Hydroxyurea-Induced Accumulation of Short Fragments During Polyoma DNA Replication

II. Behavior During Incubation of Isolated Nuclei

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The synthesis of polyoma DNA was studied in isolated nuclei from hydroxyurea-inhibited 3T6 cells infected with polyoma virus. During incubation of nuclei under conditions suitable for polyoma DNA synthesis *in vitro*, the short DNA fragments with a sedimentation coefficient of 4S formed *in vivo* (hydroxyurea fragments) became associated with preformed, replicating DNA strands. Centrifugation in dye-buoyant density gradients showed that the fragments formed part of the structure of the replicative intermediate of polyoma DNA. The proportion of "young" replicative intermediates was larger after hydroxyurea inhibition than in uninhibited controls. Hydroxyurea fragments appear to be closely related to the 4S fragments formed as normal intermediates during discontinuous synthesis of polyoma DNA.

During hydroxyurea inhibition of polyoma-infected 3T6 cells, short fragments of polyoma DNA are preferentially synthesized. These fragments which have a sedimentation coefficient of 4S are slowly converted to longer strands and may be normal intermediates in polyoma DNA synthesis which accumulate during deoxyribonucleotide starvation induced by hydroxyurea (3, 4).

The preceding communication (3) described the formation of the hydroxyurea fragments in intact cells and characterized some of their properties. Herein are described experiments using isolated nuclei obtained from polyoma virus-infected cells inhibited with hydroxyurea. These experiments contribute to the characterization of the DNA molecules containing the hydroxyurea fragments.

MATERIALS AND METHODS

Detailed descriptions of the sources of the chemicals, the handling of cells and virus, and the general methodology have been given previously (3, 5, 11).

Chemicals. The [$\alpha^{32}\text{P}$]-labeled derivatives of dCTP, dGTP, and dTTP ($4 \cdot 10^4$ counts per min per picomole) were prepared as described by Symons (10) and purified by ion exchange chromatography before use. *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Sigma Chemical Co., and the nonionic detergent Nonidet P-42 was from Shell Company.

Hydroxyurea inhibition and ^3H -thymidine labeling of cells. The conditions for hydroxyurea inhi-

bition and pulse labeling with ^3H -thymidine (^3H -dThd) of polyoma virus-infected 3T6 cultures were described previously (3).

Preparation of nuclei. Nuclei were prepared 26 to 28 h after polyoma virus infection as described previously (11). However, the cells were lysed by vigorous shaking for 4 min in isotonic HEPES buffer containing 0.5% (wt/vol) Nonidet P-42. The packed nuclei were suspended in two volumes of isotonic HEPES buffer and immediately used for *in vitro* experiments.

***In vitro* incubation of nuclei.** Nuclei were incubated at 25°C under the standard conditions for polyoma DNA synthesis described earlier (11). The [$\alpha^{32}\text{P}$]-labeled deoxyribonucleoside triphosphates and Br-dUTP were used at final concentrations of about $10 \mu\text{M}$.

Characterization of viral DNA. Viral DNA was selectively extracted by the Hirt procedure (2) and directly used for analysis in alkaline sucrose gradients as described previously (3). Equilibrium centrifugations in alkaline Cs_2SO_4 and propidium iodide-cesium chloride density gradients were also done as previously described (5). Absolute values of buoyant density in the Cs_2SO_4 gradients were based on the banding position of calf thymus DNA (1.434 g/cm^3). Before equilibrium gradient centrifugation the viral DNA was purified as described previously (3) including removal of labeled closed circular DNA by chromatography on benzoylated-naphthoylated DEAE-cellulose (BND-cellulose).

RESULTS

DNA synthesis in isolated nuclei from hydroxyurea-inhibited cells. Nuclei were isolated from polyoma virus-infected hydroxyurea-

inhibited 3T6 cells. The cells were pulsed for 10 min with ^3H -dThd 10 min after the addition of hydroxyurea. Immediately after the pulse, nuclei were prepared and incubated for 1.5, 5, or 45 min with $[\alpha^{32}\text{P}]\text{-dTTP}$ under standard conditions for polyoma DNA synthesis. Nuclei from pulse-labeled, uninhibited cells served as controls. Viral DNA was extracted by the Hirt procedure from both sets of nuclei and analyzed for total acid-insoluble ^3H and ^{32}P radioactivity. In the controls, the incorporation of ^{32}P was essentially linear during the first 5 min, but then leveled off (Fig. 1). A more detailed analysis of the kinetics of such an experiment was given earlier (11). During the first 1.5 min, the nuclei from hydroxyurea-inhibited cells showed approximately the same rate of ^{32}P incorporation as the controls. After that time, however, the process became slower, and after 45 min of incubation, the amount of dTTP incorporated was only 40% of that of the controls. The amount of acid-insoluble ^3H -label which was a measure of the DNA synthesized during the *in vivo* pulse remained constant throughout the experiments with both sets of nuclei, indicating that no significant amount of DNA synthesized *in vivo* was degraded during the *in vitro* incubations (data not shown). In a separate experiment it was found that addition of 5 mM hydroxyurea to isolated nuclei had no effect on the *in vitro* synthesis. This result precludes the possibility that the inhibition depicted in Fig. 1 was caused by hydroxyurea remaining with the nuclei after their isolation.

The effect of the length of the time period in hydroxyurea on the *in vitro* DNA synthesis is shown in Fig. 2. It is clear that the major part of the inhibition found after 45 min of incubation *in vitro* was already present after 20 min of incubation of the cells with hydroxyurea. In no instance was there a significant inhibition at the 1.5 min time point.

Sedimentation properties of viral DNA synthesized in isolated nuclei. The viral DNA obtained from hydroxyurea-inhibited cells and control cultures after 10 min of labeling with ^3H -dThd *in vivo* and additional labeling with $[\alpha^{32}\text{P}]\text{-dTTP}$ in isolated nuclei was characterized by alkaline sucrose gradient centrifugation. The sedimentation properties of the controls were similar to earlier results (4) (Fig. 3A, B, and C). At all times the ^3H -labeled material (*in vivo* incorporation) sedimented with peaks at 53S (form I) and 16S (completed viral strands). The ^{32}P incorporated *in vitro* during the first 1.5 min appeared in two radioactive peaks at 16S and at 4S (Fig. 3A). After 5 or 45 min of *in vitro* incubation (Fig. 3B and C) the ^{32}P -labeled

molecules shifted to the 16S position. The results were different when nuclei from hydroxyurea-inhibited cells were used in an identical experiment. After 1.5 min of *in vitro* incubation, both ^3H and ^{32}P appeared as major peaks at about 4S and as smaller peaks at 16S (Fig. 3D). After 5 (Fig. 3E) or 45 min (Fig. 3F) of *in vitro* incubation, the sedimentation profiles demonstrated a gradual increase in molecular weight for both ^3H - and ^{32}P -labeled chains. However, the radioactive peak at 16S never became as sharp as that found in the control experiment. With longer incubation times, a small amount

FIG. 1. Time course of viral DNA synthesis in isolated nuclei. Nuclei isolated from control cultures (○) and from hydroxyurea-inhibited cells (▼) were incubated with $[\alpha^{32}\text{P}]\text{-dTTP}$ under standard conditions for polyoma DNA synthesis. Acid-insoluble radioactivity in the Hirt supernatant fluid was measured and related to the total nuclear DNA content.

FIG. 2. Influence of the time period of hydroxyurea inhibition on DNA synthesis in isolated nuclei. Hydroxyurea was added to infected cultures, and nuclei were isolated at different times after onset of inhibition. *In vitro* incubations of nuclei were then carried out for 1.5, 5, and 45 min under standard conditions with $[\alpha^{32}\text{P}]\text{-dTTP}$, and the radioactivity of viral DNA was measured and normalized by relating it to the nuclear DNA content.

FIG. 3. Characterization by alkaline sucrose gradient centrifugation of viral DNA synthesized in isolated nuclei. Viral DNA labeled for 10 min with ^3H -dThd in vivo was further labeled by incubation of nuclei with $[\alpha^{32}\text{P}]\text{-dTTP}$ and then was centrifuged through alkaline sucrose gradients. Panels A, B, and C represent uninhibited controls, and panels D, E, and F give data from incubation of nuclei isolated from hydroxyurea-treated cells. Nuclei were incubated for 1.5 (A, D), 5 (B, E), or 45 min (C, F). The arrows give the positions of the markers (53 and 17S). $\bullet$, ^3H ; ∇ , ^{32}P .

of radioactivity also appeared at the 53S position. The results demonstrate that 4S fragments obtained from hydroxyurea-inhibited cells (hydroxyurea fragments) and labeled in vivo with ^3H -dThd were converted to longer DNA chains during in vitro incubation of nuclei isolated from these cells.

Density labeling with Br-dUTP of viral DNA synthesized in vitro. The mechanism for the transformation of hydroxyurea fragments into longer DNA chains was further investigated by using Br-dUTP as a density label during the in vitro synthesis of DNA. For this purpose, nuclei were isolated from hydroxyurea-inhibited cultures after a 10-min pulse with ^3H -dThd and incubated for 5 or 45 min with Br-dUTP and $[\alpha^{32}\text{P}]\text{-dCTP}$. The initial rate and the extent of viral DNA synthesis, as well as the sedimentation properties in alkaline sucrose gradients, were similar to those of the previous experiments. The viral DNA from the different time points was analyzed by buoyant density centrifugation in alkaline Cs_2SO_4 gradients. The DNA labeled for 10 min in vivo (0 time of

the in vitro experiment) was recovered as a broad symmetrical peak (Fig. 4A). The broad band width reflects upon the small size of the molecules. After 5 min of incubation with Br-dUTP in vitro, the major ^3H -peak was still found at the density of polyoma DNA ($\rho = 1.445$), but this peak now had a shoulder reaching to a position corresponding to a 40 mg/cm^3 increase in density (Fig. 4B). The ^{32}P -label incorporated in vitro banded with two peaks of roughly equal size. One was slightly denser than polyoma DNA, whereas the other coincided with the heavier part of the ^3H -label. After 45 min of incubation with Br-dUTP, the general distribution of the ^3H -label in the gradient (Fig. 4C) was similar to that found after 5 min of incubation. Both the main peak and the shoulder were shifted to slightly higher densities, however. The ^{32}P -label coincided closely with the ^3H -label throughout the whole gradient, suggesting that the short fragments synthesized during hydroxyurea inhibition in vivo became covalently associated with DNA synthesized in vitro.

FIG. 4. Characterization of DNA density labeled *in vitro* by alkaline Cs_2SO_4 equilibrium centrifugation. Hydroxyurea-inhibited cells were labeled for 10 min with ^3H -dThd. Nuclei were then isolated and incubated for 0, (A), 5 (B), or 45 min (C) with Br-dUTP and [$\alpha^{32}\text{P}$]-dCTP. The viral DNA was then analyzed by centrifugation in alkaline Cs_2SO_4 density gradients. Panel D presents the result of centrifugation after shearing of DNA synthesized during 45 min of *in vitro* incubation. The arrow indicate the buoyant density of authentic polyoma DNA. ●, ^3H ; ▽, ^{32}P .

When the 45-min sample was sheared by sonic treatment, the ^3H - and ^{32}P -label were partly dissociated. The labels were recovered in two broad peaks with mean density increases of about 20 and 50 mg/cm^3 , respectively (Fig. 4D). This result is consistent with the release of daughter DNA chains synthesized *in vivo* prior to the administration of ^3H -dThd. Taken together, the results depicted in Fig. 4C and 4D suggest that during *in vitro* incubation the ^3H -labeled light hydroxyurea fragments were added both onto preexisting light chains and to newly synthesized ^{32}P -labeled heavy chains.

Fate of DNA synthesized shortly before hydroxyurea treatment. The experiments described so far give no information about the replicating viral strands which exist in the infected cells immediately before addition of hydroxyurea. In the following experiment, these strands were labeled with ^3H , and their behavior was studied after density labeling *in vitro*. For this purpose, infected cells were pulsed with ^3H -dThd for 10 min, and the label was chased for 30 s with unlabeled dThd before addition of hydroxyurea. After 20 min of treatment with the drug, nuclei were isolated and incubated for 5 or 45 min with Br-dUTP and [$\alpha^{32}\text{P}$]-dGTP. The viral DNA was then analyzed by centrifugation through alkaline sucrose gradients and by equi-

librium centrifugation in alkaline Cs_2SO_4 gradients.

During alkaline sucrose gradient centrifugation of DNA from the 5-min incubation, the ^3H -label from the *in vivo* pulse sedimented as two sharp peaks at 16 and 53S, whereas the ^{32}P -label was broadly distributed between the 16S region and the top of the gradient (Fig. 5A). Upon additional incubation, no change occurred in the sedimentation profile of the ^3H -label, whereas the ^{32}P -labeled material then had a higher average sedimentation rate, as was expected from an increase in the size of the replicating chains (Fig. 5B).

The results of equilibrium centrifugation in alkaline Cs_2SO_4 of viral DNA after 5 and 45 min of *in vitro* incubation are shown in Fig. 6A and B. At both time points most of the ^3H -labeled DNA strands banded at a density close to that of light polyoma DNA, whereas the ^{32}P -labeled material showed a bimodal distribution with peaks at positions corresponding to about 10 and 50 mg/cm^3 increases in density. Figure 6C depicts the banding properties of sheared viral DNA from the 45-min time point. The prelabeled ^3H -chains remained light, whereas the ^{32}P -label was recovered as a broad asymmetrical peak with an average increase in density of about 50 mg/cm^3 .

FIG. 5. Effect of hydroxyurea treatment and *in vitro* incubation on the sedimentation properties of prelabeled DNA. Infected cells were labeled for 10 min with ^3H -dThd in the absence of hydroxyurea. After a 30-s chase with 0.10 mM dThd and 0.10 mM dCyd, the drug was added to the medium. Nuclei were isolated 20 min after onset of inhibition and incubated for 5 (A) or 45 min (B) with Br-dUTP and [$\alpha^{32}\text{P}$]-dGTP. Viral DNA was then analyzed by centrifugation through alkaline sucrose gradients. The arrows give the positions of the markers (53 and 17S). ●, ^3H ; ▽, ^{32}P .

This experiment suggests that the ^3H -label in the chains, synthesized just before addition of hydroxyurea to the cells, is not closely linked to the ^{32}P -label incorporated during *in vitro* incubation of the nuclei.

Propidium iodide-CsCl equilibrium centrifugation of viral DNA formed during hydroxyurea treatment. The replicative intermediates (RI) of polyoma DNA can be fractionated in dye-buoyant density gradients (6). "Young" molecules band at a position close to that of supercoiled, form I DNA, whereas molecules

containing very long replicating strands band at a lower buoyant density together with relaxed DNA. Centrifugation in propidium iodide-cesium chloride gradients was used to characterize the RI DNA formed during hydroxyurea inhibition. Of particular interest was to determine the degree of superhelicity of the molecules. It was not possible to analyze directly the RI molecules formed *in vivo*, for the hydroxyurea fragments synthesized during hydroxy-

FIG. 6. Effect of hydroxyurea treatment and Br-dUTP incorporation *in vitro* on the buoyant density of prelabeled DNA. Viral DNA labeled as described in the legend to Fig. 5 was chromatographed on benzoylated-naphthoylated DEAE-cellulose to remove form I DNA and then was centrifuged to equilibrium in alkaline Cs_2SO_4 gradients. Panel A shows the buoyant density of material incubated for 5 min *in vitro*, and panels B and C show the density distribution after 45 min of *in vitro* incubation before and after shearing, respectively. The arrows give the banding position of authentic polyoma DNA. ●, ^3H ; ▽, ^{32}P .

urea inhibition detached from the parental strands during extraction of DNA (5). Instead, nuclei were isolated from hydroxyurea-inhibited cells after a 10-min pulse with ^3H -dThd and incubated for 5 min in vitro with $[\alpha^{32}\text{P}]$ -dCTP to "fix" the short fragments. Under these conditions, no short fragments were released during the Hirt extraction as demonstrated by neutral sucrose gradient centrifugation (data not shown).

The viral DNA from this experiment as well as viral DNA from similarly treated nuclei

isolated from uninhibited control cells were analyzed by propidium iodide-cesium chloride centrifugation. In the control experiment the majority of the labeled molecules banded close to the position of relaxed DNA (Fig. 7A), whereas in contrast the viral DNA synthesized in nuclei from inhibited cells appeared as a broad, radioactive peak at a buoyant density intermediate between those of form I and form II DNA (Fig. 7B). The ^3H -label from the in vivo pulse and the ^{32}P -label incorporated in vitro coincided throughout the gradient indicating that the result was not due to a kinetic effect dependent on the length of the labeling time. These results indicate that the parental strands of RI molecules present after hydroxyurea inhibition do not contain nicks. The degree of superhelicity appears to be greater in these molecules than in the corresponding RI DNA obtained from controls, suggesting that RI molecules accumulating during hydroxyurea inhibition contain a relatively large fraction of young RIs.

DISCUSSION

The in vitro synthesis of polyoma DNA in isolated nuclei is a continuation of the in vivo replication process and involves the addition of all four deoxyribonucleoside triphosphates to molecules of replicative intermediates whose synthesis was begun in vivo (5). In the present work the nuclear system was used for further characterization of the RI formed in cells inhibited with hydroxyurea. When inhibited cells were pulse-labeled for 10 min with ^3H -dThd, about 80% of the label in the viral DNA was recovered at 4S fragments upon centrifugation through alkaline sucrose gradients (3). When nuclei were isolated from such cells and incubated in vitro under standard conditions for polyoma DNA synthesis, the hydroxyurea fragments increased gradually in size (Fig. 3D, E, and F). After 45 min of incubation, the fragments were converted to molecules with a sedimentation coefficient of close to 16S in alkaline sucrose gradients.

When a density label was introduced into the newly synthesized viral DNA during in vitro incubation, the ^3H -label of the hydroxyurea fragments appeared in two classes of molecules, one showing a small and the other a much larger density shift relative to light polyoma DNA (Fig. 4). In the same experiment, the in vitro synthesized ^{32}P -labeled DNA was distributed in a similar way. It seems likely that the molecules with the small density shift represent hydroxyurea fragments formed in vivo, which then attached to preformed light strands during the

FIG. 7. Characterization of replicative intermediate DNA by centrifugation in propidium iodide-cesium chloride density gradients. Nuclei isolated from uninhibited control cells or from hydroxyurea-inhibited cells, pulse-labeled for 10 min with ^3H -dThd, were incubated for 5 min with $[\alpha^{32}\text{P}]$ -dCTP in vitro. Viral DNA from the control nuclei (A) and from nuclei isolated from the inhibited cells (B) was centrifuged to equilibrium in propidium iodide-cesium chloride gradients. The arrows in panel B give the positions of ^{14}C -labeled polyoma closed circular and relaxed DNA. ●, ^3H ; ∇, ^{32}P ; ■, ^{14}C .

in vitro incubation. The origin of the second class of heavy molecules is not clear, however. They might result from elongation of hydroxyurea fragments which had failed to join preexisting light strands, or they might represent molecules which had just started replication when the nuclei were isolated and which, therefore, contained short progeny strands. The second alternative is favored by the results of equilibrium centrifugations in propidium iodide-caesium chloride gradients (Fig. 7) which suggest that nuclei from hydroxyurea-inhibited cells contained a considerably higher proportion of early RI than did normal nuclei. However, the available data do not indicate that the early RIs contain a higher proportion of hydroxyurea fragments than late RIs. These experiments also demonstrate that after hydroxyurea treatment, the parental strands of the RI still were covalently closed circles and thus were not nicked during incubation of the cells with hydroxyurea. Degradation of the daughter strands also appears to be precluded, for the ^3H -label in the hydroxyurea fragments remained acid-insoluble during incubation of the nuclei, and the density shift experiments (Fig. 6) show that the prelabeled daughter strands remained light, even after shearing the DNA.

Short fragments of viral DNA with a sedimentation coefficient of about 4S were found as intermediates during replication of both polyoma (4) and simian virus 40 DNA (1). The existence of these fragments, which resembled the "Okazaki fragments" in *Escherichia coli* (7, 9), suggests that viral DNAs are replicated by a discontinuous mechanism. Similar short fragments of viral DNA accumulate when the synthesis of precursors for DNA synthesis is limited by hydroxyurea (3) or fluoro-deoxyuridine (8). From the present results it seems clear that these fragments are synthesized de novo and do not result from drug-induced degradation of DNA. These fragments are closely related to the short DNA fragments formed as intermediates during normal replication of viral DNA. It was proposed (4) that the drug-induced accumulation of short fragments depends on a

selective inhibition of a late step in the discontinuous synthesis of polyoma DNA, with a gap-filling DNA polymerase as a possible target. An alternative explanation might be that the greatly decreased rate of DNA synthesis per se could give rise to an accumulation of short DNA fragments.

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