INHIBITION OF DNA CHAIN ELONGATION

IN A PURINE-AUXOTROPHIC MUTANT OF CHINESE HAMSTER

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ABSTRACT

DNA synthesis has been examined in a purine-auxotrophic mutant cell line of
Chinese hamster (V79 pur 1) under conditions of purine deprivation. At 6 h after
the removal of purines from the growth medium, there is a decrease in semicon-
servative DNA replication. Alkaline velocity centrifugation of the DNA synthe-
sized during a 1-min pulse under conditions of purine deprivation shows that
~50% of the newly replicated DNA is the size of Okazaki pieces. These are not
incorporated into bulk DNA during a 1-h chase. If the purine supply is restored
to the growth medium, these short DNA pieces are joined to full-sized DNA
within 1 h. DNA fiber autoradiography reveals a retardation in the rate of DNA
replication fork movement but no apparent inhibition of initiation of synthesis on
replication units within clusters actively engaged in replication. Our results indicate
that purine deprivation specifically inhibits elongation of nascent DNA chains.

DNA synthesis is an elaborately regulated process whose many steps are not yet completely understood. One of the basic requirements for DNA replication is the continuous supply of the four deoxyribonucleoside triphosphates (dNTP) that serve as building blocks. These are also thought to play a regulatory role in the replication process (26). Numerous studies have shown that treatment of cells with hydroxyurea, which depletes the intracellular purine dNTP pools (30, 37), inhibits the DNA chain elongation process and causes the accumulation of Okazaki fragments that cannot be incorporated into mature DNA strands (17-19, 35). We have available a purine-auxotrophic mutant cell line of Chinese hamster, designated as V79 pur 1, which is deficient in phosphoribosyl-pyrophosphate amidotransferase (EC 2.4.2.14), the first enzyme of the *de novo* purine biosynthetic pathway (7). In another study (39), we have shown that when V79 pur 1 cells are deprived of purines, the size of the purine dNTP pools decreases, while that of the pyrimidine dNTP pools increases. The pools that are affected the most are dATP, which decreases threefold, and dCTP, which increases twofold after withdrawal of purines from the growth medium. At the same time, DNA synthesis is markedly reduced and the cells become arrested in the S phase.

Knowing the biochemical defect in the V79 pur 1 cell line, and in light of our previous finding concerning the alterations in the dNTP pools in this mutant, we have undertaken a study to characterize the precise step in DNA replication that is affected when the supply of purines is restricted. In this paper, we present evidence that purine deprivation in these mutants delays the maturation

of newly synthesized Okazaki pieces to full-sized DNA and also inhibits the rate of DNA replication fork movement.

MATERIALS AND METHODS

Cells and Experimental Conditions

The Chinese hamster line V79 pur 1 was used. This cell line is auxotrophic for purines and has a mutation in the gene for phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14; see also reference 7). It was maintained in our laboratory in Ham's F10 medium supplemented with 5% fetal calf serum (FCS), amphotericin B (0.25 μg/ml), gentamicin (50 μg/ml), and tylocine (60 μg/ml). The details of the experimental conditions have been previously described (39). In brief, the day before the experiment the cells were transferred to plastic petri dishes at a density of 2 × 10⁵ cells per 60-mm dish and allowed to attach and grow overnight in F10 medium supplemented with 5% FCS. In experimental cultures, the cells were depleted of purines and placed under restrictive growth conditions by replacement of the growth medium with minimal essential medium (MEM) supplemented with 5% dialysed FCS. Control cultures received the same medium supplemented with 30 μM hypoxanthine.

Density Labeling and Extraction of DNA

Cell cultures that had been deprived of purines for 2 h were incubated in medium containing 5'-fluorodeoxyuridine (FdUrd; 2 × 10⁻⁶ M), bromodeoxyuridine (BrdUrd; 10⁻⁵ M), and [³H]-thymidine (10⁻⁷ M; 10 μCi/ml) for a period of 8 h in the dark. Parallel control cultures were identically treated. At the end of the incubation period, each of the cultures was mixed with an equal number of cells labeled for 16 h with [¹⁴C]thymidine, and the DNA was extracted as described by Britten et al. (3). The extracted DNA was dissolved in TNE (0.05 M Tris, pH 8.0, 0.15 M NaCl, 0.05 M EDTA) and sheared to a size of 8–9 × 10⁶ daltons by passing it four times through a 25-gauge needle.

Isopycnic Centrifugation in Neutral CsCl Gradients

An aliquot of ~3 μg of the extracted DNA was brought to a final volume of 5 ml in a solution containing TNE and CsCl (density 1.72 g/ml). Centrifugation was at 33,000 rpm for 60 h at 18°C in an S40.2 rotor in an L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions of 0.2 ml were collected from the bottom by displacement using a constant volume fractionator (MRA Corporation, Boston, Mass.). The refractive index of every fifth fraction was recorded and the radioactivity in each fraction was measured by liquid scintillation counting.

Velocity Sedimentation in Alkaline Sucrose Gradients

Samples containing ~10⁵ cells were slowly pipetted into 0.3 ml of 0.2 M NaOH–0.001 M EDTA, layered over a gradient of 5–20% sucrose in 0.9 M NaCl, 0.3 M NaOH, and 0.001 M EDTA resting on a cushion of 70% sucrose (28). Cell lysis and DNA denaturation were allowed to proceed at 4°C for 8–12 h. The gradients were then centrifuged for 16 h at 26,000 rpm at 4°C in an SW50.1 rotor in an L2-65B Beckman ultracentrifuge. Fractions of 0.2 ml each were collected from the bottom and counted

for radioactivity. ³H-labeled φX174 DNA (a gift from Dr. D. Denhardt, McGill University, Montreal, Quebec) was used as a marker.

DNA Fiber Autoradiography

The method of Hand and Tamm (12) was used. In brief, at times after exposure to purine-deprived or purine-supplemented medium, the cells were subjected to sequential 30-min pulses with [³H]thymidine, first at high specific activity (5 × 10⁻⁶ M; 50 Ci/mmol) and then at low specific activity (5.5 × 10⁻⁵ M; 5 Ci/mmol). The cells were exposed to FdUrd (2 × 10⁻⁶ M) from 30 min before the pulse to its completion. At the end of the radioactive pulses, a drop of cell suspension (~2–3 × 10³ cells) was mixed with a drop of a lysis buffer (phosphate-buffered saline containing 1% sodium dodecyl sulfate and 0.01 M EDTA) on a subbed glass slide. The DNA fibers released from the cells were spread over the surface of the slide with a glass rod, air-dried, fixed, and processed for autoradiography. Exposure time was 4–6 mo. All autoradiograms were examined using a light microscope equipped with a frosted glass screen. The autoradiographic figures were projected on the screen and traced on clear acetate.

RESULTS

Earlier studies (7, 31, 39) have shown that purine depletion inhibits DNA synthesis in V79 pur 1 cells. As measured by [³H]thymidine incorporation into acid-insoluble material, synthesis is reduced to <20% of controls by 2 h and remains at <10% of controls from 4 to 8 h after depletion. RNA synthesis shows a similar decrease, but protein synthesis is affected to a lesser extent and the decline is more gradual (31). During this time ~50% of the cells have entered S, as determined by flow microfluorography, but only ~30% of the cells are actively engaged in DNA synthesis, as determined by autoradiography (39).

Semiconservative Replication

Equilibrium density centrifugation in neutral CsCl gradients was used to investigate the type of DNA synthesis performed by the mutant cells in the purine-deprived medium. A replicative type of synthesis would be expected to result in double helices with a buoyant density higher than normal as a result of the incorporation of BrdUrd into the nascent strands. Repair synthesis would not cause a shift, because BrdUrd would be incorporated into regions too short to alter the density of the repaired stretches of DNA. Fig. 1 shows that there is no difference in the gradient profiles of the DNA extracted from cells that were either purine-deprived or purine-supplemented (control) for 10 h. DNA synthesized in the presence of BrdUrd and [³H]thymidine banded at a density of 1.75 g/ml, characteristic of the hybrid product of semi-conservative replication (8) whereas the normal
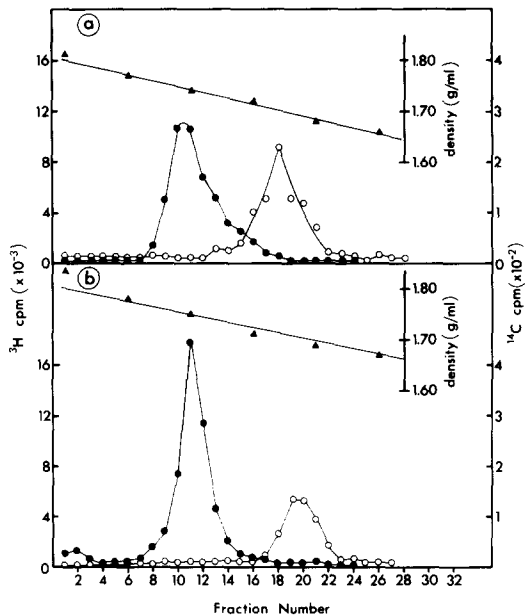
FIGURE 1 CsCl isopycnic centrifugation of DNA synthesized by purine-deprived (a) and purine-supplemented (b) V79 pur 1 cells. Cells previously deprived of purines for 2 h were incubated for an additional 8 h in medium depleted of purines containing FdUrd $(2 \times 10^{-6}$ M), BrdUrd $(10^{-5}$ M), and [^{3}H]thymidine $(10^{-7}$ M, 10 μCi/ml). Control cells were identically treated except the medium was always supplemented with 30 μM hypoxanthine. After the labeling period, the cells were mixed with cells labeled with [^{14}C]thymidine, the DNA was extracted, and an equal number of ^{3}H counts were loaded onto the gradients and centrifuged to equilibrium. (●), [^{3}H]thymidine/BrdUrd-labeled DNA; (O), ^{14}C-labeled DNA; (▲), buoyant density (g/ml).

unsubstituted marker DNA banded at a density of 1.70 g/ml. The bulk of the ^{3}H label is associated with the high-density fractions rather than with DNA of normal density. This result indicates that the residual DNA synthesized in the mutant cells deprived of purines is made in semiconservative fashion characteristic of the S phase. It also indicates that there is little or no repair replication.

Characterization of the Newly Synthesized DNA

Once we established that DNA synthesis was replicative in the absence of purines, we designed pulse-chase experiments to determine the size of nascent DNA strands under purine-depleted conditions. V79 pur 1 cells whose bulk DNA had been prelabeled with [^{14}C]thymidine were incubated in purine-deprived or purine-supplemented medium

for 6 h. [^{3}H]Thymidine $(2 \times 10^{-6}$ M; 50 Ci/mmol) was added to control and depleted cells for 1 min to pulse-label the nascent DNA. The cells were then processed immediately or incubated for an additional hour in the appropriate medium containing nonradioactive thymidine in excess $(2 \times 10^{-5}$ M) and processed for analysis on alkaline sucrose gradients.
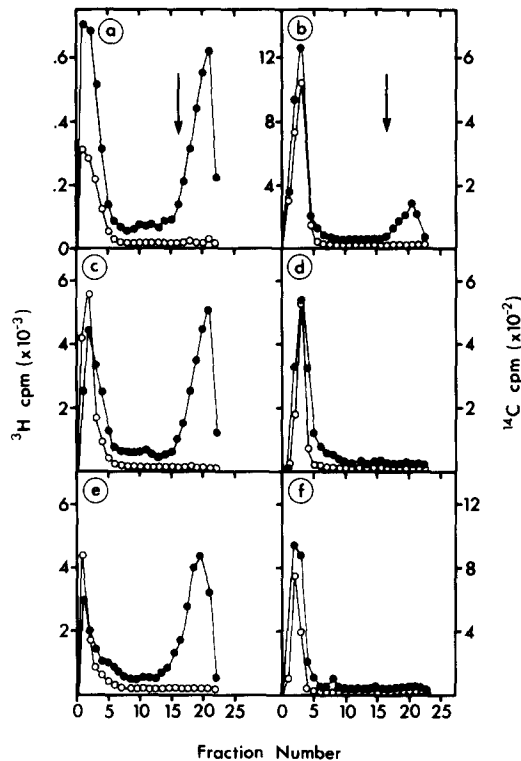
Typical gradient patterns are shown in Fig. 2.

FIGURE 2 Alkaline sucrose gradient centrifugation of DNA synthesized by purine-deprived V79 pur 1 cells in pulse-chase studies. (a) Depleted, pulse; (b) depleted, pulse-chase; (c) control, pulse; (d) control, pulse-chase; (e) replenished, pulse; (f) replenished, pulse-chase. Cells were prelabeled with [^{14}C]thymidine (0.01 μCi/ml; 50 Ci/mmol) in F10 with 5% FCS. After growth overnight, half the cultures received medium without purines and the other half received medium supplemented with 30 μM hypoxanthine (control). After 6 h, cells were pulse-labeled with [^{3}H]thymidine $(2 \times 10^{-6}$ M, 50 Ci/mmol) for 1 min and were processed either immediately (a and c) or after 1 h chase with unlabeled thymidine at 2×10^{-5} M (b and d). Some cells were deprived of purines for 4 h and then supplemented with hypoxanthine (replenished) for 1 h before the pulse (e) or pulse-chase (f) treatments. Arrow indicates position of ϕX174 marker DNA run in a parallel gradient; (●) ^{3}H cpm; (O) ^{14}C cpm.

The distribution of radioactivity is the same in the purine-deprived and purine-supplemented cells after 1 min of pulse-labeling with [³H]thymidine (Fig. 2a and c). In both, ~50% of the ³H-labeled DNA is recovered as small molecular weight material that sediments at 4–5S near the top of the gradients, while the remainder cosediments with full-sized ¹⁴C-prelabeled DNA (>46S). In the pulse-chase experiments, however, a chase period of 1 h was not enough to allow conversion of the low-molecular-weight molecules into bulk-size DNA in the purine-depleted cells (Fig. 2b), whereas in control cells, all the DNA was incorporated into full-sized molecules within this time (Fig. 2d). Although apparently some low-molecular-weight material was chased into longer strands in 1 h in purine-deprived cells, 50% of the radioactivity recovered as 4–5S material after the 1-min pulse remained as such after a 1-h chase.

If the cells were deprived of purine for 6 h and subsequently exposed to medium supplemented with hypoxanthine, the ability to chase the low-molecular-weight DNA into bulk-size was regained within 1 h (Fig. 2e and f). This ability was also present after 23 h of purine deprivation (data not shown) when replenishment of the purine ribonucleotide (31) and deoxyribonucleotide pools (39) occurs as a result of breakdown in ribosomal RNA (31). These results suggest that in the absence of purine there is a retardation in the conversion of small Okazaki-type pieces of newly replicated DNA to large molecular weight DNA.

When the samples from depleted cells were subjected to electrophoresis on agarose gels, identical results were obtained (data not shown).

Rate of DNA Replication Fork Movement

As a result of the above findings, which suggest a defect in elongation, we decided to look at the effect of purine depletion on the rate of DNA replication fork movement in V79 pur 1 cells, by use of DNA fiber autoradiography. The rate was determined by measuring the length of the high-grain-density tracks from DNA labeled during the high-specific-activity pulse of [³H]thymidine on units that initiated synthesis before the pulse, and then dividing by the duration of the pulse (12). Track lengths from control and depleted cells were approximately normally distributed at all time points. The mean values obtained and the calculated rates are shown in Table I. The rates in purine-supplemented cells are similar to those previously found for Chinese hamster cells (6, 29, 38). Within 4 h in purine-depleted medium, the rate of fork movement declined to 58% of the control rate and by 6 h reached 36% of the corresponding rate in the purine-supplemented cells. In cells deprived of purines for 4 h and then switched to purine-supplemented medium for 2 h, the rate rose again to 92% of the corresponding control. Representative autoradiograms of 6-h purine-deprived and purine-supplemented cultures are shown in Fig. 3, in which the difference in the track lengths is clearly visible. The above results indicate that purine deprivation has a direct inhibitory effect on the rate of DNA replication fork movement.

Interval between Initiation Sites

If initiation were inhibited in addition to fork movement, we might expect to see other alterations in the patterns of fiber autoradiograms in the depleted cells. Among these could be an increase in the interval between initiation sites caused by a block at some sites that would be used in the normal course of replication in nondepleted cells.

The interval between active sites was determined by measuring the center-to-center (intertrack) distances between replication units. The

TABLE I

Replication Fork Movement in Purine-deprived and Purine-supplemented V79 pur 1 Cells

Time	Medium	Track length ± SD	Rate	Percent of control	No. of tracks measured
h		μm	μm/min		
0		39.2 ± 2.87	1.3	—	198
4	Control*	37.2 ± 3.71	1.2	—	200
	Depleted	22.2 ± 1.72	0.7‡	58	202
	Depleted/replenished for 2 h	32.4 ± 3.67	1.1	92	200
6	Control*	42.8 ± 3.78	1.4	—	200
	Depleted	15.1 ± 1.33	0.5‡	36	200

* Medium supplemented with 30 μM hypoxanthine.
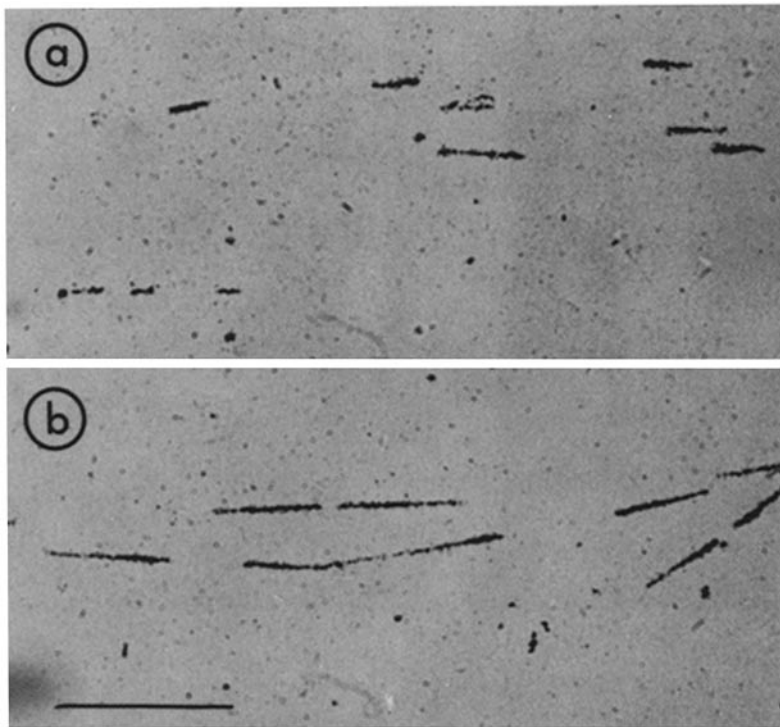‡ Values that are significantly different from the controls ($P < 0.001$).

FIGURE 3   DNA fiber autoradiograms from V79 pur 1 cells. The cells were labeled with [³H]thymidine during a 30-min high-specific-activity pulse (5 × 10⁻⁶ M; 50 Ci/mmol) followed by a 30-min low-specific-activity pulse (5.5 × 10⁻⁵ M; 5 Ci/mmol), and were processed for fiber autoradiography. (a) purine-supplemented cells; (b) purine-deprived cells. Bar, 50 μm. Both micrographs, × 450.

results are shown in Table II. They are expressed as geometric means rather than arithmetic means because the distributions of such distances are not Gaussian but skewed toward larger values, as has been observed by other investigators (2, 5, 13, 21). The geometric mean is preferred because of its "weighting" characteristics in the statistical sense, whereby it gives smaller observations relatively more importance than does the arithmetic mean (21, 23). Intertrack distances become progressively smaller with increasing time in the purine-deprived medium. The distances observed in the control cells are within the range previously reported for Chinese hamster cells (14, 38). Cumulative frequency distributions of intertrack distances are presented in Fig. 4. At all time points considered, the purine-deprived cells have higher frequencies of short intertrack distances, as indicated by the initial steep slopes in their cumulative frequency curves. The cumulative frequency curves of the purine-supplemented cells indicate a much broader distribution of intertrack distances. Thus, purine-deprivation is not associated with an increase in initiation intervals. This suggests that

initiation on active clusters of replication units is not inhibited by purine depletion.

Relative Frequency of Initiation

As an additional test of whether initiation is inhibited by purine depletion, we measured the frequency of initiations by scoring the fraction of replication units that had initiated before the beginning of the high-specific-activity pulse (prepulse units) and comparing it to the fraction of units that initiated after the beginning of the high-specific-activity pulse (postpulse units) (10, 11). Prepulse units show a central gap, which represents DNA replicated before the start of the pulse, flanked by linear tracks of heavy and light grain densities, which represent DNA replicated during the high- and low-specific-activity pulse, respectively. Postpulse units show a central track of high grain density flanked by two tracks of lower grain density. There is a continuum of postpulse to prepulse units, from postpulse ones with no apparent gaps in the high-density grain tracks to prepulse ones with increasing lengths of gap, depending on the actual time by which the initiation

Replication Unit Lengths for Purine-deprived and Purine-supplemented V79 pur 1 Cells*

Time	Medium	Geometric mean of intertrack distance		No. of intertrack distances measured
h		μm		
0		110.3	(99.2–122.7)‡	100
4	Control	111.9	(101.6–123.2)	102
	Depleted	91.2§	(82.2–99.7)	107
	Depleted/replenished for 2 h	97.2	(88.4–106.8)	113
6	Control	110.4	(99.7–122.2)	130
	Depleted	76.9‖	(69.3–85.4)	100

* Autoradiograms were collected from areas of the slides where the DNA was well spread. Two units were considered to be on the same fiber if they were aligned and in the same microscope field at a magnification of 280. The diameter of the field is 535 μm at this magnification. Controls are V79 pur 1 cells grown in medium supplemented with 30 μM hypoxanthine.

‡ 95% Confidence limits of the geometric mean based on the Student's t distribution.

§ Value significantly different from the control (P < 0.01).

‖ Value significantly different from the control (P < 0.001).

event preceded the pulse (15). The ratio of pre-pulse to postpulse units can be used to measure the relative frequency of initiation. A decrease in the proportion of postpulse units in preparations pulse-labeled after an experimental manipulation indicates decreased initiation. The results of this sort of analysis on V79 pur 1 cells are shown in Table III. Purine-deprived cells show the same proportion of postpulse units as the purine-supplemented control cells at all time points examined. We conclude, therefore, that purine deprivation does not inhibit initiation of replication on active clusters.

DISCUSSION

Our results show that under conditions of purine deprivation, DNA synthesis in V79 pur 1 cells is inhibited. Residual synthesis is replicative in nature, a characteristic of S-phase synthesis. Depleted cells lose the ability to convert small, newly replicated DNA strands (Okazaki pieces) to mature-sized chromosomal DNA. This is associated with a marked reduction in the rate of DNA replication fork movement. Purine deprivation has no apparent effect on the frequency of initiation of new DNA synthesis. All inhibitory effects observed under conditions of purine deprivation are reversible; restoration of purines to the medium results in resumption of normal DNA synthesis.

These results show that purine deprivation inhibits semiconservative DNA synthesis by directly inhibiting the rate of DNA chain elongation, as measured by replication fork progression and mat-

uration to full-sized DNA. A block in maturation has been demonstrated by other investigators in systems in which a state of purine depletion was induced through treatment with hydroxyurea (17–19, 35, 36). Purine-deprived V79 pur 1 cells are arrested in S, as we have shown (39), and this is also true for hydroxyurea-treated cells (36). Although dATP availability may be a major rate-limiting factor for DNA synthesis (39), we feel that a balance between all four dNTP pools must exist in the cells for normal DNA synthesis to occur. In addition, according to recent evidence, the nuclear ATP pools and ATP/ADP ratios seem to play an important role in the control of elongation of previously initiated replicons (25). This should be taken into consideration in interpreting our results, because the amount of purine ribonucleotides in the V79 pur 1 cellular pools drops fivefold by 12 h of purine deprivation (31).

The possibility that the small DNA persisting under purine-deprived conditions is a breakdown product of pre-existing DNA strands can be excluded for two reasons: (a) in alkaline sucrose gradients the ^{14}C-prelabeled DNA marker is recovered always as one peak at a position characteristic of bulk DNA, and (b) the small DNA could be chased into bulk DNA immediately upon replenishment of the deprived medium with purines.

We cannot completely exclude the possibility that these small DNA fragments may not represent true Okazaki pieces, but may be instead the result of an excision repair process arising by misincor-
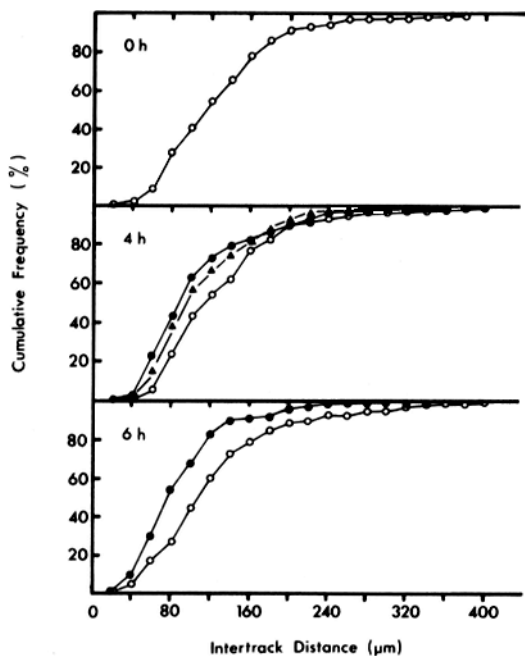
FIGURE 4 Cumulative frequency distributions of inter-track distances in purine-deprived and purine-supplemented V79 pur 1 cells. The number of intertrack distances measured are given in Table III. (●) Purine-deprived cells; (○) purine-supplemented cells; (▲) cells deprived of purines for 4 h and then supplemented with 30 μM hypoxanthine for 2 h.

poration of uracil into DNA and subsequent removal, similar to that operating in bacteria (34) and mammalian systems (4, 9, 27). Misincorporation of uracil into DNA is more frequently observed when dUTP levels are elevated (34). In mammalian cells, dUTP is synthesized by phosphorylation of dUMP formed by deamination of dCMP (20). Although normally, dUTP would be converted rapidly back to dUMP (1) and then to dTTP, this reaction may be inhibited to some extent in purine-deprived V79 pur 1 cells because of the increased size of the dCTP and dTTP pools.

Against the likelihood of repair synthesis during purine deprivation are the following observations: (a) in the absence of purines, all of the [³H]thymidine is incorporated along with BrdUrd into molecules of hybrid density, and (b) during a half-hour pulse-labeling period, ³H radioactivity is incorporated into discrete stretches of DNA measurable by DNA fiber autoradiography rather than into small areas dispersed randomly throughout the DNA. However, the possibility remains that some of the small DNA molecules seen in depleted

cells arise from excision of replicating stretches into which uracil was misincorporated.

The overall rate of DNA synthesis is determined by two factors, the rate of replication fork movement and the frequency of initiation of replication units. Our results indicate that replication fork movement is inhibited by purine depletion, but we cannot provide direct evidence that initiation is blocked. We find neither an increase in the replication unit length nor a decrease in the frequency of postpulse initiation units. These two criteria have been used in the past as evidence for a block in initiation (11, 13). However, fiber autoradiography permits examination only of those clusters of replication units active at the time of the radioactive pulse. Since purine-deprived cells are arrested in S phase (39), initiation must be inhibited at some point, probably on whole clusters of replication units (24). Moreover, the amount of total DNA synthesis in these cells is decreased 5- to 10-fold by 6 h (39), whereas the rate of replication fork movement is reduced only by 2.7-fold in the same time interval. These data also suggest that there are fewer active forks in purine-deprived cells. We are unable to detect this by fiber autoradiography.

TABLE III

Frequency of Initiation in Purine-deprived and Purine-supplemented V79 pur 1 Cells*

Time	Medium	Initiation units post-pulse (proportion)	No. of units scored
h			
0	Control	0.52	326
	Depleted	0.50	430
4	Control	0.52	349
	Depleted	0.51	334
	Depleted/replenished for 2 h	0.52	349
6	Control	0.52	373
	Depleted	0.53	341

* Autoradiograms containing 2–4 replication units were collected at a magnification of 280. No more than 50–60 units per slide were scored to ensure that the autoradiograms came from a number of different S-phase cells. Units with no apparent gap in the region of high grain density were scored as units that initiated after the beginning of the radioactive pulse (postpulse units). All others were scored as units that initiated before the pulse began (prepulse), regardless of the length of the gap. Scoring was done blind on coded slides. Controls are V79 pur 1 cells grown in medium supplemented by 30 μM hypoxanthine.

We also observed a decrease in the interval between initiation sites in the purine-depleted cells in comparison to their nondepleted counterparts. The correlation of reduced rate of replication fork movement and smaller intertrack distance has also been observed by other investigators (16, 22, 32, 33). Ockey and Saffhill (22) have postulated that a regulatory mechanism may be operating to keep the overall rate of DNA synthesis constant per nucleus. As more origins are used, the rate of synthesis per origin drops. On the other hand, Taylor and Hozier (33) have suggested that chain elongation is the prime regulator of overall DNA synthesis. When chain elongation is blocked, new initiation sites become available between those normally used. Thus they suggest that fork movement tends to stop at initiation sites and this effect is enhanced by low levels of precursors. Our data are consistent with this hypothesis. As the purine nucleotide precursor pools decrease in size, we observe a decrease in the rate of fork movement as well as in the distance between origins of replication. If the hypothesis of Taylor and Hozier is correct, then our findings of shorter intervals between initiation sites does not necessarily reflect an increase in the number of initiation events in the deprived cells. This is consistent with our finding that there is no change in the relative frequency of initiation in these cells in comparison to controls.

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