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REPLICATION OF POXVIRUS DNA IN CHICK EMBRYO FIBROBLASTS

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Summary. — Replication of vaccinia virus DNA in chick embryo fibroblasts at a high multiplicity of infection started after 4—6 hr, reached a maximum at the 12th hr and was completed 18 hr after inoculation (p.i.). An analysis of newly synthesised DNAs in the period of the highest rate of synthesis revealed the formation of short (10—30 S) fragments which within 30 min were transformed into single-stranded molecules with a sedimentation coefficient of 62 S. The final stage of closing of the ends of single-stranded molecules needed much more time and the bulk of "mature" single-stranded 90 S DNA molecules was observed 17 hr p.i.

Key words: vaccinia virus; replication of viral DNA; chick embryo fibroblasts

Introduction

Replication of DNA of poxviruses, the most complex viruses of mammals, has been attracting more and more interest (Holowczak and Diamond, 1976; Esteban and Holowczak, 1977; Pogo and O'Shea, 1978; Archard, 1979). It has been due mainly to the fact that this model apparently most closely resembles the mechanism of replication of DNA in cells of eukaryotes. However, being relatively more simple, this model makes it possible to elucidate concrete steps in the replication process and to identify the enzymes responsible for these processes (Moss, 1977; Esteban and Holowczak, 1977; Pogo and O'Shea, 1978).

One of the approaches allowing successful investigations of the replication of a number of DNA viruses is the use of conditional lethal mutants with mutations in genes responsible for DNA synthesis (Epstein *et al.*, 1963; Edgar and Lielausis, 1964; Breunwald *et al.*, 1970; Kahn and Hanawalt, 1979). In the course of several years we have collected temperature-sensitive (ts) mutants of vaccinia virus, including those with a DNA^- phenotype, differing from the original virus by their inability to synthesise DNA at 39.5 °C in chick embryo fibroblasts (CEF) (Chernos *et al.*, 1978). The aim of the

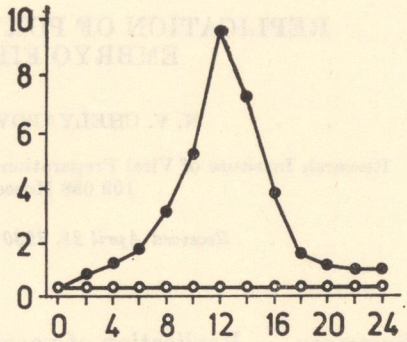

Fig. 1.

DNA synthesis in unifected (○) and vaccinia virus-infected (●) CEF
Abscissa: hr p. i.; ordinate: ^{3}H-thymidine incorporation (count/min × 10^{-3}) on a 20-min pulse

present work was an attempt to characterize the replication of DNA of the original virus in this system in view of the fragmentary data on vaccinia virus DNA replication in CEF (Magee and Levine, 1970).

Materials and Methods

Virus. The neurovariant MM of vaccinia virus was propagated on the chorioallantoic membranes of 12-day chick embryos and purified by the method of Joklik (1962). A single virus preparation with a titre of 2×10^{10} pock-forming units (PkFU) per ml was used throughout.

Radiolabelling of virus. CEF monolayers were inoculated at a multiplicity of 40 PkFU per cell. The growth medium consisted of Hanks' solution with 0.5% lactalbumin hydrolysate. From 2 to 4 hr after inoculation (p. i.) 20 μCi/ml of ^{3}H-thymidine (14—20 Ci/mmole) was added to the growth medium. From 22 to 26 hr p. i. the monolayer was washed with cold phosphate buffered saline (PBS; Dulbecco and Vogt, 1954). The cells were mechanically scraped off from the glass and pelleted. All operations were carried out in the cold. The pelleted cells were washed with a 1 mM solution of Tris. HCl pH 9.0 (buffer A), resuspended in the same buffer and allowed to stand for 10 min to make the cells swell. Thereafter the cells were disrupted in a Dounce homogenizer, the nuclei pelleted and the supernatant was placed on a linear 20—40% (w/w) sucrose gradient in buffer A and centrifuged for 45 min at 15000 rev/min in a 3 × 25 rotor of an MSE SS—65 centrifuge at 4 °C. Ultrasonication of the virus material was omitted since it could have induced additional breaks in the virion DNA (Holowczak, 1976). For further analysis of viral DNA, the material from the virus-containing zone of the gradient was diluted 7-fold ($\leqq$ 5% sucrose) with buffer A and a portion was layered on a lysing mixture placed on the top of a sucrose gradient (see below).

Kinetics of DNA synthesis. In the course of the whole replication cycle, ^{3}H-thymidine (20 μCi/ml) was added every 2 hr for 20 min to the infected cells. After the end of the incubation period, a nucleus-free cytoplasmic extract was prepared as described above, placed on a membrane filter (Synpor 3, Czechoslovakia) and washed with 5% trichloroacetic acid. Acid-insoluble radioactivity was determined in a toluene scintillator in an SL-30 spectrometer.

Vaccinia virus DNA synthesis in CEF. The DNA was isolated and analysed as described by Esteban and Holowczak (1977) with minor modifications. Infected and control cells were treated in the same way. All operations were carried out in the cold. DNA was labelled with thymidine like in the case of virus (see above). "Chase" was accomplished by removal of the label-containing medium, washing of the monolayer with fresh medium and addition of 0.5 mM unlabelled thymidine. After the end of the labelling period, the cell monolayer was washed with a solution of 0.15 M NaCl, 0.05 M Tris.HCl, pH 7.6 and 0.005 M EDTA. The cells were mechanically scraped off from the glass, washed twice with the same solution and suspended in it to a concentration of 2—5 million cells per ml. Then 0.2 ml of this suspension was placed on 0.8 ml of lysing mixture, containing 1 M NaCl, 0.01 M EDTA, 2% sodium lauryl sarcosinate (Sigma), 0.03 M β-mercaptoethanol and 0.05 M Tris.HCl, pH 7.6. The same lysing mixture was used for analysis of the materials

in neutral or alkaline sucrose gradients. The mixture was layered on the top of the gradient immediately before the addition of the test material. The sucrose gradients (15—30%, w/v) contained 0.01 M EDTA and 0.2% lauryl sarcosinate; the neutral ones also 1 M NaCl and 0.05 M Tris.HCl, pH 7.6, the alkaline ones 0.7 M NaCl and 0.3 M NaOH. Lysis proceeded at 4 °C for 16 hr in the dark. Centrifugation was done for 3 hr at 38000 rev/min in the 6 × 14 rotor of an MSE SS-65 centrifuge. Cellulose nitrate tubes from a SW-40 Beckman rotor were employed.

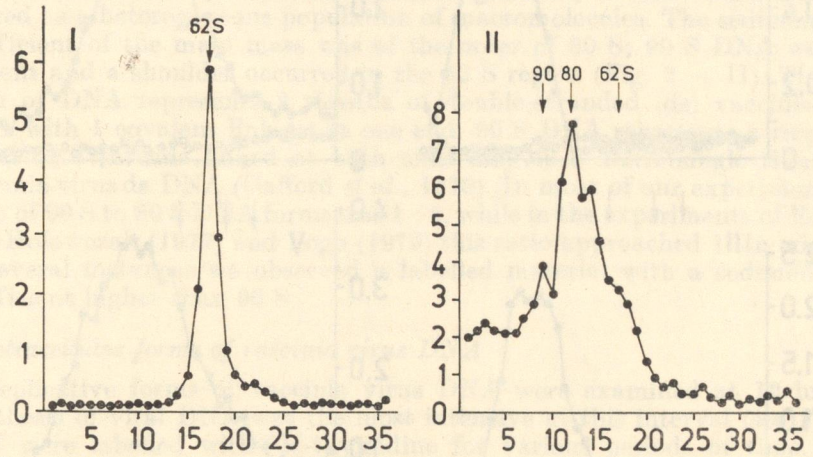

Fig. 2.

Sedimentation pattern of vaccinia virion DNA in neutral (I) and alkaline (II) sucrose density gradients

Position of phage T2 DNA (62 S) and zones with sedimentation coefficients of 80 and 90 S indicated by arrows.

Abscissa: fraction number; ordinates: ^{3}H-thymidine incorporation (count/min × 10^{-3})

T2 phage DNA with a sedimentation coefficient of 62 S (Clark and Lange, 1976) was used as a marker. Fractions were collected from the bottom of the tubes. To each fraction was added 50 μg albumin and trichloroacetic acid to a final concentration of 5%. The precipitate formed after 1 hr at 4 °C was collected on a membrane filter (Synpor 6), washed with 5% trichloroacetic acid and its radioactivity measured in a toluene scintillator.

Results

Kinetics of ^{3}H-thymidine incorporation into the cytoplasm of infected cells

The course of ^{3}H-thymidine incorporation (pulse label for 20 min) into the cytoplasm of infected and uninfected cells is illustrated in Fig. 1. The incorporation reached its maximum at 12 hr p. i. and was terminated at 18 hr p. i. These data showed that the synthesis of vaccinia virus DNA in CEF monolayer cultures was very protracted as distinct from that in L and HeLa cell suspension cultures (Holowczak and Diamond, 1976; Esteban and Holowczak, 1977).

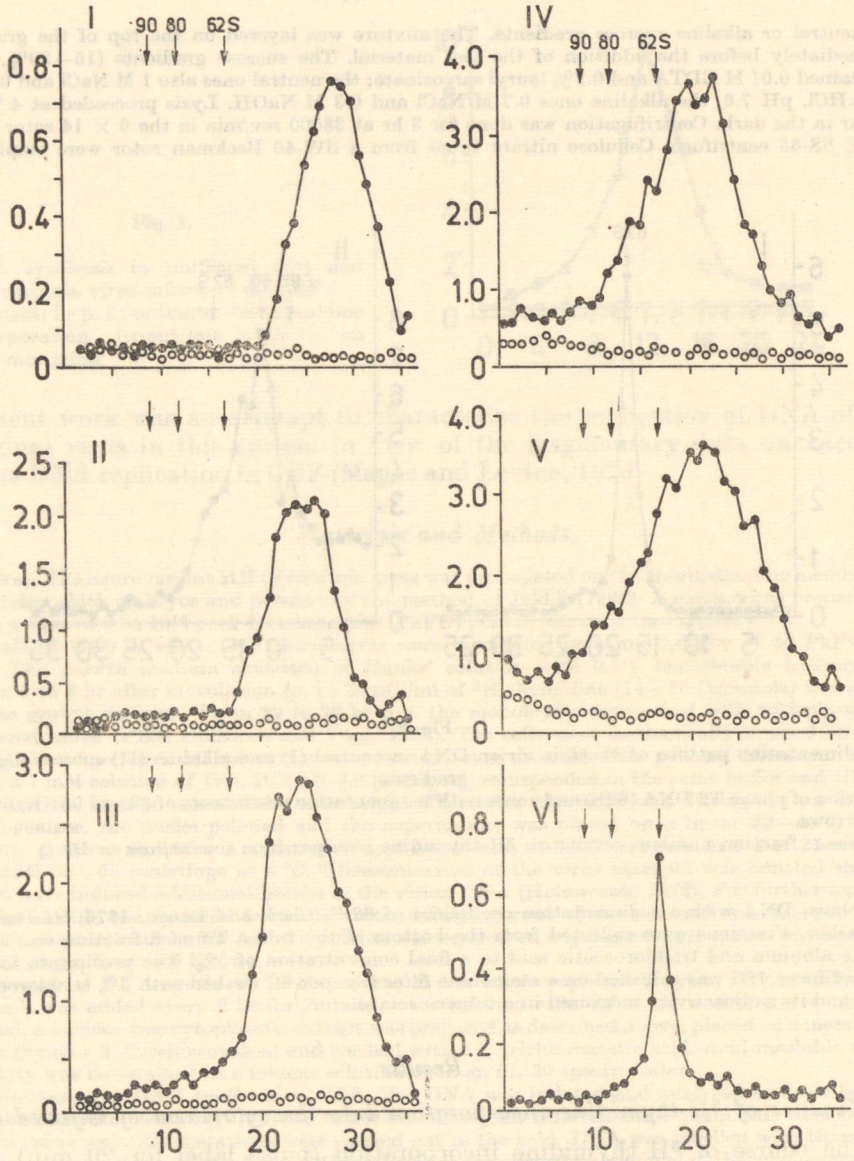

Fig. 3.

Sedimentation pattern of viral DNAs synthesised in CEF pulsed for 5 (I), 10 (II), 30 (III), 60 (IV and VI) and 75 (V) min

I — V alkaline, VI neutral sucrose density gradients ● — Infected, ○ — uninfected CEF

Virion DNA

To characterize the sedimentation properties of virion DNA, viral material from the cytoplasmic extract from infected cells was isolated by centrifugation in a 20—40% sucrose density gradient. Centrifugation of virion DNA in a neutral gradient revealed one narrow peak of radioactivity of a material co-sedimenting with phage T2 marker DNA with a sedimentation coefficient of 62 S (Fig. 2 — I). In alkaline sucrose gradient, vaccinia viral DNA appeared as a heterogeneous population of macromolecules. The sedimentation coefficient of the main mass was of the order of 80 S; 90 S DNA was also present and a shoulder occurred in the 62 S region (Fig. 2 — II). The 80 S form of DNA represents 2 threads of double-stranded (ds) vaccinia virus DNA with 1 covalent linkage at one end, 90 S DNA represents a ring composed of 2 threads linked at both ends and 62 S DNA single threads of vaccinia virus ds DNA (Gafford *et al.*, 1978). In most of our experiments the ratio of 90 S to 80 S DNA forms was 1 : 2, while in the experiments of Esteban and Holowczak (1977) and Pogo (1979) this ratio approached 1. In addition, in several instances we observed a labelled material with a sedimentation coefficient higher than 90 S.

Intracellular forms of vaccinia virus DNA

Replicative forms of vaccinia virus DNA were examined at 12 hr p. i. Synthesis of viral DNA was the most intensive at this interval (see Fig. 1). CEF were labelled with ^{3}H-thymidine for various periods of time. After incubation of the cells with the radioactive precursor, the cells were placed on ice and DNA was analysed as described in "Materials and Methods".

Ultracentrifugation in alkaline sucrose density gradients revealed a great variety of intermediate forms of single-stranded (ss) molecules in the course of viral DNA synthesis. The shortest time of labelling which made it possible to reveal reproducibly a clear-cut peak of radioactive material was 5 min. Within that period ss DNA, whose principal mass had a sedimentation coefficient of the order of 20 S, was synthesised (Fig. 3 — I). The molecular weight of ss DNAs increased with prolonged labelling (Fig. 3 — II). After a 30-min pulse the radioactive material occurred in a zone corresponding to mature ss DNA with a sedimentation coefficient of 62 S (Fig. 3 — III). One-hr pulse revealed all mature forms of DNA with sedimentation coefficients of 80 and 90 S (Fig. 3 — IV). A 75-min pulse revealed a further increase in the amount of mature forms (Fig. 3 — V).

An analysis of replicating DNAs in neutral sucrose density gradients revealed no clear-cut sedimentation profile of the radioactive materials after short (5—30 min) pulses; more than 50% of radioactivity occurred in the sediment. This was apparently due to the fact that newly synthesised short fragments of ss DNAs are closely bound to the parent matrix and are pelleted along with the replicative complex which is not destroyed by the components of the lysing mixture (Joklik and Becker, 1964; Esteban and Holowczak, 1977). After a 1-hr pulse, mature ds DNA with a sedimentation coefficient of 62 S was demonstrated (Fig. 3 — VI). Further incubation of

the cells with ^{3}H-thymidine caused no changes in the sedimentation pattern of radioactive materials in neutral sucrose density gradients.

To establish the character of vaccinia virus DNA synthesis in CEF, experiments with a short pulse with ^{3}H-thymidine and a subsequent chase with unlabelled thymidine were carried out. The shortest pulse which, together with the chase, allowed a reproducible analysis of polynucleotide structures in neutral and alkaline gradients, corresponded to 5 min. As shown in Fig. 4 — I, a 30-min chase revealed molecules with a sedimentation coefficient in the region of 40 S. After a 1-hr chase, a considerable amount of 62 S DNA along with 80 and 90 S DNA was present (Fig. 4 — II). Eighty

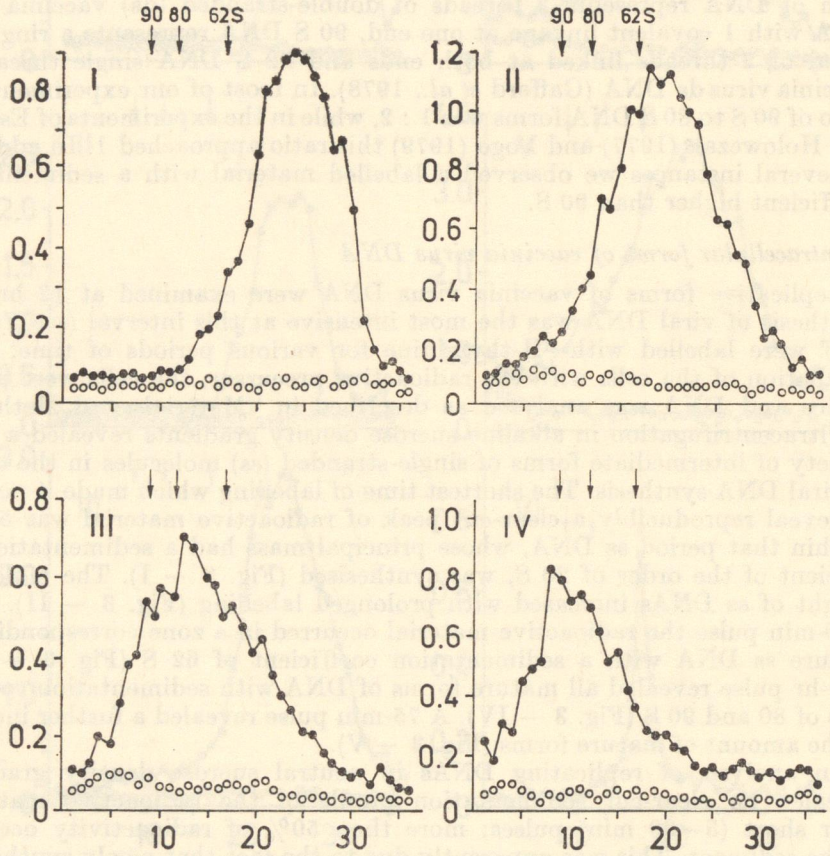

Fig. 4.
Sedimentation pattern in alkaline sucrose density gradients of viral DNAs (pulse-chase experiment)
Pulse for 5 min; chase for 30 min (I), 1 hr (II), 3.5 hr (III) and 5 hr (IV).
Other designations as in Fig. 3.

S DNA forms represented the bulk material found in alkaline sucrose density gradients after a 3.5 hr chase (Fig. 4 — III). The principal mass of DNA fragments that had been synthesised within the 5-min pulse were transformed after 5 hr into mature 90 S DNA molecules with covalent linkages at both ends (Fig. 4 — IV). In neutral gradients, like in experiments on pulse labelling, DNA materials were observed only after a 1-hr chase and at more remote intervals of incubation. They represented a homogeneous population of macromolecules with a sedimentation coefficient of 62 S, analogous to the materials shown in Fig. 3 — VI.

Discussion

According to the available experimental data, replication of vaccinia virus DNA resembles to a considerable extent that of eukaryotic cell DNA. First, short 10—12 S polynucleotides of the type of Okazaki's fragments are synthesised. These are then linked together to form fragments of intermediate size, of which mature viral DNAs are formed. In addition, the replication of viral DNA proceeds by a semiconservative mechanism symmetrically and develops in two directions (Holowczak and Diamond, 1976; Esteban and Holowczak, 1977; Esteban *et al.*, 1977; Pogo and O'Shea, 1978). These results were obtained in L and HeLa cells. Magee and Levine (1970) showed that the cytoplasmic fractions of infected CEF contained viral DNA in the form of small fragments which on completion reached a size corresponding only to 20% of the size of mature viral DNAs. CEF are known to produce complete viral progeny and such a character of reproduction of viral DNA in CEF would be incomplete. Therefore it was necessary to characterize the process of viral DNA replication in CEF. This made possible to compare the replication of viral DNA in various cell species and thus reveal any effect of the host cell on this process. In addition, this study showed the prospects of a detailed investigation of poxviral DNA replication with the use of DNA$^-$ mutants obtained previously (Chernos *et al.*, 1978) and replicating in these cells.

The present results are in general in good agreement with those obtained in L and HeLa cells. However, the delayed synthesis of viral DNA in CEF as compared with L and HeLa cells was due to a protracted course of viral DNA synthesis. The half-width of the peak of the curve illustrating the rate of viral DNA synthesis in L and HeLa cell suspension cultures as revealed by pulse labelling corresponds to 2 hr on the average (Joklik and Becker, 1964; Esteban and Holowczak, 1977), while in CEF it was 5 hr (see Fig. 1).

An analysis of intracellular intermediate forms of DNA, appearing in the course of the replication process, indicates that the synthesis of vaccinia viral DNA in monolayer CEF cultures proceeds intermittently via a number of intermediate steps. Small segments of ss fragments are completed to the order of 40 S (see Fig. 4 — I) and the latter subsequently to a mature ss molecule with a sedimentation coefficient of 62 S (see Figs 3 — III and 4 — II). This process requires 30 min for completion. After 3.5 hr the ss fragments of 62 S DNA are linked together at one end to form 80 S structures

(see Fig. 4 — III) and the complete cycle of the formation of 90 S DNA linked at both ends was accomplished after 5 hr (see Fig. 4 — IV).

Esteban and Holowczak (1977) and Pogo and O'Shea (1978) reported ss forms of viral DNA with sedimentation coefficients of 70, 92—94 and 102—106 S (estimated in alkaline sucrose density gradients) and 70 S ds DNA (in neutral gradients). In our work the respective values were 62, 80, 90 and 62 S. This discrepancy may be explained by the fact that we used another marker for estimation of the sedimentation coefficient (T2 phage DNA) and another system of calculation (reported by Gafford *et al.*, 1978). Our results are in agreement with those reported by the latter authors.

A characteristic feature of the replication of vaccinia virus DNA is the inclusion of the replicating polynucleotide strands into large cytoplasmic associations called "factories" (Joklik and Becker, 1964; Esteban and Holowczak, 1977). Replication of viral DNA is taking place in these structures. After accomplishment of the synthesis of daughter DNAs they are released from these structures to take part in the further steps of virion assembly (Joklik and Becker, 1964). Centrifugation of the structures labelled with ^{3}H-thymidine in neutral sucrose density gradients revealed a marked peak of radioactivity only after a pulse of at least 60 min. This was apparently due to the fact that the short newly synthesised ss fragments of viral DNA sedimented to the bottom of the centrifuge tube along with the replicative complex. The release of mature ds molecules from this complex could be demonstrated only after prolonging the pulse or chase periods (see Fig. 3 — VI).

A peculiarity of the CEF — vaccinia virus system was the markedly diffuse peak of viral DNA replication and the insufficient synchrony of the individual processes. This fact made the detection of the shortest structural replication units difficult. We failed to obtain evaluable data on the size of ss DNAs synthesised within short intervals (up to 5 min) and to demonstrate clearly fragments with a sedimentation coefficient of 10—12 S (Esteban and Holowczak, 1977). The reproducibility of experiments with prolonged chases (over 3 hr) was poor: in several instances we failed to obtain a clear-cut profile of radioactivity distribution in a zone with a sedimentation coefficient higher than 60 S in alkaline gradients. This was possibly due to aggregation of large ss polynucleotide threads in alkaline sucrose density gradients, as confirmed in experiments on sedimentation analysis of DNA from purified virions, in several of which we observed the labelled material in a zone with a sedimentation coefficient higher than 90 S.

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