THE EFFECT OF 5-BROMODEOXYURIDINE ON DNA REPLICATION AND CELL DIVISION IN *TETRAHYMENA PYRIFORMIS*

ANNE E. LYKKESFELDT and H. A. ANDERSEN

From the Biological Institute of the Carlsberg Foundation, 2200 Copenhagen N, Denmark

ABSTRACT

Populations of *Tetrahymena pyriformis* were grown in a chemically defined medium containing the thymidine analogue 5-bromodeoxyuridine (BUdR). About 65% of the thymidine sites in DNA were substituted by BUdR. During the first generation in the presence of BUdR, all DNA became hybrid. After the following cell division, in about 80% of the cells the second DNA replication round was initiated but no further cell division took place. The cells could be rescued by removing BUdR and adding thymidine. New replication took place before the first cell division. However, although the cells contained double heavy as well as hybrid DNA, only the hybrid DNA was replicated. After a full replication of the hybrid DNA, normal growth was restored. Melting profiles of normal, hybrid, and double heavy DNA indicated a structural change of the double heavy DNA.

INTRODUCTION

The development of the density gradient techniques for the separation of macromolecules with different buoyant densities (14) has permitted the separation of normal DNA from DNA containing 5-bromodeoxyuridine (BUdR) instead of thymidine. Consequently, BUdR has been widely used for the study of DNA replication in eukaryotic as well as prokaryotic cells (5, 15, 19). However, these studies have to some extent been handicapped by the cytotoxic and mutagenic effect of BUdR which is seen when cells are grown for a long period of time, and/or in high concentrations of BUdR (17). Furthermore, incorporation of BUdR into DNA has been shown to inhibit differentiation of specific cell types (12, 13) and to prevent normal development of embryos (8) by a selective inhibition of transcription of specific enzymes (9, 12, 20). Under most growth conditions. the highly polyploid *Tetrahymena* cells tolerate incorporation of BUdR without measurable damaging effects, as will be discussed in the present paper, and BUdR has been used in the study of the replication and organization of DNA in the macronucleus in this organism (1, 2, 3).

The present paper describes the effect of BUdR on DNA replication and cell division in *Tetrahymena pyriformis* when cells are grown on a chemically defined medium containing BUdR instead of thymidine and with simultaneous limitation of the endogenous synthesis of thymidine. Under such conditions the cells will incorporate BUdR in high amounts. After a full replication round in the presence of BUdR, cell proliferation ceases and the replication rate decreases. Double heavy DNA (containing BUdR in both strands) cannot be replicated, whereas most of the hybrid DNA (containing BUdR in one strand) can. Therefore, when the cells are released from the

inhibitory treatment by the addition of thymidine, only hybrid DNA is replicated and after a full replication round cell division reappears.

MATERIALS AND METHODS

T. pyriformis, amicronucleate strain GL, was grown axenically on the chemically defined medium described by Rasmussen and Modeweg-Hansen (16) with the modification that no tetrahydrofolic acid was present in the vitamin stock. This compound was added separately at the time of inoculation of the culture. The final concentrations were between 3×10^{-9} and 7×10^{-9} g/ml. The populations were grown in Erlenmeyer flasks sealed with screw caps and in a layer of medium not exceeding 1 cm. The flasks were kept at 28°C without shaking and aeration. Under these conditions the cells showed exponential growth to about 300,000 cell/ml and went into stationary phase at around 600,000 cells/ml. The cells were counted in an electronic cell counter after fixation with 10% Formalin.

BUdR was added after several generations of exponential growth and to a final concentration of 0.8 mM. BUdR was removed from the culture by transfer of the cells to fresh medium. The cultures were centrifuged for 1 min at 1,000 *g* and the BUdR-containing medium was discarded and replaced by the new medium. This procedure was repeated, and thymidine and tetrahydrofolic acid were added to restore normal growth.

For long-term labeling, the cells were incubated with 0.006 mCi per ml culture of [^{3}H]thymidine (Amersham/Searle Corp., Arlington Heights, Ill., specific activity 5 Ci/mmol) or 0.020 mCi per ml culture of ^{32}P as orthophosphate in isotonic saline (specific activity 7 mCi/g).

For cesium chloride gradient analysis of DNA, 5 ml of the culture containing about 250,000 cells were harvested, DNA was isolated, and gradient centrifugation was performed as described previously (1).

To estimate the density shift in the gradient, samples from the fractionated gradient were withdrawn and the refractive index was measured in a Zeiss refractometer. The position of normal DNA correlates with a refractive index of about 1.4015 (ρ = 1.695 g/ml), of hybrid DNA with a refractive index of about 1.4045 (ρ = 1.728 g/ml), and of double heavy DNA with a refractive index of 1.4075 (ρ = 1.761 g/ml).

For autoradiographic analysis 0.5-ml samples were pulse labeled with [^{3}H]thymidine, 5 μCi per sample (specific activity 5 Ci/mmol). The autoradiograms were prepared as previously described (4).

To obtain pure samples of the various types of DNA for melting-profile analysis, the DNA was isolated after density gradient centrifugation. The fractions from the gradients containing normal, hybrid, and double heavy DNA, respectively, were pooled separately and the gradient centrifugation of each fraction was repeated. The DNA fractions obtained after collection of the second gradient were diluted with SSC buffer and used for analysis of the melting profile as described in reference 11.

RESULTS

T. pyriformis grown on a chemically defined medium multiplied with a generation time of about 4–6 h, depending, for example, on the tetrahydrofolic acid concentration, which in the experiments described here was kept at a growth-limiting concentration. Tetrahydrofolic acid is kept at a low concentration in order to decrease the endogenous synthesis of thymidine and thereby increase the incorporation of exogenous thymidine or BUdR. In the experiment, the results of which are shown in Fig. 1, the cells grew exponentially with a generation time of 5 h in medium containing 7.5×10^{-6} mg/ml of tetrahydrofolic acid. At a cell density of 50,000 cells/ml, BUdR was added to a final concentration of 0.8 mM. The cells continued growth for 1.55 generations after the addition of BUdR; thereafter, cell proliferation ceased.

Unless thymidine is added, BUdR removed, or the tetrahydrofolic acid concentration increased about 10 times, the cell number remains constant for 15–20 h after the cell division has ceased, then the cell number slowly decreases.

The cells were rescued by the addition of thymidine. Cell division reappeared 8–10 h later, and normal growth rate was obtained after about 15 h. In order to follow the DNA replication during this period of time, BUdR was removed

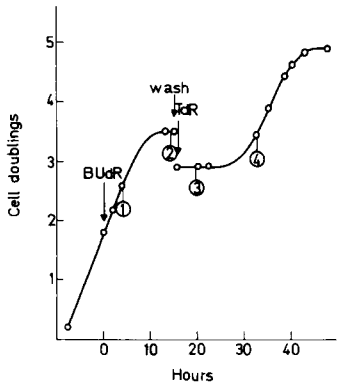

FIGURE 1 Effect of BUdR on cell division. BUdR was added at the time indicated by arrow. After the cessation of cell division, BUdR was removed by transfer of the cells to a new medium, and thymidine was added as indicated in the figure. For analysis of DNA, cells were harvested at the time points indicated by the circled numbers.

from the medium at the time of addition of thymidine.

To follow the DNA replication in the population after the addition of BUdR and again after the removal of BUdR, the DNA was uniformly labeled after several generations of growth in the presence of [^{3}H]thymidine. These labeled cells were used for inoculation 10 h before the addition of BUdR. At the time points marked ① and ② in Fig. 1, cells were harvested, and the percentage of the total amount of DNA in the population which had replicated in the presence of BUdR was assessed by a cesium chloride gradient analysis. The result is shown in Figs. 2 and 3. After 225 min, 75% of the DNA has incorporated BUdR and is located in the position of hybrid DNA, as may be seen in Fig. 2. This corresponds to the measured increase in cell number during the same period of time. [^{32}P]Orthophosphate was added with the BUdR selectively to label the BUdR-containing DNA. From the position in the gradient of the hybrid DNA, it has been calculated that 65% of the thymidine in the newly synthesized DNA strand has been replaced by BUdR.

The second sample was harvested 11.75 h later, when the cell multiplication had stopped. It is shown in Fig. 3 that all ^{3}H-labeled DNA is found in the position of hybrid DNA, and furthermore, that a ^{32}P-labeled double heavy DNA peak has appeared. The distribution of the ^{32}P-labeled DNA is 55% and 45% in the double heavy and hybrid

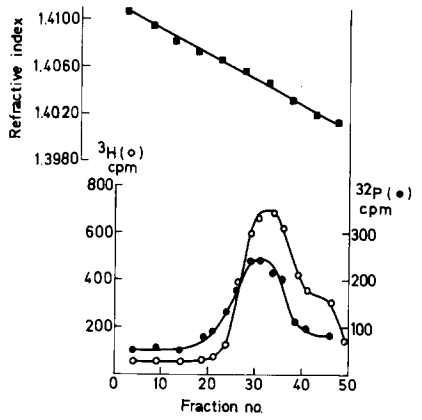

FIGURE 2 Cesium chloride gradient of DNA isolated from cells harvested 225 min after addition of BUdR and ^{32}P (①, Fig. 1). DNA was previously labeled for several generations with [^{3}H]thymidine. About 75% of the ^{3}H-labeled DNA is found in the hybrid position (○). The ^{32}P-labeled DNA is located exclusively in the hybrid position (●).

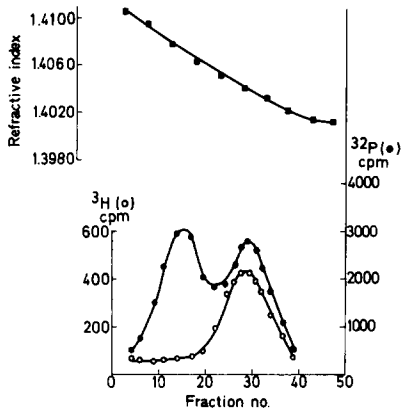

FIGURE 3 Cesium chloride gradient of DNA isolated from cells harvested 15 h after addition of BUdR and ^{32}P (②, Fig. 1). All ^{3}H-labeled DNA is found in the hybrid position. The ^{32}P-labeled DNA is distributed in hybrid and double heavy DNA, 45% and 55%, respectively.

peaks, respectively. Replication of the hybrid DNA gives rise to double heavy DNA ^{32}P-labeled in both strands and new hybrid DNA labeled in one strand. Therefore, during replication, half of the ^{32}P counts found in the double heavy peak are equivalent to the amount of ^{32}P counts in hybrid DNA arising from the second replication round. Consequently 27.5% of the counts in hybrid DNA (45%) originate from replication in the second round. In other words, 61% of the hybrid DNA has been replicated.

After addition of BUdR, the DNA replication continued at a constant rate for at least 225 min (Fig. 2). This corresponds to a full replication of DNA within 5 h, the normal generation time. However, from the results shown in Fig. 3, it was determined that in the next 11.75 h only 61% of the DNA was replicated again. To determine whether all cells were engaged in the second replication round, an autoradiographic analysis was performed. Cells were labeled with [^{3}H]thymidine for a 6-h period, beginning after the end of the first replication round in the presence of BUdR. These autoradiograms showed that only 80% of the cells were synthesizing DNA in this period. This indicates that about 20% of the cells fail to initiate the second replication round. Furthermore, autoradiograms of cells pulse-labeled for 1 h showed that after the first replication round, the percentage of cells in DNA synthesis decreased. These results also indicate that some of the cells do not take part in the second replication round. Finally, it was found that about 20% of the cells showed no

replication after the removal of BUdR, and as we shall see from the results shown in Fig. 5, about 20% of the hybrid DNA was not replicated within 17 h after the release from the BUdR inhibition, at a time when the cells had resumed exponential growth. Altogether, we may conclude that in about 20% of the cells, the incorporation of BUdR into *one* DNA strand inhibits the replication of this hybrid DNA.

To follow the DNA replication after removal of BUdR, [^{32}P]orthophosphate was added at the time when the cells were transferred to fresh medium. For this purpose, a parallel culture not previously labeled with [^{32}P]orthophosphate was used. At the time points marked ③ and ④ in Fig. 1, cells were harvested and DNA was analyzed. 5 h after removal of BUdR, [^{32}P]orthophosphate had been incorporated into DNA of normal and hybrid densities in equal amounts, as may be seen from the results shown in Fig. 4. This shows that although the cells contain hybrid DNA as well as double heavy DNA, only the hybrid DNA has been replicated. The distribution of the ^{3}H-labeled DNA in the gradient shows that 30% of the total amount of DNA has been replicated during these 5 h. 12 h later the cells grow with a normal generation time, and the sample harvested at that time (Fig. 1, ④), demonstrates that some of the cells have entered the third replication round after the removal of BUdR. The results are shown in Fig. 5. The ^{32}P-labeled DNA is now distributed in

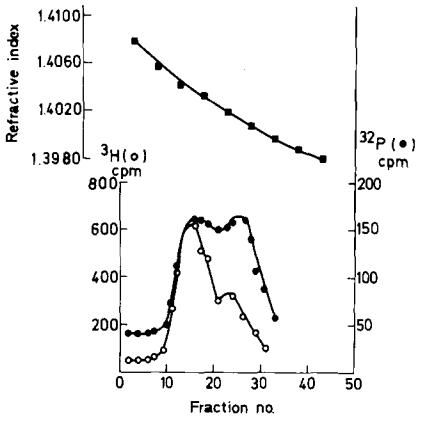

FIGURE 4 Cesium chloride gradient of DNA from cells harvested 5 h after the removal of BUdR and addition of ^{32}P (③, Fig. 1). Normal and hybrid DNA are found in the gradient, and ^{32}P is incorporated in equal amounts into these two peaks. The distribution of ^{3}H labeling indicates that about 30% of the DNA has replicated since removal of BUdR.

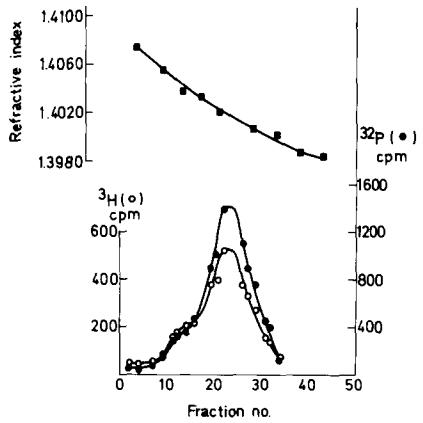

FIGURE 5 Cesium chloride gradient of DNA from cells harvested 17 h after removal of BUdR and addition of ^{32}P (④, Fig. 1). The ^{32}P-labeled DNA is distributed in hybrid and normal DNA, 12% and 88%, respectively. 20% of the ^{3}H-labeled DNA is still found in the hybrid position.

the hybrid and normal peaks 12% and 88%, respectively. This distribution is possible only if some cells have finished the second replication round, and entered the third. In spite of this, only 80% of the hybrid DNA has been replicated, indicating that in 20% of the cells replication cannot occur after the formation of hybrid DNA. In the remaining 80% of cells, replication of the hybrid DNA occurs. Thereafter, cell division reappears and normal growth continues.

The double heavy DNA, synthesized in the second replication round in the presence of BUdR, has a defective template activity both for the transcriptional processes that are necessary for cell division and for replication. Even when thymidine is again offered as the precursor for DNA synthesis, the double heavy DNA cannot function as a template. This is most probably a consequence of changes in the helical structure after incorporation of BUdR into both DNA strands, as can be seen from the melting profile of DNA (normal and BUdR-containing) shown in Fig. 6. Normal, double-stranded DNA has a melting temperature value of 90°C with a shift in optical density 260 of about 50%, which might be a little too high due to some evaporation from the cuvette during the heating. Hybrid DNA has the same melting temperature value and differs only slightly from normal DNA. However, double heavy DNA has no defined melting temperature. The separation of the two strands begins around 60°C, and a continuous change in optical density 260 is observed with

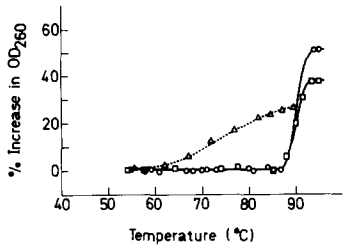

FIGURE 6 Melting profiles of normal (○), hybrid (□) and double heavy DNA (△).

increasing temperature. It should be mentioned that the increase in optical density upon melting of the double heavy DNA is significantly lower than the increase found for normal or hybrid DNA. This lower increase might indicate some single-strandedness of the double heavy DNA at the beginning of the melting profile analysis, again implying a more labile structure of the BUdR-containing DNA leading to some degree of single-strandedness during the isolation procedure.

DISCUSSION

On a proteose-peptone medium containing BUdR in the concentration used here, *Tetrahymena* cells have been grown for hundreds of generations without measurable effects. Only on a chemically defined medium, in which the low tetrahydrofolic acid concentration decreases the endogenous synthesis of thymidine, is it possible to obtain sufficiently high incorporation of BUdR into DNA to see the effect on DNA replication and cell division described in the present paper.

BUdR is known to have a mutagenic effect on many cells (6, 18, 22), and it has been proposed that the cytotoxic effect of BUdR is due to an accumulation of mutations (8, 10). However, mutations are very rare in *Tetrahymena*, possibly because of the high macronuclear ploidy implying that many copies of the same gene must be modified simultaneously to produce a mutation. Therefore, the inhibitory effect of BUdR on DNA synthesis and cell division is more probably due to the structural changes of the DNA helixes found after incorporation of high amounts of BUdR into both DNA strands. This is in agreement with the effect of BUdR on HeLa cells described by Toliver and Simon (21). These authors observed an effect of BUdR after incorporation during a full replication round. 80% of the cells containing hybrid DNA were unable to initiate the second replication round. A small fraction of the cells continued division and replication with greatly elongated generation time. After removal of BUdR these cells resumed normal growth. The authors conclude that the presence of BUdR in DNA causes structural changes responsible for the observed effect. Similar observations have been made by Gontcharoff and Mazia (7) after incorporation of BUdR into the DNA of sea urchin embryos.

In the population studied here, all cells have completed a full replication round in the presence of BUdR before any inhibitory effect on cell division appears. The increase in cell number of 1.55 doublings corresponds to two divisions of cells in G_2 and late S and one division of cells in the other phases of the cell cycle at the time of the BUdR addition. Therefore, the effect of BUdR is not seen until the cells have finished a full replication in the presence of BUdR. In most of the cells the second replication round is initiated and the hybrid DNA is replicated, but the double heavy DNA formed is not functional. At this time most cells contain twice as much DNA as a G_1 cell, but no cell division occurs unless thymidine is added and the hybrid DNA is replicated. In some cells the hybrid DNA is not replicated either in the presence of BUdR or when thymidine is added after removal of BUdR.

As mentioned above, the described effect of BUdR is seen only when more than about 60–65% of the thymidine content of DNA is substituted with BUdR. This high BUdR incorporation is only possible when the endogenous synthesis of thymidine is limited by a low tetrahydrofolic acid concentration. There is a close connection between the generation time of the cells and the tetrahydrofolic acid concentration in the medium. At lower tetrahydrofolic acid concentrations the generation time is greatly elongated and the cells may die, even when BUdR or thymidine is offered. Therefore, it is necessary to find a tetrahydrofolic acid concentration at which the needs of the cells are met with respect to methylation reactions other than thymidine synthesis, and then full repression of the thymidine synthesis is not possible. Toliver and Simon (21) discuss the existence of certain sites in DNA that prefer thymidine. In general we think that thymidine is a better precursor than BUdR for DNA synthesis. In the cells studied here the endogenous synthesis of thymidine is more or less defined by growth conditions, and cannot be repressed by exogenous BUdR. Therefore, we chose a tetrahydrofolic acid concentration that gave a relatively short generation time and at the

same time permitted the incorporation of sufficiently high amounts of BUdR to cause the inhibitory effects described.

The authors wish to thank Professor Erik Zeuthen for the excellent working facilities at the Biological Institute and for his critical reading of the manuscript. The secretarial help of Mrs E. Palludan is also gratefully acknowledged.

Received for publication 28 September 1973, and in revised form 3 April 1974.

REFERENCES

1. ANDERSEN, H. A. 1972. Induced elimination of DNA from the macronucleus of *Tetrahymena pyriformis*. *Exp. Cell Res.* **74**:610–613.
2. ANDERSEN, H. A. 1972. Requirements for DNA replication preceding cell division in *Tetrahymena pyriformis*. *Exp. Cell Res.* **75**:89–94.
3. ANDERSEN, H. A., C. F. BRUNK, and E. ZEUTHEN. 1970. Studies on the DNA replication in heat synchronized *Tetrahymena pyriformis*. *C. R. Trav. Lab. Carlsberg.* **38**:123–131.
4. ANDERSEN, H. A., and E. ZEUTHEN. 1971. DNA replication sequence in *Tetrahymena* is not repeated from generation to generation. *Exp. Cell Res.* **68**:309–314.
5. BRAUN, R., and H. WILI. 1962. Time sequence of DNA replication in *Physarum*. *Biochim. Biophys. Acta.* **174**:246–252.
6. FREESE, E. 1959. The specific mutagenic effect of base analogues on *Phage T4*. *J. Mol. Biol.* **1**:87–105.
7. GONTCHAROFF, N., and D. MAZIA. 1967. Developmental consequences of introduction of bromouracil into the DNA of sea urchin embryos during early division stages. *Exp. Cell Res.* **46**:315–327.
8. HAKALA, M. T. 1959. Mode of action of 5-bromodeoxyuridine on mammalian cells in culture. *J. Biol. Chem.* **234**:3072–3076.
9. KOTZIN, B. L., and R. F. BAKER. 1972. Selective inhibition of genetic transcription in sea urchin embryos. Incorporation of 5-bromodeoxyuridine into low molecular weight nuclear DNA. *J. Cell Biol.* **55**:74–81.
10. LITTLEFIELD, J., and E. GOULD. 1960. The toxic effect of 5-bromodeoxyuridine on cultured epithelial cells. *J. Biol. Chem.* **235**:1129–1133.
11. MANDEL, M., and J. MARMUR. 1968. Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods in Enzymol.* Tabor and Tabor, editors. 12. *b*:195–206.
12. MARZULLO, G. 1972. Regulation of cartilage enzymes in cultured chondrocytes and the effect of 5-bromodeoxyuridine. *Dev. Biol.* **27**:20–26.
13. MAYNE, R., J. ABBOT, and H. HOLTZER. 1973. Requirement for cell proliferation for the effect of 5-bromo-2-deoxyuridine on cultures of chick chondrocytes. *Exp. Cell Res.* **77**:255–263.
14. MESELSON, M., and F. W. STAHL. 1954. The replication of DNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **44**:671–682.
15. MUELLER, G., and K. KAJIWARA. 1966. Early- and late-replicating deoxyribonucleic acid complexes in HeLa nuclei. *Biochim. Biophys. Acta.* **114**:108–115.
16. RASMUSSEN, L., and L. MODEWEG-HANSEN. 1973. Cell multiplication in *Tetrahymena* cultures after addition of particulate material. *J. Cell Sci.* **12**:275–286.
17. ROY-BURMAN, P. 1970. Analogues of nucleic acid components. Recent results in cancer research. Springer-Verlag KG., Berlin, W. Germany.
18. SHAPIRO, H., and E. CHARGAFF. 1960. Severe distortion by 5-bromouracil of the sequence characterisics of a bacterial deoxyribonucleic acid. *Nature (Lond.)* **188**:62–63.
19. SMITH, D., H. SCHALLER, and F. BONHOEFFER. 1970. DNA synthesis in vitro. *Nature (Lond.)*. **226**:711–713.
20. STELLWAGEN, R., and G. TOMKINS. 1971. Differential effect of 5-bromodeoxyuridine on the concentrations of specific enzymes in hepatoma cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1147–1150.
21. TOLIVER, A., and E. H. SIMON. 1967. DNA synthesis in 5-bromouracil-tolerant HeLa cells. *Exp. Cell Res.* **45**:603–617.
22. TRAUTNER, T. A., M. N. SWARTZ, and A. KORNBERG. 1962. Enzymatic synthesis of deoxyribonucleic acid. X. Influence of bromouracil substitutions on replication. *Proc. Natl. Acad. Sci. U. S. A.* **48**:449–455.