ACCUMULATION OF SHORT DNA FRAGMENTS IN HYDROXYUREA TREATED MOUSE L-CELLS

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SUMMARY

Treatment of L-cells with hydroxyurea markedly inhibits the incorporation of [^{3}H]thymidine into DNA. The ^{3}H incorporation that persists during hydroxyurea inhibition is largely into 7S DNA chains. The labelled fragments can be chased into higher MW DNA, suggesting that they are intermediates in the replication process. This interpretation concurs with that of earlier reports which describe a similar effect of hydroxyurea on the replication of viral DNA.

INTRODUCTION

Hydroxyurea (HU) is a potent inhibitor of DNA replication in both prokaryotes and eukaryotes (1, 2). There is substantial evidence indicating that the primary biochemical effect of HU is inhibition of ribonucleoside diphosphate reductase. HU inhibits both purified E.Coli ribonucleoside diphosphate reductase (3) and reductase activity in extracts of mammalian cells (4), and the levels of dATP and dGTP are depressed in HU-inhibited cells (5). Recent reports (6-8) on the effect of HU on the replication of viral DNA provide an indication of the consequence of depressed levels of deoxynucleotides in relation to the DNA polymerases thought to be involved in a discontinuous replication process in which DNA is first synthesized as short chains (Okazaki fragments) that are subsequently joined by gap-filling and

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Footnote: Abbreviation - HU, hydroxyurea

ligation. The observed accumulation of short fragments during HU inhibition of DNA replication in polyoma virus (6), SV40 (7), and adenovirus (8), was interpreted as indicating a relatively greater sensitivity of the "gap-filling" DNA polymerase(s) to lowered deoxynucleotide pools, as compared to the "elongating" DNA polymerase(s). In this communication we report a similar effect of HU on the replication of L-cell DNA.

METHODS

Growth and labelling of cells: L-cells (from Commonwealth Serum Laboratories, Melbourne) were grown as monolayers in Modified Eagle's Medium supplemented with 10% foetal calf serum, and were free of mycoplasma contamination as monitored by relative incorporation of [^{14}C]uracil and [^{3}H]uridine (9). In all experiments, cells were in log-phase growth at the time of labelling and/or HU treatment. [^{3}H]TdR (Amersham, usually 45 ci/mmole, 1 mCi/ml) was added to a final concentration of 1 μM.

Cell lysis: With one exception, experiments were terminated by rinsing the monolayers three times with cold saline, and the addition of 2 ml of 0.5% SDS in 25 mM Tris (pH 7.5)/5 mM EDTA. After 30 minutes at 37°C, 0.2 ml of Pronase (Calbiochem, 10 mg/ml in 25 mM Tris/5 mM EDTA, pre-incubated for 2 hours at 37°C) was added and incubation continued for 1 hour at 37°C. In one case, a nuclear suspension was prepared from the rinsed monolayer by lysis and mixing in buffered saline containing 0.5% NP40. Nuclei were pelleted by centrifugation, resuspended in saline and lysed in SDS/pronase as above.

Alkaline Sucrose Gradients: Linear gradients of 5 to 20% sucrose were prepared in SW27 polyallomer tubes containing a 5 ml cushion of 45% sucrose. The gradient solutions included 0.3 M NaOH, 0.7 M NaCl, 5 mM EDTA and 0.015% Sarkosyl. Freshly prepared lysates were gently mixed with 0.1 volume of 3 M NaOH and layered onto the gradients. After centrifugation in a Beckman SW27 Rotor for 16 hours at 23,000 rpm at 20°C, the gradients were collected using an ISCO Density Gradient Fractionater and each 1 ml fraction was mixed with 50 μg of carrier DNA. TCA-insoluble material was collected on glass fibre filters (Whatman GF/B) and analysed by liquid scintillation counting using soluene and a toluene-based scintillation mixture (10).

All gradients included [^{14}C]PM2 DNA as an internal marker. The PM2 DNA was labelled and extracted from virus particles purified on a CsCl gradient (11). The marker sample, a mixture of nicked and intact molecules, was prepared by limited digestion of intact viral DNA with pancreatic DNAase (Calbiochem). S-values were calculated as described (12) assuming a value of 21S for the nicked marker (13).

RESULTS

Fig. 1a shows the size distribution of acid-insoluble material labelled during HU inhibition. The peak fraction corresponds to a sedimentation coefficient of 7S, whereas the

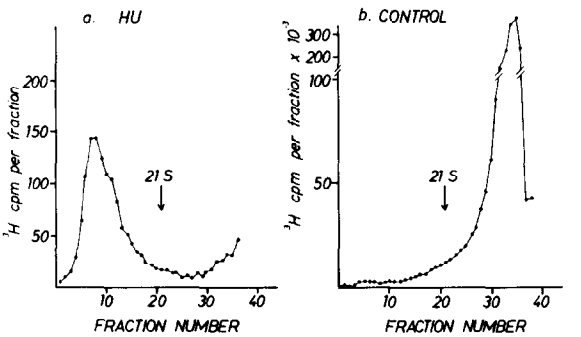

Fig. 1: Alkaline sucrose gradient analysis of labelled L-cell DNA.

(a) Hydroxyurea was added to an L-cell culture to a final concentration of 10 mM, and after 90 minutes this was followed by the addition of [^{3}H]TdR. The 20 minute labelling period was terminated by lysis as described in "Methods".

(b) A replicate culture was treated in the same way as (a) except that saline was added instead of HU.

The direction of sedimentation is from left to right.

material labelled in an uninhibited culture was much larger (Fig. 1b). The extent of labelling in the inhibited culture was 0.05% of that of the control. The material analysed was presumably DNA since it became acid-soluble after digestion with DNAase (result not shown). In a separate experiment it was found that the majority of the acid-insoluble label, in both HU-treated and control cultures, was associated with the nuclear fraction (data not shown).

In order to investigate the possibility that the HU fragments arose from endonuclease digestion of larger DNA molecules, a culture was pre-labelled with [^{3}H]thymidine for 20 minutes, chased with unlabelled deoxycytidine (0.1 mM) and thymidine (1 mM) for 30 minutes, exposed to 20 mM HU for 60 minutes, and finally lysed in SDS/pronase. Sedimentation analysis in an alkaline sucrose

gradient yielded a profile similar to that shown in Fig. 1b - no peak of small fragments was evident.

In another experiment, to be reported in detail elsewhere, a culture was pre-labelled with bromodeoxyuridine, chased in thymidine and then treated with HU and [^{3}H]thymidine as usual. Analysis of the lysate in an alkaline cesium sulphate equilibrium density gradient showed that all the labelled fragments banded as light single stranded DNA, indicating that the [^{3}H]incorporation was not repair-type synthesis.

A problem was encountered in pulse-chase experiments with HU-treated cells because the amount of [^{3}H]thymidine incorporated into DNA was several hundred-fold lower than that in acid-soluble nucleotides. This situation was presumably a consequence of both the marked inhibition of DNA synthesis by HU and also the reported expansion of the dTTP pool in HU-treated cells (5). When cells were treated with HU for 1 to 2 hours, labelled with [^{3}H]TdR for 20 minutes, and then chased in the presence of excess unlabelled thymidine (up to 1 mM) after removal of HU, there was considerable additional incorporation of [^{3}H] into DNA during the chase. In an attempt to reduce the relative extent of ^{3}H-TTP production, minimal conditions of HU exposure were sought. The detailed results of the dose-response experiments will be reported elsewhere but, in essence, it was found that short DNA fragments were still preferentially labelled by [^{3}H]thymidine after only 10-20 minutes in 5 mM HU. Additional incorporation of label during the chase was also reduced by including HU with excess unlabelled thymidine during the first part of the chase period; hence the chase protocol described in the legend of Fig. 2. In the pulse-chase experiment shown in Fig. 2, the mean [^{3}H]thymidine incorporation into acid-insoluble material in triplicate cultures

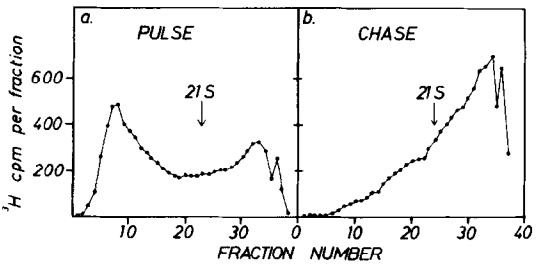

Fig. 2: Alkaline sucrose gradient analysis of a pulse-chase experiment.

(a) Hydroxyurea was added to L-cell cultures to a final concentration of 5 mM. After 10 minutes [^{3}H]TdR was added and the labelling terminated after a further 10 minutes. Lysates from triplicate cultures were pooled and analysed by sedimentation in alkaline sucrose gradients.

(b) HU and [^{3}H]TdR was added to cultures as for (a) except that the 10 minute labelling period was terminated by rinsing and incubation for 60 minutes in medium containing 5 mM HU, 0.1 mM CdR and 1 mM TdR. The saline used for rinsing contained the same additions. The chase was completed by a final 10 minute incubation in medium containing 2 mM GdR, 7 mM AdR, 0.1 mM CdR and 1 mM TdR but without HU. Lysates were prepared, pooled and analysed as for (a).

The direction of sedimentation is from left to right.

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were 13.1 x 10^3 cpm/culture for the short pulse, and 12.3 x 10^3 cpm/culture after the chase. The short chains were clearly chased into higher MW DNA but comparison of Fig. 2b with Fig. 1b reveals that the chased material is not as large as the DNA labelled in control cultures but it does sediment faster than would be expected for mitochondrial DNA (14). No attempt has yet been made to optimise chase conditions for maximum size of chased material.

DISCUSSION

The results presented here are essentially similar to those reported previously for the effect of HU on DNA replication in polyoma virus (6), SV40 (8) and adenovirus (8); the [^{3}H]thymidine incorporation that persists during HU-inhibition is largely into

short DNA chains. The major difference between the systems is in the actual size of the labelled fragments. For polyoma virus and SV40, the HU fragments are 4 to 5S, which compares well with the size of intermediate, Okazaki-type fragments reported for replication of the respective viral DNA both in vitro (15) and in vivo (16). Similarly, the 10S fragments which are labelled during HU-inhibition of adenovirus DNA replication are of comparable size to the intermediate fragments labelled in uninhibited cells both in vivo, with short [^{3}H]thymidine pulses, and in vitro in isolated nuclei (8). Preferential labelling of Okazaki-type fragments has also been reported in FUdR-inhibited SV40 (17) and L-cells (18).

The size of the L-cell HU fragments reported in this communication, namely about 7S, can only be taken as an estimate. The use of techniques with greater resolution and smaller marker DNA molecules should provide a more accurate size estimate. Moreover, it is difficult to compare the size of the L-cell HU fragments with intermediate fragments found in uninhibited cells, since the reported sizes, for various eukaryote cells, vary from short oligonucleotides (19) and 4-5S fragments (20) to 7-10S chains (21).

From the data available to us at present, we conclude that as in the viral systems, the depression of levels of dATP and dGTP by HU has a relatively greater effect on gap-filling DNA polymerase(s) than on the DNA polymerase(s) involved in generation of Okazaki fragments, so that HU treatment of L-cells and subsequent [^{3}H]thymidine labelling of nuclear DNA results in preferential labelling of Okazaki fragments.

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