in enzyme activities as well as in pool size of deoxyribonucleoside triphosphates (Nordenskjold et al. 1970). We found no great changes in the kinetics of cellular DNA synthesis in either stimulated infected or non-infected cells. However, the present cell system will be suitable for the examination of possible qualitative changes, such as the order of mouse satellite DNA synthesis (Smith, 1970; Balazs et al. 1973; Hatfield & Walker, 1973). In a second group of experiments, we tried to dissociate cellular and virus DNA synthesis. Serum stimulation of contact inhibited cells is due to as yet undefined serum proteins. We have found that such factors can be eliminated from serum which has been in contact with growing cells for different periods of time. When cells were incubated in pre-absorbed serum which had lost the stimulation factors for cell replication, unimpaired yields of vesicular stomatitis virus were obtained, indicating that the serum still contained essential nutritional factors. Similar yields of polyoma virus DNA were also obtained in cells incubated either with fresh or pre-absorbed serum and in both cases almost all cells became productively infected as judged by the capsid antigen staining method (Fig. 1). This result is unexpected as fresh serum stimulates all cells to replicate whereas this stimulation was almost completely absent in cultures incubated with pre-absorbed serum.

This is a novel situation for polyoma virus and recalls what has been observed when BSC-1 cells were infected with SV40 (Ritzi & Levine, 1970). As results with pre-absorbed serum sometimes varied, we recently combined this method with the use of either 5'-deoxy-5'-S-isobutyl adenosine (Fig. 1), an inhibitor of cell transformation (Robert-Gero et al. 1975), or methioninyl adenylate, a potent and reversible inhibitor of protein synthesis (Cassio et al. 1973). However we can conclude from our observations that complete duplication of cellular DNA is not obligatory for virus DNA synthesis. Our cell synchronization system will now be used to test the hypothesis that virus DNA synthesis may rely on biochemical programming characteristic for the S phase, which could be attained even in the absence of significant replication of host DNA.

**REFERENCES**

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