Modes of Overinitiation, dnaA Gene Expression, and Inhibition of Cell Division in a Novel Cold-Sensitive hda Mutant of Escherichia coli

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The chromosomal replication cycle is strictly coordinated with cell cycle progression in Escherichia coli. ATP-DnaA initiates replication, leading to loading of the DNA polymerase III holoenzyme. The DNA-loaded form of the β clamp subunit of the polymerase binds the Hda protein, which promotes ATP-DnaA hydrolysis, yielding inactive ADP-DnaA. This regulation is required to repress overinitiation. In this study, we have isolated a novel cold-sensitive hda mutant, the hda-185 mutant. The hda-185 mutant caused overinitiation of chromosomal replication at 25°C, which most likely led to blockage of replication fork progress. Consistently, the inhibition of colony formation at 25°C was suppressed by disruption of the diaA gene, an initiation stimulator. Disruption of the seqA gene, an initiation inhibitor, showed synthetic lethality with hda-185 even at 42°C. The cellular ATP-DnaA level was increased in an hda-185-dependent manner. The cellular concentrations of DnaA protein and dnaA mRNA were comparable at 25°C to those in a wild-type hda strain. We also found that multiple copies of the ribonucleotide reductase genes (nrdAB or nrdEF) or dnaB gene repressed overinitiation. The cellular levels of dATP and dCTP were elevated in cells bearing multiple copies of nrdAB. The catalytic site within NrdA was required for multicopy suppression, suggesting the importance of an active form of NrdA or elevated levels of deoxyribonucleotides in inhibition of overinitiation in the hda-185 cells. Cell division in the hda-185 mutant was inhibited at 25°C in a LexA regulon-independent manner, suggesting that overinitiation in the hda-185 mutant induced a unique division inhibition pathway.

The initiation of chromosomal replication is strictly coordinated with cell cycle progression in prokaryotes and eukaryotes. In Escherichia coli, ATP-bound DnaA forms a specific complex with the chromosomal replication origin, oriC (22, 41), leading to the unwinding of duplex DNA in the AT-rich region of oriC. This process is stimulated by DiaA, a DnaA-binding protein that directly promotes the formation of the specific complex consisting of ATP-DnaA and oriC (21, 33). DnaB helicase is loaded onto the exposed single-stranded region of DNA by the DnaC helicase loader. In this process, DnaA promotes the reaction by directly binding to DnaB (1, 40, 54). The loaded DnaB helicase expands the single-stranded region, where DnaG primase and DNA polymerase III holoenzyme are sequentially loaded, leading to DNA replication (49). The cellular level of ATP-DnaA fluctuates with a peak at the time of the initiation of replication (36).

To repress extra initiation events, at least three pathways are found to function in vivo. The first is the inactivation of oriC by SeqA (38, 56). The minimal oriC region contains 11 repeats of the GATC sequence. Adenine residues in both of the strands within the palindromic GATC sequence are methylated by Dam (DNA adenine methyltransferase). Immediately after the synthesis of a nascent complementary strand, hemimethylated forms are transiently generated, and SeqA preferentially binds to these sites. The binding of SeqA to oriC prevents the formation of an active initiation complex on oriC (46), thus repressing extra initiations. Also, the dnaA promoter region contains the GATC sequence, and the postinitiation stage-specific repression of dnaA transcription depends on SeqA and Dam (6). The second pathway that functions to repress extra initiation events is the titration of DnaA molecules by the datA locus (34). The chromosomal locus datA contains five DnaA boxes which are 9-mer DnaA-binding sequences. This locus can bind to a considerable number of DnaA molecules, which therefore reduces the number of DnaA molecules that are accessible to oriC. The third system is the functional regulation of DnaA termed RIDA (for regulatory inactivation of DnaA) (27). RIDA stimulates the hydrolysis of DnaA-bound ATP, yielding the inactive ADP-DnaA. This ATP hydrolysis depends on the DNA-loaded β sliding clamp and Hda (27, 29). The β sliding clamp is a subunit of the DNA polymerase III holoenzyme which is loaded on DNA during the process of DNA replication (49). Thus, cellular ATP-DnaA is converted to ADP-DnaA in a manner that is coordinated with replication and represses untimely extra initiation events.

Hda binds to the β sliding clamp via its short N-terminal region that contains the QL(S/D)LF motif that is conserved among clamp-binding proteins (10, 59). The binding of Hda to

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‖ Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
¶¶ Present address: Nipro Co., Osaka, Japan.
the β sliding clamp is required for the RIDA reaction (59). In addition, Hda carries an AAA+ domain (29, 45). AAA+ family proteins, which also include DnaA, share unique motifs associated with functions in nucleotide binding and hydrolysis. The Hda Arg-168 residue in the AAA+ box VII arginine finger motif is required for ATP-DnaA hydrolysis during the RIDA reaction (59). Loss of Hda function increases the level of ATP-DnaA in cells (29). A temperature-sensitive hda mutant (hda-86 mutant) exhibits overinitiation of chromosomal replication from oriC at a restrictive temperature, and the excessively formed replication forks stall near the oriC (29). Whole-genome microarray analysis demonstrates that hda-disrupted cells cause overinitiation of replication from oriC (7). The hda gene is essential for cell growth, although suppressor mutations can frequently occur, allowing colony formation of hda mutants (16, 29, 52).

The dnaA cos mutant, a cold-sensitive dnaA mutant, exhibits overinitiation of chromosomal DNA from oriC, resulting in overreplication of whole chromosomal DNA at a restrictive temperature (30°C) (26, 32). DnaAcos protein is resistant to RIDA and overinitiates the replication of minichromosomes in vitro (23, 25). The function of the diaA gene was first identified as a suppressor for the dnaA cos mutant (21). Cell division in the dnaA cos strain is inhibited in an sfiA-independent manner at 30°C (28). SfiA is a member of the SOS-inducible proteins and inhibits the polymerization of FtsZ, arresting cell division (11, 44, 62).

In this study, we have isolated and characterized a novel cold-sensitive hda mutant, the hda-185 mutant which carries an amino acid substitution of K185C. These mutant cells exhibited a more severe inhibition of colony formation than hda-86 cells did and therefore stimulated the analysis of suppressor mutations. Like the hda-86 strain, the hda-185 strain caused overinitiation of chromosomal replication from oriC but did not overreplicate the entire chromosomal DNA at 25°C. At this temperature, the levels of DnaA protein and dnaA mRNA were comparable to those in the wild-type hda strain. Cell division of the hda-185 strain is inhibited at restrictive temperatures in an sfiA-independent manner. Furthermore, we found that multiple copies of the ribonucleotide reductase (RRN) gene increased the deoxyribonucleotide (dNTP) levels and repressed overinitiation in the hda-185 strain.

**MATERIALS AND METHODS**

Media, bacterial strains, oligonucleotide primers, and plasmids. LB medium contains Bacto tryptone (1%), yeast extract (0.5%), and sodium chloride (1%). Thymine (50 μg/ml) was included in the medium unless indicated otherwise. The bacterial strains and plasmids that were used in this study are listed in Table 1. The sequences of the oligonucleotide primers that were used in this study are listed in Table 2. pTKM103 carries the SacI-NheI chromosomal fragment containing the dnaA gene at the corresponding restriction sites on pBR322. pTKM21 carries a promoter region upstream of ndrF and the coding region of ndrE. In order to construct this plasmid, the ndrHEF open reading frame was cloned into pBR322 using the EcoRI site, resulting in pTKM203. To remove the ndrHEF coding region from pTKM203, two DNA fragments were amplified by PCR using pTKM203 and two pairs of primers (primers TAKU19 and TAKU25 and primers TAKU20 and TAKU26). The resultant DNA fragments were digested with EcoRI and ClaI and ligated, resulting in pTKM221. The ndrF-EcoRI carrying DNA fragment was amplified by PCR using the primers TAKU32 and TAKU35; the resultant DNA fragment was cloned into the NheI and HindIII sites of pBR322, resulting in pTKM226. pMS100 was constructed using the QuickChange site-directed mutagenesis kit (Stratagene) with pTKM226 as the template and primers NdA1 and NdA2. This site-directed mutagenesis method was used throughout this study.

Isolation of cold-sensitive hda mutants. In order to screen cold-sensitive hda mutants, we utilized incompatibility in ColE1-type plasmids as follows. Plasmids carrying hda alleles were constructed by site-directed mutagenesis using pWK21-1 (wild-type hda and kan genes) and primers (HCS3 and HCS4 for V104N, HCS5 and HCS6 for K185C, HCS7 and HCS8 for R217H, and HCS9 and HCS10 for Q225H). The resultant plasmids were introduced into strain WKO01 (ΔdnaA::cat) harboring pBAD/Hda (wild-type hda and bla genes) (59), and colonies were formed on LB plates containing kanamycin (750 μg/ml) at 42°C. Loss of pBAD/Hda was tested in the resultant colonies using LB plates containing ampicillin (100 μg/ml). The selected cells (KanR AmpR) were incubated at 25°C and 30°C on LB plates containing kanamycin (750 μg/ml) in order to assess cold sensitivity. pWK21-1 is a derivative of pBAD/Hda carrying the kan gene that was derived from pUC4K and was inserted at the Scal site within the hda gene. Strain WKO01 had been constructed previously by P1 transduction using the recipient strain KHS402-1 and a donor strain bearing ΔdnaA::kan nhle::Tn3 (29).

Construction of the hda-185 strain. The DNA fragment containing an hda coding region and its upstream (2 kb) and downstream (2 kb) regions was amplified by PCR using genomic DNA and the HCS1 and HCS2 oligonucleotides as primers. The resultant fragment was digested with NheI and BamHI and then cloned into pBR322 using the corresponding sites, resulting in pHC83-1. An hda region was amplified by PCR using pHS1-1 and the KM1 and KM2 oligonucleotides as primers. The resultant fragment, together with a kanamycin resistance gene-containing region of DNA, was digested with Ascl and NcoI and then ligated, resulting in pHCS2-1. The kanamycin resistance gene had been prepared by PCR using pUC4K and the KMI and KM2 oligonucleotides as primers. To introduce the mutation K185C into hda on pHCS2-1, we performed site-directed mutagenesis using pBR322 and the KM5 and KM6 oligonucleotides as primers, resulting in pHCS1-1. After digestion with NdeI and BamHI, pHCS2-1 (wild-type hda) and pHCS1-1 (hda-185) were introduced into strain 708M016 (recD::mini-tet), and colonies were formed on LB plates containing kanamycin (50 μg/ml) at 42°C. After screening for ampicillin sensitivity, insertion of the kanamycin resistance gene was verified by colony PCR. The kanamycin resistance gene-linked wild-type hda gene and hda-185 were introduced into the MG1655 thyA rpsL strain using P1 transduction, resulting in strains YH014 and YH103, respectively.

Construction of diaA, lexA, sfiA, and slmC mutants. The wild-type diaA strain MG1655 thyA rpsL was replaced with ΔdnaA::FRT kan by P1 transduction using strain JW3118, and the kanamycin resistance gene (kan) was removed as described previously (3, 12), resulting in strain YH105 (ΔdnaA::FRT). The kan-linked wild-type hda or hda-185 was introduced into strain YH015 by P1 transduction using strain YH014 or YH103, resulting in YH106 or YH107, respectively. To construct the ΔsfiA-linked sfiA mutant which carries an SfiA gene, strain WK001 had been constructed previously by P1 transduction using strain YH014 or YH103, respectively, using P1 transduction.

Determination of the cellular levels of dCTP and dATP. DNA polymerase-based assay for the dCTP level was performed as previously described (63). Briefly, cells were grown in LB medium (10 ml) until an optical density (A600) reached 0.4, collected by centrifugation, and suspended in 60% methanol. The suspension was boiled for 5 min and then centrifuged, and the supernatant was isolated. The sample was dried under vacuum and dissolved in water (20 μl). For analysis of the dCTP level, a portion (0.3 μl) was subjected to a polymerase assay using [γ-32P]dATP, the DNA polymerase I type enzyme, and a primed DNA template consisting of only thymine and guanine nucleotides.

The level of dATP was determined using two-dimensional thin-layer chromatography as described previously (5) with minor modifications. Briefly, cells were grown at 25°C in modified TG medium containing 13C2H4O6 (0.4 mM/ml) (36) until an optical density (A600) reached 0.2. Portions (300 μl) of the cultures were mixed and centrifuged, and 25 μG of 11 O-formic acid, and supernatants were obtained from the sample. The suspension was boiled for 5 min and then centrifuged, and the supernatant was isolated. The sample was dried under vacuum and dissolved in water (20 μl). The selected cells were supplemented by centrifugation, and suspended in 0.4 M HCl. The suspension was boiled for 5 min and then centrifuged, and the supernatant was isolated. The sample was dried under vacuum and dissolved in water (20 μl). The selected cells were subjected to a polymerase assay using [γ-32P]dATP, the DNA polymerase I type enzyme, and a primed DNA template consisting of only thymine and guanine nucleotides.

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Quantitative PCR was performed according to the manufacturer's instructions (Takara Bio). Sybr Premix (Takara Bio) was used for each reaction to amplify and quantify the plasmid Genotype or relevant characteristic(s) Reference or source

**TABLE 1. List of E. coli strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Genotype or relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KKH502-1</td>
<td>ivy thyA tyrA(Am) thrE829(Am) metE deo supF6(Ts)</td>
<td>24</td>
</tr>
<tr>
<td>KKW01</td>
<td>KKH502-1 ΔdnaA::cat</td>
<td>This study</td>
</tr>
<tr>
<td>MGG1655 thyA rpsL</td>
<td>ivy G rph thyA rpsL</td>
<td>J. Kato*</td>
</tr>
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<td>YH014</td>
<td>MG1655 thyA rpsL hda'* kan</td>
<td>This study</td>
</tr>
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<td>YH013</td>
<td>MG1655 thyA rpsL hda-185 kan</td>
<td>This study</td>
</tr>
<tr>
<td>KA413</td>
<td>KKH502-1 dnaA46 ivy*</td>
<td>24</td>
</tr>
<tr>
<td>NA001</td>
<td>KKH502-1 dnaA cos</td>
<td>24</td>
</tr>
<tr>
<td>ME9018</td>
<td>MG1655 recD1903::mini-tet</td>
<td>NIG*</td>
</tr>
<tr>
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<td>KKH502-1 seqa::Tn10 zig-131::Tn5</td>
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</tr>
<tr>
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<td>KKH502-1 zig-167::Tn10</td>
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</tr>
<tr>
<td>NA169</td>
<td>KKH502-1 recA::Tn10</td>
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<tr>
<td>KW262</td>
<td>MG1655 mha::Tn3 ΔoriC::tet</td>
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</tr>
<tr>
<td>MK86</td>
<td>KW262 Δhda::cat</td>
<td>29</td>
</tr>
<tr>
<td>JW3118</td>
<td>BW25113 Δdia::FRT kan</td>
<td>3'</td>
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<tr>
<td>YH105</td>
<td>MG1655 thyA rpsL Δdia::FRT</td>
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</tr>
<tr>
<td>YH106</td>
<td>YH105 hda' kan</td>
<td>This study</td>
</tr>
<tr>
<td>YH107</td>
<td>YH105 hda-185 kan</td>
<td>This study</td>
</tr>
<tr>
<td>MZ002</td>
<td>YH014 ΔgsfA::cat</td>
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<td>MZ001</td>
<td>YH013 ΔgsfA::cat</td>
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<tr>
<td>HSR2005</td>
<td>AB1157 except for lexA3::Tn10</td>
<td>55d</td>
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<td>YH101</td>
<td>YH014 lexA3::Tn10</td>
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<tr>
<td>YH102</td>
<td>YH013 lexA3::Tn10</td>
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<tr>
<td>YH103</td>
<td>YH014 ΔslmA::cat</td>
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<td>YH104</td>
<td>YH013 ΔslmA::cat</td>
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</tr>
<tr>
<td>KP7364</td>
<td>KP245 ΔdnaA::spec mha::kan</td>
<td>36</td>
</tr>
</tbody>
</table>

| **Plasmids**                |                                        |                     |
| pBR322                      | tet bla                                | Laboratory stock   |
| pACYC177                    | bla kan                                | Laboratory stock   |
| pACYC184                    | cat tet                                | Laboratory stock   |
| pUC4K                       | kan bl                                 | Laboratory stock   |
| pWK21-1                     | ColElori PBAD hda kan                   | This study          |
| pBAD/Hda                    | ColElori PBAD hda bla                   | 59                  |
| pYHM1                       | pWK21-1 except for hda V104N substitution | This study          |
| pYHM2                       | pWK21-1 except for hda K185C substitution | This study          |
| pYHM3                       | pWK21-1 except for hda R217H substitution | This study          |
| pYHM4                       | pWK21-1 except for hda Q225H substitution | This study          |
| pHCS1-1                     | pHCS1-1 except for carrying kan downstream of hda | This study          |
| pHCS2-1                     | pHCS2-1 except for hda K185C substitution | This study          |
| pHCS4-1                     | pACYC177-hda                           | This study          |
| pTKM221                     | pBR322-nrdEF                           | This study          |
| pTKM226                     | pBR322-nrdAB                           | This study          |
| pTKM103                     | pBR322-dnaB                            | This study          |
| pMS100                      | pTKM226 except for nrdA C439A substitution | This study          |
| pTSO182                     | pBR322-oriC                            | 26                  |

* Gift from J. Kato.

b NIG, National Institute of Genetics, Japan.


d Gift from T. Hishida.

determined by UV absorbance. The positions of ATP and GTP were determined by the method of Bochner and Ames (5).

**Determination of the oriC/terC ratio by Southern blotting and a quantitative PCR method.** The dosage of the oriC and terC genes in cells was measured by Southern blotting or a quantitative PCR method. Chromosomal DNA from the cells was prepared following a standard protocol. Southern blot analysis was performed essentially as described previously (47) except the chromosomal DNA was digested with StyI and EcoRI, and oriC and terC probes were amplified using chromosomal DNA as a template and primers ORI_1 and ORI_2 for the oriC probe and primers TER_1 and TER_2 for the terC probe. These probes were labeled using the Megaprime DNA labeling system (Amersham) and [α-32P]dCTP. Quantitative PCR was performed according to the manufacturer's instructions (Takara Bio). Sybr Premix Ex Taq (Takara Bio) was used for each reaction to amplify and quantify the oriC and terC regions. The reaction mixtures (25 μl) contained chromosomal DNA (1 ng) and primers (5 pmol each) (primers ORI_1 and KWoRiCRev for oriC; primers SuEterFw1 and TER_2 for terC). The amplification and quantification were carried out using the Thermal Cycler Dice real-time system (Takara Bio). The oriC/terC ratio was normalized using strain KA413 (dnaA46) incubated at 42°C for 2 h.

**Measurement of DNA synthesis.** DNA synthesis was measured as described previously (28). Whole chromosomal DNA was labeled while the cells were growing in LB medium containing [3H]thymine (25 μg/ml, 3 μCi/ml). The cells were withdrawn and kept on ice for at least 15 min in the presence of trichloroacetic acid (7%). Insoluble materials were collected on a GF/C filter (Whatman), and [3H]thymine incorporation was quantified using a liquid scintillation counter.

**Measurement of DnaA content.** DnaA content in the cells was determined by immunoblot analysis using an anti-DnaA antibody as described previously (30, 31). Briefly, aliquots (200 μl at an A600 of 0.1) of the cells were collected during exponential growth, immediately transferred to chilled trichloroacetic acid
(10%), and kept on ice for at least 15 min. Precipitates were collected by centrifugation, dissolved in sodium dodecyl sulfate (SDS) sample buffer, and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Purified proteins were centrifuged, dissolved in sodium dodecyl sulfate (SDS) sample buffer, and kept on ice for at least 15 min. Precipitates were collected by centrifugation.

**Flow cytometry analysis.** The flow cytometry experiment was performed as described previously (31, 33). To determine the number of oriC in cells, the cells were exponentially grown for 10 generations until the optical density (OD660) reached 0.1 to 0.2, and then rifampin (300 μg/ml) and cephalexin (10 μg/ml) were added. After further incubation for 4 h to complete ongoing replication, the cells were fixed in 70% ethanol. The fixed cells were washed and stained with 2 μM Sytox green (Invitrogen), followed by analysis using a FACSCalibur (Becton Dickinson). To determine the cell mass and DNA content, cells that were in the stationary growth phase were incubated in 70% ethanol, washed, and suspended in buffer as described above, followed by analysis using a FACSCalibur.

**Quantification of the dnaA mRNA level.** Total cellular RNA was isolated by the hot phenol method (50). Quantitative reverse transcription–PCR (RT-PCR) was performed using the One Step SYBR PrimeScript RT-PCR kit and Thermal Cycler Dice real-time system (Takara Bio). The reaction mixtures (25 μl) contained DNase I-treated total RNA (1 ng) and primers (5 pmol each) (primers R-rpoA1 and R-rpoA2 for rpoA mRNA; primers R-dnaA1 and R-dnaA2 for dnaA mRNA). An S1 nuclease assay was performed as described previously (58). The dnaA probe (5'-CGGGCGAACAGACCTGGCAAGGAAAAGTGGACACGGGGAGACTCCATCCGACACCACCGGCACACA-3') and the rpoA probe have been described previously (60) and were end labeled with 32P. After hybridization of these probes with total RNA and S1 nuclease digestion, the products were analyzed using 8 M urea-10% PAGE and quantified with a BAS2500 image analyzer (Fuji Film).

**RESULTS**

**Construction of a cold-sensitive hda mutant.** We had previously isolated several cold-sensitive dnaA mutants bearing the single-amino-acid substitutions I219N, R302C, R334H, and R342H (19). These mutations reside in the AAA domain, which is shared by DnaA and Hda. Indeed, Hda carries the same residues or residues that are chemically similar to those in DnaA (Fig. 1). We therefore speculated that substitutions in Hda that were identical to those resulting in cold-sensitive dnaA mutants might also render Hda cold sensitive. By site-directed mutagenesis, we introduced these substitutions in the hda gene of strain MUTANT 5371bearing a wild-type hda gene (MUTANT 5371). The resultant plasmids carrying an hda mutation (V104N, K185C, R217H, or Q225H) were introduced into the chromosomal hda-deleted strain bearing a wild-type hda gene on an ampicillin-resistant plasmid derived from CoE1. The transformed cells that were resistant to kanamycin and sensi-
tive to ampicillin were then selected at 42°C and tested for colony formation at 42°C, 30°C, or 25°C (Table 3). Moderate inhibition of colony formation was observed for the hda-185 (K185C) mutant at 25°C. We next replaced the hda-gene on the chromosome with the hda-185 allele (see Materials and Methods for details). The resultant strain, YH013, exhibited cold sensitivity during colony formation (Table 4). This cold-sensitive colony formation was complemented by a plasmid bearing the wild-type hda-gene (pHCS4-1) to a level that was comparable to that of the wild-type strain (Table 4), confirming that cold sensitivity depends on the hda mutation.

**Overinitiation of replication in the hda-185 mutant.** We next asked whether the hda-185 strain causes the overinitiation of chromosomal replication at 25°C. The copy number ratio of oriC to terC (replication termination region) was analyzed using Southern blot hybridization (Fig. 2A). The oriC/terC ratio increased upon the temperature shift down to 25°C in the hda-185 strain but not in the wild-type control strain, suggesting that overinitiation occurs in the hda-185 strain at this temperature. Consistent with the previous observations in hda-86 mutants (29), overall chromosomal DNA synthesis did not increase in the hda-185 strain after the shift to 25°C (Fig. 2B), which suggests that the progression of replication forks is blocked near oriC in these mutants. Reduction of overall DNA synthesis was not significantly observed at 25°C even in the hda-185 strain (Fig. 2B), which suggests that overinitiation indeed occurred and that the replication fork block was not the only cause of the increase in the oriC/terC ratio. In this experiment, the dnaA cos mutant, which causes overreplication of the entire chromosome (32), was also used for comparison.

For an independent approach, we performed flow cytometry experiments (Fig. 2C). Cells were exponentially grown at 42°C, transferred to 25°C, and incubated for 2 h at 25°C, followed by further incubation at 42°C for 4 h in the presence of rifampin and cephalaxin. If the hda-185 cells cause overinitiation at 25°C and the resultant blocked replication forks are reactivated and proceed during incubation at 42°C, the overall DNA content should have increased. After incubation at 25°C for 2 h, the DNA content observed was basically similar to the DNA contents of the wild-type and hda-185 cells (Fig. 2C), consistent with the data of Fig. 2B. Only a small population of the hda-185 cells showed DNA contents equivalent to eight or more chromosomes. After subsequent incubation at 42°C for 4 h, overall DNA contents in the hda-185 cells were evidently higher at 42°C than those in the wild-type cells (Fig. 2C). In

**TABLE 3. Colony formation ability of cells bearing a mutant hda allele***

<table>
<thead>
<tr>
<th>Amino acid substitution on hda allele on plasmid</th>
<th>CFU/ml (10^9) for cells grown at the following temp:</th>
<th>Ratio of CFU/ml for cells grown at the following temp:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42°C</td>
<td>30°C</td>
</tr>
<tr>
<td>None (wild type)</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>V104N</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>K185C</td>
<td>1.0</td>
<td>0.084</td>
</tr>
<tr>
<td>R217H</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Q225H</td>
<td>1.1</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* WK001 (Δhda) cells harboring a plasmid carrying wild-type hda or the indicated mutant allele were grown overnight in LB medium at 42°C. These cells were incubated on LB plates at 42°C for 12 h, at 30°C for 18 h, or at 25°C for 27 h. Kanamycin (750 μg/ml) was included in the medium, with the exception of the medium for WK001 cells carrying the wild-type hda-plasmid when ampicillin (100 μg/ml) was included.
We first measured the rpoA42°C and 25°C using the a temperature shift down from 42°C, the defects in used real-time PCR to quantitatively examine whether the forms of DnaA affect its repressor activity in vitro (57). We cells and change (Fig. 2D).

Cellular DnaA contents in response to a similar temperature slightly increased, which is consistent with the result of the increase in the contents might be present after a temperature shown), indicating that the negative regulation of initiation is not complete even at 42°C.

A similar analysis was performed using hda-185 cells grown at 42°C. These cells were incubated in the presence of rifampin and cephalexin. DNA histograms showed a broad distribution in chromosome equivalents of up to eight or more (data not shown), indicating that the negative regulation of initiation is not complete even at 42°C.

DnaA molecules are not oversupplied in hda-185 cells. Western blot analysis revealed that the cellular contents of DnaA protein in hda-185 cells were similar at 25°C to those in the wild-type control strain (Fig. 2D). In both strains, a slight increase in the contents might be present after a temperature shift from 42°C, but at 25°C the overall levels in the both strains were similar. These results indicate that overinitiation in the hda-185 strain is independent of the DnaA dosage.

DNA A transcription level in hda-deficient strains. dnaA transcription is autoregulated by DnaA protein, and the nucleotide forms of DnaA affect its repressor activity in vitro (57). We used real-time PCR to quantitatively examine whether the defects in hda could affect the dnaA transcription level (Fig. 3). We first measured the dnaA mRNA levels in hda-185 cells at 42°C and 25°C using the rpoA mRNA as a normalization control (Fig. 3A). At 25°C, the dnaA mRNA levels in the wild-type hda cells and hda-185 cells were comparable. After a temperature shift down from 42°C, the dnaA mRNA level might be slightly increased, which is consistent with the result of the cellular DnaA contents in response to a similar temperature change (Fig. 2D).

We also measured the dnaA mRNA levels in ΔoriC ΔrnhA mutants bearing the wild-type hda or Δhda allele. Chromosomal replication in the ΔrnhA mutant occurs in a manner independent of DnaA and oriC (29, 35). The dnaA mRNA levels in the wild-type hda cells and Δhda cells were comparable (Fig. 3B). These results are consistent with the idea that hda did not significantly affect dnaA transcription.

The growth defect in the hda-185 strain is stimulated by disruptions in seqA or recA. SeqA preferentially binds to hemimethylated oriC DNA and represses the initiation at oriC (38, 46, 56). We performed P1 transduction using ΔseqA::Tn10. Transduction into the hda-185 mutant was severely inhibited even at 42°C, unlike control transductions using the wild-type strain as a recipient or using Δdel-16::Tn10 as a donor (Table 5). These results suggest that overinitiation of replication in hda-185 cells is not completely repressed even at 42°C, consistent with flow cytometry analysis using the hda-185 cells grown at 42°C. Most likely the SeqA function, which acts in an RDA-independent manner, is required for inhibiting severe overinitiation and for rescuing cell growth.

Stalled replication forks can lead to double-strand breaks, which if they are not repaired, cause cell death (17). RecA is responsible for double-strand break repair (9). We found that P1 transduction using ΔrecA::Tn10 into the hda-185 strain was severely inhibited even at 42°C (Table 5); this result is in contrast to that obtained using the dnaA cos mutant (28). RecA-dependent recombination might be required for the stalled fork repair in the hda-185 cells.

Growth of the hda-185 strain is rescued by disruption of the diaA gene and the multicopy oriC. When an oriC plasmid was introduced into the hda-185 strain, cell growth at 25°C was restored (Table 6); this result is similar to that observed with the dnaA cos mutant (26). In addition, cells bearing hda-185 and ΔdiaA double mutations exhibited colony formation at 25°C in a manner similar to that of the control wild-type hda strain (Table 6). This is consistent with the previous observation that diaA deletion suppresses the growth defect in the dnaA cos mutant cells (21) and in cells expressing the ATP hydrolysis-defective mutant of DnaA, DnaA R334A (33). Taken together, these results support the idea that the hda-185 mutant causes overinitiation due to an elevated level of DnaA activity.

Elevated ATP-DnaA level in the hda-185 ΔdiaA cells. In a culture of wild-type cells, the ATP-DnaA level is repressed to

---

**TABLE 4. Colony formation ability of cells bearing an hda-185 allele**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>CFU/ml (10^6) for cells grown at the following temp:</th>
<th>Ratio of CFU/ml for cells grown at the following temp:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>42°C</td>
<td>30°C</td>
</tr>
<tr>
<td>MG1655 thyA rpsL</td>
<td>hda^+</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>YH014</td>
<td>hda^- kan</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>YH013</td>
<td>hda^-185 kan</td>
<td>1.7</td>
<td>1.0 × 10^-3</td>
</tr>
<tr>
<td>YH013/pACYC177</td>
<td>hda-185</td>
<td>2.6</td>
<td>1.0 × 10^-3</td>
</tr>
<tr>
<td>YH013/pHCS4-1</td>
<td>hda-185</td>
<td>2.6</td>
<td>3.0</td>
</tr>
<tr>
<td>YH014/pACYC177</td>
<td>hda^-</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>YH014/pHCS4-1</td>
<td>hda^-</td>
<td>2.3</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a Cells from the indicated strains were grown overnight at 42°C and incubated on LB plates at 42°C for 12 h, at 30°C for 18 h, or at 25°C for 27 h. The medium contained kanamycin (50 µg/ml) for strains YH013 and YH014. Ampicillin (100 µg/ml) was included when a plasmid (pACYC177 vector or pHCS4-1) was present. pHCS4-1 is a derivative of pHCS4-1 carrying wild-type hda (hda^+).
and mean DNA content of YH014 cells incubated at 25°C for 2 h were measured as described in Materials and Methods. The mean cell mass in Rif and Cfx. Relative cell mass and DNA contents were estimated by incubation at 25°C for 2 h and analysis using flow cytometry (25°C, hda-185). Some cells were further incubated at 42°C for 4 h in the presence of rnhA. Relative cell mass and DNA contents were used for the RT-PCR analysis of dnaA and rpoA mRNA levels. Four measurements were performed, and standard deviations are also shown. (B) Relative dnaA mRNA levels in the hda-null strain. Total RNA was isolated from KW262 (rnh::Tn3 ΔoriC::tet (hda-185)) and MK86 (rnh::Tn3 ΔoriC::tet Δhda::cat (hda mutant) cells that were grown at 37°C until the optical density (A660) reached 0.4. (C and D) dnaA mRNA level in DnaB-overexpressed cells. Cells of strain YH014 (WT), YH014 carrying pTKM103 (dnaA), and YH013 (hda-185) carrying pTKM103 were grown at 25°C until the optical density (A660) reached 0.5. Total RNA was isolated, and the relative dnaA mRNA levels were analyzed by RT-PCR (C) or S1 nuclease assay (D).
The transduction frequency of the recipient strains was indicated. Colonies of transductants were counted after incubation at 42°C for 12 h on LB plates. These plates contained tetracycline (15 μg/ml) and kanamycin (50 μg/ml) for strains YH014 and YH013 or tetracycline (15 μg/ml) for strain MG1655 thyA rpsL. The zid-16T::Tn strain was used as a positive control.

The transduction frequency is the number of transductant colonies per P1 phage. * Efficiency relative to the transduction frequency of the strain using a DNA library.

25°C (Fig. 4), a lower level than above. This might be caused by a residual activity of Hda-185 or moderated production of ATP-DnaA at low temperatures.

Overexpression of DnaB or RNRs suppresses the cold-sensitive growth of the hda-185 mutant. We have previously isolated multicity suppressors for the dnaA cos strain using a DNA library containing chromosomal fragments cloned into pBR322 (28). Restriction enzyme analysis distinguishes these suppressor plasmids into seven groups (group A and groups C to H) (28). Deletion analyses of plasmids in groups D and F revealed that the nrdE genes are responsible for the dnaA cos suppression, respectively (T. Ishida and T. Katayama, unpublished data). Notably, we found that pBR322 carrying either the dnaB gene or the nrdE genes also suppresses cold-sensitive colony formation in the hda-185 mutant (Table 6). The presence of excessive numbers of DnaB molecules in the hda-185 mutant might abnormally interact with DnaA, resulting in the inhibition of overinitiation (see below).

The nrdE operon and the nrdAB operon encode an RNR that functions at a rate-limiting step for synthesizing dNTPs. NrdAB is the major RNR, and NrdE is an alternative enzyme that is expressed under specific conditions (48). When we

### Table 5: Effect of the combination of hda-185 with a disruption in seqA or recA

<table>
<thead>
<tr>
<th>Donor strain genotype</th>
<th>Recipient strain</th>
<th>Transduction frequency (42°C)</th>
<th>Relative efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>zid-16T::Tn10</td>
<td>MG1655 thyA rpsL (hda·)</td>
<td>5.9 × 10⁻⁷</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YH014 (hda·)</td>
<td>2.6 × 10⁻⁷</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>YH013 (hda-185)</td>
<td>2.9 × 10⁻⁸</td>
<td>4.9 × 10⁻²</td>
</tr>
<tr>
<td>ΔseqA::Tn10</td>
<td>MG1655 thyA rpsL (hda·)</td>
<td>1.4 × 10⁻⁸</td>
<td>2.4 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>YH014 (hda·)</td>
<td>1.7 × 10⁻⁸</td>
<td>2.8 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>YH013 (hda-185)</td>
<td>&lt;7.5 × 10⁻¹¹</td>
<td>&lt;1.3 × 10⁻⁴</td>
</tr>
<tr>
<td>ΔrecA::Tn10</td>
<td>MG1655 thyA rpsL (hda·)</td>
<td>27 × 10⁻⁷</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>YH014 (hda·)</td>
<td>2.4 × 10⁻⁷</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>YH013 (hda-185)</td>
<td>&lt;6.7 × 10⁻¹⁰</td>
<td>&lt;1.1 × 10⁻³</td>
</tr>
</tbody>
</table>

* P1 transduction was performed using P1vir phages propagated in strain KA470 for zid-16T::Tn10, strain KA483 for seqA::Tn10, and strain NA169 for recA::Tn10. The recipient strains and their relevant genotypes are indicated. Colonies of transductants were counted after incubation at 42°C for 12 h on LB plates. These plates contained tetracycline (15 μg/ml) and kanamycin (50 μg/ml) for strains YH014 and YH013 or tetracycline (15 μg/ml) for strain MG1655 thyA rpsL. The zid-16T::Tn10 strain was used as a positive control.

### Table 6: Effects of deletion of diaA and multicopy supply of oric, dnaB, nrdAB, and nrdE

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>CFU/ml (10⁷) for cells grown at the following temp:</th>
<th>Ratio of CFU/ml for cells grown at 25°C/42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>YH014</td>
<td>hda·</td>
<td>4.3</td>
<td>0.9</td>
</tr>
<tr>
<td>YH013</td>
<td>hda-185</td>
<td>3.8</td>
<td>3.7 × 10⁻⁴</td>
</tr>
<tr>
<td>YH106</td>
<td>hda· ΔdiaA::FRT</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>YH107</td>
<td>hda-185 ΔdiaA::FRT</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>YH014/pBR322</td>
<td>hda·</td>
<td>1.9</td>
<td>0.84</td>
</tr>
<tr>
<td>YH013/pBR322</td>
<td>hda-185</td>
<td>2.6</td>
<td>&lt;3.8 × 10⁻⁴</td>
</tr>
<tr>
<td>YH014/pTSO182 (oricC)</td>
<td>hda·</td>
<td>1.7</td>
<td>0.94</td>
</tr>
<tr>
<td>YH013/pTSO182 (oricC)</td>
<td>hda-185</td>
<td>1.4</td>
<td>0.64</td>
</tr>
<tr>
<td>YH013/pTKM103 (dnaB)</td>
<td>hda-185</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>YH013/pTKM226 (nrdAB)</td>
<td>hda-185</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>YH013/pTKM221 (nrdE)</td>
<td>hda-185</td>
<td>2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>YH013/pMS100 (nrdA(C439A) nrdB·)</td>
<td>hda-185</td>
<td>1.9</td>
<td>&lt;1.1 × 10⁻³</td>
</tr>
</tbody>
</table>

* Cells of the indicated strains were grown overnight in LB medium at 42°C and then incubated on LB plates at 42°C for 12 h or at 25°C for 27 h. Ampicillin (100 μg/ml) was included when plasmids were present.
introduced pBR322 carrying the \( nrdAB \) genes into the \( hda-185 \) strain, suppression of cold sensitivity was also observed (Table 6). The Cys-439 residue within NrdA is located in the catalytic pocket in which the substrates bind and is essential for RNR activity (2, 13). The NrdA C439A mutant protein exhibits no enzymatic activity in vivo or in vitro, whereas the stability of the protein is not affected (2, 13). pBR322 bearing \( nrdA \) C439A and wild-type \( nrdB \) was impaired in \( hda-185 \) suppression activity (Table 6). These results support the idea that an active form of NrdAB or an increased level of dNTPs contributed to the restoration of cell growth in the \( hda \) mutant. This is the first time an \( nrdA \) catalytic site mutant has been used to suggest that an elevated dNTP level is important for the rescue of an overinitiating mutant.

**Cellular dNTP levels are elevated in cells bearing the \( nrdAB \) plasmid.** We assessed cellular levels of dNTPs using two different methods (Fig. 5). First, we used a method in which DNA polymerase activity is assessed in vitro using a cell extract as the only source of dCTP (63). Cells were grown at 42°C and incubated at 25°C for 8 h, followed by preparation of dNTP extracts and DNA replication assay. The dCTP levels in the cell extracts were deduced using a standard dCTP solution (Fig. 5A). The dCTP level in the \( hda-185 \) cells was elevated about twofold, depending on pBR322 bearing \( nrdAB \). In the wild-type cells, the dCTP level was elevated about 1.5-fold, depending on the same plasmid. These results are consistent with a previous study that used pBR322 carrying \( nrdAB \) (63). In the presence of pBR322 excluding \( nrdAB \), the dCTP level in the \( hda-185 \) cells was significantly lower than that in the wild-type cells. This might be caused by the elevated ATP-DnaA level and transcriptional repression of the chromosomal \( nrdAB \) genes in the \( hda-185 \) cells. ATP-DnaA represses the \( nrdAB \) transcription more effectively than ADP-DnaA did (16).

Second, we assessed cellular dATP levels using two-dimensional thin-layer chromatography. Cells were grown at 25°C in a phosphate-limited medium in the presence of \( ^{32}P\)O\(_4\), the
total cell volume was normalized, and the cell extracts were analyzed. The dATP level in the wild-type cells was elevated considerably, depending on pBR322 bearing nrdAB (Fig. 5B), consistent with a previous report (63). In the presence of the same plasmid, the dATP level in the hda-185 cells was comparable to that in the wild-type cells (Fig. 5B), which is consistent with the dCTP level data (Fig. 5A). The levels of ATP and GTP were not significantly changed, which supports the specific effect of nrdAB. The hda-185 cells bearing only vector pBR322 did not grow in a phosphate-limited medium.

Oversupply of DnaB or RNRs suppresses overinitiation in the hda-185 mutant. We then asked whether the oversupply of DnaB or RNR inhibits overinitiation by assessing the oriC ratio using quantitative real-time PCR (Fig. 6A). Introduction of pBR322 derivatives carrying dnaB or nrdAB into hda-185 cells repressed the oriC ratio to levels that were essentially the same as those of wild-type cells. Similar results were also obtained for the pBR322 derivative carrying nrdEF, but in this case, moderate inhibition was observed.

The function of these suppressors to repress the oriC ratio in the hda-185 cells can be explained by either the repression of overinitiation or the release of the fork progression block. To distinguish these possibilities, we investigated DNA synthesis and found that an increase in overall chromosomal replication was not observed at 25°C in the hda-185 strain harboring the dnaB plasmid or the nrdAB plasmid or in the strain harboring a control vector (Fig. 6B). These results indicate that the suppressor genes repress overinitiation in the hda-185 strain. These results are the first to demonstrate that an oversupply of NrdAB, NrdEF, or DnaB inhibits overinitiation of chromosomal replication.

We further assessed using flow cytometry replication modes in the suppressed cells. If the oversupply of DnaB or NrdAB enhanced the replication fork movement without repressing overinitiation, the suppressed hda-185 cells should have an increased ratio of DNA/mass at 25°C compared to the ratio of the wild-type cells. However, the DNA/mass ratio of the suppressed cells at 25°C was similar to that of the wild-type cells (Table 7). These results agree with the overall DNA synthesis/total cell volume data (Fig. 6B) and the idea that overinitiation is repressed in the suppressed cells. We do not exclude the possibility that the oversupply of DnaB or NrdAB enhanced the replication fork movement at 25°C in hda-185 cells.

Abnormal interactions between DnaA and DnaB might affect the repressor function of DnaA (42, 57). Indeed, dnaA transcription is negatively autoregulated by DnaA protein. We examined the dnaA mRNA level in hda-185 cells harboring the dnaB plasmid. Even at 25°C, the level of dnaA mRNA in these cells was comparable to the level in wild-type hda cells harboring the dnaB plasmid (Fig. 3C). Similar results were also obtained using the quantitative S1 nuclease assay (Fig. 3D).

Initiation modes in the wild-type cells bearing multiple copies of nrdAB or dnaB. We further examined the effects of multiple copies of the dnaB and nrd genes on the initiation of replication in the wild-type hda strain. Growing cells were incubated for 4 h in the presence of rifampin and cephalexin, followed by analysis by flow cytometry (Fig. 6C). The wild-type strains harboring pBR322, pBR322-nrdAB, or pBR322-nrdEF

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relative DNA content per cell</th>
<th>Relative DNA per mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322 (vector)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>pTKM221 (nrdEF)</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>pTKM226 (nrdAB)</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>pTKM103 (dnaB)</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>pTKM221 (nrdEF)</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>pTKM226 (nrdAB)</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>pTKM103 (dnaB)</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>pTKM221 (nrdEF)</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>pTKM226 (nrdAB)</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>pTKM103 (dnaB)</td>
<td>1.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Cells of the indicated strains were grown at 25°C in LB medium containing ampicillin (50 μg/ml) until an optical density (A₅₆₀) reached 0.2. Cell mass and DNA content per cell were determined using flow cytometry as described in Materials and Methods. The values obtained for strain YH014 bearing pBR322 are defined as 1, and relative values are shown compared to this value. Plasmids were pBR322 (vector), pTKM221 (nrdEF), pTKM226 (nrdAB), and pTKM103 (dnaB).*
predominantly contained four or eight chromosomes in a single cell at 25°C. In contrast, the wild-type strain harboring pBR322-dnaB showed a decreased DNA content and contained peaks of three, five, or six chromosomes, indicating that excessive DnaB inhibits initiation and thus causes asynchronous initiations. Excessive interaction between DnaA and DnaB might inhibit the actions of DnaA in the initiation processes, which might result in repression of overinitiation (Fig. 6A and B).

**Effect of the LexA regulon on the hda-185 strain.** Microscopic analysis of the hda-185 cells revealed that after incubation at nonpermissive temperatures for 8 h, this mutant forms filamentous cells (Fig. 7A). At 25°C, filamentation of the hda-185 cells could be observed when the cells were incubated for 4 h or longer (data not shown). Flow cytometry suggested that after incubation at 25°C for 8 h, the hda-185 cells were approximately seven times longer than the wild cells (Fig. 7B).

Cell division in the dnaA cos mutant is inhibited at 30°C in an sfiA-independent manner (28). In order to determine whether the SOS pathway affects cell division in the hda-185 strain, we introduced an sfiA deletion or a lexA3 mutation. The lexA3 mutation renders LexA resistant to RecA*-induced cleavage, and thus, the SOS regulon is constitutively repressed (43). When we examined colony formation at 25°C, the ΔsfiA hda-185 mutant exhibited a more severe inhibition of cell growth than the wild-type sfiA hda-185 mutant did (Fig. 7C). The lexA3 mutation also enhanced growth inhibition in the hda-185 mutant at 25°C (Fig. 7C). It is conceivable that by the fork progression block, SOS genes, such as sfiA, are induced at low levels in the hda-185 mutant at 25°C, enhancing survival of the mutant cells (see Discussion).

The ΔsfiA hda-185 mutant formed filamentous cells at 25°C, and its average cell mass was similar to that of the wild-type sfiA hda-185 strain (Fig. 7A and B). A similar result was obtained using the lexA3 hda-185 strain (Fig. 7A and B). Thus, these results indicate that an sfiA-independent inhibition of cell division occurs due to defects in hda function.

The SlmA protein blocks FtsZ ring formation in the vicinity of nucleoids, which subsequently prevents untimely cell division (4). This form of regulation is termed nucleoid occlusion (4). We did not observe any significant effects of slmA deletion on cell size or cold sensitivity in the hda-185 mutant at 25°C (Fig. 7A to C). These results suggest that the pathway blocking cell division in the hda-185 mutant is independent of the nucleoid occlusion system.

**DISCUSSION**

In this study, we isolated a novel cold-sensitive hda allele, hda-185, and analyzed modes of chromosomal replication, dnaA expression, and cell division in the hda-185 mutant. Notably, we found that multicopy supply of dnaB, nrdAB, or nrdEF inhibits overinitiation. Previously, oversupply of NrdAB or DnaB has been shown to repress the growth defect of hda-deleted cells or that of dnaA219 overinitiation mutant cells, respectively (16, 54); however, it has not been demonstrated whether overinitiation of chromosomal replication is inhibited in the suppressed cells. Furthermore, we demonstrated that multiple copies of nrdAB increase the cellular dATP and dCTP levels in hda-185 cells and a critical residue in

![FIG. 7](image-url)
the NrdA catalytic center is required for the *hda-185* suppression. These findings suggest a possible link between the repli-
cational initiation system and the dNTP pool level. In addition,
we found that cell division is inhibited at 25°C in *hda-185* cells in
an *sfiA*-independent manner. This *sfiA*-independent system
is unique in operating independently of the LexA regulon. An
unrevealed regulatory mechanism would inhibit cell division
upon overinitiation of chromosomal replication in the *hda*
mutant.

The increased dNTP level or an active form of NrdAB might
be related to the repression of overinitiation in the *hda-185*
cells (Fig. 5 and 6; Table 6). The repression would be specific
to extra initiation events because initiation in the wild-type
cells was not inhibited by multiple copies of *nrdAB* (Fig. 6C).
A possible linkage mechanism between the dNTP levels and the
initiation regulatory systems might be that the elevated dNTP
levels result in functional inhibition of a protein involved in
replication initiation. For example, in *Saccharomyces cerevi-
siae*, it has been reported that constitutively elevated dNTP
levels result in inhibition of entry into S phase in a manner
concomitant with reduced chromatin loading of Cdc45, a com-
ponent of replication initiation (8). Alternatively, the dNTP
levels might affect the expression of DNA replication-related
genes. For example, NrdR is a transcriptional repressor for
*nrdAB* and *nrdEF* (61), and the NrdR homolog in *Streptomyces
coeiolor* shows dATP/ATP-dependent binding to a specific
DNA in vitro (18). The NrdR-binding consensus (NrdR box) is
seen in the promoter region of *dnaA* in *Shewanella* spp. and in
*Myxococcus xanthus* and *topA* in *Pseudomonas* spp. (53).

The *hda-185* mutant causes *sfiA*-independent inhibition of
cell division at nonpermissive temperatures (Fig. 7). Previ-
ously, mitomycin has been shown to induce an *sfi*-independent
division inhibition in a manner depending on a gene included
in the LexA regulon (20). The filamentation of *hda-185* mutant
cells was independent of the LexA regulon, indicating that a
division inhibition pathway in this mutant is distinct from the
previously reported *sfi*-independent pathway. Nalidixic acid or
thymine starvation, which causes inhibition of the replication
fork progression, is reported to lead to a reduction in the
transcription of the FtsZ operon (37). Although the mech-
anism causing this reduction remains to be elucidated, it could
be related to our observations in this study. At present, only
limited investigations have been performed for the mecha-
nisms regulating cell division in cells causing overinitiation of
chromosomal replication (28).

Also, we noticed an unexpected role for SfiA in the survival
of *hda-185* cells at 25°C: the colony formation defect in the
*hda-185* mutant at 25°C was enhanced by sfiA disruption (Fig.
7C). The replication fork block in the *hda-185* strain could
activate the SfiA division inhibition system via the SOS re-
sponse. SfiA inhibition of FtsZ ring formation is reported to be
reversible (39). We speculate that the SfiA system keeps cells
viable, resulting in an enhanced occurrence of suppressor mu-
tations. In the absence of *sfiA*, an *sfiA*-independent system
would be complementarily induced in the *hda-185* cells. It is
conceivable that this system inhibits the cell division process at
a point later than the SfiA-inhibiting point and causes adverse
effects on the viability of the cells.

The cellular levels of *dnaA* mRNA and DnaA in the *hda-185*
cells at 25°C were similar to those in the wild-type cells (Fig.
2D and 3). Also, a moderate increase in the ATP-DnaA level
was seen in an *hda-185*-dependent manner (Fig. 4). Thus,
factors other than ATP-DnaA might sustain the *dnaA* tran-
scriptional regulation more effectively in vivo, whereas in vitro,
ATP-DnaA represses the *dnaA* promoter activity more tightly
than ADP-DnaA does (57). Indeed, in vivo experiments using
replication cycle-synchronized cells demonstrate that the *dnaA*
promoter activity is tightly repressed in the postinitiation pe-
riod in which the ATP-DnaA level is decreased and the ADP-
DnaA level is elevated by RIDA (6, 36). A recent report has
indicated that the DnaA protein level is reduced in *hda*-defi-
cient cells to ~50 to 60% of the wild-type level (52); however,
the *hda*-deficient cells used in that study carry the *hsm-1*
suppressor mutation (*hsm* suppressor mutation). The gene bearing
the *hsm-1* mutation has not been identified. The possibility that
the wild-type *hsm* gene product directly or indirectly regulates
the *dnaA* transcription and that the *hsm-1* mutation involves the
*dnaA* transcription level in the absence of Hda, resulting in sup-
pression of the growth defect in *hda*-deficient cells, might be
important.

The mutation site (*K185C*) within the *hda-185* allele is lo-
cated in the AAA+ domain that Hda and DnaA share (29).
The basic moiety at this position is highly conserved among the
orthologs of DnaA and Hda in many bacterial species. The
crystal structure of the *Aquifex aeolicus* DnaA AAA+ domain
shows that the residue (Arg-246) corresponding to the Hda
Lys-185 residue resides on an α-helix of the C-terminal sub-
domain (domain IIIb) and its basic side chain is located inside
the protein structure (14, 15). The substitution of K185C might
destabilize the whole conformation of the C-terminal subdo-
main and thus render the Hda function temperature depen-
dent.

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