Accumulation of an Intermediate in DNA Synthesis by HEp.2 Cells Treated with Methyl Methanesulfonate

(alkylating agents/replication/single-stranded DNA)

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ABSTRACT A portion of the DNA synthesized by HEp.2 cells after short incubation in the presence of BrdU bands in a CsCl gradient at a distinct peak of density 1.715–1.718 g/cm³. Inhibition of DNA synthesis by methyl methanesulfonate results in an increased proportion of this “intermediate density” DNA. Cells labeled and subsequently chased in nonradioactive BrdU yield only hybrid DNA. Treatment with single-strand-specific deoxyribonuclease converts a portion of the intermediate density material to molecules of hybrid density (1.755 g/cm³). The data suggest an intermediate in DNA replication that contains a single-stranded region in the parental strand and that accumulates when DNA synthesis is blocked by an alkylation-induced lesion.

Bacterial DNA replication involves as intermediates structures with single-stranded regions at the growing point (1–3). Newly synthesized, pulse-labeled DNA that behaves on hydroxyapatite and/or nitrocellulose as though it contained single-stranded regions has also been observed in mammalian cells (4–8). Chromatography on benzoylated, naphthoylated DEAE-cellulose permits the separation of such single-strand-containing intermediates in DNA synthesis that accumulate after treatment with the monofunctional alkylating agent, methyl methanesulfonate (MMS, ref. 9). We report here the isolation from HEp. 2 cells incubated with BrdU of a fraction of newly synthesized DNA whose banding characteristics in CsCl gradients and response to incubation with a single-strand-specific nuclease indicate that it contains single-stranded regions in both parental and newly synthesized DNA. Treatment of cells with MMS at concentrations that inhibit DNA synthesis increases the proportion of pulse-labeled material appearing in this fraction. We propose that the fraction collected at a density of 1.715 g/cm³ in CsCl is enriched for normal intermediates in DNA synthesis and that alkylation-induced lesions have the effect of immobilizing and accumulating a replicating structure.

MATERIALS AND METHODS

Cells. HEp. 2 cells (10) were cultured in monolayers as reported (11). Our cultures have a median chromosome number of 74 and a doubling time of 22–24 hr. BrdU does slow the rate of growth, but all of the cellular DNA is found at the hybrid density (1.755 g/cm³) within 48 hr from the start of incubation in the presence of halogenated pyrimidine.

Labeling and Treatment. Template DNA was labeled by incubating cells for 30 hr in 0.1 μ Ci/ml of [¹⁴C]dT (Schwarz-

Mann; 54.8 mCi/mmol). Plates were washed and incubated an additional 18 hr in medium containing 1 μ g/ml of unlabeled dT (Calbiochem). BrdU and FdU were used at concentrations of 16 μ M and 1 μ M, respectively. Methyl methanesulfonate (Eastman Organic Chemicals) was vacuum redistilled and diluted into medium containing BrdU and FdU immediately before use. After a 1-hr treatment, cells were washed and labeled in this same medium containing [³H]dT (Schwarz-Mann; 16.6 μ Ci/ml; 55 Ci/mmol except where indicated). Treatment with 2.5 mM MMS reduces [³H]dT incorporation to about 30% of the control. Roberts *et al.* (12) have shown that a mixture of BrdU and [³H]dT may be substituted for [³H]BrdU as a density label in mammalian cells, and we have confirmed this observation with MMS-treated HEp.2 cells. Hydroxyurea (Calbiochem) was diluted into BrdU-FdU medium to a concentration of 10 mM. Cells were harvested as described (11).

DNA Extraction. Cells were harvested and washed in phosphate-buffered saline (11), washed in 0.15 M NaCl + 0.015 M sodium citrate, and resuspended in saline-citrate at approximately 4.0×10^6 /ml. Single-stranded, newly synthesized DNA is preferentially adsorbed to the interface of organic solvent extractions (13, 14). Our preparations must, therefore, be done at high cell density (15), which makes it possible to avoid the losses in recovery reported by Hanawalt and Ray (16). Sodium dodecyl sulfate was added to 0.2%, and extraction was carried out as reported (11).

CsCl Density Centrifugation. DNA (30–60 μ g), sheared by passage three times through a 22-gauge needle, was centrifuged through a CsCl gradient that was collected and processed as described (11). Alkaline gradients were prepared by the dropwise addition of 0.1 M NaOH to the DNA sample to bring the pH to 12 before addition of CsCl.

Exonuclease Treatment. Fractions from CsCl gradients were pooled and dialyzed against 0.13 M glycine buffer, pH 8.4. Samples were incubated in a reaction mixture of 1.5 ml with 3 units/ml of *Escherichia coli* exonuclease I (supplied by Dr. N. Cozzarelli) in 13 mM MgCl₂ and 3.3 mM 2-mercaptoethanol for 1.5 hr at 37°. Nuclease susceptibility was measured by comparison of the radioactivity solubilized by cold and hot 5% trichloroacetic acid. This preparation of exonuclease I, although completely specific for single-stranded DNA, includes some endonuclease activity (9). At least 90% of denatured DNA was converted to acid-soluble fragments after treatment with enzyme.

Abbreviation: MMS, methyl methanesulfonate.

FIG. 1. CsCl density gradients of DNA synthesized in the presence of bromodeoxyuridine after treatment of HEP.2 cells with MMS. HEP.2 cells were incubated for 2 hr with BrdU (16 μ M) and FdU (1 μ M), treated for 1 hr with 2.5 mM MMS in the presence of BrdU and FdU, washed three times with medium, and labeled for 30 min with a mixture of BrdU and [3 H]dT. The final specific activity of the mixture is 1.02 Ci/mmole of deoxynucleoside and the ratio of BrdU:dT is 53:1. Extracted DNA was centrifuged in CsCl (average density 1.70 g/cm 3) at 30,000 rpm for 60 hr in an SW 50.1 rotor. Collected fractions were assayed for absorbance at 260 nm (O) and acid-precipitable counts (●). (A) Control; (B) MMS-treated. Values on ordinates have been multiplied by 10^{-3} or 10^{-2} , as indicated.

Nitrocellulose Adsorption. DNA from CsCl gradients was either precipitated and filtered as described (11), or filtered without precipitation through presoaked nitrocellulose filters (Millipore), washed with 10 ml of 0.5 M KCl-0.01 M Tris-HCl, pH 7.3. After addition of sample, each filter was washed with 25 ml of this buffer before it was dried and counted.

Sucrose Gradient Sedimentation. DNA (0.5 ml) in glycine buffer was layered on a 5-ml, 5–20% (w/v) neutral sucrose gradient containing 1 M NaCl. Samples were centrifuged for 4 hr at 30,000 rpm, 20°, in a Beckmann-Spinco SW50.1 rotor. Gradients were collected from the bottom, precipitated, filtered, and counted. S values were calculated from McEwen (17); molecular weights from Studier (18).

RESULTS

HEP.2 cells were treated with MMS in BrdU-containing medium and then incubated with a mixture of [3 H]dT and BrdU for 30 min. The DNA was extracted and centrifuged to equilibrium in a CsCl gradient. We expected to observe a peak of radioactivity at the hybrid density (1.755 g/cm 3) due to semiconservative replication and a minor peak at the light density (1.70 g/cm 3) due to repair synthesis. Instead we found that much of the radioactivity in the DNA produced by MMS-treated cells appeared at a density of about 1.715 g/cm 3 , significantly displaced from the peak of absorbance at 260 nm, which indicates the position of unsubstituted DNA (Fig. 1). We will refer to the material of density 1.715 g/cm 3 , intermediate between light and hybrid, as DNA_{int}. DNA_{int} can be observed in preparations of DNA obtained from purified nuclei prepared from cells lysed with NP40, indicating that it does not represent some cytoplasmic artifact.

When a DNA preparation containing 60% DNA_{int} (Fig. 1) was centrifuged in an alkaline CsCl gradient, most of the radioactivity banded at a density characteristic of a completely BrdU-substituted strand, with only a small amount of radioactivity in the light strand (Fig. 2). We interpret this experiment as meaning that the density of DNA_{int} is not due to repair, since patches of repair would not be large enough

FIG. 2. Alkaline CsCl density gradient. DNA from BrdU-incubated, MMS-treated cells (Fig. 1) was denatured by addition of 0.1 M NaOH to a pH of 12.0 and centrifuged at that pH in CsCl.

to occupy a whole fragment of single-strand molecular weight more than 8.5×10^6 (see below). The proportion of DNA_{int} increased as a function of the concentration of MMS (Table 1), although at the highest MMS concentration the label incorporated into DNA was distributed through a broader density range, indicating a greater proportion of repair synthesis. [3 H]dT remaining in the intracellular pools makes it impossible to prevent further incorporation of label during a chase period, particularly in the MMS-treated cells, which accumulate a large pool of [3 H]dT presumably because of

TABLE 1. Accumulation of "intermediate density" DNA (DNA_{int})

Sample	Labeling time (hr)	Chase time (hr)	DNA _{int} *	
			cpm	% of total radioactivity
(A) Control	1	0	2271	5.0
1.0 mM MMS	1	0	2112	9.6
2.0 mM MMS	1	0	6827	32.5
3.0 mM MMS	1	0	9438	52.9
5.0 mM MMS	1	0	3768	66.6
(B) 2.5 mM MMS	1	0	7913	32.3
2.5 mM MMS	1	2	5885	16.9
2.5 mM MMS	1	4	4334	9.7
(C) (Hydroxyurea chase)				
2.5 mM MMS	1	0	4506	39.6
2.5 mM MMS	1	2	5419	41.0
2.5 mM MMS	1	4	5265	41.5

(A) Treatment, extraction, and centrifugation as described in the legend in Fig. 1.

(B) After labeling, cells were washed and reincubated in medium containing BrdU and FdU.

(C) Hydroxyurea (10 mM) was added to the labeling medium after a 30-min incubation with isotope. After an additional 30 min, the medium was removed, cells were washed with BrdU-FdU-hydroxyurea medium, and reincubated in that medium.

* DNA_{int} = radioactivity from fractions with a density of 1.69–1.73 g/cm 3 with a peak at 1.715 g/cm 3 .

FIG. 3. Exonuclease I-induced transfer of intermediate density DNA to the hybrid position. Intermediate density DNA was isolated from CsCl, dialyzed against glycine buffer, treated with exonuclease I for 1 hr, and recentrifuged in CsCl. (A) No nuclease treatment; (B) exonuclease I-treated.

the inhibition of DNA synthesis. However, if isotope is removed after 1 hr and incubation is continued for an additional period in medium containing unlabeled BrdU, there is a decrease in the total counts in the DNA_{int} region and a shift towards a hybrid density (Table 1B). This is the behavior to be expected of a replicative intermediate. Inhibition of semi-conservative synthesis with hydroxyurea during the chase period immobilized DNA_{int} and no transfer of radioactivity to hybrid density occurred. We interpret the stability of DNA_{int} in the absence of DNA synthesis to mean that transfer to hybrid density is not the result of degradation.

Structure of the Intermediate. DNA_{int} was isolated by pooling fractions from CsCl gradients, and the pooled, dialyzed fractions were treated with *E. coli* nuclease specific for single-stranded DNA. Enzyme treatment converted a large portion of the radioactivity to fragments that banded in the position of hybrid DNA (Fig. 3). Hybrid fractions, subjected to the same treatment, showed no change in density. The density profile given by DNA_{int} molecules after enzyme digestion is that expected from a mixture of hybrid molecules, molecules with end-to-end joining of BrdU-substituted and unlabeled segments, and by light molecules with regions of repair synthesis.

Although the density of DNA_{int} is altered by treatment with single-strand-specific nuclease, only a small fraction of the ³H counts are made acid-soluble. In separate experiments, 12.7, 16.2, and 10.1% of the ³H radioactivity in isolated DNA_{int} was solubilized by nuclease treatment, as compared to 3.2, 2.3, and 3.5% of the radioactivity from hybrid DNA. The low nuclease susceptibility of DNA_{int} suggested that most of the BrdU was incorporated into molecules with the structure of native DNA. When fractions from the CsCl gradient were passed through hydroxyapatite columns, 92.6% of the label in the DNA_{int} region required phosphate concentrations above 0.16 M for elution, as expected if most of the labeled fragments contained double-stranded regions.

DNA isolated at a density of 1.715–1.72 g/cm³ and that had been prelabeled with [¹⁴C]dT before alkylation and incubation with BrdU released 6–8% of its radioactivity after nuclease treatment. Only 2% of the ¹⁴C-labeled parental strand was released from DNA isolated at a light (1.70) or hybrid (1.755) density. This value of 6–8% must be corrected for the contamination of DNA_{int} with main-band, light DNA. We used the specific activity (cpm per unit of absorbance at 260 nm) of the hybrid peak as an indication of the amount

FIG. 4. Adsorption of [¹⁴C]dT-labeled parental DNA from MMS-treated cells to nitrocellulose filters. Cells were labeled for 30 hr with [¹⁴C]dT (0.1 μ Ci/ml; 54 mCi/mmol), chased for 18 hr in the presence of 1 μ g/ml of unlabeled dT, and exposed to BrdU and treated with MMS as described in the legend to Fig. 1. Extracted DNA was centrifuged in duplicate CsCl gradients. (A) Gradient was precipitated with trichloroacetic acid and filtered. (B) Gradient was filtered without precipitation, as described in Methods. (C) ¹⁴C counts were plotted as unprecipitated/precipitated for each fraction.

of ³H activity associated with a given amount of double-stranded DNA_{int} in an experiment involving [¹⁴C]dT pre-labeling and incubation with [³H]dT and BrdU after alkylation. We estimated that a maximum of 10% of the ¹⁴C counts at a density of 1.715 g/cm³ are actually associated with the hybrid portions of DNA_{int} rather than with the main-band, nonreplicated DNA. If the proportion of single-strandedness in DNA_{int} can be calculated from the ¹⁴C counts solubilized by nuclease treatment (after the 2% nuclease susceptibility of the light or hybrid DNA has been subtracted), then 4–6% of the ¹⁴C counts are associated with single-stranded regions of DNA_{int}. If DNA_{int} were composed only of hybrid DNA associated with parental single-stranded DNA, then the percentage of single-stranded regions in DNA_{int} would be approximately $4/(10 + 4) = 29$ or $6/(10 + 6) = 37\%$. This is a maximum, since ¹⁴C counts in light double-stranded structures from parental DNA would reduce the value.

If single-stranded regions are present, DNA_{int} should adsorb to nitrocellulose filters (19). Newly synthesized DNA in the intermediate density portion of the gradient was, in fact, completely retained on nitrocellulose filters without precipitation, whereas the remainder of the DNA was largely washed through. Parental DNA, prelabeled with [¹⁴C]dT, also adhered preferentially to nitrocellulose in the intermediate region of the density gradient (Fig. 4). Furthermore, if the DNA_{int} structure is not an artifact produced by incorporation of the halogenated pyrimidine, a corresponding molecular species should be identifiable in DNA from cells incubated in analog-free medium. When the nitrocellulose filtration technique was applied to unsubstituted [³H]dT

Fig. 5. Sedimentation analysis of intermediate density and hybrid DNA before and after exonuclease I digestion. Fractions of intermediate and hybrid density DNA were isolated from several CsCl gradients, pooled, and dialyzed against glycine buffer. Half of each sample was treated for 1 hr with exonuclease I. Controls were incubated without enzyme. DNA (0.5 ml) was layered on a 5-ml, 5–20% neutral sucrose gradient containing 1 M NaCl. Gradients were centrifuged for 4 hr at 30,000 rpm at 20° in an SW 50.1 rotor. Sedimentation is from right to left. (O) No nuclease; (●) exonuclease I-treated. (A) Intermediate density DNA; (B) hybrid DNA.

pulse-labeled DNA, the unprecipitated ^3H was adsorbed to the filter in a skewed fashion with a peak near 1.710 g/cm 3 , whereas the precipitated radioactivity coincided with the peak of absorbancy.

We have analyzed the molecular size of the DNA $_{\text{int}}$ fragments before and after treatment with nuclease by sedimentation through sucrose gradients. The sedimentation profile of hybrid DNA was hardly changed by nuclease treatment, but the DNA $_{\text{int}}$ fraction behaved as though a substantial amount of material had been digested away (Fig. 5). Molecules sedimenting near the bottom of the gradient before nuclease treatment were lost upon digestion and an increase in counts was observed in the more slowly sedimenting region. We are unable to calculate the size of a molecule made up of single- and double-stranded regions from these data, but after nuclease digestion the material from both intermediate and hybrid density regions sedimented at an s_{20} of about 28, corresponding to a molecular weight of 1.7×10^7 for a native DNA molecule.

Some idea of the relative amount of BrdU substitution in DNA $_{\text{int}}$ can be obtained by variation of the BrdU:dT ratio in the incubation mixture. If the density of DNA $_{\text{int}}$ is primarily determined by its content of single-stranded and unsubstituted parental native regions, then the per cent substitution with BrdU should have little effect on its banding position. In contrast, the density of hybrid DNA should be sensitive to such changes. As expected, the hybrid peak does become progressively less dense as the BrdU:dT ratio decreases in the labeling mixture. At a BrdU:dT ratio of 53:1, the hybrid position is 1.755 g/cm 3 ; at 3:1 it is 1.750; and at 1:1 it moves to 1.740. DNA $_{\text{int}}$, however, maintains its position at about 1.715 g/cm 3 . This insensitivity of DNA $_{\text{int}}$ to the proportion of BrdU indicates that its density is primarily determined by both native and single-stranded parental DNA.

DISCUSSION

Our results are best interpreted in terms of the Okazaki hypothesis (20), that discontinuous synthesis along one of the antiparallel parental strands accompanied by an unwinding of the helix exposes a region of single-stranded template DNA. Such structures have been identified in the replication

Fig. 6. Model of events at the growing point showing the generation of DNA $_{\text{int}}$ fragments by shear, the liberation of hybrid DNA by nuclease treatment, and the release of newly synthesized and parental DNA susceptible to solubilization by single-strand-specific nuclease. Solid lines: unsubstituted parental DNA; broken lines, newly synthesized BrdU-substituted DNA.

of bacteriophages T2, T4, and λ and of adenovirus 5 (21–26). Newly synthesized DNA can become single-stranded, according to this hypothesis, by a process of strand displacement (27) in which the reassociation of the single-stranded template strand with its parental complement displaces the newly synthesized strand from the double helix. When synthesis is blocked by an alkylation lesion, an equilibrium exists between the two states, with template and newly synthesized daughter DNA alternately in the single-stranded state (9).

The intermediate density material has both the structural and kinetic features that establish its role as an intermediate in replication: DNA $_{\text{int}}$ accumulates as DNA synthesis is inhibited and is chased into hybrid when DNA synthesis is allowed to continue. According to this view, DNA $_{\text{int}}$ would be generated by shearing at the replication fork (Fig. 6).*

Any structure for DNA $_{\text{int}}$ must account for the following: (a) newly synthesized DNA is present in mainly a double-stranded configuration but with about 13% accessibility to single-strand-specific nucleases; (b) the average amount of single-stranded template in DNA $_{\text{int}}$ is no more than 30–40% of the length of the associated hybrid stretch; (c) treatment with single-strand-specific nuclease results in liberation of a fraction with hybrid density; and (d) the density of DNA $_{\text{int}}$ indicates that the major portion of the molecule must consist of light DNA. We conclude, therefore, that there is a significant contribution of parental, native DNA to the structure. A variety of possible structures can be drawn (Fig. 6), and the heterogeneity of the fragments in sucrose gradients indicates that such a mixture is present.

Structures of intermediate density derived from growing points substituted with BrdU have been reported by Hanawalt and Ray (16), Taylor (28), and Souleil and Panijel (29). Introduction of lesions that inhibit DNA-synthesis permits the accumulation of these intermediates. It is probable that the DNA $_{\text{int}}$ that we report in this paper is analogous to the DNA with single-stranded regions (DNA $_{\text{ss}}$) isolated by benzoyletated, naphthoylated DEAE-cellulose chromatography

* Structures similar to those postulated in this paper have been seen by Kriegstein and Hogness (30).

(9). It does seem necessary to postulate some special mechanism that will yield fragments of a particular density rather than the distribution of densities between hybrid and light expected from random shearing around transition points. It has been suggested (28) that there may be definite break points, or nuclease susceptible points, in the parental DNA that would account for specific fragmentation, but the molecular basis for such susceptibility is completely unknown.

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