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A Retarded Rate of DNA Chain Growth in Bloom's Syndrome

(DNA replication/chromosomes/Fanconi's anemia/DNA fiber autoradiography)

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ABSTRACT **The cytogenetic observation that homologous chromatid interchange occurs in Bloom's syndrome more often than normal prompted an investigation of DNA replication in that rare genetic disorder. Using DNA fiber autoradiography, an estimation was made of the rate of one component of ongoing DNA replication, DNA chain growth. The rate in Bloom's syndrome dermal fibroblasts in tissue culture was found to be significantly slower than that in normal control cells. (The rate was found to be normal in Fanconi's anemia cells.) The explanation for the retarded chain growth may be either that an enzyme concerned directly with semiconservative DNA replication is defective or that a defective enzyme not itself concerned directly with replication results in disturbed cellular metabolism, which in turn affects replication.**

Bloom's syndrome (1) is a rare, autosomal recessive disorder characterized clinically by intrauterine and postnatal growth retardation, sun-sensitive facial telangiectasia, disturbed immune function, and a predisposition to cancer. Lymphocytes and dermal fibroblasts from homozygous-affected individuals exhibit increased chromosome instability (2, 3). Interphase cells proliferating *in vitro* show many more nuclear irregularities and micronuclei than normal, and mitotic cells display increased numbers of broken and rearranged chromosomes. The enzymatic defect in the syndrome is unknown.

A chromosome abnormality that is highly characteristic of dividing Bloom's syndrome cells is an interchange between chromatids of homologous chromosomes at apparently homologous sites. (See refs. 4 and 5 for pictured examples.) Such a chromatid interchange, which is represented at metaphase by a quadriradial configuration of symmetrical proportions, is present in from 0.5 to 14.0% of Bloom's syndrome lymphocytes, whereas, it is found only rarely in lymphocytes from normal persons. The regions affected in such interchanges are nonrandomly distributed throughout the chromosome complement; certain regions of certain homologous chromosomes, particularly regions near the centromeres, are affected preferentially (4). Exchanges between sister chromatids also are greatly increased in frequency in Bloom's syndrome cells, but these are more randomly distributed (6). This unusual propensity to exchange between both sister and homologous chromatids is thought to be specific for Bloom's syndrome, although the homologous chromatid interchanges occasionally are found along with various other chromatid aberrations in Fanconi's anemia and the Louis-Bar syndrome (7), two other rare genetic disorders. (These three disorders are sometimes considered together because all show chromosome instability and predisposition to cancer.) The characteristic chromatid interchanges can be induced in normal cells by mitomycin C (8, 9) and certain other chromosome-breaking chemicals.

The chromatid interchange that leads to the characteristic metaphase quadriradial configuration of Bloom's syndrome is best interpreted as having taken place between homologous regions of two chromosomes during or following the period in the cell cycle when they engaged in semiconservative DNA replication. The exact spatial relationship of the two parental strands in the DNA duplex at the time it is replicating is unknown. However, we reasoned that at the time of replication, strands probably separate in some way, so that a chance for exchange between strands of two chromosomes might exist when "paired" (i.e., closely associated), genetically homologous regions replicate synchronously. Under these circumstances, a growing DNA fiber in one chromosome might erroneously become continuous with the homologous fiber in another chromosome and lead to chromatid interchange. Furthermore, the possibility of such an exchange of strands would be increased should the process of replication itself be abnormally retarded along individual replicating units, so that the spatially approximated replicating regions would remain "open" for a longer than normal period of time. In this way, the cytogenetic disturbances characteristic of Bloom's syndrome could be caused by unusually slow DNA chain propagation.

Two components of ongoing semiconservative DNA replication in mammalian cells are recognized: (a) multifocal initiation of new DNA chain synthesis and (b) chain growth. The second, rate of DNA chain growth, can be quantitated using the technique of DNA fiber autoradiography after [^{3}H]dThd pulse-labeling (10–12). We have tested the above hypothesis by measuring the rate of DNA chain growth in dermal fibroblasts in culture, comparing that in Bloom's syndrome fibroblasts with that in fibroblasts from normal persons. In addition, fibroblasts derived from persons with Fanconi's anemia were examined, because they as well as those from Bloom's syndrome display chromosome instability (3); however, the distribution of types of chromosome aberrations in Fanconi's anemia is different from that in Bloom's syndrome (13, 14). Symmetrical quadriradial configurations are far less common, open chromatid gaps and breaks being characteristic; interchanges, when they occur, usually affect nonhomologous chromosome regions. The hypothesis that slow chain growth is responsible for the increased number of homologous chromatid interchanges seemed applicable to Bloom's syndrome only, not to Fanconi's anemia.

MATERIALS AND METHODS

Cell Lines. Diploid fibroblast cell lines were derived from skin biopsies taken from five individuals with Bloom's syn-

drome: cell line HG 46 from individual 3(HoCo) (see ref. 1 for identification of individuals); HG 149 from 5(JaOa); HG 469 from 26(SaTi); Hg 666 from 47(ArSmi); and HG 709 from 32(MiKo). Each of the Fanconi's anemia lines, identified as HG 261, HG 280, and HG 293, was derived from a different child with classical features of that syndrome, including chromosome instability. The control lines were derived from skin mainly of normal laboratory personnel; included as a control was one line from the skin of a child with trisomy 21 (line no. 7 of the controls in Fig. 3).

The methods of derivation and maintenance of the lines have been described elsewhere (9). The lines had been inactivated in liquid nitrogen prior to the present experiments. When reactivated, they were maintained in glass flasks till confluency, then passed and seeded into petri dishes at a density of approximately 5000 per cm^2. The tissue culture medium was Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. Experiments were performed 24 hr after seeding when the cells were in a logarithmic growth phase. Generation times were measured in several cell lines according to the method of Puck and Steffen (15). In three normal lines, times were 30.0 hr, 40.0 hr, and 43.0 hr and in two Bloom's syndrome lines, 25.6 hr and 39.5 hr. All experiments were performed between passage levels 5 and 17, when the cells were in Hayflick stage II (16). Determinations of chain growth were performed from two to four times in each Bloom's syndrome or Fanconi's anemia cell line, each determination being made at a different passage level. For each determination, a control line comparable in number of subculture generations was tested concomitantly. (No correlation was found between rate of DNA chain growth and subculture generation through passage level 17.)

Pulse-Labeling Protocol. The method, described only briefly here, was that used previously for determinations of rate of chain growth in other experiments with mammalian cell lines (17, 18). Fluorodeoxyuridine, (FdUrd) (2 μM), was added to the culture medium 30 min before [^{3}H]dThd to exhaust the cells' endogenous dThd and ensure immediate high density labeling of DNA fibers at the beginning of the [^{3}H]dThd pulse. The FdUrd was left in the medium during the pulse in order to increase the incorporation of exogenous [^{3}H]dThd. The cells were first given a "hot" pulse of 30-min duration, using [^{3}H]dThd of a high specific activity (50 Ci/mmol, 5 μM), following which they were immediately given a "warm" pulse of 30-min duration, using [^{3}H]dThd of a lower specific activity (5 Ci/mmol, 5.5 μM). At the end of the warm pulse the cells were washed and processed for DNA fiber autoradiography.

DNA Fiber Autoradiography. This also was performed as described previously (17, 18). The cells were dropped gently on to a glass slide where they were lysed with sodium dodecyl sulfate. The DNA fibers released from the cells were spread with a glass rod over the surface of the slide. When dry, the preparations were coated with NTB-2 nuclear track emulsion (Kodak) which was exposed 4–8 months, developed, and examined.

Scoring of Autoradiograms and Determination of Rate of DNA Chain Growth. The pulse-labeling protocol outlined produces two characteristic types of autoradiographic patterns, each reflecting DNA synthesized along individual replication units during both the hot and the warm pulses (Fig. 1). In those units in which replication was initiated during the hot pulse, a linear array of high silver grain density is flanked on either end by arrays of low grain density. The heavily labeled center section represents DNA replicated during the hot pulse, the flanking lightly labeled arrays, DNA replicated during the warm pulse (postpulse-initiation figure) (Fig. 1b,y). In those

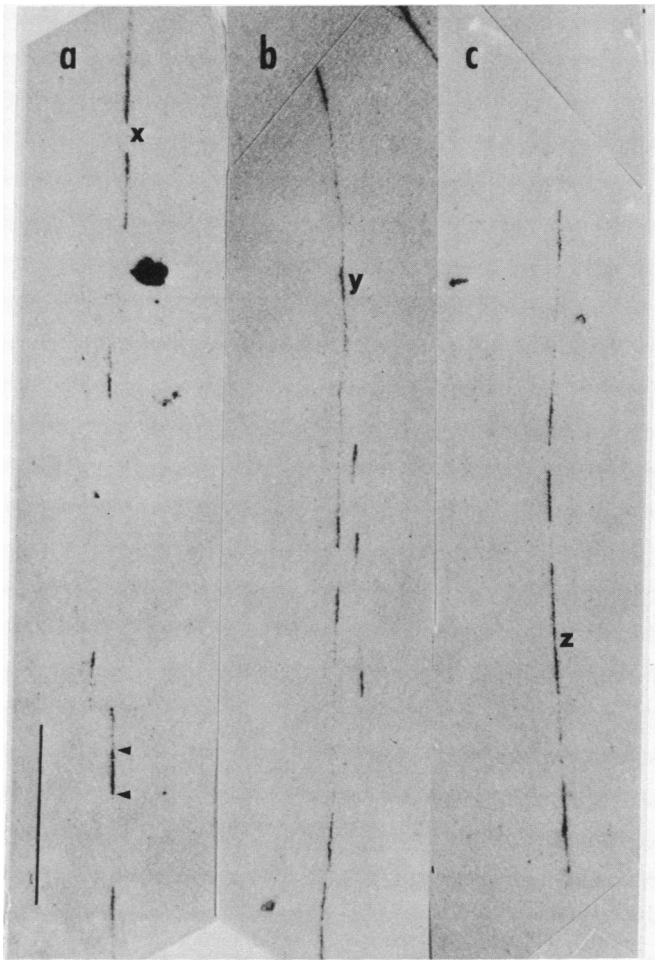

FIG. 1. Autoradiograms of replicating DNA from human dermal fibroblasts: (a) Bloom's syndrome; (b) normal control; (c) Fanconi's anemia. The DNA was pulse-labeled with [^{3}H]-dThd and prepared for autoradiography as described in *Materials and Methods*. The bar represents 100 μm. In (a) x marks a prepulse-initiation figure; in (b), y marks a postpulse-initiation figure. In (c), z marks a pattern that may represent two adjacent sections that completed replication during the hot pulse and became continuous. In (a), the two arrowheads near the lower prepulse-initiation figure delineate a length of DNA that would have been measured to determine the rate of DNA chain growth. In this prepulse-initiation figure, replication had been initiated prior to the beginning of the pulse-labeling period, at some point in the clear (grain-free) area below the lower arrowhead. Replication had continued, and at the start of the hot pulse, marked by the lower arrowhead, the growing chain began to incorporate [^{3}H]dThd of high specific activity. This incorporation continued for the duration of the hot pulse (30 min). When the hot pulse was discontinued and the warm pulse begun, the growing chain incorporated [^{3}H]dThd of lower specific activity; this moment is marked by the transition from heavy to light grain density, indicated by the upper arrowhead. The length of the heavy grain density thus can be used to measure the rate of chain growth on this particular replication section.

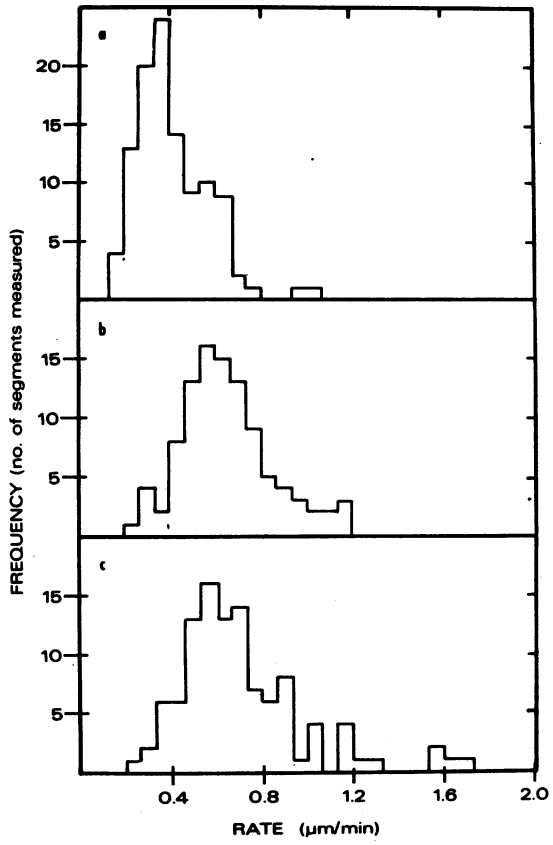

FIG. 2. Frequency distributions of rates of chain growth on individual replication sections in dermal fibroblasts: (a) Bloom's syndrome; (b) normal controls; (c) Fanconi's anemia. Each histogram represents one determination, i.e., measurements made from one cell line during a single experiment.

units in which replication had been initiated before the beginning of the pulse, a clear segment (representing unlabeled DNA) is flanked by two segments of high grain density, followed by the segments of low grain density (Fig. 1a,x). Both types of autoradiographic patterns represent DNA replication initiating at the center of a replication unit (in the second type, the initiation point is unlabeled) and proceeding bidirectionally outward (10, 17, 19) during the hot and warm pulses. About equal numbers of the two types of patterns are found in any single autoradiographic preparation (18). The patterns representing units in which replication had been initiated before beginning of the pulse can be used to measure the rate of DNA chain growth, because in these patterns, the beginning of the hot pulse is marked by the appearance of grains and the end by the transition from a heavy to a light density of grains. These prepulse-initiation figures are traced and measured, and the lengths of the heavy grain density segments are used to calculate the rate of chain growth during the hot pulse.

TABLE 1. *Rate of DNA chain growth in dermal fibroblasts*

Cells	No. of determinations	Rate, μm/min mean ± SEM
Bloom's syndrome	14	0.47 ± 0.024
Control	24	0.64 ± 0.014
Fanconi's anemia	10	0.65 ± 0.019

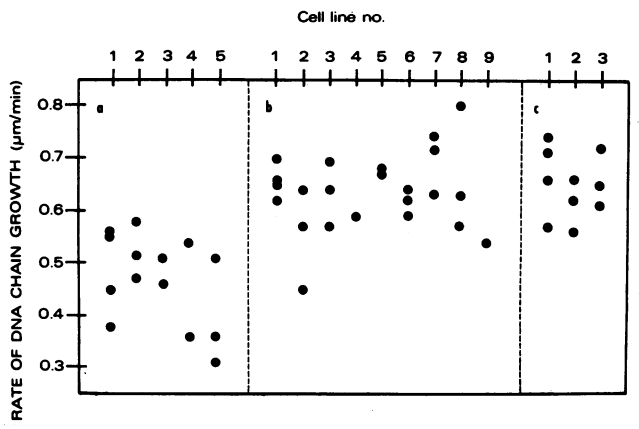

FIG. 3. Rates of DNA chain growth in five Bloom's syndrome, nine control, and three Fanconi's anemia dermal fibroblast cell lines: (a) Bloom's syndrome; (b) controls; (c) Fanconi's anemia. Each filled circle represents a single determination (like each of the three in Fig. 2), which is the mean of the measurements of 100 (in some experiments 50) replicating sections of DNA fibers. Determinations on the same cell line performed at different passage levels are represented by the vertical array of circles under the cell line number. Standard errors of the means of individual determinations were usually 5% or less.

The autoradiographs were examined by light microscopy, and appropriately labeled patterns were traced and measured. The method of selection of autoradiograms for measurement minimizes the chance of selection bias. Pre-selected microscopic fields were examined in search of appropriate autoradiographic patterns. The pattern closest to an arbitrary position in the microscopic field (3 o'clock at the periphery) was traced. Only one autoradiographic pattern from each microscopic field and a maximum of 25 from any one slide were used. Three to five slides produced from each experimental cell suspension were examined; this procedure maximizes the chances that the various autoradiographic patterns examined come from many different cells. The length of DNA replicated during the hot pulse was measured and the rate of DNA chain growth was calculated by dividing the length (in μm) by the duration of the pulse (in minutes). In the present study, a determination of chain growth for a single culture involved the tracing and measuring of 100 (in some experiments, 50) autoradiographic patterns. (Rates of chain growth derived using this method (10–12, 17) are in close agreement with those obtained using equilibrium sedimentation methods (20–23)).

RESULTS

The general patterns produced by the arrays of grains over labeled DNA fibers in the Bloom's syndrome and the Fanconi's anemia cell lines were similar to those produced in the control lines (Fig. 1). Furthermore, they were similar to those produced in earlier experiments using various established mammalian cell lines (18).

During the course of several experiments, the mean rates of DNA chain growth were determined for the various Bloom's syndrome and Fanconi's anemia cell cultures and compared with the rates in the controls. Fig. 2 shows the frequency distributions of the rates found in individual replication units from a single experiment in which one cell line of each type was used. In this experiment, the rates of chain growth on individual replication sections were slower in the Bloom's syn-

drome line than in the normal or Fanconi's anemia. The data from all lines, combined from the several experiments, are summarized in Fig. 3. The mean rates from the 14 determinations for the five Bloom's syndrome lines ranged from 0.31 to 0.58 μm/min. The means of 22 of the 24 determinations from the eight normal lines and the trisomy 21 line were in excess of 0.55 μm/min, as were those of all 10 determinations from the three Fanconi's anemia lines. Thus, little overlap exists between the mean rates of DNA chain growth of Bloom's syndrome cells and those of either control or Fanconi's anemia cells. Table 1 shows the mean rates of chain growth for all the determinations from the Bloom's syndrome, control, and Fanconi's anemia lines. Bloom's syndrome cells showed a reduction in the rate by 26.6% of that of control cells, whereas Fanconi's anemia cells resembled the controls. The difference observed between the Bloom's syndrome cells and both the control and the Fanconi's anemia cells is significant statistically ($P < 0.001$). The rate of chain growth in normal diploid fibroblasts is similar to that in established mammalian tissue culture lines (10–12, 17, 20–23).

DISCUSSION

The experiments represent an attempt to elucidate in molecular terms an unusual cytogenetic phenomenon that is characteristic of a rare genetic disorder of growth, Bloom's syndrome. The beginning observation was that homologous chromosomes exchange chromatids at apparently homologous sites much more often in Bloom's syndrome than in normal cells. The phenomenon itself is unusually interesting, pertaining as it does to somatic chromosome pairing and exchange of genetic material between chromosomes. The new finding here is that propagation of newly forming DNA chains in individual replication units proceeds slower in Bloom's syndrome than in the other cells tested. This indicates, therefore, that DNA replication itself is disturbed in Bloom's syndrome cells in culture. The question then arises as to whether the disturbance is primary, i.e., the consequence of a defective replicative mechanism, or secondary to a defect farther removed from metabolism of the genetic material itself.

A recognized limitation of these experiments is that diploid skin fibroblasts in culture may represent a heterogeneous population of cells. Conceivably, the predominant type of cell which grows out from explants from Bloom's syndrome skin is different from that which grows out from normal or Fanconi's anemia skin. Because of this, we employed a relatively large number of cell lines. Even with this limitation, our data suggest that most if not all replication units in S-phase cells in Bloom's syndrome tissue cultures exhibit a reduced rate of DNA chain growth. Differences have been detected in the percentage of cells in the S-phase in Bloom's syndrome versus normal cultures (Hand and German, unpublished results), but these differences do not apply to the present findings, which relate only to events occurring in individual replication units in cells themselves actively engaged in semiconservative DNA synthesis at the time of observation.

A possible explanation for the demonstrated disturbance in semiconservative DNA replication is a decrease in DNA polymerase activity. Complete absence of this enzyme's activity would prove lethal to the developing embryo, and even a reduction of mild degree theoretically should have serious consequences, even though several DNA polymerase activities exist in mammalian cells (24–26) and there could possibly be a backup enzyme system for semiconservative replication if activity of the enzyme normally employed is defective. The present experiments point not to absence but to a significant decrease in enzymatic activity, reflected by decreased rate of chain propagation.

An alternate interpretation of our data is that polymerization is reduced indirectly as result of inhibition of some other step of importance in DNA synthesis, i.e., one not immediately involved in chain propagation. It has been shown that inhibition of cellular protein synthesis or DNA precursor synthesis reduces the rate of DNA chain elongation (17) as well as inhibiting other aspects of DNA synthesis and cellular metabolism in general. Also, because FdUrd was used in these experiments to block the endogenous synthesis of dThd and the main source of dThd for DNA replication was exogenously supplied, it is conceivable that genetically determined inhibition of uptake, transport, or phosphorylation of dThd in our experiments would have manifested itself as a reduction in the rate of chain growth. Of the two components of ongoing DNA replication, initiation and chain growth, we have measured only the second. Now, the first, initiation, should be studied, probably using sedimentation (27, 28) as well as autoradiographic techniques. If an inhibition of initiation also is found, it, in combination with the disturbance of chain propagation, would favor there being some indirect effect on semiconservative replication in Bloom's syndrome.

In addition to the studies in initiation proposed above, direct assay of the various DNA polymerases of Bloom's syndrome fibroblasts appears to be indicated.

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