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# Analysis of DNA chain growth rates in Bloom syndrome B-lymphoblastoid cell lines using DNA fiber autoradiography

BY Takahiro TAGUCHI and Yukimasa SHIRAISHI

*Laboratory of Human Cytogenetics, Department of Anatomy, Kochi Medical School, Nankoku-City*, 781-51, *Kochi*

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ABSTRACT

The rates of DNA chain growth in four Bloom syndrome (BS) and one normal B-lymphoblastoid cell lines (B-LCLs) were studied using DNA fiber autoradiography. Four BS cell lines were classed into two groups, one with high sister chromatid exchange (SCE) character, the other with normal (low) SCE. Hot pulse warm chase (hot-warm) labeling was used to measure the replicon sizes, and pulse labeling with three different durations (30, 60 and 120 mins) was used to estimate DNA chain growth rates. There was no significant difference in the rate of chain growth per minute among all of the cell lines studied. The finding that there is no difference of DNA chain growth rate between normal and BS cells with both high and normal SCE which originated from the same blood sample strongly indicates that BS DNA chain growth is basically normal and is independent of the SCE character.

## 1. INTRODUCTION

Bloom syndrome (BS) is an autosomal recessive genetic disorder characterized by pre- and post-natal growth retardation, sun-sensitive eruption of the face and predisposition to cancer (German 1969). The most prominent cytogenetic characteristic in BS is an increased rate of sister chromatid exchange (SCE) in cells labeled with 5-bromo-2′-deoxyuridine (BrdU) for two cell cycles, though the exact molecular mechanism involved in BS SCE is still unclear (Shiraishi *et al.* 1982, 1983b). The formation of SCE has been postulated to be associated with the action of DNA replication (Kato 1974), possibly being related to the process of DNA replication fork movement (DNA chain growth) (Painter 1980). Hand and German (1975, 1977) reported a retarded rate of DNA chain growth in BS skin fibroblasts as well as in phytohemagglutinin (PHA) stimulated BS blood lymphocytes, using a protocol in which BS cells were cultured in media containing 5-fluoro-2′-deoxyuridine (FudR) to reduce the DNA precursor pool size of endogenous thymidine triphosphate. However, Ockey (1979) pointed out that FudR reduces the mean rate of DNA chain growth in BS cells as well as in mammalian cells and that culture conditions are major factors which influence chain growth rates in BS fibroblasts because of low growth rates in vitro and has reported that there was no dif-

ference of chain growth rates in normal and BS skin fibroblasts. Even though the exact cause of these discrepancies remains unknown, the difference of culture conditions and of cell growth rate may be reflected in these different results. Two protocols for measurement of DNA chain growth rate through fiber autoradiography have been reported (Huberman and Riggs 1968). The hot-warm labeling method (Hand and German 1975, 1977; Ockey 1979), which is especially suited to the measurement of replicon sizes, labels the cells for a short period (15-20 min) in concentrated doses of $^3$H-thymidine ($^3$H-TdR) and for another period of equal length in a dilute solution, then measures the length of the labeled DNA for these relatively short periods. The pulse labeling method uses continuous labeling with $^3$H-TdR at the higher concentrations for periods of 10 to 560 min to measure the labeled distance and chain growth rate per min (Hori and Lark 1973). The use of these longer labeling periods yields values that can be measured with much greater accuracy than those of the hot-warm method because of the difficulty of measuring the transition point from hot to warm. Therefore, we have applied the pulse labeling protocol and have used permanent BS B-lymphoblastoid cell lines (B-LCLs) to evaluate DNA chain growth rates. Fortunately, we have established several BS B-LCLs which grow constantly *in vitro*, using Epstein-Barr virus (EBV) (Shiraishi 1982, 1983a). Interestingly, in two BS cases, a cell line with a basically normal level of SCE and a normal karyotype and a cell line with high SCE and an abnormal karyotype were obtained from the same blood sample (Shiraishi 1983a). In the present study, we have undertaken to clarify the exact chain growth rate in BS B-LCLs with high and normal SCE, as well as a normal B-LCL, using DNA fiber autoradiography.

## 2. MATERIALS AND METHODS

1. *Cell lines and cell culture*: Permanent BS B-lymphoblastoid cell lines (B-LCLs: $BS_{1-1}$, $BS_{1-2}$, $BS_{2-1}$ and $BS_{2-2}$) were established from the patients with BS (Shiraishi 1982, 1983a) and a normal B-LCL (KS86) from a normal subject by EBV. As previously described (Shiraishi 1983a), $BS_{1-2}$ and $BS_{2-2}$ were found to have an elevated rate of SCE (average SCE: $70.1\pm2.44$ in $BS_{1-2}$, $71.3\pm2.31$ in $BS_{2-2}$) in cells labeled with BrdU for two cell cycles. They also have abnormal karyotypes: $BS_{1-2}$; 46, XY, 7p+, 12q+, +14, t(15;15)(p11;p12), $BS_{2-2}$; 47, XY, 7p+, t(3;15)(p25;q15), t(7;11)(q11;p15), +m, while $BS_{1-1}$ and $BS_{2-1}$ had a normal level of SCE frequency ($6.1\pm0.20$ and $5.5\pm0.22$, respectively) and normal karyotypes. The karyotype of KS86 was normal and the base line level of SCE per cell was $5.2\pm0.18$. These cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS) at 37°C in 5% $CO_2$ humidified incubation. All of the cell lines studied were subcultured one day before $^3$H-thymidine ($^3$H-TdR) labeling experiment.

2. *DNA fiber autoradiography*: Two protocols were employed; one was hot pulse warm chase labeling (protocol 1) to measure replicon sizes and the other was pulse labeling with three different durations (30, 60 and 120 mins) to evaluate DNA chain growth rates (protocol 2). In protocol 1, growing cells ($2\times10^5$ cells) were labeled in 2 ml media (3 cm petri dish) with 100 $\mu$Ci/ml $^3$H-TdR with high specific activity (48 Ci/mmol) for 20 min followed by incubation with 20 $\mu$Ci/ml $^3$H-TdR with low specific activity (9.6 Ci/mmol) for 40 min. In the protocol 2, cells ($2\times10^5$/ml), cultured in 3 cm petri dish containing 2 ml media, were labeled with 100 $\mu$Ci/ml $^3$H-TdR only with high specific activity (48 Ci/mmol) for 30, 60 and 120 mins, then chilled Phosphate-buffered saline (PBS) containing 20 $\mu$g/ml cold TdR was added to each culture. Cells were centrifuged, transferred to chilled PBS containing cold TdR (20 $\mu$g/ml) washed three times by centrifuging and resuspended in the mixture of 0.5 ml ice cooled PBS and 2 ml 0.07 M KCl. A part (0.5 ml) of 2.5 ml cell suspension was used for cell counting and measurement of $^3$H-TdR incorporation into DNA in protocol 2. The amounts of $^3$H-TdR incorporated into DNA were measured according to routine liquid scintillation counting (Houldsworth and Lavin 1980). A drop containing approximately $3\times10^3$ cells was placed on a glass slide previously coated with 0.5% serum albumin and mixed with 2 drops of 1% sodium dodecyl sulfate (SDS) in 0.05 M EDTA and fibers were drawn following the procedure of Hori and Lark (1973). After air drying, the slides were fixed with 10% ice-cooled trichloro acetic acid (TCA) for 5 min and washed once with water and twice with ethanol (99.5%). The dried slides were then coated with nuclear emulsion (Sakura NR-M2). After coating with emulsion, the slides were kept at 4°C in light-proof boxes. Slides for DNA fibers were exposed for nearly 8 months. And then slides were developed in Kodak D-19 developer for 5 min at 20°C and observed under light microscope. Analysis of DNA fiber autoradiograms was done according to the method of Hori and Lark (1973). Measurements for both replicon sizes and rates of DNA chain growth were made by direct observation under the microscope with aid of an ocular micrometer and on photographs using a dial caliper and pencil.

## 3. RESULTS

### *Replicon size*

The replicon sizes were measured with center to center distances between neighboring hot tracks using 50 pairs of them in each cell line. Only hot tracks followed on both sides by warm tracks or center prepulse gaps followed on both sides by hot tracks continuing with warm tracks were taken into consideration. Fig. 1 shows the schematic representation of five types of replicon pairs measured in the present study. Fig. 2 exhibits examples of alignments of typical hot-warm tracks in KS86, $BS_{1-1}$ and $BS_{1-2}$ cells. As

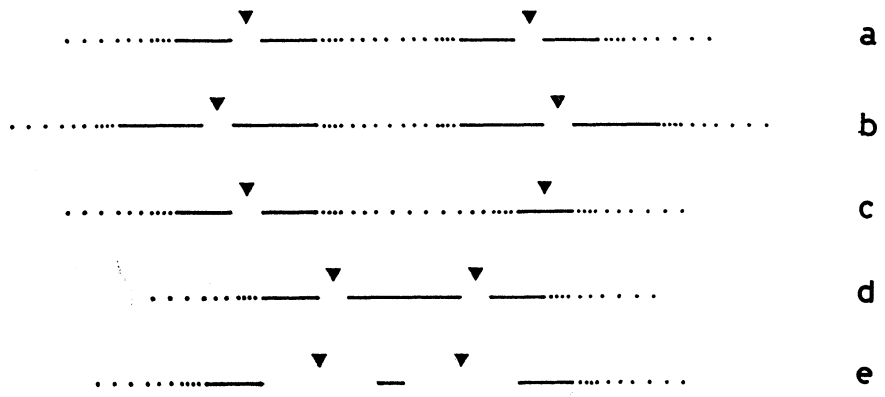


Fig. 1. Diagram of the five typical replicon pairs found in the DNA fiberautoradiographs of human B-LCLs. Triangle arrows indicate several initiation sites.

Table 1. *Replicon sizes in Bloom syndrome B-lymphoid cell lines with high SCE ($BS_{1-2}$, $BS_{2-2}$) and normal SCE ($BS_{1-1}$, $BS_{2-2}$) and a normal cell line (KS86) Protocol 1 (hot-warm labeling)*

| Cell lines | Average replicon size ($\mu$m) Mean ± S.E. |
|---|---|
| KS86 | 69.5±2.9 |
| $BS_{1-1}$ | 62.5±3.4 |
| $BS_{1-2}$ | 68.4±2.5 |
| $BS_{2-1}$ | 68.1±3.1 |
| $BS_{2-2}$ | 74.0±3.2 |

Table 2. *DNA chain growth rates in Bloom syndrome B-lymphoblastoid cell lines with high SCE ($BS_{1-2}$, $BS_{2-2}$) and normal SCE ($BS_{1-1}$, $BS_{2-1}$) and a normal cell line (KS86)*

| Cell lines | Rates of chain growth per minutes ($\mu$m/min)±S.E. | | | Mean ($\mu$m/min) |
|---|---|---|---|---|
| | 30 min | 60 min | 120 min | |
| KS86 | 0.76±0.03 | 0.70±0.03 | 0.68±0.03 | 0.71±0.03 |
| $BS_{1-1}$ | 0.76±0.02 | 0.70±0.03 | 0.71±0.03 | 0.72±0.03 |
| $BS_{1-2}$ | 0.84±0.02 | 0.72±0.03 | 0.71±0.03 | 0.76±0.03 |
| $BS_{2-1}$ | 0.82±0.03 | 0.75±0.03 | 0.77±0.03 | 0.78±0.03 |
| $BS_{2-2}$ | 0.79±0.02 | 0.72±0.03 | 0.76±0.03 | 0.76±0.03 |

shown in Fig. 2, there was almost no difference of the average values of replicon unit, though there was variation of the replicon sizes even in the

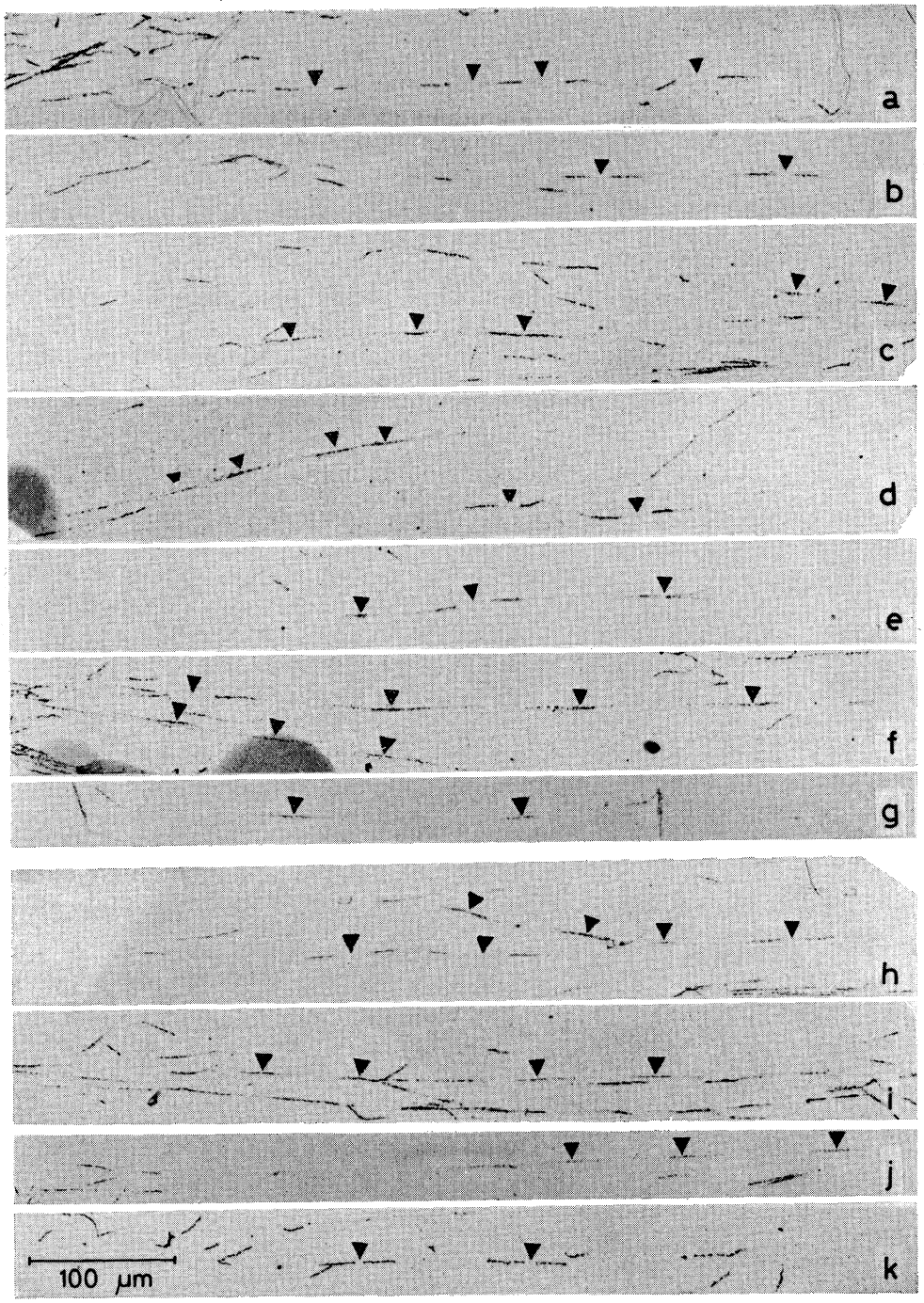


Fig. 2. Arrays of replicons (hot-warm labeling) in KS86 (a, b, c), $BS_{1-1}$ (d, e, f, g) and $BS_{1-2}$ (h, i, j, k). Triangle arrows indicate several initiation sites of replicons with or without fusion occurring during the pulse. Replicon sizes were measured with the distance between neighbouring two initiation sites.

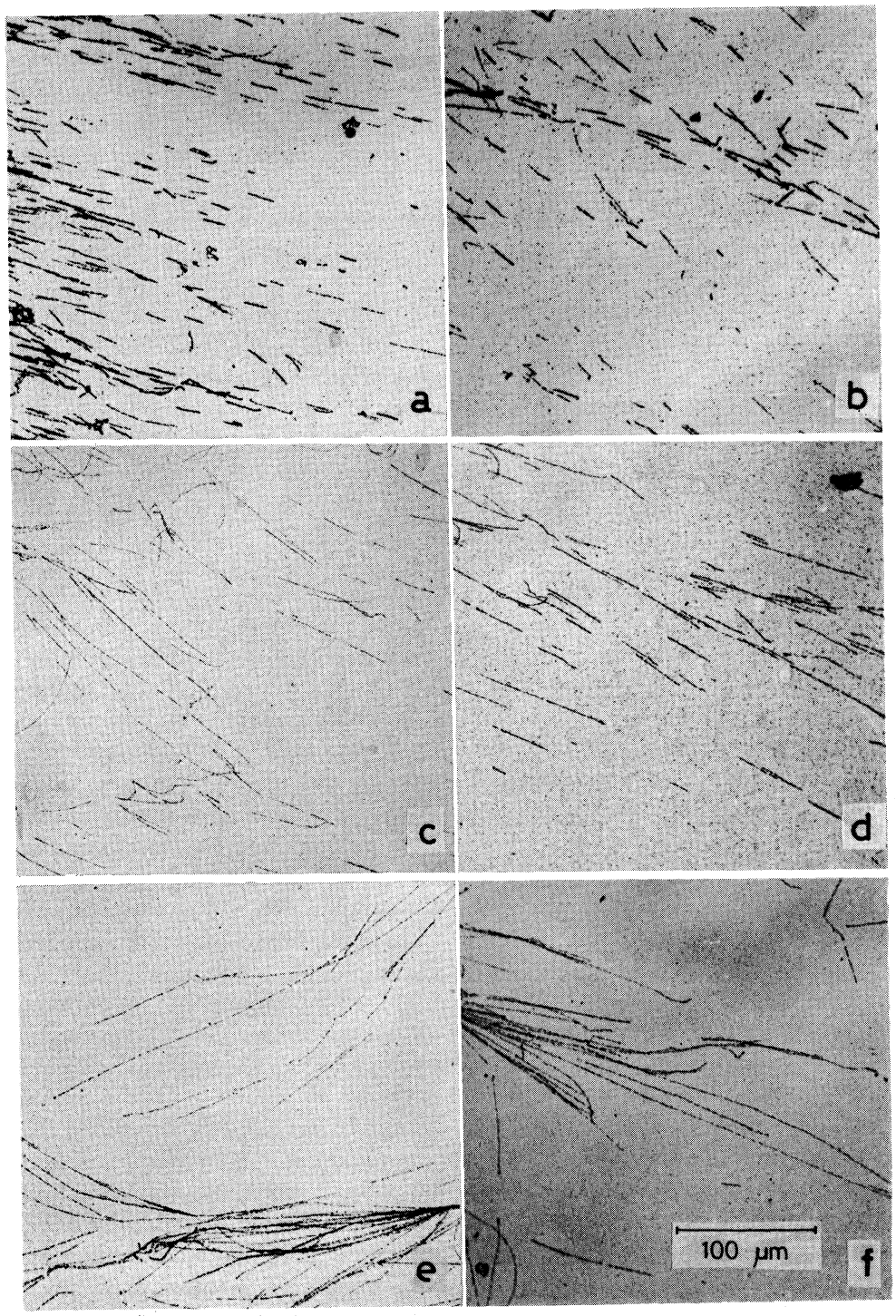


Fig. 3. Autoradiographs of dense track length (pulse labeling) labeled for 30, 60 and 120 min. in $BS_{1-1}$ (a, c, e) and $BS_{1-2}$ (b, d, f). Dense track length increased with the labeling duration.

same cell line. Average center to center distance (replicon size) was 64.5 $\mu$m in KS86, 62.5 $\mu$m in $BS_{1-1}$, 68.4 $\mu$m in $BS_{1-2}$, 68.1 $\mu$m in $BS_{2-1}$ and 74.0 $\mu$m in $BS_{2-2}$ (ranging from 40.0-110.0, 20.0-110.0, 20.0-160.0, 20.0-120.0 and 20.0-130.0 $\mu$m, respectively) (Table 2). The average replicon size in $BS_{1-1}$ is slightly smaller than those of the other cell lines.

*Rate of DNA chain growth*

In the pulse labeling protocol (30, 60 and 120 mins), the average dense track length (labeled DNA) was taken as the distance traveled by the moving forks during the pulse duration. All suitable arrays of labeling contained at least three tandemly aligned dense tracks of silver grains formed during each pulse labeling. Fig. 3a-f exhibits typical examples of dense tracks due to pulse

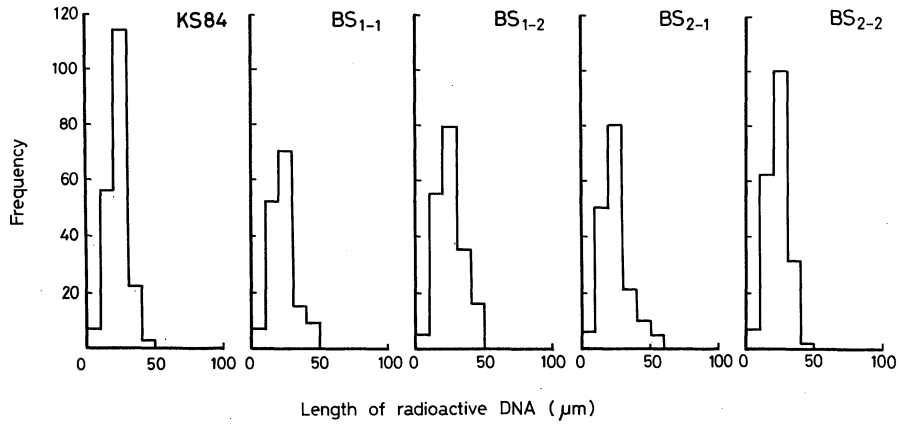


Fig. 4. Frequency distribution of the length of DNA labeled (pulse labeling) for 30 min. in KS86, $BS_{1-1}$, $BS_{1-2}$, $BS_{2-1}$, $BS_{2-2}$ cells.

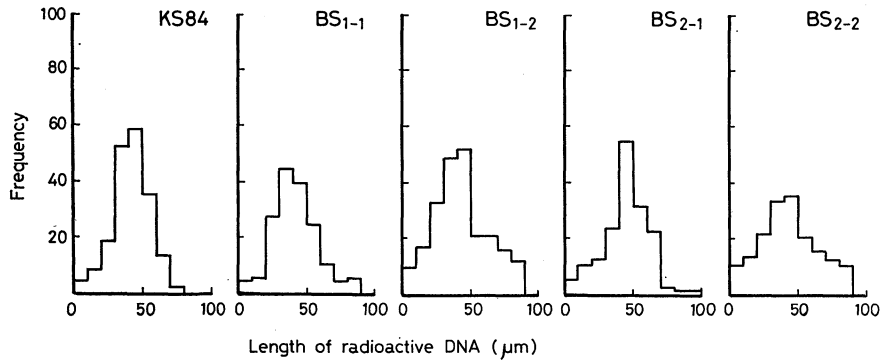


Fig. 5. Frequency distribution of the length of DNA labeled (pulse labeling) for 60 min. in KS86, $BS_{1-1}$, $BS_{1-2}$, $BS_{2-1}$ and $BS_{2-2}$ cells.

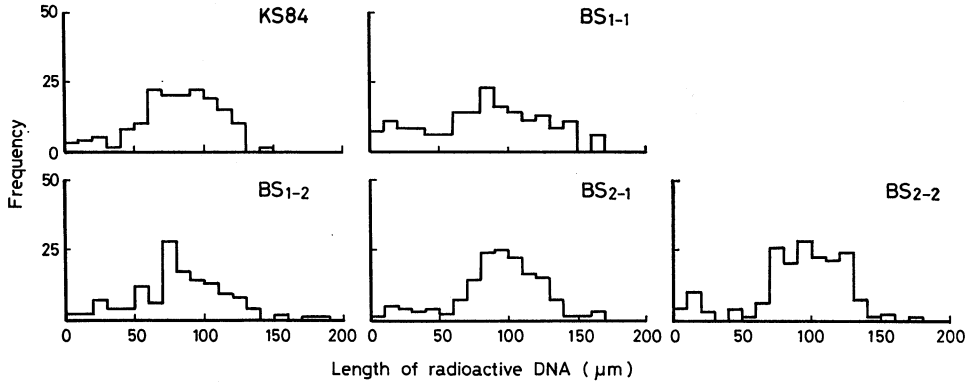


Fig. 6. Frequency distribution of the length of DNA labeled (pulse labeling) for 120 min. in KS86, $BS_{1-1}$, $BS_{1-2}$ and $BS_{2-1}$ cells.

labeling (30, 60 and 120 mins) in $BS_{1-1}$ and $BS_{1-2}$ cells. Figs. 4, 5 and 6 present the frequency distributions of the length of radioactive pieces of DNA replicated during 30, 60 and 120 min labelings from each cell line. As shown in these figures, the dense track length was elongated with time, though there existed variations of labeled DNA lengths. Chain growth rates were calculated as the ratio of dense track length to pulse labeling time (30, 60 and 120 mins). The average rate of chain growth was 0.71, 0.72, 0.76, 0.78 and 0.76 $\mu$m/min in KS86, $BS_{1-1}$, $BS_{2-1}$, $BS_{1-2}$ and $BS_{2-2}$, respectively (Table 2). These measure-

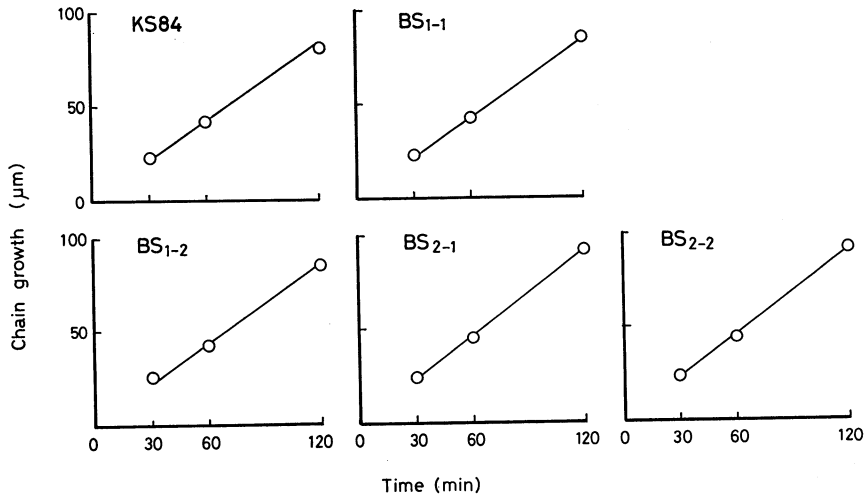


Fig. 7. Correlation between chain growth and pulse labeling period (30, 60 and 120 mins). Chain growth rates were expressed as a function of the pulse duration with $^3$H-TdR measured on DNA fibers isolated from each cell line.

ments showed that the rate of chain growth did not differ in all these cell lines.

In Fig. 7 the chain growth is expressed as a function of pulse time and the curve produced is linear with a positive slope. The chain growth is shown to increase linearly with pulse time in each cell line. Extraporation of the curve to zero pulse time gives a value of near zero, showing linear relationship between pulse duration and chain growth distances with approximately equal slants (0.71-0.78).

### *$^3$H-TdR incorporation into DNA*

The amounts of $^3$H-TdR incorporated into DNA for 30 and 60 minutes were measured in normal and BS B-LCLs, since cell growth rates and S phase labeling were closely correlated among normal and BS B-LCLs (Shiraishi *et al.* 1983b). As shown in Table 2, there was no significant difference in the values of $^3$H-TdR incorporated for 30 and 60 minutes among these four cell lines. The data indicate no significant decrease in $^3$H-TdR incorporation rates in $BS_{1-2}$ and $BS_{2-2}$ when compared with $BS_{1-1}$ and KS86 cells.

Table 3. *$^3$H-TdR incorporation into DNA in normal and BS B-LCLs*

| Cell lines | Incorporated amount of $^3$H-TdR(cpm) into DNA ($\times 10^3$) | |
|---|---|---|
| | 30 min | 60 min |
| KS86 | 80 | 170 |
| $BS_{1-1}$ | 76 | 169 |
| $BS_{1-2}$ | 82 | 170.1 |
| $BS_{2-1}$ | 81.6 | 171 |
| $BS_{2-2}$ | 81.5 | 178 |

## 4. DISCUSSION

The present study has shown that there is no significant difference in the rates of DNA chain growth between the normal and BS B-LCLs, which were examined by DNA fiber autoradiography. The present results were obtained from the pulse labeling protocol (Hori and Lark 1973) using four BS B-LCLs (two with high SCE and the other two with normal SCE) which grew constantly *in vitro*. Measurements of DNA chain growth rates were evaluated from the average growth rate based on the DNA fiber length labeled with $^3$H-TdR for 30, 60 and 120 min. The DNA chain elongated with time, and there was a linear relationship between the labeled time and the elongation of the DNA chain, and there was no significant difference of chain growth rate among different lengths of pulse labeling in all of the cell lines studied.

This supports the previous results obtained by Ockey (1979). The use of B-LCLs is particularly well suited to evaluate the exact rates of DNA chain growth in BS as well as in normal cells, since the B-LCLs used grew constantly *in vitro* culture and there was no difference of cell growth rates between normal and BS B-LCLs (Shiraishi 1983b). The cell growth rate of BS skin fibroblasts seems unstable judging from the low plating efficiency and poor $^3$H-TdR incorporation rates (Giannelli *et al.* 1977). The unstable growth property of BS skin fibroblasts may be reflected in the divergent results reported for DNA chain growth rates (Henson *et al.* 1981; Hand and German, 1975, 1977; Giannelli *et al.* 1977; Ockey 1979; Kapp 1982). Therefore cell lines with poor growth properties seem to exhibit retarded chain growth as observed in BS skin fibroblasts. The finding that there was little difference between $BS_{1-1}$ and $BS_{1-2}$, and between $BS_{2-1}$ and $BS_{2-2}$ is especially important, since each of these pairs of cell lines was derived from the same blood sample although the first member has normal SCE and the second has high SCE when cultured in the presence of BrdU for 2 cell cycles. This strongly indicates that BS chain growth is independent of the SCE character of the cell line. The present finding is of special interest considering our previous reports (Shiraishi 1982; 1983b) that most of BS SCEs were caused by BrdU and that the spontaneous SCE level was almost normal in BS cells. Also considering that X-ray (Ockey 1983) and inhibitors of DNA (hydroxyurea, FudR) and protein synthesis (puromycin) (Hand and Tamm 1973; Hori and Lark 1973) caused a retarded rate of chain growth and that though no difference of chain growth was detected in non-treated BS and normal cells, UV irradiated BS skin fibroblasts showed retarded chain growth using alkaline sucrose gradient profile (Henson *et al.* 1981), the present finding that there is no difference of chain growth rates in non-treated BS and normal cells is reasonable. Further studies are now in progress to examine the chain growth rate in BS cells treated with BrdU and other mutagens.

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