STIMULATION OF MITOCHONDRIAL DNA SYNTHESIS AS AN EARLY FUNCTION OF HERPES SIMPLEX VIRUS*

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1. Introduction

Infection of HeLa cells by herpes simplex virus type 1 (HSV) results in an enhancement of thymidine incorporation into mitochondrial DNA during the initial period post infectionem (p.i.) [1] whereas that into cellular DNA of nuclear origin is progressively inhibited [2]. Since earlier findings did not exclude a possible involvement of virus-induced nucleotide pool alterations as a basis for the mitochondrial reaction [3] thymidine-kinase-less (tk^-) cells were employed in the present study to examine this possibility in a suitable cell culture system. Furthermore, pertinent to the analysis of the phenomenon, was to clarify whether enhancement of mitochondrial DNA synthesis in virus-infected cells could proceed in the absence of viral DNA replication.

Our results show an enhanced thymidine incorporation into mitochondrial DNA as compared to appropriate controls by HSV-infected tk^- mouse cells and isolated mitochondria of infected cells as well. In addition, whereas the effect of virus infection on mitochondrial DNA synthesis could not be abolished by cytosine abrabinoside (ara-C) cycloheximide (CHI) treatment of infected cultures, however, prevented the mitochondrial reaction in vivo as well as in vitro.

2. Materials and methods

tk^- Mouse cell cultures [4], a generous gift of Dr. H. Koprowski (Wistar Institute, Philadelphia, USA), were cultivated in Eagle's Basal Medium supplemented with 10% calf serum, 50 μg aureomycin and 10 μg 5-bromodeoxyuridine (BUdR) per ml. The type of herpes simplex virus used (type 1) and the infection procedure were identical to those described previously [1]. Multiplicity of infection in all experiments was approximately 10 infectious units per cell. Mock infection was carried out with serum-less medium already used for cultivation. Virus titrations were performed as outlined before [5].

The basic experimental schedule consisted of infection of 10^8 Cl-1D cells from 0–1 hr, labelling from 1–6 hr with [^{14}C]thymidine (0.1 μCi per ml culture medium) and homogenization of the cells with a Dounce type glass homogenizer according to Albring et al. [6]. In the case of drug treatment of mock and virus-infected cultures ara-C was added to the cultures at a final concentration of 2 μg or CHI

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at a final concentration of 25 μg per ml culture medium simultaneously with [^{14}C]thymidine. Isolation of mitochondria from the same cell homogenate was performed following the previously described procedure [7]. In vitro labelling of isolated nuclei with [^{3}H]thymidine triphosphate was carried out according to the method of Hershey et al. [8]. Isolated mitochondria were labelled in vitro with [^{3}H]thymidine as described by Koike and Kobayashi [9].

DNA of isolated nuclei was characterized by isopycnic centrifugation in cesium chloride (CsCl) [2] after lysis of doubly labelled nuclei in 2 percent Sarkosyl. Mitochondrial DNA was extracted from isolated doubly labelled organelles following essentially the method of Hirt [1,10] and characterized by isopycnic centrifugationin cesium chloride–ethidium bromide (CsCl–EthBr) [11]. DNA and protein were quantitated and radioactivity assayed as described before [12].

3. Results

Table 1 compares thymidine uptake into mitochondrial DNA by mock-infected and virus-infected cells as well as by isolated organelles of mock- and virus-infected cells under various experimental conditions. In the initial experiment which was performed without administration of metabolic inhibitors from 1–6 hr p.i. [^{14}C]thymidine incorporation into mitochondrial DNA was not determined because contamination of mitochondria by labelled viral DNA could not be excluded, even when DNAse treatment of isolated organelles was employed prior to characterization of DNA. When ara-C which is known to exhibit a considerably more pronounced inhibitory effect on nuclear and viral [13] than on mitochondrial DNA synthesis [14] was added to mock- and virus-infected cultures from 1–6 hr p.i. ^{14}C-label of mitochondrial DNA from virus-infected cells was found to exceed that of the controls approximately five-fold. Under these conditions there was no labelling of either nuclear or viral DNA (fig. 2D and F). Organelles isolated 6 hr p.i. from the cultures which were either not pretreated or treated with ara-C from 1–6 hr p.i. continued to incorporate [^{3}H]thymidine at a considerably enhanced rate (table 1) when compared to the controls also under in vitro conditions. CHI pretreatment, on the other hand, abolished the effect of HSV-infection on mitochondrial DNA synthesis in vivo as well as in vitro. Characterization of ^{3}H-label from isolated mitochondria by isopycnic centrifugation in CsCl–EthBr (fig. 1 A,B,C) revealed a radioactivity distribution typical for replicating mitochondrial DNA [15].

In order to analyze nuclear and viral DNA synthesis of the same cells the nuclear fraction was subjected to in vitro labelliing with [^{3}H]thymidine triphosphate in parallel. Doubly labelled DNA was subsequently characterized by isopycnic centrifugation in CsCl. As shown in fig. 2 ^{14}C-radioactivity (fig. 2B) could be recovered only from infected nuclei in the position

Table 1
Picomoles of thymidine incorporated into 1 μg of mitochondrial DNA* of mock- and virus-infected Cl-1D cells

Pretreatment of cells in vivo (1–6 hr p.i.)	In vivo [^{14}C]**		In vitro [^{3}H]**	
	Mock-infected	Virus-infected	Mock-infected	Virus-infected
None	6.1070	–***	0.1702	0.3645
Ara-C 2 μg/ml Culture medium	3.3870	19.7727	0.1785	0.4462
Cycloheximide 25 μg/ml culture medium	5.0733	6.0117	0.0911	0.0812

* Calculated on the basis of the value of 0.5 μg mitochondrial DNA/mg mitochondrial protein.
** Calculated on the basis of dpm recovered from CsCl–ethidium bromide gradients loaded with mitochondrial DNA corresponding to 2 mg of mitochondrial protein.
*** Not determined because of contamination with labelled viral DNA.

of viral DNA (1.725 g/ml). Most of the ^{3}H-label (fig. 2A) from infected nuclei which exceeded that of control nuclei considerably was found in the position of viral DNA as well, that of nuclei from mock-infected tk^{-} cells, on the other hand, in the position of cellular DNA of nuclear origin (1.698 g/ml). Pretreatment of mock-infected and HSV-infected cultures with either ara-C or CHI from 1–6 hr p.i. prevented incorporation of ^{14}C-label into nuclear as well as viral DNA (fig. 2D and F) in vivo. In vitro labelling of isolated nuclei from ara-C pretreated mock-infected and viral-infected cultures proceeded, however, at a rate comparable to that from untreated cultures. Gradient radioactivity profiles of ^{3}H-label of DNA from mock- and virus-infected nuclei of ara-C pretreated cultures were essentially identical to those of nuclei from untreated cultures (fig. 2C), e.g. predominant labelling of viral DNA in infected nuclei and labelling of cellular DNA of nuclear origin in mock infected nuclei. [^{3}H]Thymidine-triphosphate uptake into acid-insoluble material by nuclei from HSV-infected cultures following CHI-treatment, on the other hand, did not exceed that of nuclei from CHI-treated mock-infected nuclei. However, the radioactivity distribution in CsCl-gradients of ^{3}H-labelled material differed in that DNA from infected nuclei banded in part in the position of viral DNA, whereas DNA of control nuclei was recovered from the position of cellular DNA (fig. 2E).

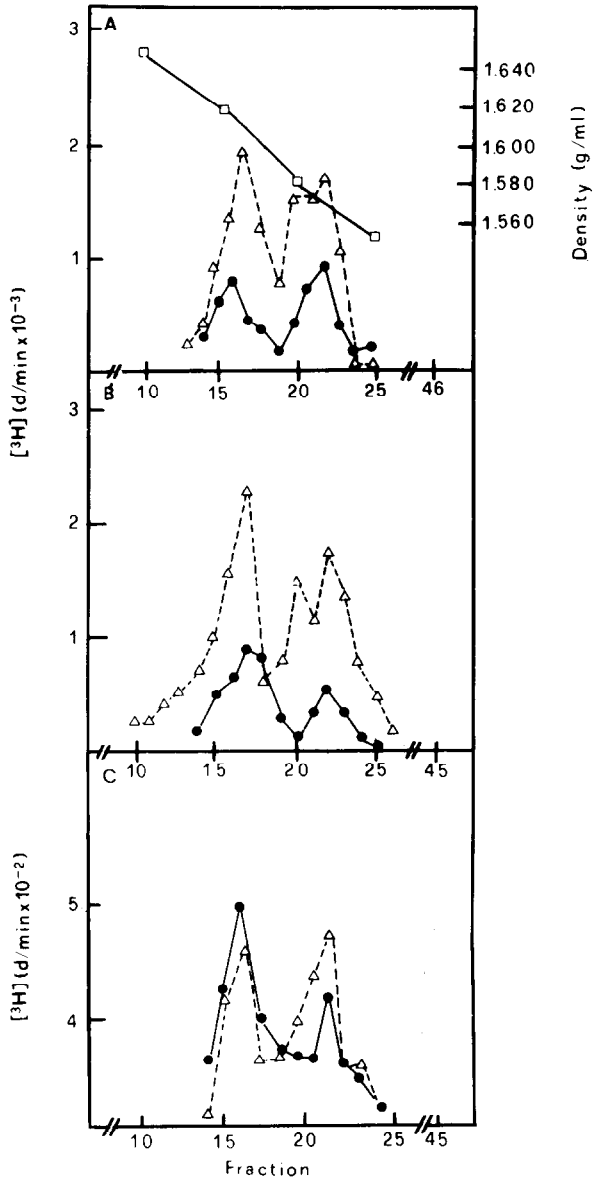

Fig. 1. Isopycnic centrifugation in cesium chloride–ethidium bromide (250 μg/ml) of mitochondrial DNA from equal amounts of mock- (●——●) and virus-infected (△– – –△) Cl-1 D cells. Cultures were labelled with 0.1 μCi [^{14}C]thymidine/ml culture medium from 1–6 hr p.i. Cell fractionation at 6 hr was followed by in vitro labelling of isolated mitochondria for 60 min with [^{3}H]thymidine and extraction of DNA: (A) shows radioactivity profiles for ^{3}H-label when cultures were not pretreated from 1–6 hr p.i.; (B) represents an identical experiment after pretreatment of cultures in vivo with 2 μg ara-C/ml culture medium from 1–6 hr p.i.; (C) identical experiment except for pretreatment of cultures in vivo with 25 μg CHI/ml culture medium from 1–6 hr p.i. Top of the gradient is to the right. Circular double stranded DNA = 1.608 g/ml, Linear double stranded DNA = 1.570 g/ml. Centrifugation conditions: 44 000 rpm, 24 hr, 20°C in a 65 fixed angle rotor of a Beckman centrifuge.

4. Discussion

The data reported here indicate that the enhancing effect of HSV-infection on mitochondrial DNA synthesis in tk^{-} cells does not involve virus-induced nucleotide pool alterations [3] since organelles of infected cells continue to incorporate DNA precursors into their DNA at an elevated rate as compared to controls also under in vitro conditions. The observa-

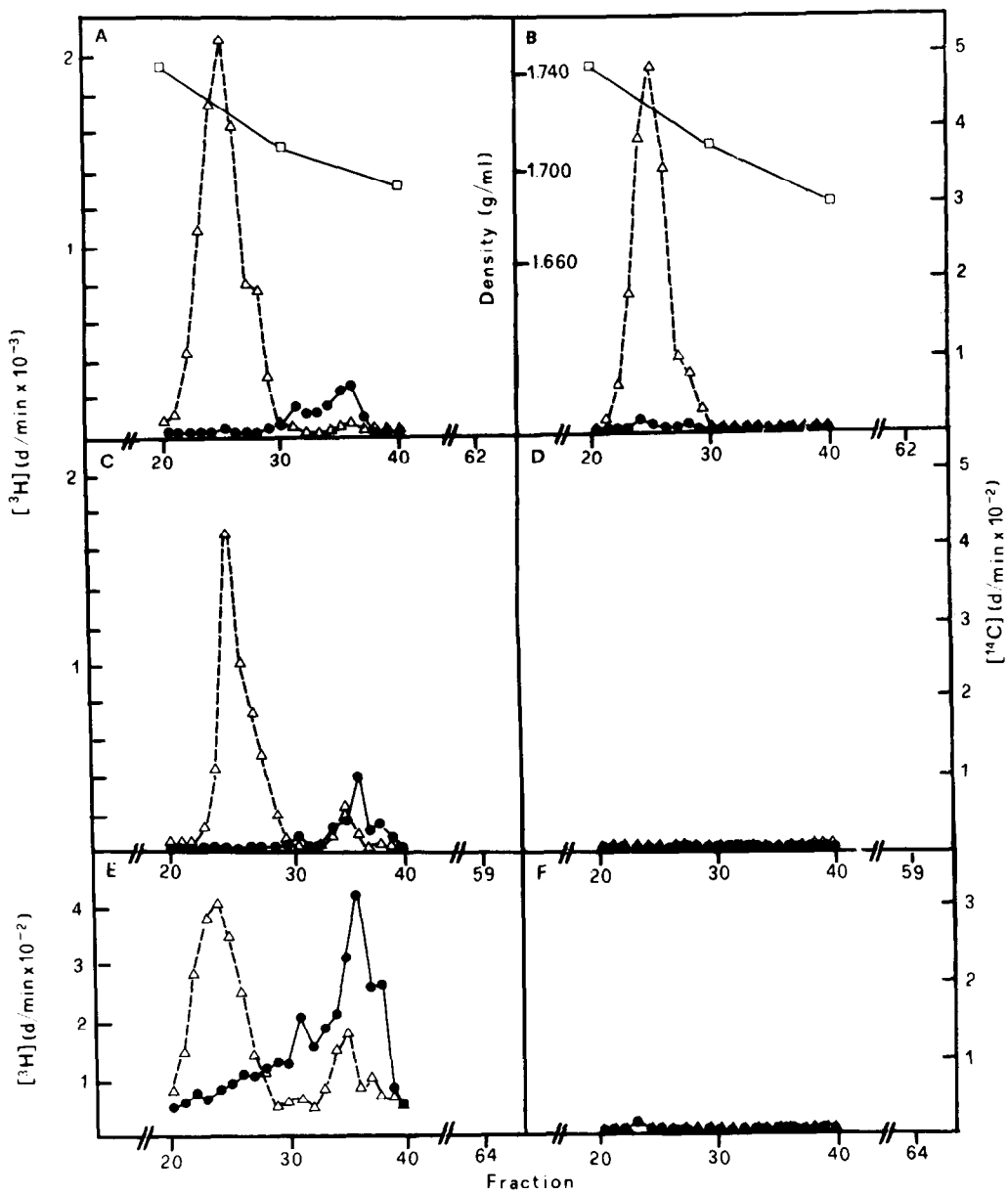

Fig. 2. Isopycnic centrifugation in cesium chloride of DNA of isolated nuclei from equal amounts of mock- (●——●) and virus-infected (△– – –△) Cl-1D cells. Cultures were labelled with 0.1 μCi [^{14}C]thymidine/ml culture medium from 1–6 hr p.i. Cell fractionation at 6 hr was followed by in vitro labelling of isolated nuclei for 30 min with [^{3}H]thymidine triphosphate and lysis of nuclei. (A) shows radioactivity profiles for ^{3}H and (B) for ^{14}C when cultures were not pretreated from 1–6 hr p.i. (C) and (D) represent an identical experiment showing radioactivity distribution for ^{3}H and ^{14}C, respectively, after pretreatment of cultures in vivo with ara-C (2 μg/ml culture medium) from 1–6 hr p.i. (E) and (F) demonstrate radioactivity pattern for ^{3}H-label and ^{14}C-label, respectively, of an experiment following an identical schedule except for pretreatment of cultures in vivo with 25 μg CHI/ml culture medium from 1–6 hr p.i. Top of the gradients is to the right. Viral DNA = 1.725 g/ml, cellular DNA 1.698 g/ml. Centrifugation conditions: 44 000 rpm, 60 hr, 20°C in a 65 fixed angle rotor of a Beckman centrifuge.

tion that CHI but not ara-C abolishes the virus-induced mitochondrial reaction suggests the phenomenon to be a consequence of an early function of HSV [7], because the stimulatory effect of virus infection was also found in the absence of viral DNA replication but was prevented when nuclear and viral DNA- and protein synthesis were inhibited.

A tentative interpretation of these findings has to take into account that nuclear-directed cellular macromolecular synthesis decreases progressively in the same cells following infection by HSV as a result of virus-induced 'inhibitors' [2,16]. Activation of cell-specific synthetic processes in infected cells can therefore not be due to an induction of enzymes translated on cytoplasmic ribosomes under the control of the nuclear genome. Hence, an induction of mitochondrial DNA polymerase, for instance, as a basis for the mitochondrial reaction in infected cells can essentially be excluded since this enzyme has been reported to be of nuclear-cytoplasmic origin [17]. In view of the knowledge of the HSV–host cell reaction [1,2], on one hand, and the present concept of nuclear–mitochondrial interaction in eukaryotic cells [18], on the other hand, activation of mitochondrial DNA synthesis in HSV-infected tk^- cells may reflect the progressive elimination by 'virus-induced inhibitors' of a so hypothetical nuclear repressor system with a short half-life for mitochondrial DNA replication. Prerequisite for this assumption would be the insensitivity of mitochondrial DNA synthesis to 'viral inhibitors' [1,7].

However, on the basis of these preliminary experiments it cannot be excluded that permeability differences between mock-infected and virus-infected mitochondria might contribute to the mitochondrial reaction observed. Experiments are in progress to determine and characterize activity of mitochondrial DNA polymerase from infected cells directly in comparison to that of mock-infected cells.

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