Heat shock proteins are induced when cells are exposed to heat shock, and the relaxed state of DNA rapidly reverts to the original state with exposure to conditions of heat shock. We have now obtained genetic and biochemical evidence indicating that DnaK heat shock protein of E. coli, a prokaryotic homologue of hsp70, is involved in this re-supercoiling of DNA. As re-supercoiling of DNA did not occur in an rpoH amber mutant, it seems likely that heat shock proteins are required for this reaction. Plasmid DNA in a dnaK deletion mutant relaxed excessively after temperature shift-up, and the re-supercoiling of DNA was not observed. DNAs incubated with a crude cell extract prepared from the dnaK mutant were more relaxed than seen with the extract from its isogenic wild-type strain, and the addition of purified DnaK protein to the mutant extract led to an increase in the negative supercoiling of DNA. Moreover, reaction products of purified DNA gyrase more negatively supercoiled in the presence of DnaK protein. Based on these results, we propose that DnaK protein plays a role in maintaining the negative supercoiling of DNA against thermal stress.

The physical environment of living organisms is ever changing, and such changes can cause stress, damage, or even death to these organisms, which respond at the cellular level to unfavorable conditions such as heat shock or other situations and maintain intracellular homeostasis (1). The manner in which cells sense changes in the physical environment is unknown (2).

Heat shock proteins are induced when cells are exposed to environmental stress (3–5). These proteins are members of a family called “molecular chaperones,” and function of these proteins is considered to protect other proteins from thermal inactivation and to reactivate aggregated forms of proteins subjected to stress. In Escherichia coli, heat shock response results from increased transcription of heat shock genes (6–9), which depends on an rpoH gene product, σ32 (7, 10); the concentration of σ32 immediately and transiently increases about 10-fold after temperature shift-up (11, 12), resulting in both increased synthesis (12–16) and stabilization of σ32 (12). One heat shock protein, DnaK, a prokaryotic homologue of hsp70, was proposed to be a “cellular thermometer” that transduces the signal after increasing the temperature to other cellular factors (17); DnaK protein, along with DnaJ protein, releases from σ32 and binds to unfolded or denatured proteins that accumulate upon stress, an event which presumably leads to a transient stabilization of σ32 (18–20). In addition, DnaK protein seems to serve in a manner so as to directly sense the environmental temperature on the basis of the highly temperature dependence of its ATPase and autophosphorylating activities (21).

DNA supercoiling could also be a sensor for changes in the physical environment. The level of DNA supercoiling can be altered by shifts in the environment such as temperature (22) and available nutrients (23), and the extent of DNA supercoiling affects various steps of DNA events such as replication, transcription, and recombination (24–26). In accordance with this notion, Wang and Syvanen (27) proposed that the DNA twist serves as a transcriptional sensor for environmental changes; successful transcription initiation of many bacterial promoters is strongly dependent on supercoiling of the template DNA molecule.

We reported that plasmid DNA in exponentially growing E. coli immediately relaxes after heat shock and the relaxed DNA quickly supercoils even when heat shock conditions are not interrupted (28–30). The finding that the extent of relaxation of plasmid DNA was greater at higher temperatures (30) implies that changes in DNA supercoiling could transduce to other cellular factors the signal of change in the environmental temperature. Since relaxation of DNA correlates with induction of heat shock proteins (28, 29, 31), the reaction may function as a signal in the heat shock response. In the present study, we investigated the molecular mechanism of this heat shock-induced, immediate, and transient relaxation of plasmid DNA. In earlier attempts to identify DNA topoisomerases that catalyze the reaction, we found that the relaxation of DNA is catalyzed by DNA topoisomerase I and by DNA gyrase (30), while the subsequent re-supercoiling of DNA is catalyzed by DNA gyrase (28); the DNA relaxation was inhibited by nalidixic acid or by oxolinic acid in a DtopA mutant but not in its isogenic wild-type strain, while the re-supercoiling of DNA did not occur in a temperature-sensitive gyrA42 mutant.

We report here genetic and biochemical evidence that DnaK heat shock protein, in addition to DNA gyrase described above, is also involved in the re-supercoiling of DNA. Plasmid DNA in a ΔdnaK52 mutant relaxed excessively after heat shock, and re-supercoiling of DNA did not occur. DNAs incubated with a crude cell extract prepared from the ΔdnaK52 mutant were more relaxed than seen with the extract from its isogenic wild-type strain and negatively supercoiled by adding purified DnaK protein. These findings lead to the notion that DnaK

**DnaK Heat Shock Protein of Escherichia coli Maintains the Negative Supercoiling of DNA against Thermal Stress*\)**

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protein contributes to a system to maintain homeostasis of DNA supercoiling in cells exposed to thermal stress.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Bacterial strains used in this study are listed in Table I. Strains HB101 and W3110 were from our laboratory stocks. Strains MC4100, KY1429, KY1472, NRR156, and BB1553 were kindly provided by T. Yura. Strains YO07, YO08, YO09, YO10, YO11, YO12, and YO13 were constructed by P1 transduction in this work.

**Plasmids**—Plasmid pUC118 (39) was prepared from *E. coli* HB101 harboring this plasmid and purified as described (32). Plasmid pMOB45-dnaK (40) was kindly provided by S. Yasuda, and plasmid pBR322-dnak (39) was kindly provided by Y. Sakakibara. Plasmid pYO01 was constructed by inserting the HindIII-BglII dnak fragment from plasmid pBR322-dnak (40) into the HindIII-BglII site of a mini-R vector, pIP1673 (41). The resultant plasmid, pYO01, complemented an amphenicol-resistant transductant which was temperature sensitive (37,42) and cold sensitive (37),42). This work.

**P1 Transduction**—The source of the phage P1 vir has been described (43). P1 phages were grown in KY1429, NRR117, KY1472, NRR156, and BB1553 and harvested by centrifugation in a Hitachi centrifuge at 3,000 rpm for 5 min at 0°C. This immediate chilling of cells is essential for detecting an immediate and transient DNA relaxation after heat shock (28–30). Purification of Dnak Protein was purified by the method previously described (40, 46, 47) by collecting the activity that stimulates DNA supercoiling reaction in the fraction II extract prepared from the dnaK52 mutant (37), did not exist in the fresh dnaK52 mutant (37), did not exist in the fresh dnaK52 mutant (37).

**Preparation of Crude Cell Extracts**—Crude cell extracts, fraction II, were prepared as described (44) but with some modifications. W3110 and YO13 were grown to late logarithmic phase in 100 ml of LB broth supplemented with 0.4% glucose in a 1-liter flask (Iwaki) at 30°C, diluted 1:100 into 500 ml of the same medium in a 2-liter flask (Iwaki), and grown at 30°C until *A*$_{650}$ values of 0.5 were reached. Doubling times were 36 and 42 min, respectively. These cultures (2 liters) were harvested by centrifugation in a Beckman JA-20 rotor at 8,000 rpm for 15 min at 2°C. Pellets were suspended in buffer A (25 mM HEPES, pH 7.6, 1 mM EDTA) and frozen in liquid nitrogen, and stored at −85°C. Frozen cell suspensions were thawed on ice and adjusted to 80 mM KCl, 2 mM dithiothreitol, and 300 μg/ml egg lysozyme (Seikagaku Kogyo) in a Beckman centrifuge tube (25 × 89 mm). After incubation on ice for 30 min, the cells were again frozen in liquid nitrogen and thawed on ice. Lysates were clarified by centrifugation in a Beckman Type 42.1 rotor at 40,000 rpm for 20 min at 4°C (fraction I). 0.28 μg/ml ammonium sulfate was added slowly to fraction I (4.0–4.5 ml) on ice with stirring over a 10-min period and further stirred for 20 min. Precipitations were collected by centrifugation in a Beckman JA-20 rotor at 18,000 rpm for 20 min at 2°C. The pellets, placed directly into a tube (20×32, Viskase) without adding a buffer were dialyzed against 1 liter of buffer B (25 mM HEPES, pH 7.6, 0.1 mM EDTA, and 2 mM dithiothreitol) on ice for 70–80 min. The dialysates (fraction II, 150 μl), with a protein concentration of 30–50 mg/ml, were distributed as 50-μl aliquots, frozen in liquid nitrogen, and stored at −85°C. Protein concentration was determined by the method of Lowry et al. (45), using bovine serum albumin (Boehringer Mannheim) as a standard.

**Purification of Dnak Protein**—Dnak protein was purified by the method previously described (40, 46, 47), but with some modifications, and by measuring the activity that stimulates DNA supercoiling reaction in the fraction II extract prepared from the dnaK52 mutant (37). W3110 harboring pMOB45-dnak (40) was grown overnight at 30°C in 400 ml of LB broth supplemented with 0.4% glucose and 20 μg/ml chloroquine in a 2-liter flask (Iwaki), diluted 1:10 into 500 ml of the same medium in a 2-liter flask (Iwaki), and grown at 30°C until *A*$_{650}$ values of 1.0 were reached. Doubling period was 45 min. The culture (7 liters) was centrifuged in a Beckman JA-10 rotor at 7,000 rpm for 15 min at 2°C, and the bacterial pellets were suspended in 80 ml of 10% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0, frozen in liquid nitrogen, and stored at −85°C. A frozen cell suspension was thawed on ice, and 10 ml of buffer K (180 mM spermidine-HCl (Sigma) (pH 7.5), 50 mM dithiothreitol, 50 mM EDTA, and 0.9 mM ammonium sulfate) (40) with fresh lysozyme (2 mg/ml, Seikagaku Kogyo) was added. After incubation on ice for 45 min, the mixture was further incubated at 37°C for 5 min, then on ice for 20 min, and spun in a Beckman type 42.1 rotor at 30,000 rpm for 30 min at 2°C. The supernatant (88 ml) was passed through a DE52 cellulose column (3 × 40 cm) (Whatman) equilibrated with buffer A (100 mM potassium phosphate (pH 7.0), 100 mM ammonium sulfate, 5 mM 2-mercaptoethanol, 5 mM EDTA, and 10% (w/v) glycerol) (40) at a

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**Table I**

**Bacterial strains**

| Strain     | Relevant genotype          | Reference or source |
|------------|---------------------------|---------------------|
| HB101      | supE44 hsdR50 (F- rpsL150 mcrB2 mrr1 recA14 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 mecrB::Tn10 rpsL20 xyl-5 mtl-1 mecrB) | 32                   |
| MC1400     | F' araD193 (λargF-lacI189 repL150 relA1 fib5301 deloC1 phs256 rbsR) | 33                   |
| KY1429     | as MC4100 except rpmH8(am) zhy50::Tn10 | 34                   |
| NRR117     | as MC4100 except dnaK52::Tn10 | 35                   |
| KY1472     | as MC4100 except groE572 zid::Tn10 | 36                   |
| NRR156     | as MC4100 except dnaK756 thr-34::Tn10 | 37                   |
| BB1553     | as MC4100 except ΔdtnK32::Cam^+ sidB1 | 37                   |
| W3110      | F' | 38                   |
| YO07       | as W3110 except zje::Tn10 | This work            |
| YO08       | as YO07 except groEL44 | This work            |
| YO09       | as W3110 except zid::Tn10 | This work            |
| YO10       | as YO09 except groEST2 | This work            |
| YO11       | as W3110 except zhy55::Tn10 | This work            |
| YO12       | as YO11 except rodR6 (am) | This work            |
| YO13       | as W3110 except ΔdnaK52::Cam^+ | This work            |

1 T. Yura, unpublished data.
flow rate of 6.4 ml/10 min. The flow-through fractions (125 ml) were dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 3 mM MgCl₂, and 10% (v/v) glycerol) and loaded onto a 5-ml ATP-agarose affinity column (Sigma) equilibrated with buffer C. The column was washed with buffer C containing 500 mM NaCl. After re-equilibration of the column with buffer C, DnaK protein was eluted with 10 mM ATP in buffer C, adjusted to pH 7.6. The active fraction was dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 3 mM MgCl₂, and 10% (v/v) glycerol) and loaded onto a Mono Q HR 5/5 column (Pharmacia Biotech Inc.) equilibrated with buffer D. Bound proteins were eluted with a linear gradient of 0.05–0.6 M KCl. DnaK protein eluted at approximately 0.35 M KCl. Fractions were separately dialyzed against 2 (v/v) glycerol, and bromphenol blue, and analyzed by 1% agarose gel phenol and 200

newly synthesized protein, we examined the effect of chloramphenicol on DNA and the subsequent re-supercoiling of DNA would require new protein syntheses. On the other hand, taking into consideration that it is of an almost identical magnitude to that induced by inhibitors of DNA gyrase, which catalyzes the re-supercoiling of DNA in chloramphenicol-treated cells, we determined by densometric analysis of SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R250 (Nacalai Tesque).

**Purification of A and B Subunits of DNA Gyrase—**A and B subunits of DNA gyrase were purified by measuring the DNA supercoiling activities. The A subunit was purified by ammonium sulfate precipitation of a cell lysate and chromatographies on DEAE-cellulose and phenyl Sepharose. The B subunit was purified by the method previously described (48). The obtained fraction was further purified by Mono S column chromatography. The specific activity of the final fractions of A and B subunits were 2 × 10⁶ units/mg and 4 × 10⁴ units/mg, respectively. The final fractions showed single bands on SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R250 (Nacalai Tesque).

**DNA Supercoiling Reaction in Vitro—**The standard reaction was done as described (49) but with some modifications. The reaction mixture (16.5 μl) contained 35 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 24 mM KCl, 1.8 mM spermidine–HCl, 0.36 mg/ml bovine serum albumin (Boehringer Mannheim), 0.14 mM EDTA, 5 mM diithiothreitol, 6.5% (v/v) glycerol, 1.4 mM ATP, 100 μM/mg of yeast RNA (Boehringer Mannheim), 0.1 mg/ml creatine kinase, 10 mM creatine phosphate, and 230 ng of relaxed pUC118 DNA, a reaction product obtained with calf thymus DNA topoisomerase I (Life Technologies, Inc.), and crude cell extracts, fraction II (1.56–25.0 μg of protein) or 13.8 ng of A and 8.7 ng of B subunits of DNA gyrase with or without DnaK protein (1.25–10.0 μg/mg).

The fraction II extracts or A and B subunits of DNA gyrase were diluted into a solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 5 mM diithiothreitol, 50% glycerol, and 3.6 mg/ml bovine serum albumin (Boehringer Mannheim) and preincubated with or without DnaK protein at 25°C for 5 min. The reaction mixture, except the fraction II extracts or A and B subunits of DNA gyrase, was prewarmed at 25°C for 5 min. The reaction was initiated by combining them, which was continued at 25°C for 60 min, and terminated by adding 200 μl of phenol and 200 μl of a solution containing 100 mM Tris–HCl, pH 9.0, 10 mM EDTA, 2% SDS, and 20 μg of yeast RNA (Boehringer Mannheim) and extracted with ethanol, dissolved in a solution containing 100 mM Tris borate, 100 mM boric acid, 2 mM EDTA, 20% (v/v) glycerol, and bromphenol blue, and analyzed by 15 μg/ml chloroquine. Less negatively supercoiled topoisomers before electrophoresis migrate more rapidly at this concentration of chloroquine. +Cm, cells treated with chloroquine; −Cm, control without the drug.

**RESULTS**

**Requirement of Concomitant Protein Syntheses for Re-supercoiling of DNA after Heat Shock—**We earlier found that the linking number of plasmid DNA in exponentially growing E. coli immediately and transiently increased after heat shock (28–30). This relaxation of plasmid DNA is apparently small, however, taking into consideration that it is of an almost identical magnitude to that induced by inhibitors of DNA gyrase, which is known to have a significant effect on gene expression (50); the relaxation of DNA observed here can alter the pattern of gene expression. To determine whether the relaxation of DNA induced by the subsequent re-supercoiling after heat shock is caused by newly synthesized protein, we examined the effect of chloramphenicol on these reactions. As shown in lanes 1 and 2 of Fig. 1A, pUC118 plasmid DNA in chloramphenicol-treated cells immediately relaxed after temperature shift-up from 30 to 50°C. Therefore, the relaxation of plasmid DNA does not require new protein syntheses. On the other hand, pUC118 plasmid DNA in the chloramphenicol-treated cells excessively relaxed after temperature shift-up; the re-supercoiling of DNA was not observed (lanes 3–6). Thus, this reaction apparently requires concomitant protein syntheses.

There remained the possibility that excessive relaxation of DNA in the chloramphenicol-treated cells is due to an irreversible inactivation of DNA gyrase, which catalyzes the re-supercoiling of DNA (28). The relaxed DNA in the chloramphenicol-treated cells supercoiled again when the temperature was again decreased to 30°C (Fig. 1B, lanes 3–6), a finding which suggests that DNA gyrase remains active in chloramphenicol-treated cells, even after heat shock treatment. Therefore, the excessive relaxation of DNA in the chloramphenicol-treated cells may not be due to an irreversible inactivation of DNA gyrase; rather, it may be caused by inhibition of concomitant protein syntheses required for the re-supercoiling of DNA after heat shock.

**Requirement of Heat Shock Proteins for the Re-supercoiling of DNA after Heat Shock—**The observations that re-supercoiling of DNA after heat shock requires concomitant protein syntheses (Fig. 1) and that the reaction follows the induction of heat shock response (28, 29) raised a possibility that induced heat shock proteins are required for this reaction. To examine this possibility, we investigated the effect of an rpoH mutation on the re-supercoiling of DNA after heat shock. As shown in lanes 1 and 2 of Fig. 2, pUC118 DNA in an rpoH6 (am) mutant, YO12, immediately relaxed after temperature shift-up. This observation confirms our previous finding that the DNA relaxation induced by re-supercoiling after heat shock is not the result of heat shock response (28). On the other hand, the plasmid DNA in the rpoH6 (am) mutant excessively relaxed after temperature shift-up, while, in its isogenic wild-type strain, YO11, the DNA

**Participation of DnaK Protein in DNA Supercoiling**

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we asked whether the wild-type DNAK protein is apparent
ecessary for the re-supercoiling of DNA after heat shock (data not shown). Thus, DnaK protein is transiently
ient strain, W3110, was transient. Moreover, pUC118 in a
pUC118 in a D

the fraction II extracts at 25°C as described (49). Fig. 4 shows
tions eluted from Mono Q column were subjected to SDS-poly-
cations on this reaction. As shown in lanes 3–6 of Fig. 3A,
pUC118 in a ΔdnaK52 mutant, YO13, excessively relaxed after
temperature shift-up, whereas the DNA relaxation in its par-
Y13 harboring the mini-R plasmid would recover the re-supercoiling of DNA in the presence of 15 μg/ml chloroquine.

To further confirm the requirement of DnaK protein for this
reaction, we asked whether the wild-type dnaK gene cloned on a
mini-R plasmid would recover the re-supercoiling of DNA in the
ΔdnaK52 mutant. pUC118 plasmid DNA in the ΔdnaK52
mutant harboring a plasmid pYO01, a low copy mini-R plasmid
carrying the wild-type dnaK gene, did not excessively relax after
temperature shift-up, while DNA in YO13 harboring the
mini-R vector, pKP1673, excessively relaxed (data not shown).

Subsequently, we investigated whether GroEL and GroES
proteins would be also responsible for the re-supercoiling of DNA after heat shock. For this purpose, the effects of a
groEL or a groES mutation on the reaction were examined. pUC118 in a
groEL44 mutant, YO08 (Fig. 3B), or a groES72 mutant, YO10
(data not shown), did not excessively relax after temperature shift-up. Thus, GroEL and GroES proteins seem to be dispensable for the reaction.

Comparison of the Linking Number of DNA Incubated with a
Crude Cell Extract Prepared from a ΔdnaK52 Mutant with That from Its Isogenic Wild-type Strain—The findings described above suggest that DnaK protein participates in DNA supercoiling. To obtain biochemical evidence to support this
notion, we prepared a crude cell extract, fraction II (44), from
the ΔdnaK52 mutant or from its isogenic wild-type strain and compared the linking number of plasmid DNA incubated with the fraction II extracts at 25°C as described (49). Fig. 4 shows topoisomser patterns of pUC118 incubated with the fraction II extract from the ΔdnaK52 mutant, YO13, or from the
dnaK52 mutant was larger than that with the extract from the wild-type strain, i.e. the DNAs incubated with the extract from the ΔdnaK52 mutant were more relaxed than seen with the extract from the wild-type strain.

Effect of Purified DnaK Protein on DNA Supercoiling Reaction in the Extract from the ΔdnaK52 Mutant—The result described above suggests that DnaK protein participated in DNA supercoiling reaction in the fraction II extracts. To examine this possibility, we asked whether purified DnaK protein would decrease the linking number of the plasmid DNA incubated with the extract from the ΔdnaK52 mutant. As shown in Fig. 5, DnaK protein did decrease the linking number in a dose-dependent manner.

To further confirm the participation of DnaK protein in the
decrease in linking numbers of DNA in the mutant extract, we
observed whether or not this activity would co-migrate with DnaK protein on Mono Q column chromatography. The fractions eluted from Mono Q column were subjected to SDS-poly-

acrlylamide gel electrophoresis (Fig. 6A) and used in DNA supercoiling assays (Fig. 6B). The result indicates that the

from the ΔdnaK52 mutant was larger than that with the extract from the wild-type strain, i.e. the DNAs incubated with the extract from the ΔdnaK52 mutant were more relaxed than seen with the extract from the wild-type strain.

Fig. 2. Effect of an rpoH6 (am) mutation on re-supercoiling of DNA after heat shock. YO11 (rpoH6) or YO12 (rpoH6 (am)) harboring pUC118 was grown at 30°C in L broth supplemented with 0.4% glucose and 100 μg/ml ampicillin. Exponentially growing cells (A650 of 0.5) (lane A) were exposed to 50°C for 2, 4, 6, 8, and 10 min (lanes 2–6). DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.

Fig. 3. Influence of ΔdnaK52 or groEL44 mutations on the re-supercoiling of DNA after heat shock. W3110 (dnaK) and YO13 (ΔdnaK52) (A) or YO07 (groEL) and YO08 (groEL44) (B) harboring pUC118 was grown at 30°C in L broth supplemented with 0.4% glucose and 100 μg/ml ampicillin. Exponentially growing cells (A650 of 0.5) (A and B, lane 1) were exposed to 50°C for 2, 5, 10, 20, and 30 min (A, lanes 2–6) or for 2, 10, 15, 20, and 30 min (B, lanes 2–6). DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.

Fig. 4. DNA supercoiling reaction in a crude cell extract, fraction II, prepared from a ΔdnaK52 mutant or its isogenic wild-type strain. DNA supercoiling reaction in a crude cell extract, fraction II, prepared from W3110 (dnaK) or from YO13 (ΔdnaK52) was carried out as described under “Materials and Methods.” DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.
activity of the decrease in the linking number of DNA co-migrated with DnaK protein on Mono Q column chromatography (Fig. 6C). Thus, DnaK protein is responsible for this activity. Since there was no detectable change in the linking number of plasmid DNA incubated with DnaK fraction in the absence of the extract from the ΔdnaK52 mutant (data not shown), the possibility that DnaK protein alone (or an activity of a putative contamination in DnaK fraction) changes the linking number of the plasmid DNA can probably be ruled out.

**Effect of DnaK Protein on DNA Supercoiling Reaction of Purified DNA Gyrase**—Since DNA gyrase is the only known enzyme that introduces negative supercoiling into DNA in *E. coli* (24–26, 51), we then investigated whether DnaK protein would affect the DNA supercoiling reaction of purified DNA gyrase. As shown in Fig. 7, the linking number of reaction products at 25 °C of DNA gyrase decreased in a DnaK protein-dependent manner. The same results were obtained in the reaction at 45 °C (data not shown). This apparent stimulation...
of DNA supercoiling reaction of DNA gyrase co-migrated with DnaK protein on Mono Q column chromatography (Fig. 8), as well as in case of the assay for the fraction II extract from the ΔdnaK52 mutant (Fig. 6). Thus, DnaK protein is indeed responsible for this activity.

As DnaK protein does not bind to DNA (40) and does not introduce negative supercoils into circular duplex DNA in the presence of eukaryotic DNA topoisomerase I (data not shown), DnaK protein is not likely to constrain negative supercoils in DNA. DnaK protein apparently acts directly on DNA gyrase.

**DISCUSSION**

To elucidate the molecular basis of immediate and transient relaxation of plasmid DNA induced by heat shock, we searched for factors that participate in the process. We reported elsewhere that relaxation of DNA is catalyzed by both DNA topoisomerase I and DNA gyrase (30), while re-supercoiling of DNA is catalyzed by DNA gyrase (28). The experiments described here provide genetic and biochemical evidence indicating that DnaK heat shock protein, as well as DNA gyrase described above, contributes to the re-supercoiling of DNA. Plasmid DNA in a ΔdnaK52 mutant immediately relaxed after heat shock, but the re-supercoiling of DNA was not observed. The finding that plasmid DNA carried by the ΔdnaK52 mutant was more relaxed than that carried by its isogenic wild-type strain implies that DnaK protein participates in DNA supercoiling. We attempted to obtain biochemical evidence to support this notion and found that DNAs incubated with a crude cell extract prepared from the ΔdnaK52 mutant were more relaxed than seen with the extract from the wild-type strain, while addition of purified DnaK protein to the mutant extract led to an increase in the negative supercoiling of DNA.

With regard to the manner in which DnaK protein functions in the re-supercoiling of DNA after heat shock, at least one mechanism can be considered: DnaK protein acts directly on DNA gyrase after heat shock, and, as a result, the reaction products of this enzyme supercoil more negatively. Recently, we found that reaction products of purified DNA gyrase, under physiological concentrations of ATP, immediately relax upon temperature shift-up from 30 to 45 °C (52). This observation can explain our previous finding that DNA gyrase contributes to the heat shock-induced DNA relaxation in vivo (30). The relaxed state of the reaction products at 45 °C quickly reverted to the original state when the temperature was decreased to 30 °C (52), a finding which suggests that DNA gyrase remains active, even after heat treatment. These results indicate that relaxation of the products is not due to an irreversible inactivation of DNA gyrase; rather, it is caused by the intrinsic nature of this enzyme. Thus, the linking number of reaction products...
products of DNA gyrase can reversibly be altered upon temperature shift-up and -down, i.e. the linking number of the reaction products is a function of temperature. Since reaction products of purified DNA gyrase more negatively supercoiled in the presence of DnaK protein (Figs. 7 and 8), DnaK protein seems to alter thermal equilibrium of the reaction of DNA gyrase. The molecular mechanism through which DnaK protein acts on DNA gyrase remains obscure.

Numbers of DnaK molecules needed to stimulate DNA supercoiling reaction in vitro using the extract from the \textit{dnaK}52 mutant (Fig. 5) or purified DNA gyrase (Fig. 7) were relatively high; however, the range is almost identical to that seen in reactivation of heat-inactivated \textit{E. coli} RNA polymerase holoenzyme (53, 54). DnaK protein functionally interacts with DnaJ and GrpE proteins (55); DnaJ and GrpE proteins stimulate ATPase activity of DNA gyrase proteins up to 50-fold (56) and regulate the autophosphorylating activity of DnaK protein (57). Taking into consideration that DnaJ and GrpE proteins substantially reduce the level of DnaK protein needed in reactivation of heat-inactivated RNA polymerase (54), these proteins may lower the effective concentration of DnaK protein needed for maximal activity of stimulation of DNA supercoiling reaction in vitro.

Re-supercoiling of DNA caused by DnaK protein seems to play a role in the negative regulation of heat shock response; the re-supercoiling of DNA (Fig. 3A) and the recovery from heat shock response (19, 58) did not occur after temperature shift-up in \textit{dnaK} mutants. The correlation between DNA relaxation and heat shock response has been reported (28, 29, 31); both the DNA relaxation and the induction of heat shock proteins are simultaneous and transient in wild-type cells, while both events are continuous in the \textit{gyrA42} mutant (28) or in anaerobically growing \textit{E. coli} cells (29). Moreover, chemicals such as inhibitors of DNA gyrase that induce heat shock proteins also induced DNA relaxation (28), and expression of LetD (CcdB) protein, an inhibitor for DNA gyrase encoded by the \textit{F} factor of \textit{E. coli}, co-induced DNA relaxation and synthesis of heat shock proteins (31). Since the temperature shift (Fig. 2) and chemicals (28) induced DNA relaxation in the \textit{rhoH6} (am) mutant, DNA relaxation is not the result of heat shock response. Based on these findings, we propose a putative model of heat shock response: DNA relaxation induced by heat shock stimulates synthesis of heat shock proteins, including DnaK protein that is involved in the re-supercoiling of DNA, which in turn shuts off the heat shock response (Fig. 9). While this model does provide a good explanation for its transience, the molecular mechanism through which changes in DNA supercoiling transduce the signal responsible for the induction of heat shock response to other cellular factors will need further study.

Temperature-sensitive growth of \textit{dnaK} mutants may be partially explained by failure to maintain the negative supercoiling of DNA at high temperatures, since the negative supercoiling of DNA is essential to maintain normal cell function (24–26), especially in cellular processes such as replication (59, 60), transcription (59), and recombination. Physiological studies showed that the \textit{Delta}dnaK52 mutant blocks DNA synthesis, RNA synthesis, and cell division after temperature shift-up (42) and reduces homologous recombination in a plasmid at a semi-permissive temperature (61).

The \textit{dnaK} gene was originally identified when its mutation caused defects in bacteriophage \textit{A} DNA replication (62, 63). A \textit{dnaK111} mutant was seen to be defective in initiation of DNA replication from the origin of \textit{E. coli} chromosome, \textit{oriC}, at a non-permissive temperature, thereby suggesting that the \textit{dnaK} gene is also required for initiation of chromosomal replication in \textit{E. coli} (64). However, the function of the \textit{dnaK} gene product in initiation of DNA replication from \textit{oriC} remains uncertain, since enzymatic replication of plasmids containing \textit{oriC} is independent of DnaK protein. Biochemical studies revealed that DnaK protein activates aggregated DnaA protein (47) or mutant forms of DnaA protein, DnaA5 and DnaA46, which are thermolabile in initiation of DNA replication from \textit{oriC} (65, 66). Our present study provides insight into the manner in which DnaK protein contributes to form the negatively supercoiled DNA template, which is required for formation of an active initiation complex (59, 60).

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\[\text{Participation of DnaK Protein in DNA Supercoiling}\]
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