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Uracil incorporation into nascent DNA of thymine-requiring mutant of *Bacillus subtilis* 168

(accumulation of short DNA chains/*thy*$^-$ and *thy*$^+$ cells/uracil-DNA glycosidase/deoxyuridylate)

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ABSTRACT A thymine-requiring mutant of *Bacillus subtilis* strain 168 accumulates short DNA chains after brief pulses with [^{3}H]thymidine. Reversion of the *thy* mutation to *thy*$^+$ abolishes the accumulation of short DNA chains, suggesting that the accumulation is related to the *thy* mutation. The reason for this accumulation has been further investigated by analysis of a mutant with a defective uracil-DNA glycosidase activity (*urg*). The accumulation of short DNA chains in *thy*$^-$ cells is abolished by the deficiency of uracil-DNA glycosidase activity. In *thy*$^+$ cells, the deficiency of the glycosidase activity does not change the sedimentation profile of pulse-labeled DNA. DNA isolated from *thy*$^-$*urg*$^-$ cells is fragmented by successive treatment with purified uracil-DNA glycosidase and alkali, indicating that uracil residues are present in this DNA. DNA isolated from *thy*$^+$*urg*$^-$ cells is not fragmented by the same treatment. Significant radioactivity is detected in the dUMP region, when [^{3}H]uridine-labeled DNA from *thy*$^-$*urg*$^-$ cells is hydrolyzed and analyzed by thin-layer chromatography. Only a trace amount of radioactivity, which is not influenced by the deficiency of uracil-DNA glycosidase activity, is found in the dUMP region in DNA hydrolysates from *thy*$^+$ cells. These results suggest that, in *thy*$^-$ cells, uracil is incorporated into DNA and the accumulation of short DNA chains results from the excision-repair of this uracil whereas in *thy*$^+$ cells, uracil is seldom, if ever, incorporated into DNA.

Studies in this and other laboratories support the idea that one or both strands of DNA are synthesized by a discontinuous mechanism whereby short DNA chains are synthesized and subsequently joined into long DNA chains (1). Short DNA chains accumulate in *Escherichia coli* cells in which DNA ligase or DNA polymerase I is inhibited, suggesting a role of these enzymes in the synthesis and joining of the short chains (2–7). Recently, Tye *et al.* (8) observed that mutants defective in dUTPase accumulate short (4–5 S) DNA chains. They suggested that this accumulation resulted from the excision-repair of uracil incorporated into DNA.

In the course of investigations on the discontinuous replication of *Bacillus subtilis* DNA, we noticed that a thymine-requiring mutant of *B. subtilis* strain 168 accumulated short DNA chains. Moreover, the accumulation was not observed in the spontaneous *thy*$^+$ revertant, suggesting that the accumulation is related to the thymine-requiring mutation. The studies described here were undertaken in order to determine if the accumulation of short DNA chains in strain 168T cells resulted from the excision-repair of uracil incorporated into DNA. Uracil-DNA glycosidase is thought to be responsible for the excision of uracil residues in DNA (9, 10). Therefore, a *B. subtilis* mutant with a defective uracil-DNA glycosidase activity (11) was analyzed. Results obtained from the analyses of this mutant suggest that, in *B. subtilis thy*$^-$ cells, uracil is incorporated into DNA. The present work also suggests that, in *B. subtilis thy*$^+$ cells, uracil is seldom, if ever, incorporated into DNA.

MATERIALS AND METHODS

Organisms and Chemicals. *B. subtilis* 168T (*thyAthyB*) and TKJ6901 (*thyAthyB urg*-1) were kindly provided by F. Makino (11). Spontaneous *thy*$^+$ revertants, 168R (*thy*$^+$) and TKJ6901R (*thy*$^+$*urg*-1), were isolated on minimal agar (12). Cells were grown in modified Spizizen medium containing 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% sodium citrate $\cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 1% glucose, and 1% Casamino Acids (13). Thymine was added (5 μg/ml) in the case of *thy*$^-$ cells. [*methyl*-^{3}H]Thymidine (20 or 50.8 Ci/mmol) was obtained from New England Nuclear Corp. and [5-^{3}H]uridine (29.65 Ci/mmol) from the Radiochemical Centre. *E. coli* uracil-DNA glycosidase, purified by phase partition, CM-Sephadex chromatography and hydroxyapatite column chromatography, was a gift of M. Sekiguchi (14). This preparation had no endonuclease activity under the condition used in this study as checked by the size change of δA-DNA. Bovine pancreatic RNase IA, bovine pancreatic DNase I, and snake venom phosphodiesterase were obtained from the Worthington Biochemical Corp. RNase T1 was obtained from Sankyo, Japan. RNases were heated at 90° for 10 min in 10 mM sodium acetate buffer (pH 5.0).

Analyses of Nascent DNA. Pulse-labeling with [^{3}H]thymidine, extraction of DNA in the denatured state, and alkaline sucrose gradient sedimentation of DNA were as described (13).

To prepare nascent short DNA chains for uracil-DNA glycosidase treatment, an 80-ml culture of TKJ6901 or TKJ6901R, grown at 30° to 10^8 cells per ml in modified Spizizen medium, was pulse-labeled for 30 sec with 0.1 μM [^{3}H]thymidine (50.8 Ci/mmol). The pulse was terminated with an ethanol/phenol mixture (15) and the cells were collected by centrifugation. These cells were suspended in 7.6 ml of 27% sucrose/20 mM EDTA/0.15 M NaCl/0.015 M sodium citrate and the DNA was extracted by a modified Thomas procedure (15), except that lysozyme treatment was carried out for 10 min at 37° with 500 μg of lysozyme per ml. DNA was precipitated by ethanol, dissolved in 9.4 ml of 0.015 M NaCl/1.5 mM sodium citrate/1 mM EDTA/0.1% sodium dodecyl sulfate and dialyzed against the same buffer. After heat denaturation (100°, 4 min), the sample was layered on two 27.5-ml sucrose gradients (5–20% in the above buffer) made on a 5-ml cushion of 80% sucrose and centrifuged in Beckman SW 27 rotor for 16 hr at 24,000 rpm and 18°.

After fractionation into 31 fractions, 2 fractions each corresponding to about 1500 and 3900 nucleotides were pooled and

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dialyzed against 10 mM Tris·HCl, pH 7.4/1 mM EDTA, concentrated by evaporation to 0.5 ml, and dialyzed against 10 mM Tris·HCl, pH 8.0/1 mM EDTA. To treat the nascent short DNA pieces with uracil-DNA glycosidase, samples (50–70 μl) were incubated at 37° for 120 min in a reaction mixture (90 μl) containing 50 mM Tris·HCl (pH 8.0), 10 mM EDTA, 28.7 units (16) of enzyme per ml, and 44 μM δA [^{14}C]DNA (700 cpm/nmol). δA [^{14}C]DNA was included in each reaction tube in order to ensure that neither endonucleolytic cleavage nor breakage of DNA occurred in uracil-free DNA. The reaction was stopped by the addition of 10 μl of 2% Sarkosyl. Cleavage of the chains at apyrimidinic acid sites was carried out by adding 100 μl of 2 M glycine/NaOH (pH 13.1) (the pH of the final 1 M solution was 12.8) and incubating for 4 hr 15 min at 25° (17).

Preparation of [^{3}H]Uridine-Labeled DNA. A 100-ml culture, grown at 30° to 8 × 10^7 cells per ml in modified Spizizen medium, was labeled with 1 μM [^{3}H]uridine (7.9 Ci/mmol). The labeling was terminated with ethanol/phenol mixture and the cells were collected by centrifugation. These cells were suspended in 9.5 ml of 0.15 M NaCl/0.015 M sodium citrate/27% sucrose/20 mM EDTA, and DNA was extracted as described above, precipitated by ethanol, dissolved in 4 ml of 0.015 M NaCl/1.5 mM sodium citrate, and dialyzed against 0.015 M NaCl/1.5 mM sodium citrate. After adjustment to 0.15 M NaCl and 0.015 M sodium citrate, pancreatic RNase IA and RNase T1 were added to final concentration of 50 μg/ml and 1345 units/ml, respectively, and incubated for 90 min at 37°. After addition of sodium dodecyl sulfate to 1%, the sample was treated with Pronase and phenol and passed through a column of Sephadex G-100 (2.1 × 35 cm). The excluded material was concentrated by evaporation and dialyzed against 5 mM Tris·HCl, pH 7.4/1 mM EDTA, then against 10 mM Tris·HCl, pH 7.4/0.1 M NaCl/1 mM EDTA, and finally against 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA. By this method, radioactivity incorporated into RNA could be decreased to 0.001–0.01% of the starting material.

Degradation of DNA to Deoxyribonucleoside 5′-Monophosphate and Chromatographic Separation of the Deoxyribonucleotides. DNA (180 nmol) was first incubated for 2 hr 40 min at 37° in a reaction mixture (0.95 ml) containing 20 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, and 60 μg of pancreatic DNase I. Then, 0.1 ml of 1 M glycine·KOH (pH 9.0) and 263 μg of snake venom phosphodiesterase were added and the mixture was incubated for 90 min at 37°. Thin-layer chromatography was done on a polyethyleneimine-cellulose plate (Polygram Cel 300 PEI, Macherey-Nagel, Germany). To separate the four deoxyribonucleoside 5′-monophosphates, development was carried out first with 0.25 M formic acid up to 12 cm beyond the origin and then, after drying, with 0.5 M lithium formate (pH 3.1) up to 18 cm (18) (solvent A). To separate rUMP and dUMP, development was carried out with 0.5 M LiCl/0.15 M ammonium borate (solvent B). To separate rUMP, dUMP, and TMP, development was carried out with 95% ethanol/1 M ammonium acetate, pH 7.5 (7:3) (solvent C) by continuous-flow chromatography (19).

RESULTS

Effect of *thy*$^-$ and *urg*$^-$ Mutation on the Accumulation of Nascent Short DNA Chains. Sedimentation analysis of the pulse-labeled DNA revealed the accumulation of nascent short DNA chains in a *thy* mutant of *B. subtilis* strain 168. *B. subtilis* 168T (*thy*$^-$*urg*$^+$) cells were grown in modified Spizizen medium plus 5 μg of thymine per ml and pulse-labeled with [^{3}H]thymidine at 30°. The labeled DNA was analyzed by alkaline sucrose gradient centrifugation. Virtually all the radioactivity incorporated into DNA by 168T cells during 20- and 60-sec pulses was recovered in the short chains sedimenting at average rates of 8.5 and 13 S, respectively (Fig. 1*A*). Because the generation time at 30° is about 60 min, a 60-sec pulse would label 1.7% of the whole chromosome. It is thus obvious that discontinuity persists in a large portion of the daughter strands in this mutant.

To determine whether the accumulation of short DNA chains in 168T cells is due to the thymine-requiring mutation, spontaneous *thy*$^+$ revertants of 168T were isolated. These revertants were grown in modified Spizizen medium and pulse-labeled for 20 and 60 sec at 30° with [^{3}H]thymidine, and the labeled DNA was analyzed by alkaline sucrose gradient centrifugation (Fig. 1*B*). In marked contrast with 168T, 54% and 74% of the radioactivity incorporated into DNA during 20- and 60-sec pulses, respectively, was recovered in the long DNA chains with sedimentation coefficients larger than 30 S.

The accumulation of short DNA chains in 168T cells was not observed with a deficiency of uracil-DNA glycosidase activity. A mutant of 168T with a defective uracil-DNA glycosidase activity, *B. subtilis* TKJ6901 (*thy*$^-$*urg*$^-$), was grown at 30° in modified Spizizen medium containing 5 μg of thymine per ml. After pulse-labeling for 20- or 60-sec with [^{3}H]thymidine, the labeled DNA was analyzed by alkaline sucrose gradient centrifugation (Fig. 1*C*). Similar to the sedimentation profile of *thy*$^+$ revertants of 168T, a majority of the radioactivity incorporated into DNA by TKJ6901 cells during the 60-sec pulse was recovered in the long DNA chains. Therefore, the deficiency of uracil-DNA glycosidase activity resulted in a significant alteration of the sedimentation profile of the pulse-labeled DNA of 168T.

Fig. 1*D* shows the sedimentation profile of the pulse-labeled DNA of spontaneous *thy*$^+$ revertants of TKJ6901. The sedimentation profile of the 20-sec labeled DNA of TKJ6901R (*thy*$^+$*urg*$^-$) was nearly the same as that of 168R (*thy*$^+$*urg*$^+$). A slight difference was observed in the case of the 60-sec pulse.

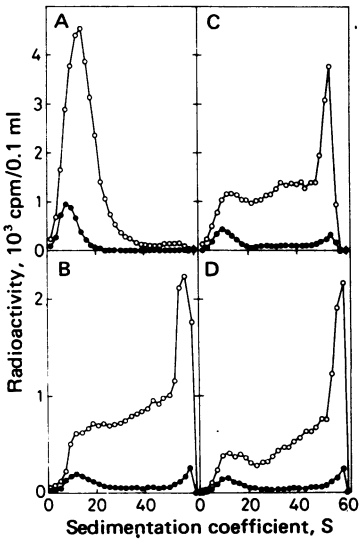

FIG. 1. Alkaline sucrose gradient sedimentation of DNA from *B. subtilis* 168T (*A*), 168R (*B*), TKJ6901 (*C*), and TKJ6901R (*D*) pulse-labeled with [^{3}H]thymidine. Cells were grown to a titer of 10^8 cells per ml at 30°. Portions (5 ml) of each culture were pulse-labeled with 0.1 μM [^{3}H]thymidine (20 Ci/mmol) for 20 sec (●) or 60 sec (○) at 30°. The pulse was terminated by the addition of ethanol/phenol mixture. DNA was extracted in the denaturated state and sedimented through an alkaline sucrose gradient (15) in a SW 41 rotor for 15.3 hr at 23,000 rpm.

Detection of Uracil-DNA Glycosidase-Susceptible Sites in DNA of TKJ6901. The above results support the hypothesis that uracil is incorporated into DNA in *thy*$^-$ cells and that the uracil residues incorporated are removed by an excision-repair mechanism, the first step of which is catalyzed by uracil-DNA glycosidase. If so, then we could expect uracil residues to be present in DNA of *thy*$^-$*urg*$^-$ cells. In order to investigate this point, we isolated known sizes of DNA chains and treated them successively with uracil-DNA glycosidase and alkali. If uracil residues are present in the DNA, uracil-DNA glycosidase will remove uracil bases and the apyrimidinic acid sites produced will be cleaved by alkali, resulting in the fragmentation of the DNA (9).

TKJ6901 (*thy*$^-$*urg*$^-$) and TKJ6901R (*thy*$^+$*urg*$^-$) cells were pulse-labeled with [^{3}H]thymidine for 30 sec at 30°. DNA was extracted by a modified Thomas procedure and then fractionated according to its chain length by neutral sucrose gradient centrifugation. The size distributions of the labeled DNA of TKJ6901 and TKJ6901R were almost identical. DNA chains corresponding to about 1500 and 3900 nucleotides were pooled, treated with or without uracil-DNA glycosidase, incubated in 1 M glycine·NaOH (pH 12.8) for 4 hr 15 min at 25° to cleave the chains at apyrimidinic acid sites, and then analyzed by alkaline sucrose gradient centrifugation. As shown in Fig. 2 *A* and *C*, a marked reduction of the size of the labeled DNA of TKJ6901 resulted from treatment with uracil-DNA glycosidase. DNA chains of average 1500 and 3900 nucleotides isolated from TKJ6901 were fragmented to those of average 800 and 1400 nucleotides, respectively. In contrast, the size of the labeled DNA of TKJ6901R remained unchanged by the same treatment (Fig. 2 *B* and *D*). In addition ^{14}C-labeled DNA from bacteriophage δA remained intact throughout uracil-DNA glycosidase and alkali treatments. These results indicate that uracil residues are present in the DNA of TKJ6901 but not in the DNA of TKJ6901R.

Detection of dUMP by Labeling DNA with [^{3}H]Uridine. Evidence for the presence of uracil residues in DNA of TKJ6901 was also obtained by labeling DNA with [^{3}H]uridine. TKJ6901

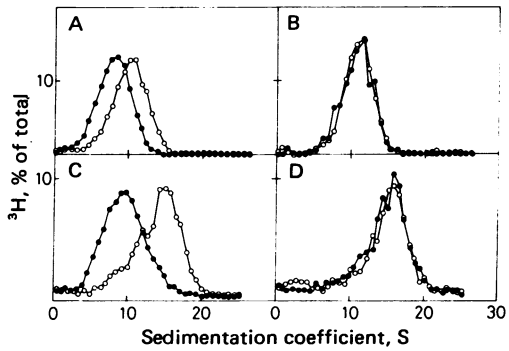

FIG. 2. Successive treatment of DNA with uracil-DNA glycosidase and alkali. ^{3}H-Labeled nascent DNA chains corresponding to average 1500 and 3900 nucleotides were prepared, subjected to successive treatment with uracil-DNA glycosidase and alkali (●) or alkali alone (○) and then analyzed by alkaline sucrose gradient centrifugation. Centrifugation was for 12 hr at 35,000 rpm and 4° in a Spinco SW 56 Ti rotor. (*A*) DNA chains of average 1500 nucleotides from TKJ6901; (*B*) DNA chains of average 1500 nucleotides from TKJ6901R; (*C*) DNA chains of average 3900 nucleotides from TKJ6901; (*D*) DNA chains of average 3900 nucleotides from TKJ6901R. (●-●) Incubated with uracil-DNA glycosidase and then incubated in 1 M glycine·NaOH (pH 12.8); (○-○) incubated without uracil-DNA glycosidase and then incubated in 1 M glycine·NaOH (pH 12.8). Total radioactivities were: (*A*) ●, 2560 cpm; ○, 2556 cpm; (*B*) ●, 587 cpm; ○, 586 cpm; (*C*) ●, 2179 cpm; ○, 2045 cpm; (*D*) ●, 592 cpm; ○, 572 cpm.

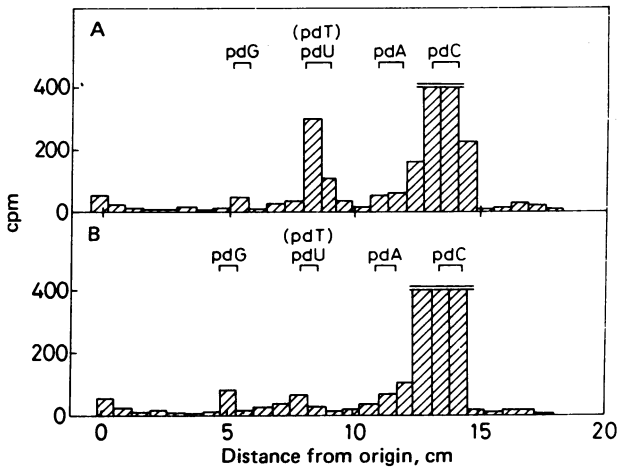

FIG. 3. Chromatography of [^{3}H]uridine-labeled DNA hydrolysates from TKJ6901 (*A*) and 168T (*B*). Each 100-ml culture was labeled with 1 μM [^{3}H]uridine (7.9 Ci/mmol) for 16 min at 30°. DNA was isolated (radioactivity incorporated into DNA: *A*, 7.07 × 10^5 cpm; *B*, 7.37 × 10^5 cpm), and hydrolyzed with pancreatic DNase I and snake venom phosphodiesterase to deoxynucleoside 5′-monophosphates. After precipitation of enzymes with $HClO_4$ and neutralization with KOH, the sample was desalted (recovery, 90%). A portion of the sample was applied to a strip of polyethyleneimine-cellulose with nonradioactive markers and the chromatogram was developed with solvent A. Radioactivity in the dCMP region was: *A*, 30,269 cpm; *B*, 28,396 cpm.

(*thy*$^-$*urg*$^-$) and 168T (*thy*$^-$*urg*$^+$) cells were labeled for 16 min at 30° with 1 μM [5-^{3}H]uridine. DNA was extracted and treated with pancreatic RNase IA and RNase T1, followed by Sephadex G-100 column chromatography to remove RNA. The DNA thus isolated was degraded to its constituent nucleoside 5′-monophosphates by pancreatic DNase I and snake venom phosphodiesterase. As shown in Fig. 3, the majority of the radioactivity incorporated into DNA was found in the dCMP region. In the case of TKJ6901, a significant portion of the radioactivity (about 1.4% of total) was also found in the dUMP region (Fig. 3*A*), whereas in the case of 168T less than 0.3% of the radioactivity was found in this region (Fig. 3*B*). When *thy*$^+$ revertants TKJ6901R and 168R were labeled for 16 min at 30° with 1 M [5-^{3}H]uridine and analyzed as above, less than 0.3% and 0.25% of the total radioactivity incorporated into TKJ6901R and 168R DNA, respectively, was found in the dUMP regions (Fig. 4).

In order to verify that the radioactivity detected in the dUMP region with the TKJ6901 DNA hydrolysate represented the radioactivity of dUMP, the radioactivity in this region was eluted and rechromatographed on polyethyleneimine-cellulose thin-layer plates. Development was with 0.5 M LiCl/0.15 M ammonium borate (solvent B) in order to separate dUMP from rUMP. As shown in Fig. 5*A*, most of the radioactivity was recovered in the dUMP region. The small amount of radioactivity found in the rUMP region might be due to contamination of the DNA preparation by RNA. The radioactivity in the dUMP region was eluted again and chromatographed in ethanol/1 M ammonium acetate, pH 7.5 (solvent C) (this system separates dUMP from TMP and rUMP). Again in this system, almost all the radioactivity was recovered in the dUMP region and hardly any radioactivity was detected in the TMP region (Fig. 5*B*). On the other hand, about half of the radioactivity found in the dUMP region of 168T DNA hydrolysate (Fig. 3*B*) was due to rUMP (data not shown).

In order to determine the time course of the incorporation of radioactivity into uracil residues in DNA, TKJ6901 cells were labeled for 3, 8, 16, and 32 min at 30° with [5-^{3}H]uridine. The

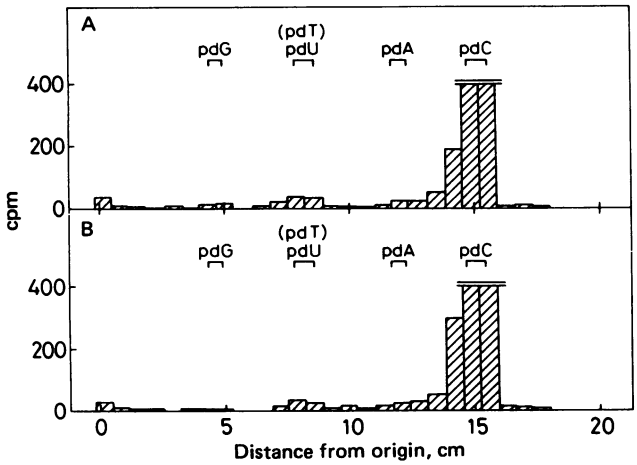

FIG. 4. Chromatography of [^{3}H]uridine-labeled DNA hydrolysates from TKJ6901R (*A*) and 168R (*B*). Each 100-ml culture was labeled with 1 μM [^{3}H]uridine (7.9 Ci/mmol) for 16 min at 30°. DNA was isolated (radioactivity incorporated into DNA: *A*, 3.59 × 10^6 cpm; *B*, 3.60 × 10^6 cpm), hydrolyzed, desalted, and chromatographed as in Fig. 3. Radioactivity in the dCMP region was: *A*, 24,578 cpm; *B*, 22,780 cpm.

DNA was isolated and the radioactivity incorporated into uracil residues in DNA was determined after chromatography of each hydrolysate in solvent A and then solvent B. As shown in Fig. 6, the incorporation of radioactivity into DNA continued to increase for 32 min, whereas the radioactivity incorporated into uracil residues in DNA reached a plateau at approximately 16 min. This suggests that the uracil residues incorporated into DNA are gradually excised in TKJ6901 cells. The radioactivities incorporated into uracil residues and cytosine residues in DNA show ratios of 1:5.8, 1:49, 1:82, and 1:142 after 3, 8, 16, and 32 min of labeling, respectively. The high dUMP/dCMP ratio observed at the short pulse times might be due to differences in the specific radioactivities of dCTP and dUTP.

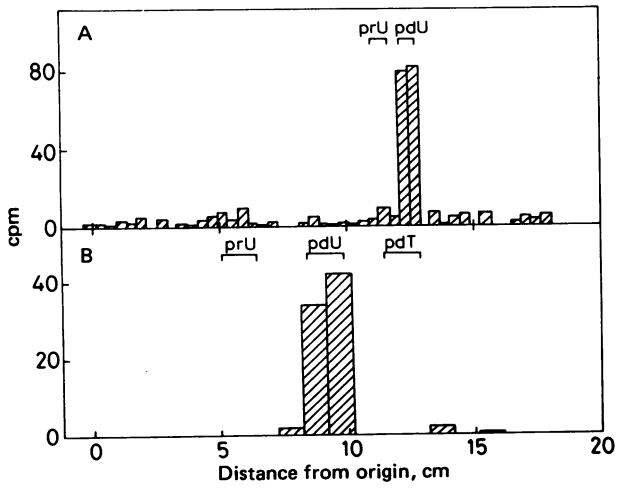

FIG. 5. Identification of dUMP. The radioactivity in the dUMP region of Fig. 3*A* was eluted with 2 M triethylammonium bicarbonate, evaporated, and dissolved in distilled water. (*A*) The sample was applied to a strip of polyethyleneimine-cellulose with nonradioactive markers and the chromatogram was developed with solvent B. (*B*) The radioactivity in the dUMP region of *A* was eluted again and applied to polyethyleneimine-cellulose with nonradioactive markers and the chromatogram was developed with solvent C.

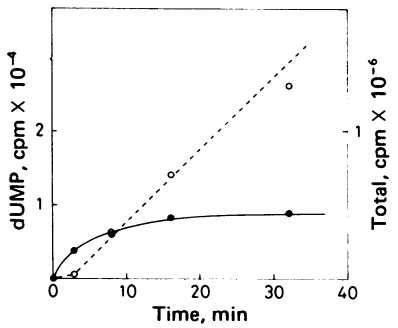

FIG. 6. Time course of incorporation of radioactivity into uracil residues in DNA of TKJ6901. *B. subtilis* TKJ6901 was grown at 30°. Each 100 ml culture was labeled with 1 M [^{3}H]uridine for 3, 8, 16, and 32 min. DNA was isolated and hydrolyzed to deoxynucleoside 5′-monophosphates, and mononucleotides were separated by thin-layer chromatography on polyethyleneimine-cellulose as in Fig. 3. The radioactivity in the dUMP region was eluted and rechromatographed as in Fig. 4*A*. The radioactivity in the dUMP region was determined.

DISCUSSION

We have carried out sedimentation analyses of DNA, pulse-labeled with [^{3}H]thymidine, from several strains of *B. subtilis* wild-type cells including JH406 (13, 20), SB1058 (21), BC26 (22), and HA101 (23). In every case, a certain portion of radioactivity incorporated into DNA even after a very brief period was found in long DNA chains. The sedimentation profile was always similar to that of 168*thy*$^+$ shown in Fig. 1*B*. Hybridization of the pulse-labeled DNA from JH406 or 168*thy*$^+$ with H and L fractions of chromosomal DNA revealed that almost all the radioactivity incorporated into H fraction DNA during a brief pulse is recovered in short DNA chains, whereas that incorporated into L-fraction DNA is recovered in both short and long DNA chains (unpublished data).

In the case of 168*thy*$^-$, however, the situation is very different. Pulse-labeling with [^{3}H]thymidine revealed that almost all the radioactivity incorporated into DNA is recovered in short DNA chains, even after a relatively long pulse such as 1 min at 30°. Virtually all the radioactivity incorporated into L-fraction DNA, as well as into H-fraction DNA during a brief pulse, is recovered in short DNA chains. Thus, abnormal accumulation of short DNA chains is observed in this mutant.

Analysis of a mutant defective in uracil-DNA glycosidase activity has provided an explanation for this abnormal accumulation of short chain. The [^{3}H]thymidine pulse-labeled DNA from a thymine-requiring, uracil-DNA glycosidase activity-defective double mutant was found to be similar to that of 168*thy*$^+$ by sedimentation analysis. Furthermore, uracil residues were detected in DNA from this double mutant by using uracil-DNA glycosidase. On the other hand, no uracil residues were detected in DNA from a *thy*$^+$ revertant of this double mutant. Thus, it seems reasonable to speculate that the accumulation of short DNA chains observed in 168*thy*$^-$ cells is a result of the excision-repair of the uracil incorporated into its DNA.

Successive treatment with uracil-DNA glycosidase and alkali resulted in the fragmentation of DNA from TKJ6901 cells. DNA chains having sedimentation coefficients of 10 and 15 S were fragmented to 7.8 and 9S, respectively. Based on the former value, a maximum of 1 uracil is incorporated for each 800 nucleotides. The accumulated short DNA chains in 168*thy*$^-$ cells demonstrate a mean sedimentation coefficient of 7.5 S when pulse-labeled for 10 sec at 30°. The incorporation of uracil

residues into DNA in 168*thy*$^-$ cells might not be so frequent as in *dut*-1 or *sof*-1 mutants of *E. coli*, which accumulate 4–5S short chains (8).

It was possible to detect uracil residues by labeling DNA with [^{3}H]uridine. Significant radioactivity was found to migrate together with authentic dUMP in three different solvent systems in the case of TKJ6901 DNA hydrolysate. Three lines of evidence suggest that this radioactivity resides in uracil incorporated into DNA. First, the radioactivity is found only in *thy*$^-$*urg*$^-$ cells. In *thy*$^+$ cells, only a trace amount was detected even though uracil-DNA glycosidase activity was defective. Second, the incorporation of radioactivity into uracil residues in DNA reached a plateau, whereas the incorporation of radioactivity into cytosine residues in DNA continued to increase. Thus, it is not likely that the radioactivity migrating together with dUMP arises by the deamination of cytosine residues in DNA. Third, when we treated TKJ6901 DNA labeled with [^{3}H]uridine for 8 min at 30° with uracil-DNA glycosidase, 2.26% of total radioactivity was released as acid-soluble product. This value is in good agreement with the percentage of the radioactivity identified as dUMP in DNA hydrolysate, 1.94%. (The same treatment released less than 0.097% of total radioactivity as acid-soluble radioactivity from 168T DNA labeled for the same period.) The uracil-DNA glycosidase treatment, at the same time, caused the fragmentation of TKJ6901 DNA to short DNA chains having an average sedimentation coefficient of 10 S after alkali treatment. A trace amount of radioactivity was found in the dUMP region in 168T, 168R, or TKJ6901R DNA hydrolysates. This trace amount of radioactivity was found in a nearly constant ratio ($^1/_{1000}$ to $^1/_{600}$ of the total radioactivity) in DNA labeled for different periods and thus we think that this does not represent the radioactivity of the misincorporated uracil but is a background level of the present analysis.

Our results with *thy*$^-$ cells demonstrate that short DNA chains can in fact be formed by a repair process involving the transient incorporation of uracil into DNA. On the other hand, our results with *thy*$^+$ cells suggest that this process is not playing a major role in the generation of short DNA chains in *thy*$^+$ cells. Almost the same amount of short DNA chains are formed in *thy*$^+$ cells irrespective of the deficiency of uracil-DNA glycosidase activity. It was not possible to obtain any indications that uracil was incorporated into DNA in *thy*$^+$ cells. Our current interpretation is that, in *thy*$^+$ cells, short DNA chains are formed by *de novo* synthesis whereas in *thy*$^-$ cells, short DNA chains are formed as a result of uracil incorporation into DNA in addition to those formed by *de novo* synthesis.

In contrast to the present results with *B. subtilis*, a thymine-requiring mutant of *E. coli* W3110 does not accumulate unusually large amounts of short DNA chains (5). Alkaline sucrose gradient sedimentation profiles of [^{3}H]thymidine pulse-labeled DNA from *E. coli* BD10 (*thy*$^-$*ung*$^-$), an uracil-DNA glycosidase-defective mutant isolated by B. Duncan, and from W3110 (*thy*$^-$*ung*$^+$) are almost the same. When DNA from BD10 is treated with uracil-DNA glycosidase and alkali, fragmentation of DNA is hardly detected with DNA smaller than 5000 nucleotides, although with DNA larger than that a slight fragmentation is detected. Moreover, DNA smaller than 10,000 nucleotides from a *thy*$^+$ revertant of BD10 is scarcely fragmented by the same treatment (Y. Machida and T. Okazaki, unpublished data). Therefore, it appears that uracil is incorporated into DNA much less frequently in *E. coli* than in *B. subtilis* and thus only a few, if any, of the 1500-nucleotide nascent fragments in *E. coli* arise from the repair of misincorporated uracil.

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