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Cycloheximide inhibits cellular, but not SV40, DNA replication

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We have prepared extracts from cycloheximide-treated cells for the study of simian-virus-40 (SV40)-DNA replication in vitro. When supplemented with the viral initiator protein (large T antigen), these extracts fully supported SV40-DNA replication. We also determined that SV40-DNA replication in vivo is much more resistant to cycloheximide than cellular DNA replication. SV40 encodes its own initiator protein, T antigen, which also functions as a DNA helicase, but depends on cellular functions for all additional replication reactions. Therefore, it appears to be quite likely that cycloheximide affects cellular DNA replication by blocking the synthesis of (a) cellular function(s) that is(are) performed by T antigen in SV40-DNA replication. Indeed, DNA fiber autoradiography and alkaline sucrose gradient centrifugation of pulse-labeled cellular DNA showed that cycloheximide treatment almost completely suppressed replicon initiation and reduced the rate of replication fork movement to about one third of the control.

1. INTRODUCTION

It has been known for two decades that mammalian DNA replication depends on ongoing protein synthesis. This conclusion is based on observations showing that cycloheximide and other drugs, interfering with protein synthesis, cause a rapid reduction of [^{3}H]thymidine incorporation into the DNA of proliferating mammalian cells in culture. Since translation inhibitors of different structures and different mechanisms of action have identical effects on DNA replication it appears to be highly unlikely that essential replication proteins are directly affected, e.g. by protein-bound drugs. Early experiments had also excluded the possibility that cycloheximide and related drugs may affect cellular nucleotide metabolism. Therefore, the inhibition of DNA replication by cycloheximide and other translation inhibitors are best explained by assuming that one or more essential replication factors are rapidly inactivated or degraded, and that these factors have to be synthesized de novo to allow DNA replication to continue [1,2].

Recent experiments have shown that translation inhibitors induce the expression of certain genes. However, it is unlikely that this could play a part in replication inhibition since at least 100-fold higher drug concentrations are needed to effectively block protein synthesis and DNA replication [3].

The nature of the replication factors that are either inactive or missing in drug-treated cells is presently unknown. However, with the establishment of assays for the cell-free replication of SV40-DNA [4] it is now possible to reinvestigate this problem.

In these in vitro assays, protein-free viral DNA or plasmid DNA, carrying the SV40 origin of replication [4], as well as SV40 minichromosomes [5–7] are replicated by a classical semiconservative mechanism; DNA replication is initiated at a fixed origin of replication where two replication forks are established which then proceed bidirectionally to meet each other at a termination site just opposite on the circular DNA template. These reactions require only one virus-encoded protein, large T antigen, whereas all other functions are provided by a protein extract from proliferating 'permissive' human cells (reviewed in [8,9]).

T antigen binds specifically to sequence motifs in the SV40 origin of replication where it induces a conformational change in the double helix. In the presence of ATP hydrolysis, T antigen unwinds the complementary DNA strands at the origin and then serves as a helicase to propagate replication forks (reviewed in [10]).

All other reactions, required for SV40 DNA replication, are performed by cellular proteins. These include the DNA polymerase α–primase complex for lagging and the DNA polymerase δ for leading strand synthesis as well as several auxiliary factors such as the 'proliferating cell nuclear antigen' (PCNA) and replication factor C (RF-C). Additional essential cell proteins are the human single-strand-specific binding protein (replication factor A, RF-A), DNA topoisomerases, RNase H and ligase (for recent reviews, see [11,12]).

If one of these proteins were missing or inactive in extracts, prepared from cells treated with the translation

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Abbreviations: SV40, simian virus 40; CHX, cycloheximide.

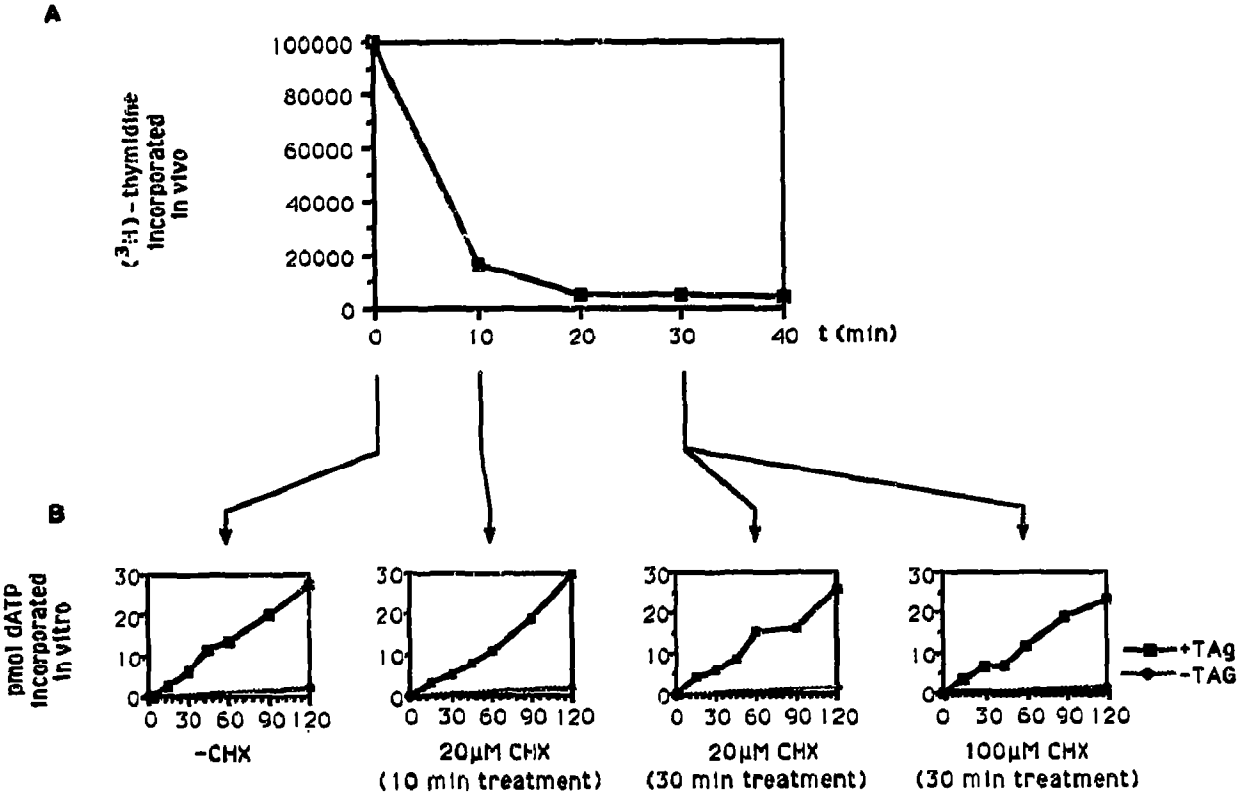

Fig.1. Extracts from CHX-treated HeLa cells support origin-dependent DNA replication in vitro. (A) Pulse-label with [^{3}H]thymidine. We present an experiment with 20 μM CHX, added at t = 0 min, to proliferating HeLa cells. The data, obtained with 50 and 100 μM CHX, were virtually identical (not shown). (B) DNA replication in vitro. Incubations were performed using 100 ng pSVMO1-DNA, about 250 ng cellular proteins and 1 μg purified T antigen in 0.05 ml reaction volumes. Control incubations were performed in parallel assays in the absence of T antigen. Aliquots of 5 μl were removed at the indicated times (min, abscissa) to determine the incorporated radioactivity by acid precipitation. The remainder of the samples were processed for agarose gel electrophoresis (see Fig. 2).

inhibitor cycloheximide (CHX), the chain elongation reaction in the in vitro SV40-DNA replication assay would be impossible. On the other hand, if replication proceeded undisturbed in extracts from CHX-treated cells, it would be concluded that, in the in vitro system, T antigen replaces the cellular protein(s) the amount or activity of which is limited in CHX-treated cells. In this case, we would also expect that CHX has less severe effects on SV40 DNA replication in vivo as it is known that the nuclei of SV40-infected cells contain high amounts of T antigen. The experiments, reported in this communication, were performed to investigate these possibilities.

2. MATERIALS AND METHODS

2.1. *Cells, virus and radioactive labeling*

Human HeLa cells (American Type Culture Collection *CCL 2.2*) were used for the preparation of cell extracts. The TC7 subline of monkey CV1 cells (American Type Culture Collection *CCL70*) was used for viral infection. The cells were grown on plastic dishes in 5% fetal calf serum under standard cell culture conditions. Infection with SV40 and DNA extraction has been described before [13]. Uninfected HeLa or CV1 cells at 24 h after infection were labeled with 0.04 μCi [^{14}C]thymidine (Amersham) for 10–16 h (long-term label). Medium was then removed and replaced by 2 ml fresh medium (plus 5% fetal calf serum), containing CHX in the concentrations given below. After various times in CHX, pulse-labeling was performed by the addition of 10 μCi/ml [^{3}H]thymidine for 8–10 min. The cells were then washed with ice-cold phosphate-buffered saline and collected in the same buffer before lysis in 0.5% sodium dodecylsulfate and precipitation of DNA by trichloroacetic acid. Viral DNA was extracted by the Hirt procedure [14] and precipitated by trichloroacetic acid. The long-term ^{14}C-label served as an internal standard. Labeled cellular DNA was investigated by alkaline sucrose gradient sedimentation as previously described [18]. Pulse-labeled viral DNA was denatured in 0.1 M NaOH and also analyzed by alkaline sucrose gradient sedimentation [13].

2.2. *SV40 DNA replication in vitro*

Protein extracts were prepared from untreated and CHX-treated HeLa cells as described in [4] with minor modifications [7]. T antigen was purified from crude extracts by an immunoaffinity procedure [16]. As a template for in vitro DNA replication, we used plasmid pSVMO-1, a pUC9-derivative carrying the SV40 origin of replication (SV40 nucleotide coordinates: 5171 to 42). Replication was performed according to published procedures [4,7] using [α-^{32}P]dATP as a radioactive tracer. The replication products were purified from the reaction mixture and investigated by agarose gel electrophoresis and autoradiography [7].

2.3. *DNA fiber autoradiography*

Untreated and, in parallel experiments, CHX-treated HeLa cells (2×10^5 cells/35-mm dish) were incubated in 1 ml prewarmed medium

with 1 μM [^{3}H]thymidine (methyl-[^{3}H]; 82 Ci/mmol, Amersham) ('hot pulse'). After 20 min, a 'warm pulse' was started by the addition of non-radioactive thymidine to dilute the specific radioactivity to 1/10. After an additional 20 min incubation period, the cells were washed with phosphate-buffered saline, trypsinized and processed for autoradiography as previously described [17]. After a 6-month exposition, the autoradiographs were developed and evaluated as described [18].

3. RESULTS AND DISCUSSION

3.1. *Replication efficiencies of protein extracts prepared from CHX-treated cells*

We used CHX at concentrations between 10 and 100 μM CHX which are sufficient to reduce the rate of protein synthesis within 10 min to less than 1% of the control as determined by [^{35}S]methionine or [^{3}H]leucine incorporation (not shown). As demonstrated in Fig. 1, the rate of [^{3}H]thymidine incorporation into cellular DNA also rapidly decreased to 10% or less of the control value. Similar data were obtained using 80 μM puromycin as the protein inhibitor (not shown).

Protein extracts were prepared from normal and from CHX-treated HeLa cells [4]. These extracts were added to assay mixtures containing purified T antigen and, as a template, plasmid pSVMO1, carrying the SV40-origin. Incubation at 37°C revealed that the incorporation of ^{32}P-labeled nucleotides into acid precipitable material was identical regardless of whether the protein extracts were prepared from untreated cells or from cells, treated with CHX for different lengths of times or at different drug concentrations (Fig. 1).

It is unlikely that the in vitro DNA synthesis observed was due to repair since the incorporation of ^{32}P-labeled nucleotides depended on T antigen (Fig. 1) and required the SV40 origin of replication as plasmids without the viral origin were inactive as templates for in vitro replication (not shown; see [4]).

However, to clearly demonstrate that replication was identical in extracts from untreated and CHX-treated cells we isolated the in vitro synthesized ^{32}P-labeled DNA for analysis by agarose gel electrophoresis and autoradiography. As demonstrated in Fig. 2, radioactively labeled material appeared after 15 min incubation time as slowly migrating replicative intermediates and, at later times, as closed circular form II DNA in relaxed and slightly superhelical configuration. In addition, we observed in these (and other similarly performed) experiments a large fraction of radioactivity in slowly migrating, large DNA structures. These forms include replicative intermediates and catenated DNA forms as well as rolling circle type intermediates [7] which may arise after artefactual breakage of one fork of in vitro replicating DNA.

For the present discussion, it is important to note that the replication products were very similar for extracts from untreated and from CHX-treated cells.

Thus, all cellular replication elongation functions, known to be essential for SV40 DNA replication in vitro, are quire active in extracts from CHX-treated cells. Similar data were obtained using puromycin which inhibits protein synthesis by an entirely different mechanism (not shown).

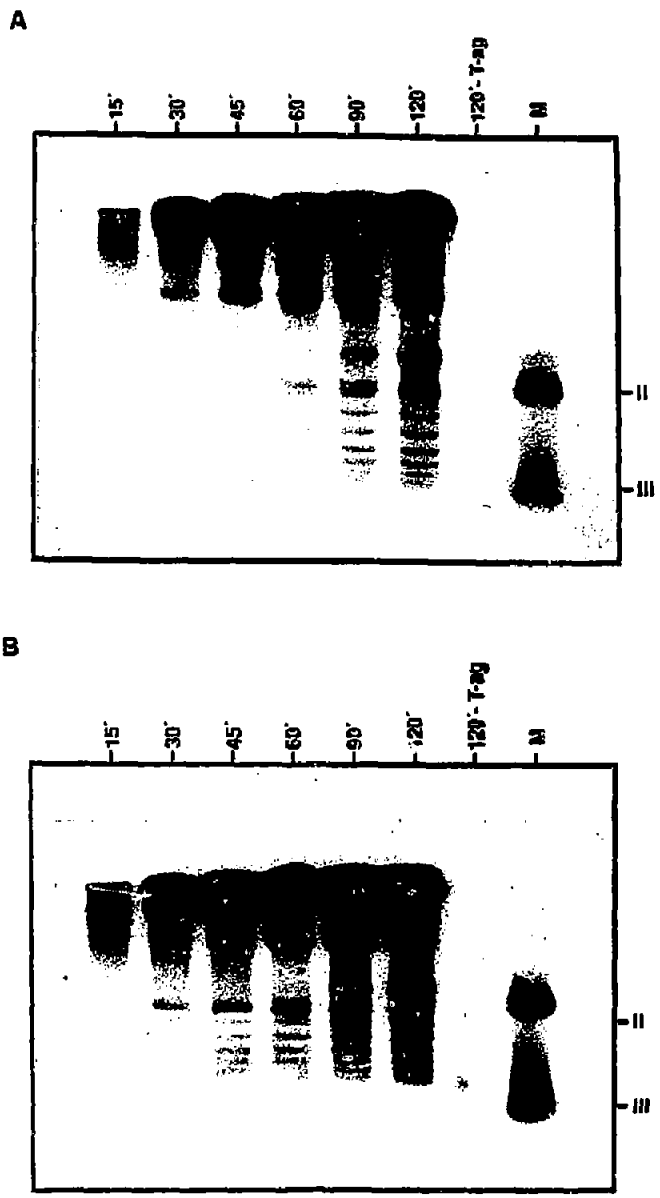

Fig. 2. Replication products, synthesized with extracts from untreated and from CHX-treated HeLa cells. Aliquots were removed at the indicated times from in vitro assay mixtures and treated first with RNase A (100 μg/ml) and then with proteinase K (100 μg/ml) before the DNA was purified by extraction with phenol and chloroform. The DNA was precipitated twice with ethanol and analysed on 1% agarose gels and autoradiography. (A) DNA synthesis in extracts, prepared from untreated HeLa cells; (B) DNA synthesis in extracts, prepared from cells, treated for 30 min with 20 μM CHX (see Fig. 1). In vitro replication times (in min) are indicated. The results of control incubations (120 min in the absence of T antigen) are also shown (−T-ag). The electrophoresis markers (M) are nicked circular (form II) and linear (form III) pSVMO1 DNA.

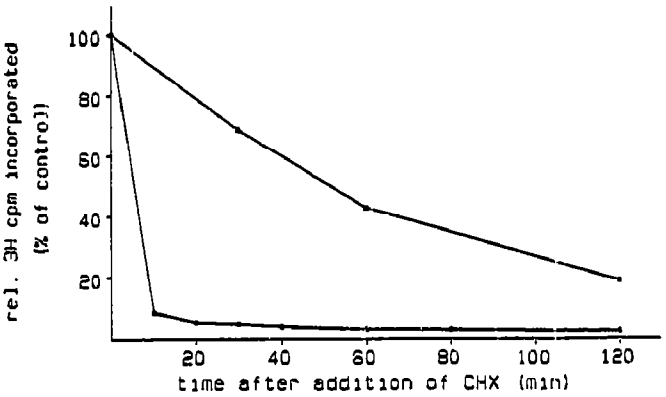

Fig. 3. Effect of CHX on SV40 DNA replication in vivo. SV40-infected CV1 cells were long-term labeled with [^{14}C]thymidine, treated with 30 μM CHX and pulse-labeled with [^{3}H]thymidine at the times indicated. SV40-DNA was extracted by the Hirt procedure, acid-precipitated and counted (□). For comparison, we show the results of a parallel experiment, measuring the incorporation of pulse-label into the DNA of uninfected cells (○).

A prediction, derived from the data of Figs. 1 and 2, is that SV40 DNA replication in vivo should be less vulnerable to CHX treatment than cellular DNA replication because nuclei of SV40-infected cells contain high amounts of T antigen. The experiments, reported in the next section, were performed to investigate this possibility.

3.2. *Effects of CHX on SV40-DNA replication in vivo*

As described above (Fig. 1), the rate of cellular DNA synthesis, determined by pulse-labeling with [^{3}H]thymidine, decreases to less than 10% within 10 min after addition of CHX. In contrast, SV40-DNA replication was found to be far less sensitive to CHX; incorporation of [^{3}H]thymidine decreased to only 70% and 50% of the control at 30 and 60 min after addition of CHX, respectively; and even at 120 min, the rate of ^{3H} incorporation was still about 20% of the control (Fig. 3).

To determine whether the mode of SV40-DNA replication changes in the presence of CHX, we investigated the structure of pulse-labeled SV40-DNA by alkaline sucrose gradient sedimentation. Under these conditions, mature covalently closed SV40-DNA sediments as a random coil with >50 S whereas nicked circular and replicative intermediate DNA is denatured to give linear DNA strands of different lengths. Using this technique, we were unable to detect significant differences between the structures of pulse-labeled SV40-DNA, synthesized before and at increasing times after addition of CHX (Fig. 4). An interesting, but unexplained observation was that the fraction of pulse-labeled DNA, appearing in mature, fast sedimenting supercoiled DNA, increased from 6% to about 14% of total incorporated radioactivity upon treatment with CHX (Fig. 4).

In sum, the results of the in vivo experiments show that SV40 DNA replication is far less sensitive to CHX-

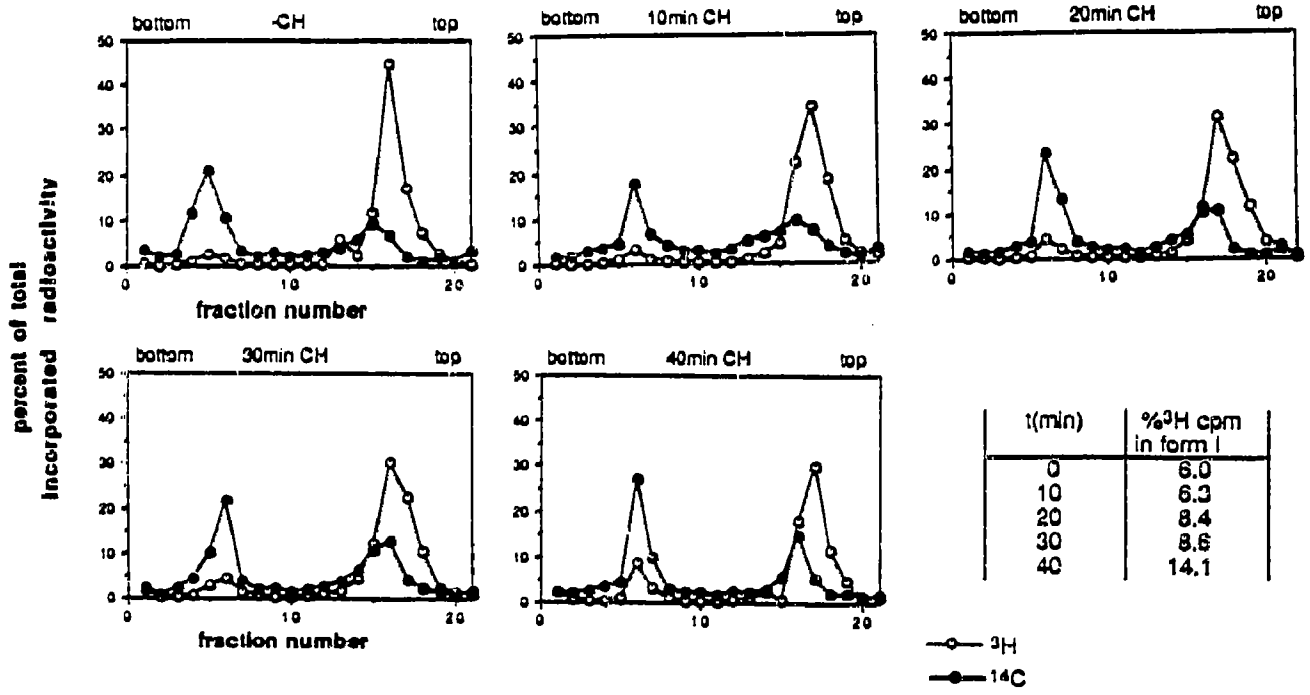

Fig. 4. Analysis of SV40-DNA by alkaline sucrose gradient centrifugation. SV40-DNA was long-term labeled with [^{14}C]thymidine and pulse-labeled with [^{3}H]thymidine before and at the indicated times after addition of CHX as described in section 2. Hundred-percent values are: ^{14}C, ca. 1200 cpm; ^{3}H, 181,000 cpm (control); 155,000 cpm (10 min in CHX); 135,000 cpm (20 min); 110,000 cpm (30 min); and 68,000 cpm (40 min). The table (insert) gives the fraction of pulse-labeled DNA in mature supercoiled form I SV40-DNA.

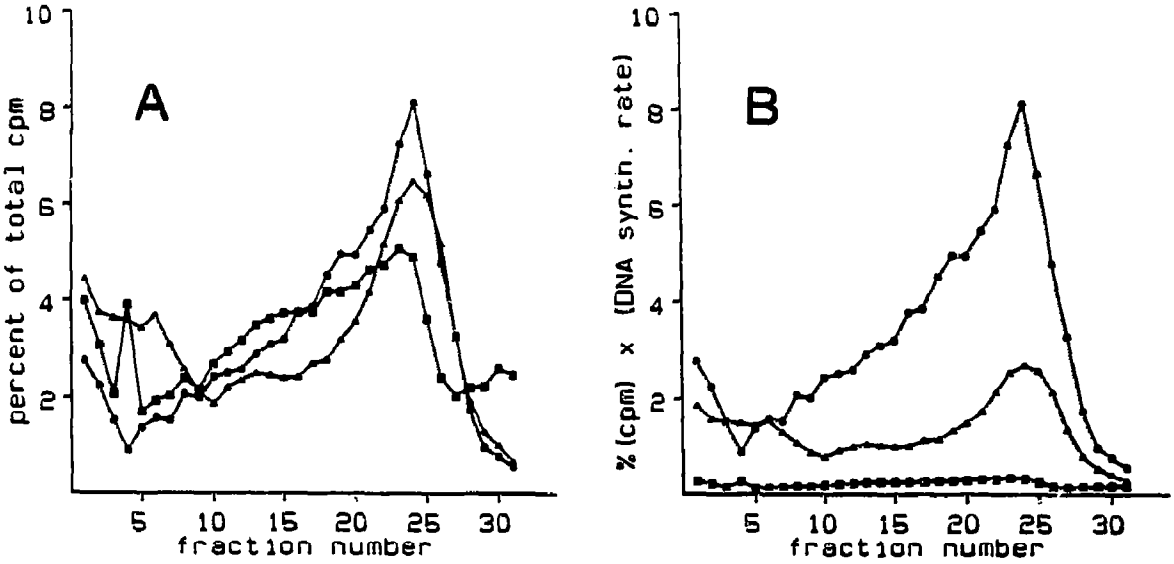

Fig. 5. Alkaline sucrose gradient sedimentation of labeled cellular DNA strands in untreated and CHX-treated HeLa cells. Untreated and CHX-treated (30 μM) HeLa cells were pulse-labelled for 8 min with [^{3}H]thymidine. The cells were washed with cold phosphate-buffered saline, trypsinized and lysed on top of an alkaline sucrose gradients which were made up and centrifuged exactly as described [21]. Direction of sedimentation is from the right to the left. ^{14}C-labeled phage λ DNA served as a marker (37–40 S) (not shown). (A) Untreated control (●); 2 h after CHX (■); 2 h in CHX, followed by 40 min in CHX-free medium (▲). The ordinate gives the percentage of recovered radioactivity. The total radioactivity was: untreated control, 183,600 cpm; 2 h in CHX, 12,630 cpm; 40 min recovery after CHX-treatment, 76,100 cpm. (B) Data of (A) were multiplied by the quotient of the (separately determined) rates of DNA synthesis in CHX-treated and control cells. Sedimentation was from the right to the left.

treatment than cellular DNA replication. The most likely reason for this is the high concentration of T antigen, the viral initiator protein, in the nuclei of infected cells.

However, T antigen is subjected to post-translational modifications; and it is known that highly phosphorylated 'old' T antigen is unable to properly bind to the viral origin and to induce replication (reviewed in [19]). Thus, in the presence of CHX, the synthesis of new and replication-competent T antigen is prevented, and stored T antigen is inactivated by phosphorylation. This explains why the rate of SV40-DNA replication slowly decreased with time after addition of CHX.

In general, however, the results of the in vivo experiments agree with those obtained from DNA replication in vitro. They suggest that replication elongation functions are not depleted in CHX-treated cells. Since both viral and cellular DNA replication depend on the same set of replication functions, it appears that the lack of (an) active cellular initiator(s) may be responsible for the drastic effects of CHX on cellular DNA replication.

3.3. *Cellular DNA replication in CHX-treated cells*

As a first attempt to determine the nature of this function, we used DNA fiber autoradiography [20] to investigate the frequency of cellular replication initiation events and the fork movement rate in untreated and CHX-treated HeLa cells [18].

The relative frequency of replication initiation [18,22] was determined as the ratio of the number of grain pattern indicative of initiation within the 20-min 'hot' labeling period and the number of post-initiation grain pattern (Table I). We found a reduction of this ratio from 1.44 before to 0.09 after CHX-treatment showing that replicon initiation was severely inhibited in CHX-treated HeLa cells. The fork movement rate, determined as the lengths of the autoradiographic silver grain tracks, was also reduced from 0.86 μm/min in normal to 0.3 μm/min in CHX-treated cells. However, the arrangement of active replicons along the parental

Table I

Results of DNA fiber autoradiography

	Control	20 min CHX
Relative initiation frequency*	1.44 n = 523	0.09 n = 199
Fork movement rate (μm/min)	mean = 0.86 n = 427 S.D. = 0.31	mean = 0.30 n = 357 S.D. = 0.12
Inter-initiation distance (μm)	mean = 67.0 n = 172 S.D. = 31.5	mean = 44.9 n = 75 S.D. = 30.3

Post-pulse initiation pattern, —■—; pre-pulse initiation pattern, —■ ■—. * The 'relative initiation frequency' is the ratio of post-pulse initiation to pre-pulse initiation pattern.

DNA as represented by inter-initiation distances appeared not to be significantly affected by CHX (Table I) suggesting that clusters of replicons fail to be simultaneously activated in CHX-treated cells.

The autoradiographic data were supported by the results of an entirely different approach, the analysis of chain length distribution by alkaline sucrose gradient sedimentation [21]. In untreated control cells, most of the DNA strands, pulse-labeled with [^{3}H]thymidine for 8 min, sedimented with a peak at about 40 S (Fig. 5). After CHX treatment, the amount of incorporation of [^{3}H]thymidine-labeled DNA strands was drastically reduced as expected (see Fig. 1). However, the residual radioactivity appeared in DNA strands that were clearly longer than the DNA strands, made in the absence of CHX, suggesting a continued growth of initiated DNA strands and a reduction of initiation events. This is not due to a general toxic effect on cell metabolism since [^{3}H]thymidine-labeled 40 S DNA strands reappeared soon after removal of CHX (Fig. 5) indicating that new replicons were reinitiated.

Thus, the results of both experiments, fiber autoradiography and alkaline sucrose gradient centrifugation, suggested an almost complete block of replication initiation. In addition, fiber autoradiography indicated a reduced fork movement rate in HeLa cells as noted before by others [1,2]. Interestingly, in Ehrlich ascites cells, suppression of initiation appears to be the only response of the replication machinery to CHX treatment [15].

4. CONCLUSION

Our data show that cellular functions, responsible for replicative chain elongation in the in vitro SV40 replication system, remain active after treatment of HeLa cells with CHX. In addition, SV40 DNA replication in vivo is far less sensitive to CHX-treatment than cellular DNA replication. Except for the virus-encoded, T-antigen-associated DNA helicase, the viral and the cellular replication apparatus share the same group of elongation functions. Therefore, the reduction of cellular DNA replication in CHX-treated cells is best explained assuming that cellular functions, responsible for the initiation of replication, are absent or inactive in these cells.

Indeed, fiber autoradiography demonstrated an almost complete block of replication initiation, and, in addition, a reduced fork movement rate in HeLa cells. It will be noted that these two activities, an activation of origins and the propagation of replication forks, are performed by T antigen in SV40 DNA replication. And it is an intriguing possibility that the CHX-sensitive cellular function may be a cellular counterpart of T antigen.

However, since almost nothing is known about the nature of cellular initiator proteins we can only speculate about possible mechanisms of their inactivation after CHX treatment. The proteins could be degraded, or they could be sequestered by being tightly bound to DNA sites in the origin or in the termination region of a replicon where they remain unavailable for the activation of origin sequences in other replicons. It is also possible that the proteins are inactivated by modifications. The inactivation of T antigen by hyperphosphorylation may serve as an example [19]. In any case, our experiments have implications for a further analysis of the mammalian replication machinery as the concentration of active initiator proteins may be quite low and difficult to investigate by standard biochemical procedures.

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