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Antibody to σ^{32} cross-reacts with DnaK: Association of DnaK protein with *Escherichia coli* RNA polymerase

(heat shock/σ factor)

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ABSTRACT **A polyclonal antibody to σ^{32}, the heat shock σ factor, has been used to show the presence of low levels of σ^{32} in *Escherichia coli* RNA polymerase preparations ($E\sigma^{70}$), which explains the observed *in vitro* activity of $E\sigma^{70}$ towards heat shock genes. The σ^{32} antibody cross-reacts with DnaK, and DnaK has been found associated with purified preparations of both $E\sigma^{70}$ and the heat shock RNA polymerase, $E\sigma^{32}$.**

In *Escherichia coli* at least 17 proteins have been identified as members of the heat shock (HS) regulon (1). When *E. coli* cells growing at 30°C are shifted to 42°C, the rates of synthesis of HS proteins increase 5- to 20-fold, reaching a peak within 5 to 15 min after the temperature shift. Thereafter, synthesis declines until a new rate characteristic of the higher temperature is attained.

It is now well established that the HS response in *E. coli* requires the presence of the *htpR* gene product, a 32-kDa protein (2–4). This protein functions as a σ factor (σ^{32}) by binding to core DNA-directed RNA polymerase (E; EC 2.7.7.6) (5, 6), to form a HS RNA polymerase ($E\sigma^{32}$) that can recognize specific promoter sequences on HS genes (7). $E\sigma^{32}$, relatively free of $E\sigma^{70}$ [RNA polymerase containing the normal 70-kDa σ factor (σ^{70})], has been purified from *E. coli* transformed with a plasmid containing the σ^{32} gene (6). Previous *in vitro* studies using $E\sigma^{32}$ showed that it could transcribe HS genes in a transcription assay (6) and also function in the expression of HS genes in a highly defined DNA-directed protein synthesis system (8). It was also noted in these latter studies that $E\sigma^{70}$ preparations unexpectedly showed significant activity towards HS genes, such as *dnaK*. It was not determined at that time whether this was due to contamination of $E\sigma^{32}$ in the $E\sigma^{70}$ preparations or activity of $E\sigma^{70}$ itself. This question is relevant to the fact that HS proteins such as DnaK and GroEL are major products of *E. coli*, even when the cells are grown at non-HS temperatures (1). Thus, it is not clear whether $E\sigma^{70}$ can transcribe HS genes under some conditions. In the present report, we have used a polyclonal antibody (Ab) to σ^{32} to examine this question in more detail. In the course of these studies we have demonstrated that the σ^{32} Ab cross-reacts with DnaK and that DnaK is associated with purified preparations of both $E\sigma^{70}$ and $E\sigma^{32}$

MATERIALS AND METHODS

Plasmids, *E. coli* Strains, and Antibodies to σ^{32}, $E\sigma^{70}$, and DnaK. Plasmid pCG203, which contains the *dnaK* gene, was kindly supplied by C. Georgopoulos (University of Utah). pBR322 was used as template for β-lactamase synthesis. Purified plasmid DNAs were prepared from cleared lysates after two successive centrifugations through CsCl/ethidium bromide. An *htpR* strain (CAG9301) lacking σ^{32} was kindly supplied by C. Gross (University of Wisconsin, Madison). $E\sigma^{32}$ was separated into its subunits on a 10% polyacrylamide gel containing $NaDodSO_4$. The σ^{32} was cut out of the gel and eluted with buffer (9). Rabbit polyclonal Abs to σ^{32} and $E\sigma^{70}$ were prepared as described previously (9, 10). DnaK was purified by the procedure of Zylicz and Georgopoulos (11), and rabbits were injected with 100 μg of the protein with booster doses at 2 and 4 weeks as outlined elsewhere (10).

DNA-Directed *in Vitro* Protein Synthesis. A partially defined *E. coli* DNA-directed *in vitro* protein synthesis system was used in this study (10, 12) with the exception that 30–50 μg of protein of a 0.25 M salt eluate from DEAE-cellulose (13) was used in place of the purified aminoacyl-tRNA synthetases and RNasin (18 units; Promega Biotec, Madison, WI) was substituted for the RNase inhibitor present in ascites extracts. Each reaction mixture contained 0.5–1.0 μg of plasmid DNA as the template, and incubations were at 37°C for 1 hr. Aliquots were removed and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (14). After electrophoresis, the gels were soaked in Enlightning (New England Nuclear), dried, and fluorographed at −70°C. For quantitation, the protein gels were electroblotted (2 hr at 0.5 A) to a sheet of nitrocellulose (0.1 μm pores, Schleicher & Schuell) and an autoradiogram of the nitrocellulose was used to visualize the protein bands. The autoradiogram was superimposed over the sheet of nitrocellulose and the bands of interest were excised from the nitrocellulose sheet and dissolved in Bray's scintillation fluid (National Diagnostics, Somerville, NJ). The amount of radioactivity was then determined in a liquid scintillation counter.

Isolation of RNA Polymerase. $E\sigma^{70}$ was purified by the method of Burgess and Jendrisak (15) and $E\sigma^{32}$ was prepared as described previously (6, 8), using, as a final step, chromatography on a Bio-Gel A-1.5m column (100–200 mesh, Bio-Rad) as described previously (15). This final Bio-Gel chromatography step was not included in the purification of $E\sigma^{70}$ from the σ^{32} mutant strain (CAG9301).

For identification of DnaK in the polymerase preparations, chromatography of the purified RNA polymerase preparations on a Bio-Gel P-150 column was employed (50–150 mesh, Bio-Rad). For these experiments 200–500 μg of RNA polymerase in a total volume of 100 μl was applied to the Bio-Gel column (0.7 × 50 cm) equilibrated with buffer containing 20 mM Tris·HCl at pH 8.0, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.15 M NaCl, and 5% (vol/vol) glycerol. Elution was carried out with the same buffer at a flow rate of 2 ml/hr. Fractions (0.26 ml) were collected and aliquots were assayed for RNA polymerase activity by using a modified

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Abbreviations: HS, heat shock; σ^{32} and σ^{70}, 32- and 70-kDa σ factors; Ab, antibody.

procedure of Burgess (16) and analyzed for DnaK by immunoblot analysis and autophosphorylation as described below.

Immunoblotting of RNA Polymerase and DnaK. RNA polymerase or DnaK was electrophoresed on 10% polyacrylamide gels in the presence of 0.1% $NaDodSO_4$. The proteins were electroblotted (2 hr at 0.5 A) to a sheet of nitrocellulose (0.1 μm pores, Schleicher & Schuell) as described elsewhere (17). The nitrocellulose blot was soaked in 2% gelatin containing 20 mM Tris·HCl at pH 7.5 and 0.15 M NaCl (TBS) for 1 hr at 37°C. The blot was rinsed three times with 0.02% $NaDodSO_4$ in TBS and then three times in TBS for a total of 30 min. All subsequent incubations were done at room temperature with shaking. The nitrocellulose blot was then incubated for 16 hr with the Ab to σ^{32}, DnaK, or $E\sigma^{70}$ and then washed as described above. ^{125}I-labeled staphylococcal protein A (Amersham; 36 mCi/mg, 1332 MBq/mg) was diluted 1:5000 in TBS containing 2% gelatin and incubated with the blot for 2 hr. The nitrocellulose was then washed, dried, and exposed to x-ray film.

Autophosphorylation of DnaK Protein. DnaK autophosphorylation was performed as described by Zylicz *et al.* (18). The incubations were for 30 min at 37°C and the mixtures (50 μl) contained 50 mM Mes buffer at pH 6.2, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10% (vol/vol) glycerol, 50 pmol of [γ-^{32}P]ATP (10 μCi, 3000 Ci/mmol), and either DnaK (1–5 μg) or RNA polymerase (50 μg). In some experiments the protein samples were heated at 90°C for 10 min prior to addition to the incubation mixtures. The reaction was stopped by the addition of 200 μl of 20% (wt/vol) Cl_3CCOOH and 1 μl of 10% (wt/vol) sodium deoxycholate. After 30 min at 0°C, the sample was centrifuged for 10 min at 15,000 × *g*. The pellet was washed with acetone, dried, and suspended in sample buffer (14) and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. After electrophoresis, the gel was dried and autoradiographed at −70°C.

RESULTS

***In Vitro* Gene Expression of $E\sigma^{32}$ and $E\sigma^{70}$.** We recently reported that $E\sigma^{70}$ can transcribe HS genes (8), and the initial goal of this study was to examine this reaction in more detail. A partially defined DNA-directed protein synthesis system (see *Materials and Methods*) was used to study the activity of $E\sigma^{32}$ and $E\sigma^{70}$ towards HS and non-HS genes. Fig. 1*A* shows that, when $E\sigma^{70}$ is used in the *in vitro* system, maximal expression of β-lactamase (a non-HS gene) is obtained when 1.0–2.0 μg of RNA polymerase is present. However, only about 0.5 μg of this polymerase is required for maximal expression of the HS *dnaK* gene. As seen in Fig. 1*B*, $E\sigma^{32}$ shows no activity toward non-HS genes in this system, confirming previous results (8). $E\sigma^{32}$ gives maximal activity with *dnaK* as template when 0.1–0.2 μg is used; however, levels of $E\sigma^{32}$ above 0.5 μg show significant inhibition of *dnaK* expression (Fig. 1*B*). Results similar to those seen with DnaK were obtained when $E\sigma^{32}$ was used with another HS gene, *groEL* (data not shown). At the optimal concentrations the activity of $E\sigma^{70}$ towards HS genes was about 50% of that seen with $E\sigma^{32}$.

Presence of σ^{32} in $E\sigma^{70}$ Preparations and Effect of σ^{32} Antibody on $E\sigma^{70}$ Activity. One explanation for the ability of $E\sigma^{70}$ to transcribe HS genes (see Fig. 1*A*) could be the presence of $E\sigma^{32}$ in the $E\sigma^{70}$ preparations. Recently, Lesley *et al.* (19) reported that their $E\sigma^{70}$ preparations contained low levels of σ^{32}, and we have confirmed these findings. Immunoblot analyses of $E\sigma^{70}$ and $E\sigma^{32}$ using Ab against either $E\sigma^{70}$ holoenzyme or the σ^{32} subunit are shown in Fig. 2. The $E\sigma^{70}$ Ab readily detects the $\beta\beta'$ and α subunits of E in both $E\sigma^{70}$ and $E\sigma^{32}$, (Fig. 2*A*, lanes 1 and 2, respectively), as well as the σ^{70} subunit in $E\sigma^{70}$. No σ^{70} is detected in $E\sigma^{32}$. When equivalent amounts of $E\sigma^{32}$ and $E\sigma^{70}$ are probed with the σ^{32} Ab (Fig. 2*B*), a prominent σ^{32} band is seen in $E\sigma^{32}$ (lane 4) but σ^{32} also is observed in $E\sigma^{70}$ (lane 3). In this preparation of $E\sigma^{70}$ ≈5% of the holoenzyme could be $E\sigma^{32}$. Several different $E\sigma^{70}$ preparations, either prepared in our laboratory or obtained commercially, were analyzed, and all showed significant levels of σ^{32} in the $E\sigma^{70}$. It was estimated that the various $E\sigma^{70}$ preparations contained between 1% and 10% $E\sigma^{32}$. It can also be seen (lanes 3 and 4) that the σ^{32} Ab reacts with another protein of about 69 kDa in both $E\sigma^{70}$ and $E\sigma^{32}$. This protein has been identified as DnaK as shown below.

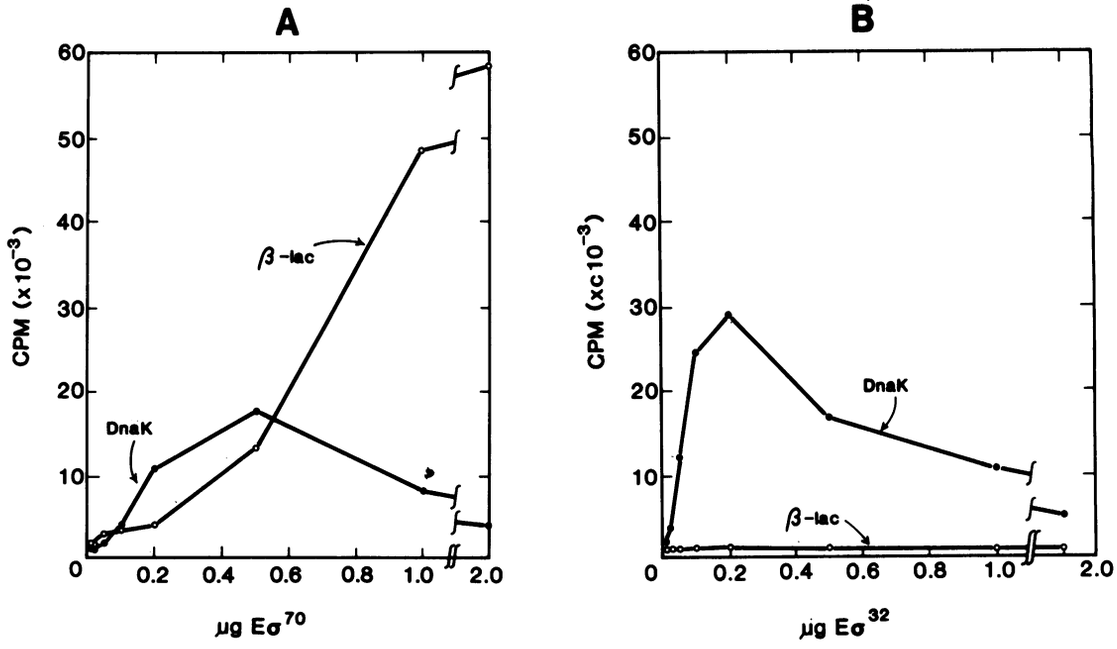

FIG. 1. Effect of $E\sigma^{70}$ (*A*) and $E\sigma^{32}$ (*B*) on the synthesis of DnaK and β-lactamase (β-lac). Plasmid DNAs (either pBR322 or pCG203) were incubated in a partially defined DNA-directed protein synthesis system. After 60 min at 37°C, 5-μl samples were removed and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and then the proteins were transferred to nitrocellulose paper. The protein bands were identified by autoradiography, and after excision from the nitrocellulose the radioactivity in the bands was determined.

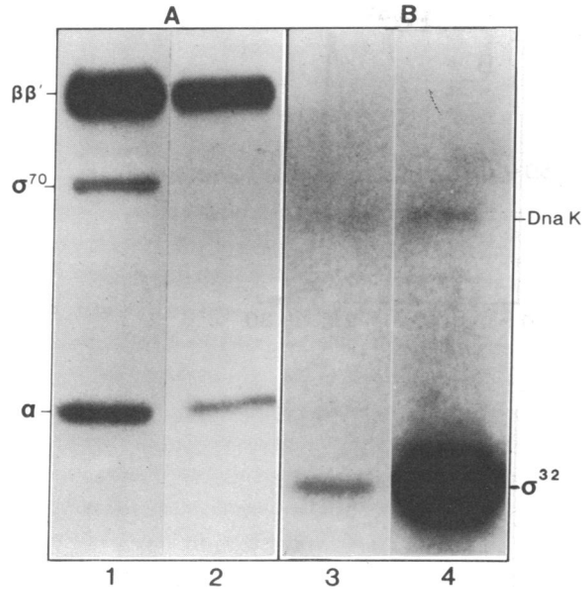

FIG. 2. Immunoblot analysis of $E\sigma^{70}$ and $E\sigma^{32}$ using Ab against either $E\sigma^{70}$ holoenzyme (*A*) or σ^{32} subunit (*B*). Lanes 1 and 3, $E\sigma^{70}$; lanes 2 and 4, $E\sigma^{32}$. Lanes 1, 3, and 4 contained 5 μg of protein and lane 2 contained 2.5 μg.

The presence of σ^{32} in $E\sigma^{70}$ strongly suggested that the *in vitro* activity of $E\sigma^{70}$ towards HS genes might be due to the $E\sigma^{32}$ in these preparations. σ^{32} Ab was used to show this directly. As seen in Fig. 3, when σ^{32} Ab is included in the *in vitro* protein synthesizing system, it has no effect on the synthesis of β-lactamase by $E\sigma^{70}$ (lanes 1 and 2); however, it almost completely inhibits the synthesis of DnaK with either $E\sigma^{70}$ (lanes 3 and 4) or $E\sigma^{32}$ (lanes 5 and 6). Preimmune sera at the same concentration showed either no inhibition or only a slight nonspecific effect (data not shown).

Association of DnaK with RNA Polymerase. We have recently reported preliminary results that an Ab to σ^{32} reacts weakly with DnaK (9). As seen in Fig. 2*B*, both $E\sigma^{70}$ and $E\sigma^{32}$ contain

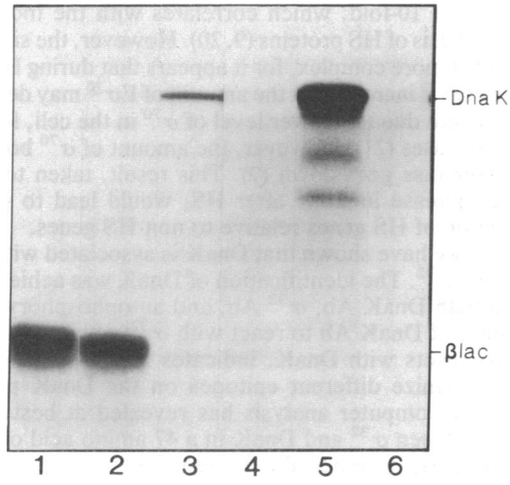

FIG. 3. Effect of σ^{32} Ab on the activity of $E\sigma^{70}$ and $E\sigma^{32}$. Plasmid DNAs were incubated in a partially defined DNA-directed protein synthesis system containing either $E\sigma^{70}$ (0.5 μg) or $E\sigma^{32}$ (0.2 μg). After 60 min at 37°C, 5-μl samples were removed, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and fluorographed. Lane 1, pBR322 (β-lactamase) and $E\sigma^{70}$; lane 2, pBR322, $E\sigma^{70}$, and σ^{32} Ab; lane 3, pCG203 (DnaK) and $E\sigma^{70}$; lane 4, pCG203, $E\sigma^{70}$, and σ^{32} Ab; lane 5, pCG203 and $E\sigma^{32}$; lane 6, pCG203, $E\sigma^{32}$, and σ^{32} Ab. The incubation mixtures used for lanes 2, 4, and 6 contained 1 μl of σ^{32} Ab.

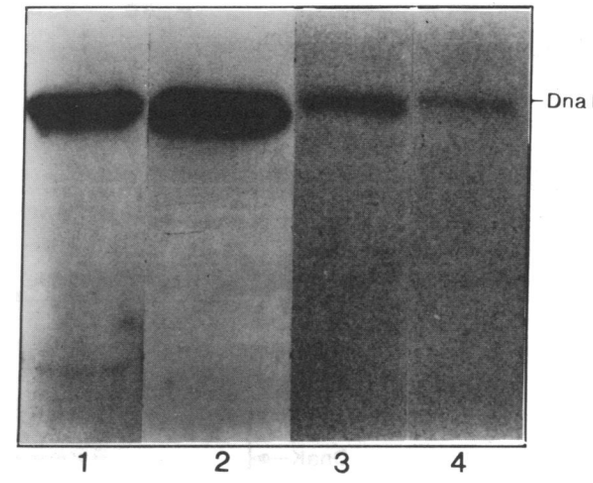

FIG. 4. Immunoblot analysis of DnaK protein and RNA polymerase preparations by using Ab against either the σ^{32} subunit or DnaK protein. Lanes 1 and 2, 3 μg and 0.5 μg of DnaK protein, respectively; lanes 3 and 4, 5 μg of $E\sigma^{70}$ and $E\sigma^{32}$, respectively. σ^{32} Ab was used in lane 1 and DnaK Ab was used in lanes 2–4.

a protein of ≈69 kDa that reacts with σ^{32} Ab. This protein has now been identified as DnaK and appears to be associated with RNA polymerase. Fig. 4 shows that σ^{32} Ab and DnaK Ab both react with purified DnaK (lanes 1 and 2, respectively) and that the DnaK Ab also reacts with the same 69-kDa protein present in $E\sigma^{70}$ and $E\sigma^{32}$ (lanes 3 and 4) that reacted with σ^{32} Ab (Fig. 2*B*).

An analytical sizing column (Bio-Gel P-150) was used to obtain further evidence that the DnaK is associated with the RNA polymerase. This column readily separates RNA polymerase from DnaK. Chromatography of both $E\sigma^{70}$ and $E\sigma^{32}$ showed that both enzymes eluted between fractions 18 and 24 (Fig. 5 *A* and *B*). Immunoblots (Fig. 5 *C* and *D*) using DnaK Ab detected DnaK in the fractions that had enzymatic activity. The ability of DnaK to be autophosphorylated (18) provided further proof of its presence in the polymerase preparations. As shown in Fig. 6, incubation of $E\sigma^{70}$ and $E\sigma^{32}$ with [γ-^{32}P]ATP showed phosphorylation of the 69-kDa protein in both preparations (lanes 1 and 3, respectively). The incubations were carried out at 37°C, but similar results were obtained after the RNA polymerase preparations were heated for 10 min at 90°C (lanes 2 and 4). These findings are consistent with the known heat stability of the DnaK autophosphorylation reaction (18). It can also be seen in Fig. 6 (lane 5) that RNA polymerase isolated from a σ^{32} mutant strain (CAG9301) that is also lacking DnaK (data not shown) does not contain a 69-kDa protein that can be phosphorylated. In other experiments not shown, the DnaK remained associated with RNA polymerase after electrophoresis under nondenaturing conditions and after centrifugation in a 5–30% (vol/vol) glycerol gradient.

DISCUSSION

In the present report, we have used a partially defined *in vitro* system to study the ability of purified $E\sigma^{32}$ and $E\sigma^{70}$ to transcribe HS and non-HS genes. The results confirm previous *in vitro* results demonstrating that $E\sigma^{32}$ could efficiently transcribe HS genes such as *dnaK* but could not transcribe a non-HS gene such as that encoding β-lactamase (8). In contrast, $E\sigma^{70}$ not only transcribes non-HS genes but also weakly transcribes HS genes in this *in vitro* system. The original purpose of this study was to understand how $E\sigma^{70}$ can transcribe a HS gene that does not appear to contain a typical $E\sigma^{70}$ promoter. All of the present evidence indicates

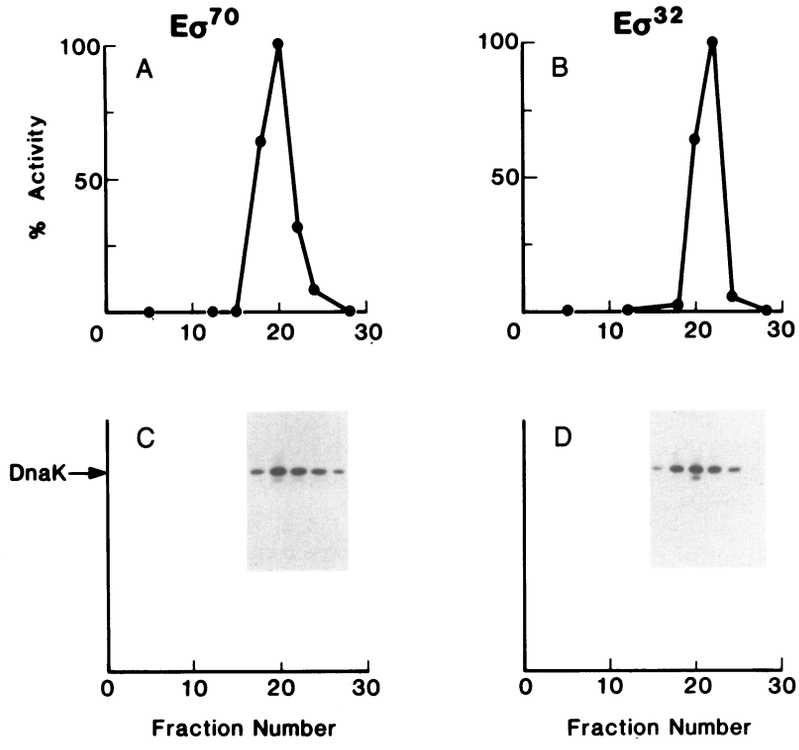

FIG. 5. Chromatography of $E\sigma^{70}$ and $E\sigma^{32}$ preparations on a Bio-Gel P-150 column. RNA polymerase activity of $E\sigma^{70}$ (*A*) and $E\sigma^{32}$ (*B*) fractions was determined by [^{3}H]UTP incorporation into Cl_3CCOOH-insoluble material (16). $E\sigma^{70}$ (*C*) and $E\sigma^{32}$ (*D*) fractions were analyzed by immunoblotting using DnaK Ab. Free DnaK is eluted between fractions 60 and 72 (data not shown).

that the observed activity of $E\sigma^{70}$ towards HS genes is due to low levels of $E\sigma^{32}$ contamination in the $E\sigma^{70}$ preparations. It was estimated by immunoblot analysis that the $E\sigma^{70}$ used in these studies contained about 5% $E\sigma^{32}$. In addition, all other preparations of $E\sigma^{70}$ that we examined contained various amounts of $E\sigma^{32}$. That the $E\sigma^{32}$ contaminating the $E\sigma^{70}$, and not $E\sigma^{70}$ itself, was responsible for the transcription of HS genes was shown by the ability of σ^{32} Ab to specifically inhibit the activity of $E\sigma^{70}$ towards HS genes. These results are in agreement with those of Grossman *et al.*

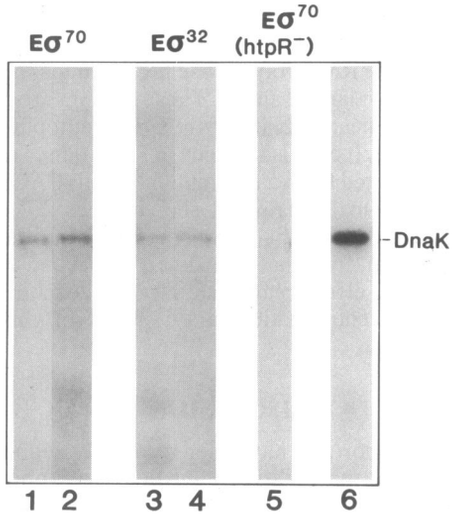

FIG. 6. Autophosphorylation of the DnaK protein in the RNA polymerase preparations. The autophosphorylation reaction was performed as described in the text and reaction products were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lanes 1 and 2, $E\sigma^{70}$; lanes 3 and 4, $E\sigma^{32}$; lane 5, $E\sigma^{70}$ from the σ^{32} mutant strain (CAG9301); lane 6, DnaK protein. In lanes 2 and 4 the polymerase preparations were heated to 90°C for 10 min prior to addition to the incubations.

(6), who showed in a run-off transcription assay that $E\sigma^{70}$ could not transcribe HS genes. It is also of interest that, in the *in vitro* system used in these studies, the optimal concentration of $E\sigma^{32}$ for transcription of HS genes is approximately 1/5th to 1/10th the optimal concentration of $E\sigma^{70}$ for a non-HS gene. This apparent higher activity of $E\sigma^{32}$ may account for the high levels of DnaK and GroEL proteins that accumulate in *E. coli* grown at non-HS temperatures (1).

Although the absolute amount of $E\sigma^{32}$ in cells at non-HS temperatures is relatively low, during HS the levels of $E\sigma^{32}$ increase 5- to 10-fold, which correlates with the increased rate of synthesis of HS proteins (9, 20). However, the situation is somewhat more complex, for it appears that during HS, not only does $E\sigma^{32}$ increase but the amount of $E\sigma^{70}$ may decrease (9). This is not due to a lower level of σ^{70} in the cell, for σ^{70}, in fact, increases (21). However, the amount of σ^{70} bound to core polymerase goes down (9). This result, taken together with the increase in $E\sigma^{32}$ after HS, would lead to greater transcription of HS genes relative to non-HS genes.

Finally, we have shown that DnaK is associated with both $E\sigma^{70}$ and $E\sigma^{32}$. The identification of DnaK was achieved by reaction with DnaK Ab, σ^{32} Ab, and autophosphorylation. The failure of DnaK Ab to react with σ^{32}, although the σ^{32} Ab cross-reacts with DnaK, indicates that these two antibodies recognize different epitopes on the DnaK protein. Preliminary computer analysis has revealed at best a 15% identity between σ^{32} and DnaK in a 47 amino acid overlap. It is not clear whether the presence of DnaK on RNA polymerase has any physiological significance, since the bulk of the DnaK in the cell is free. One could speculate that only a specific modified form of DnaK is found associated with RNA polymerase. To study the interaction of DnaK with either $E\sigma^{70}$ or $E\sigma^{32}$, it will be necessary to purify these polymerases from DnaK mutants and investigate the requirements for DnaK binding. Our preliminary findings indicate that there are no major differences in the amount of DnaK bound to $E\sigma^{70}$ as compared to $E\sigma^{32}$. However, it should be noted that the amount of DnaK associated with the RNA

polymerase preparations decreased significantly during the purification of both $E\sigma^{70}$ and $E\sigma^{32}$. In fact, we have not quantitated the amount of DnaK that is associated with RNA polymerase in the original cell extract. However, in the final purified polymerase preparations, we estimate that less than 10% of the RNA polymerase has bound DnaK.

The association of DnaK with RNA polymerase may be a clue to how the HS response is modulated. It is known that temperature-sensitive *dnaK* mutants are defective in DNA and RNA synthesis (22) and also have an extended HS response (23), suggesting that the DnaK protein is involved in turning off the HS response. Fujita *et al.* (24) have shown, *in vitro*, an exchange of σ^{70} subunit with $E\sigma^{32}$ to form functional $E\sigma^{70}$. Although DnaK was not tested in these latter studies (24), one possibility is that DnaK mediates the exchange of σ^{70} and σ^{32} with core polymerase and in this way modulates the HS response. Ishihama *et al.* (25) have noted the presence of several proteins that copurify with *E. coli* RNA polymerase. They also isolated some of these factors from crude RNA polymerase preparations (25), and they reported that three of the proteins appeared to be HS proteins. One was GroEL (26), but the other two were not identified.

It is clear that further studies are needed to determine whether the association of DnaK, GroEL, and perhaps other HS proteins with RNA polymerase is related to the HS response.

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