Replication of Herpes Simplex Virus DNA after removal of Hydroxyurea Block from Infected Cells

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SUMMARY

Hydroxyurea (HU) treatment of HSV-infected cells markedly inhibits the synthesis of virus DNA. Only 0.36% of the 3H-thymidine label was incorporated into virus DNA in the presence of HU as compared to untreated infected cells. Removal of HU resulted in a renewed synthesis of virus DNA as determined by the gradual increase in the incorporation of 3H-thymidine into HSV DNA. The labelled virus DNA molecules were isolated and chromatographed on benzoylated napthoylated DEAE (BND)-cellulose columns to separate the replicative intermediates that have single-stranded (ss) sequences from the mature double-stranded (ds) DNA genomes. Mature radioactive dsDNA molecules were found to appear at 22 min after removal of HU and gradually increased in amount thereafter. The virus DNA molecules synthesized during the initial 20 min after removal of HU, constitute the replicative intermediates of HSV DNA. It was calculated that the synthesis of HSV DNA proceeds at the rate of about $5 \times 10^6$ daltons per min.

Studies on the mode of replication of herpes simplex virus (HSV) DNA revealed the presence of replicative intermediates of virus DNA which banded in CsCl density gradients at the same density as mature virions. The replicative intermediates of HSV DNA were isolated and characterized by chromatography on BND-cellulose columns and by electron microscopy (Shlomai, Friedmann & Becker, 1976a; Shlomai et al. 1976b; Friedmann, Shlomai & Becker, 1977). HSV DNA molecules that have a replicative loop, DNA molecules that are Y-shaped and DNA molecules that have single-stranded filaments attached to them were identified (Friedmann et al. 1977).

In a previous study (Shlomai et al. 1976a) it was determined that 20 min was the minimal time required for labelled HSV DNA molecules containing single-stranded sequences to become fully double-stranded. However, since this study was made in infected cells at the time that virus DNA synthesis was maximal but not synchronized, it was desirable to establish conditions that would permit HSV DNA replication to be initiated simultaneously on all the virus DNA molecules undergoing replication. This requirement was fulfilled by the use of hydroxyurea (HU; reviewed by Becker, 1976) at a concentration that reversibly inhibits the biosynthesis of HSV DNA (Rosenkranz & Becker, 1973). Under such conditions, removal of the HU leads to an immediate resumption of incorporation of nucleotides into DNA. In HU-treated cells there is transcription of the parental virus DNA (A. Ben-Zeev & Y. Becker, unpublished results) and many of the virus-coded proteins are synthesized (Olshevsky, 1974; Powell, Purifoy & Courtney, 1975). HU seems to prevent DNA synthesis by inhibiting the ribonucleotide diphosphate reductase (Krakoff, Brown & Reichard, 1968; Lewis & Wright, 1974). However, its inhibitory effect can be effectively removed by washing the infected cultures. In the present study, we demonstrate that after the removal of HU from infected cells, 3H-thymidine is incorporated into the replicative intermediates of HSV DNA.
Fig. 1. BND-cellulose chromatography of HSV DNA labelled for different time periods after reversion of inhibition with HU. BSC-1 cells were infected with HSV in the presence of $5 \times 10^{-2}$M-HU. At 12 h after infection these cells were washed extensively with pre-warmed medium to remove the HU and re-incubated for (a) 2.5, (b) 10, (c) 15, (d) 22 and (e) 30 min with 390 $\mu$Ci/ml of $^3$H-thymidine. The cells were harvested and the virus DNA (density 1.718 g/ml) isolated in CsCl gradients (Shlomai et al. 1976a), was chromatographed on BND-cellulose columns. NET buffer (0.3 M) consists of 0.3 M-NaCl, 10$^{-3}$ M-EDTA and 0.01 M-tris/HCl, pH 8.1. NET buffer (1.0 M) consists of 1.0 M-NaCl, 10$^{-3}$ M-EDTA and 0.01 M-tris/HCl, pH 8.1.
DNA during the initial period of 22 min. The first mature dsHSV DNA molecules appear 22 min after removal of HU and the initiation of HSV DNA biosynthesis.

BSC-1 cells were infected with 10 p.f.u./cell of the HF strain of HSV type 1, using Dulbecco's modified Eagle's medium in the absence and presence of $5 \times 10^{-2} \text{ m-HU}$. An initial experiment was done to determine whether treatment of HSV-infected cells with HU at a concentration of $5 \times 10^{-2} \text{ m}$ inhibits the synthesis of virus DNA. Infected cells were labelled with $^3\text{H-thymidine}$ (sp. act. 16.5 Ci/mmol, Nuclear Research Centre, Negev, Israel) and incubated for 12 h at 37 °C with and without HU. The cells were separated by centrifugation in CsCl density gradients (Shlomai et al. 1976a, b). In the infected, untreated cells most of the radioactivity was present in HSV DNA ($1.3 \times 10^6 \text{ ct/min}$) although about 15% of the total radioactivity ($0.2 \times 10^6 \text{ ct/min}$) was also incorporated into cellular DNA. Treatment of infected cells with HU markedly inhibited the incorporation of $^3\text{H-thymidine}$ into virus and cellular DNA (about 99.6%). Radioactive DNA from HU-treated infected cells banded at the density of HSV DNA (0.36% of the virus DNA found in untreated cells) and also at the density of cellular DNA (1.2% of the radioactivity found in infected untreated cells). The reason for the incorporation of radioactive thymidine into parental HSV DNA in the presence of HU is not yet known.

To study the time course of HSV DNA replication in HSV-infected, HU-treated cells, the inhibitor was removed by careful washing and the cultures were re-incubated in fresh medium and labelled with $^3\text{H-thymidine}$ for different time intervals. After exposure to the radioactive isotope, the cells were harvested and the DNA was extracted and centrifuged in CsCl gradients. The virus DNA was then pooled and chromatographed on BND-cellulose columns (Shlomai et al. 1976a, b). The results of a typical experiment are presented in Fig. 1. It is of interest to note that some dsHSV DNA molecules were found 2-5 min after the reversal of HU inhibition. The reason for this is not known although it may be due to the repair of parental DNA molecules that were present in the nuclei of HU-treated cells during the 12-h period of HU treatment. No radioactive dsHSV DNA molecules were found 10 or 15 min after removal of the HU but the amount of radioactive HSV DNA that eluted with caffeine gradually increased (Fig. 1). Labelled dsHSV DNA molecules that eluted with 0.0 M-NET buffer appeared only at 22 min and gradually increased in amount, in parallel with a decrease in the percentage of radioactive HSV DNA molecules that eluted with caffeine (ssHSV DNA). The results, summarized in Fig. 2, show that after 22 min the amount of dsHSV DNA increased linearly during a subsequent period of 100 min. Thus it may be concluded that during the initial 22 min after the removal of HU, $^3\text{H-thymidine}$ was incorporated into HSV DNA molecules having single-stranded sequences as determined by chromatography on BND-cellulose columns. This is a property of the replicative intermediates of HSV DNA. The first mature dsHSV DNA molecules appear at 22 min after initiation of HSV DNA replication. This result is in agreement with our previous finding that about 20 min are required for the semi-conservative replication of a HSV DNA molecule (Shlomai et al. 1976a).

From this and the previous study (Shlomai et al. 1976a) it can be concluded that on average, $5 \times 10^6$ daltons of virus DNA are synthesized during 1 min of DNA replication. This means that about 15000 nucleotides/min are polymerized on each HSV DNA strand. The rate of HSV DNA replication is ten times faster than that of the DNA of CELO adenovirus which was found to be $0.5 \times 10^6$ daltons/min (Bellet & Younghusband, 1972) or type 2 adenovirus, $0.7 \times 10^6$ daltons/min (Pearson, 1975) and SV40 DNA synthesis which was found to be $0.6 \times 10^6$ daltons/min (Nathans & Danna, 1972). The synthesis of Escherichia coli DNA was found to proceed at a rate of 40 kilobases/min (Kornberg, 1974). The
rate of DNA replication in the polytene chromosomes in eukaryotic cells was 0·025 μm/min (about 0·05 × 10^6 daltons/min at 24 °C; Cordeiro & Meneghini, 1973).

Replicative intermediates of HSV DNA having replicative loops were studied by electron microscopy (Friedmann et al. 1977). It was possible to calculate that the initiation site for DNA biosynthesis was either 10 or 20 μm from one of the molecular ends of the DNA. We may therefore assume that part of the HSV DNA molecule replicates bidirectionally and becomes Y-shaped when one replication fork reaches the molecular end closest to the initiation site. Thus, until the Y-shaped molecules of HSV DNA are formed, the replication is bidirectional and subsequently the synthesis of the virus DNA is unidirectional.

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