Near-UV Stress in *Salmonella typhimurium*: 4-Thiouridine in tRNA, ppGpp, and ApppGpp as Components of an Adaptive Response

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We have examined the role of 4-thiouridine in the responses of *Salmonella typhimurium* to near-UV irradiation. Mutants lacking 4-thiouridine (*nuv*) and mutants defective in the synthesis of ppGpp (guanosine 5'-diphosphate-3'-diphosphate) (*relA*) were found to be sensitive to killing by near-UV. Near-UV induced the synthesis of a set of proteins that were not induced in the *nuv* mutant. Some of these proteins were identified as oxidative defense proteins, and others were identified as ppGpp-inducible proteins. Over 100-fold increases in ApppGpp (adenosine 5', 5'''-triphosphoguanosine-3'''-diphosphate, the adenylylated form of ppGpp) were observed in wild-type cells after near-UV irradiation but not in the 4-thiouridine-deficient mutant. These data support a model in which ppGpp and ApppGpp, a dinucleotide proposed to be synthesized by tRNA-aminoacyl synthetases as a response to the cross-linking of 4-thiouridine in tRNA by near-UV, induce the synthesis of proteins necessary for resistance to near-UV irradiation.

One of the most common stresses confronted by organisms is solar radiation. The most energetic part of the solar spectrum which penetrates the atmosphere is the near-UV region (300 to 400 nm) (22). Studies on the environment mortality of bacteria have found that die-off is dependent on light exposure, especially the near-UV portion of the solar spectrum (10, 16, 17). Since enteric bacteria such as *Escherichia coli* must adapt to exposures of near-UV during transmission between hosts (8), they are an ideal system for characterizing mechanisms involved in protection from the toxic effects of near-UV.

The irradiation of growing *E. coli* cells with near-UV results in the cessation of growth long before lethal effects are observed (21, 22). This growth lag is dependent on the tRNA-modified base 4-thiouridine (s^4U), which is found in 65% of tRNA species (22, 40, 45). This base is unusual in that it has an absorption maximum in the near-UV region (334 nm). After exposure to near-UV, s^4U participates in a photochemical reaction which generates intramolecular cross-linking between s^4U and a specific cytosine (13, 14, 29). These cross-linked tRNAs have been shown to be poor substrates for aminoacylation (51). The accumulation of nonaminoacylated tRNA serves to trigger the stringent response via the *relA* gene product, which is involved in the synthesis of ppGpp (guanosine 5'-diphosphate-3'-diphosphate) (9, 15), which then acts to inhibit stable RNA synthesis while inducing amino acid biosynthesis (15, 43). Inhibition of protein synthesis after near-UV exposure in *E. coli* is a result of both the stringent response and decreased aminoacylation of tRNA (44).

Near-UV exerts toxic effects on *E. coli* and *Salmonella typhimurium* at higher fluences, primarily through oxidative mechanisms (22, 23). Macromolecules which absorb in the near-UV region (i.e., heme and flavins) may act as endogenous photosensitizers, transferring the absorbed energy to O_2 and forming toxic oxygen species (10, 22, 23, 48). The response of bacteria to oxidative stress (such as H_2O_2) includes inhibition of growth and the induction of proteins involved in defense against the stress (11, 12, 30). Similarly,

near-UV has also been reported to induce the synthesis of specific proteins in *E. coli* (36).

The mechanisms by which bacteria defend themselves against near-UV killing are largely uncharacterized, although the induction of oxidative defense pathways is involved (23). Since the initial effect of near-UV on bacteria is to induce s^4U-mediated tRNA cross-linking, an s^4U-dependent response could serve to protect the cells from killing by near-UV. The isolation of mutants lacking s^4U (*nuv*) (39) has facilitated investigation of the function of this base. *E. coli nuv* mutants have been reported to be more resistant to near-UV killing, with s^4U apparently acting as a photosensitizer (34, 47). However, the types of exposures used in these experiments (high- or low-intensity monochromatic light) are not environmentally relevant, since monochromatic light focuses all of the energy on a single or small group of chromophores, whereas sunlight is a broad-band light source. The use of monochromatic near-UV at the λ_{max} of s^4U (34, 47) leads to an overestimation of the importance of this base as a photosensitizer under physiological conditions. Since it has been shown that the biological effects of near-UV depend on the fluence rate as well as the total dose (24, 33), it was unclear whether the results obtained with high-intensity and/or monochromatic light sources represented the responses exhibited by cells upon exposure to sunlight.

We therefore investigated the sensitivity of an *S. typhimurium nuv* mutant to a broad-band near-UV source at a fluence rate of 35 W/m² (23) as compared with 35 to 50 W/m² for the sun (36, 47). We found, under these conditions, that a *nuv* mutant which lacked s^4U was in fact more sensitive to near-UV killing. Mutants in the *relA* gene were also more sensitive to near-UV, indicating that ppGpp was involved in this protective response. We examined the pattern of protein synthesis during a near-UV exposure; the *nuv* mutant was defective in the induction of a set of proteins that were induced in the wild type. This induction was independent of the stringent response. These results are consistent with a model in which s^4U acts as a sensor for near-UV and mediates cellular responses to this stress.

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MATERIALS AND METHODS

Bacterial growth. The following bacterial strains from this laboratory were used: TA4187 (*zaj-1034*::Tn*10*), TA4330 (*nuvA1*), TA4331 (*zaj-1034*::Tn*10 nuvA1*), and TA2438 (*relA2*). Unless otherwise noted, liquid bacterial cultures were grown in minimal VBC salts medium (50) containing 0.4% glucose. Overnight cultures were grown without shaking at 37°C in minimal VBC salts containing 0.08% glucose. Viable cells were determined by plating dilutions of cells onto nutrient broth plates (0.8% Difco NB, 0.5% NaCl, and 1.5% agar) and counting colonies after 14 to 20 h at 37°C.

Near-UV exposure. Near-UV exposures were performed as described previously (23). Cells were grown in 100-ml flat-bottomed petri plates. Illumination was initiated by suspending three 15-W General Electric F15T8BLB black light bulbs over the cultures. Thus, the only perturbation of the growing conditions was the imposition of the near-UV light stress. The fluence rate was 35 W/m² (23). Once the optical density (650 nm) reached 0.15, irradiation was started. Viability was determined at various times by removing samples and performing viable cell counts. Killing curves were generated by averaging the results of two to six separate experiments.

Isolation and characterization of a *nuv* mutant. Mutants lacking s⁴U were isolated by a procedure similar to that of Ryals et al. (41). Wild-type *S. typhimurium* LT2 was diluted and spread onto NB plates at a density of approximately 300 to 600 colonies per plate. The plates were preincubated at 37°C for 5 h, followed by irradiation. The near-UV light source was as described previously (23). The plates were exposed to near-UV in cycles of 30 min of exposure and 90 min of dark for a period of 41 h. Single-colony isolates were purified from large colonies obtained in the initial isolation. The modified base content of suspected *nuv* mutants was analyzed by the method of Buck et al. (7).

Analysis of protein induction. Cells were labeled with [³⁵S]methionine (Dupont-New England Nuclear Corp. Products) and prepared for electrophoresis as described by Morgan et al. (30). Two-dimensional gel electrophoresis was done as described by O'Farrell (31). Proteins were classified by using an alphanumeric system similar to that used by Pedersen et al. (35). Near-UV-induced proteins were determined by removing 400-μl samples and adding the labeled methionine. Exposure was continued with stirring in a small vessel. Proteins induced by the stringent response were determined by labeling cells after the addition of 0.6 mM serine hydroxamate (Sigma Chemical Co.). This concentration was chosen because the overall incorporation of label was similar to that seen with near-UV exposure. All data reported are for proteins which were seen to be induced in both of two separate experiments.

Nucleotide analysis. Bacterial cultures (80 ml; optical density at 650 nm, 0.2) were harvested by rapid filtration through Whatman GF-F filters. The filters were immersed in 4 ml of cold 8% HClO₄ for 20 min on ice and washed with another 4 ml of 8% HClO₄. After the addition of 2 ml of 4 M KOH and 2 ml of 2 M KHCO₃, the samples were centrifuged, and the supernatants were decanted and adjusted to pH 6.2 with 8% HClO₄ before application to quaternary-amine solid-phase extraction columns (J. T. Baker Chemical Co.). Retained material was eluted from the solid-phase extraction columns in 2 ml of 1 M NH₄HCO₃ (in the case of ppGpp analysis) or 2 ml of 1 M NH₄CH₃COOH, pH 9.0 (in the case of ApppGpp [adenosine 5′, 5′′′-triphosphoguanosine-3′′′-diphosphate] analysis). Eluates intended for ppGpp analysis were lyoph-

ilized to dryness, suspended in 300 μl of water, and subjected to analysis by high-pressure liquid chromatography (HPLC). Eluates intended for ApppGpp analysis were incubated with 50 U of bovine alkaline phosphatase (Boehringer Mannheim Biochemicals) per ml for 105 min at 37°C, conditions optimized to provide quantitative conversion of Appp Gpp to adenosine 5′,5′′′-triphosphoguanosine (ApppG). Samples were then subjected to affinity chromatography on dihydroxyboryl-Bio-Rex 70, a resin which quantitatively and selectively retains compounds containing two or more sets of *cis*-diols (1). Bound nucleotides were eluted from this resin in 2 ml of water, lyophilized to dryness, and suspended in 300 μl of water for HPLC analysis.

Recoveries of ApppGpp and its stability throughout the assay were routinely determined by the addition of a radiolabeled standard. [³H]ApppGpp was synthesized from [³H] ATP (ICN Pharmaceuticals) in a reaction containing 100 μM ATP, 4 mM ppGpp (Pharmacia Fine Chemicals), 5 mM lysine, 0.1 mM dithiothreitol, 5 mM MgCl₂, 10 mM phosphoenolpyruvate, 20 mM Tris hydrochloride (pH 7.4), 0.5 U of pyruvate kinase (Sigma), 0.5 U of inorganic pyrophosphatase (Sigma), and 4 μl of lysyl tRNA synthetase purified by O. Goerlich (18). After incubation for 15 min at 37°C, [³H]ApppGpp was purified by HPLC and characterized by digestion with alkaline phosphatase to ApppG.

HPLC was performed using Waters 510 pumps, a Waters U6K injector, and a Whatman Solvecon solvent conditioning system. Nucleotides were resolved on a Whatman Partisil-10 SAX column with 0.8 M KH₂PO₄ (pH 6.5) as a mobile phase for the analysis of ppGpp and 100 mM KH₂PO₄ (pH 6.5) as a mobile phase for the analysis of ApppGpp. Elution profiles were monitored with a Kratos 773 detector at 254 nm, and the data were analyzed with a Hewlett-Packard 9816 computer and Nelson Analytical software. Nucleotides were quantitated by comparison of corresponding peak areas with those of standards (obtained from Pharmacia) and intracellular concentrations were calculated assuming a cellular volume of 10^{-15} and 8×10^8 cells per ml per unit of optical density at 650 nm. ApppGpp levels were calculated by subtracting the ApppG concentration in cells (from nonphosphatase-treated samples) from the ApppGpp plus ApppG concentration in cells (from phosphatase-treated samples). The detection limit of this assay for ApppGpp was <60 nM. When we were attempting to measure basal levels, samples were prepared in quadruplicate and combined on one 1.5-ml boronate column for further analysis. The detection limit with this modification was <15 nM.

RESULTS

Isolation of a *nuv* mutant in *S. typhimurium*. To evaluate the possible role of s⁴U in protecting cells from near-UV, it was first necessary to isolate a mutant which is unable to synthesize this base. Mutants defective in s⁴U synthesis were isolated by a modification of the method of Ryals et al. (41). After being spread onto NB plates at dilutions appropriate to allow single-colony formation, cultures of wild-type *S. typhimurium* (LT2) were exposed to near-UV in cycles of 30 min of light and 90 min of dark for a period of 41 h total in an effort to select for mutants which have an attenuated s⁴U-dependent growth delay. Colonies containing such mutants were found to be much larger than wild-type colonies by the end of this procedure. Since *relA* mutants, which also have an attenuated growth delay, have been isolated by similar procedures (38), all mutants isolated were screened for being *relA* mutants by methyl tryptophan sensitivity (41).
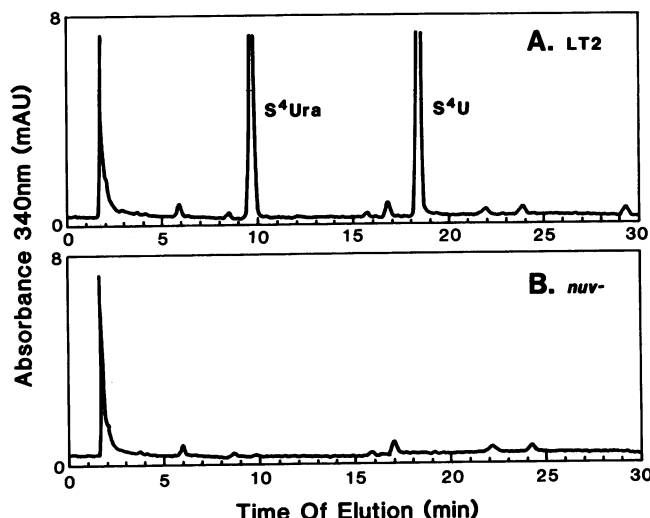
FIG. 1. HPLC analysis of digests of bulk tRNA extracted from (A) LT2 and (B) TA4330 (nuv). tRNA was purified, and reversed-phase HPLC was performed as described elsewhere (7). Analysis of the UV profile at 340 nm allows specific determination of s⁴U and s⁴U-derived compounds. The identity of the s⁴U and s⁴uracil peaks were confirmed by spectral analysis using an HP1040a diode array detector.

Mutants which had an attenuated growth delay and which were not relA mutants were selected for further study.

When the tRNA-modified base content of potential nuv mutants was characterized by the method of Buck et al. (7), one isolate (TA4330) contained no detectable s⁴U (Fig. 1). The biosynthesis of s⁴U has been shown to involve at least two distinct gene products in E. coli (28). One of these genes, nuvA, has been mapped to 9 min on the E. coli chromosome (28). E. coli nuvA mutations have been reported to be complemented by F-factor 254 (28). The mutation in our S. typhimurium nuv mutant, TA4330, is judged to be in nuvA because it is also complemented by F-factor 254 and is linked to a Tn10 insertion in the 9-min region (zaj-1034::Tn10).

We have also characterized the growth delay in response to near-UV of our S. typhimurium nuv mutant in liquid culture. A growth delay was observed in wild-type cells whereas the growth of the nuv mutant was unaffected (Fig. 2). When exposed for longer periods (4 h), the nuv mutant exhibited a growth delay, but entry into this growth lag was delayed as compared with the wild type (Fig. 2). The response of the nuv mutant to a 15-min near-UV exposure was similar to that seen in E. coli nuv mutants (22). This s⁴U-independent growth delay may have been mediated by near-UV-induced cellular damage. The initial target of hyperbaric O₂ toxicity has been shown to be leucine-isoleucine-valine biosynthesis; i.e., the addition of these amino acids protected E. coli from this stress (5, 6). Additionally, oxidizing agents have been shown to induce synthesis of ppGpp, indicating damage to amino acid biosynthesis (4). We have found that the addition of branched-chain amino acids (leucine, isoleucine, and valine) to the medium when exposing the nuv mutant to near-UV greatly delayed the entry into the s⁴U-independent growth lag, whereas other amino acids (methionine, arginine, and glutamate) had no effect (data not shown). The near-UV-induced growth delay in this strain thus may involve damage to branched-chain amino acid biosynthesis.

Sensitivity of nuv mutant to near-UV. Continuous exposure

FIG. 2. Effect of nuv mutation on near-UV-induced growth delay. Cells were grown in minimal glucose medium and exposed to near-UV for 15 min (A) or 4 h (B) as described in the text. Symbols: (●) TA4187, (○) TA4331 (TA4187 nuvA1). The arrows represent the beginnings and the ends of the near-UV exposures.

to near-UV for 4 h induces a biphasic killing phenomenon in strain LT2 (23). Between 4 and 5 h the cells begin to die at a rapid rate, whereas from 6 to 8 h the rate of killing is much lower. To determine whether s⁴U was involved in protecting cells, we characterized the sensitivity of the nuv mutant to near-UV. This mutant was more sensitive than the wild-type strain (Fig. 3). Although both strains began to die at approximately the same time, the nuv cells were killed to a greater extent (over 10-fold at 8 h). This result may be due to the inability of the nuv mutant to efficiently defend against accumulation of near-UV-induced damage. These results indicate that the function of s⁴U in tRNA may be to protect cells from killing by near-UV.

Sensitivity of relA mutant to near-UV. s⁴U is involved in triggering ppGpp production in response to near-UV (22). Since a mutant lacking s⁴U was more sensitive to near-UV killing, ppGpp may be a key signal in this protective response to near-UV. We have tested this possibility by characterizing the near-UV sensitivity of a mutant with an impaired ability to synthesize ppGpp (relA). The mutant began to die at a much earlier time (1 to 2 h) and was killed to a much greater extent (Fig. 3), indicating that ppGpp is also involved in protecting cells from near-UV.
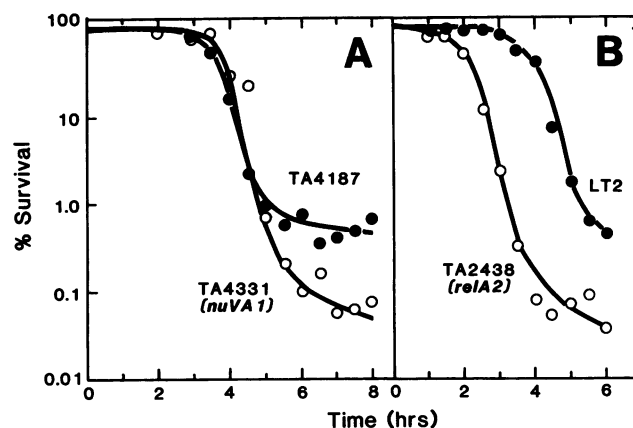

FIG. 3. Effect of nuv and relA mutations on near-UV sensitivity. Cells were grown in minimal glucose medium. Viability was determined throughout a 6- to 8-h exposure as described in Materials and Methods.
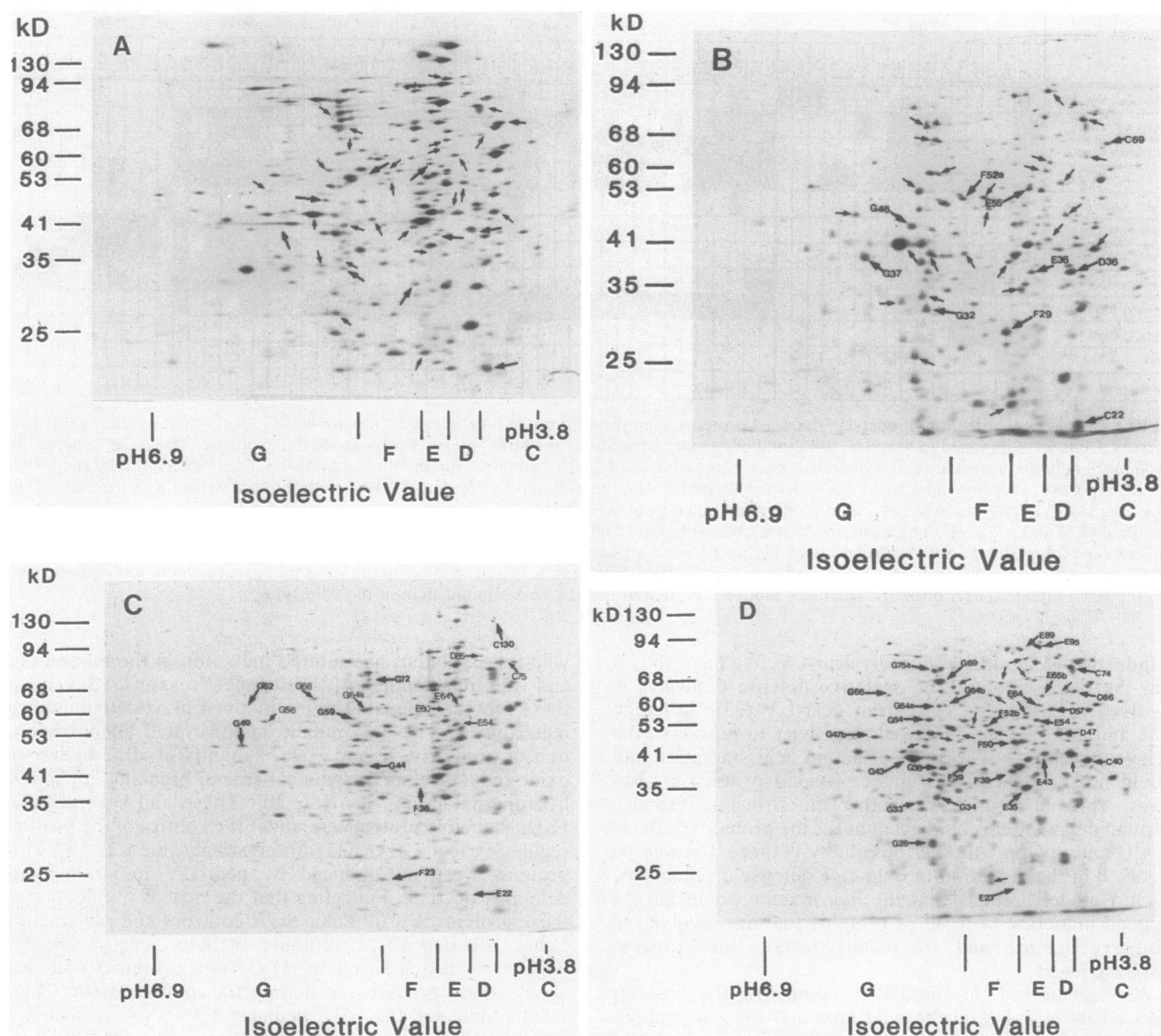
FIG. 4. Schematic diagram of near-UV-induced proteins from the wild type (TA4187) and a *nuv* mutant (TA4331) and overlap with other stresses. Cells were exposed to near-UV for a total of 80 min and labeled for 20-min intervals throughout the exposure. Thus, the "+near-UV 60-80 min" column represents cells which had been exposed to near-UV for 60 min without labeling and were then labeled during the last 20 min of exposure. Proteins induced by the stringent response were determined by treating TA4187 with 0.6 mM serine hydroxamate. Data regarding H_2O_2, *oxyR*, and heat-induced proteins were taken from the results of Morgan et al. (30). Proteins marked with asterisks have been identified (C22 and F52a, alkyl hydroperoxide reductase subunits; C69, DnaK protein). Filled boxes indicate that the protein was induced by the corresponding stress. Set A, Proteins not induced by the stringent response which have altered induction in the *nuv* mutant; set B, stringency-induced proteins which have altered induction in the *nuv* mutant; set C, proteins with the same pattern of induction in the wild type and the *nuv* mutant; set D, proteins which are induced to greater extent in *nuv* mutant than in the wild type.

Induction of specific proteins by near-UV. We have shown that the rapid induction of oxidative defense pathways is involved in protecting cells from near-UV (23). Although *relA* mutants showed increased sensitivity to near-UV, the stringent response is a result of amino acid starvation and would not be expected to induce oxidative defense pathways. Thus, it is unclear whether the stringent response (growth delay) alone could account for the protective effects of s⁴U and ppGpp. Another possibility is that s⁴U could be involved in the induction of oxidative defense mechanisms. Such a model predicts that the *nuv* mutant would have a delayed induction of a set of proteins that are involved in oxidative defense and are independent of the stringent response alone.

We have tested this model by comparing the proteins induced by near-UV in the wild type and the *nuv* mutant. Time-course experiments were performed in which protein induction was determined by two-dimensional gel analysis for 20-min intervals throughout the first 80 min of exposure. Near-UV was found to induce at least 57 proteins in wild-type cells (Fig. 4 and 5). Comparison of the pattern of protein induction between the wild type and the *nuv* mutant revealed that at least 48 proteins had altered induction in the mutant (Fig. 4, sets A and B). Of these proteins, 35 were not induced in the *nuv* mutant; those that were typically were induced at later times. The stringent response induces the synthesis of a large number of proteins in *E. coli* (32). Thus, many of the near-UV-induced proteins would be expected to be dependent on the stringent response. Proteins induced during the stringent response in *S. typhimurium* were determined by repeating the two-dimensional gel analysis on cells that had been treated with 0.6 mM serine hydroxamate, an amino acid analog that induces the stringent response (46). Of the proteins induced by near-UV in wild-type cells, 33 were found to be stringent-response-dependent proteins (Fig. 4 and 5D). The *nuv* mutant was defective in the induction of most of these stringency-induced proteins, as expected from the mechanism of growth delay. However, 17 proteins (Fig. 4, set A) were observed that were induced by near-UV in

wild-type cells but had altered induction in the *nuv* mutant and were independent of the stringent response. Several of these proteins appeared to be involved in oxidative defense mechanisms. The *nuv* mutant demonstrated altered induction of proteins C22 and F52a, subunits of alkyl hydroperoxide reductase, an enzyme capable of breaking down lipid hydroperoxides in vitro (11, 30). These and several other H_2O_2-inducible proteins are under the control of the positive regulatory locus *oxyR* (11). Interestingly, the C22 and F52a proteins were not induced by near-UV in strains with deletions of *oxyR*, indicating that the near-UV induction of these proteins is also under *oxyR* control (data not shown). Other s⁴U-dependent proteins include several proteins which are also induced by H_2O_2 (six proteins) and heat shock (three proteins), including the *dnaK* protein. These results indicated that s⁴U mediates a response to near-UV which involves induction of protection from near-UV killing that is independent of the stringent response. This response may include the induction of proteins involved in protection from oxidizing agents.

Induction of specific dinucleotides by near-UV. 5'-Adenylyl dinucleotides of the general structure AppppN (adenosine-5', 5'''-ribonucleotide tetraphosphate) or ApppN have been suggested to be alarmones for oxidative stress in bacteria (4). It was also suggested that the synthesis of these dinucleotides by tRNA synthetases could be mediated through changes in tRNA-modified bases (4). The synthesis of these compounds by aminoacyl-tRNA synthetases has recently been shown to be stimulated by alterations in tRNA base modification (20). We have thus examined the possibility that dinucleotide induction may be altered in mutants lacking s⁴U. Since ppGpp induction by near-UV in *E. coli* has been demonstrated (21), we determined the levels of ApppGpp as well as ppGpp itself in our system. ApppGpp was induced by near-UV, and the *nuv* mutant was defective in this induction (Table 1). The induction of the stringent response by serine hydroxamate could itself also result in ApppGpp production (Table 1). However, the ApppGpp/ppGpp ratio is much higher with near-UV than with the stringent response alone,

FIG. 5. Representative two-dimensional gels. Cells were labeled for 20 min with [^{35}S]methionine. Molecular sizes (in kilodaltons) are indicated at the left. Isoelectric values are listed at the bottom. G, F, E, D, and C refer to the alphanumeric system described elsewhere (30). (A) Untreated wild-type cells (TA4187). (B) Near-UV-treated wild-type cells labeled from 40 to 60 min of exposure. Arrows show near-UV-induced proteins with altered induction in *nuv* mutant. Dark arrows show proteins not induced by the stringent response (members of set A, Fig. 4). Light arrows show proteins which are also induced by the stringent response (members of set B, Fig. 4). (C) Near-UV-treated *nuv* mutant cells labeled from 40 to 60 min of exposure. Bold arrows show near-UV-induced proteins which are not induced in the wild type (members of set D, Fig. 4). Light arrows show proteins which are also induced in the wild type (members of set C, Fig. 4). (D) Stringency-induced proteins. Wild-type cells were labeled from 40 to 60 min after addition of 0.6 mM serine hydroxamate. Bold arrows show stringency-induced proteins which were also induced by near-UV; light arrows show stringency-induced proteins which appeared not to be induced by near-UV.

indicating that near-UV specifically induces ApppGpp accumulation. This defect in ApppGpp accumulation in the *nuv* mutant could be a result of a defect in dinucleotide induction or could simply result from lower induced levels of ppGpp (Table 1). After 30 min of near-UV exposure, the ppGpp levels in the *nuv* mutant cells were induced to 78 μM. Based on the ApppGpp/ppGpp ratio in wild-type cells, the ApppGpp concentration observed when ppGpp levels reached 78 μM would be 150 nM if dinucleotide synthesis were unaffected. However, we have found that ApppGpp levels in the *nuv* mutant cells remained below 15 nM, indicating that this mutant has a specific defect in the

induction of dinucleotide synthesis. We have also measured AppppN levels in response to near-UV. A 15-min exposure results in a fourfold AppppN induction in the wild type, whereas no increases in AppppN levels were observed in the *nuv* mutant, further indicating that the absence of s^{4}U in tRNA results in a defect in the accumulation of adenylylated dinucleotides after near-UV stress.

DISCUSSION

Solar near-UV light is one of the most common environmental stresses to which most organisms need to adapt.

TABLE 1. Effect of *nuvA* mutation on stress-induced changes in intracellular ppGpp and ApppGpp

Conditions	Strain	Concn (μM)[a]		ApppGpp/ppGpp ratio (10³)
		ppGpp	ApppGpp	
Control	Wild type	23	<0.015	<0.65
	nuv	19	<0.015	<0.79
Near-UV[b]	Wild type	790	1.53	1.94
	nuv	37	<0.015	<0.41
Stringent[c]	Wild type	1,040	0.35	0.33
	nuv	1,120	ND[d]	ND

[a] Values shown represent the means of two or three separate experiments containing a standard error of less than 20%.

[b] Cultures were irradiated for 20 min with near-UV light as described in Materials and Methods.

[c] Cells were treated with serine hydroxamate (600 μM) as described in Materials and Methods.

[d] ND, Not determined.

Near-UV exposure is a physiologically relevant stress for many bacterial species, such as coliforms, since transmission between hosts can involve environmental exposure (8). It is reasonable to predict that bacteria have evolved mechanisms to protect themselves from near-UV. We have characterized such protective mechanisms in *S. typhimurium*.

When exposed to sublethal doses of near-UV, *E. coli* enters a growth delay (22). This response is mediated by the tRNA-modified base s⁴U (22, 40), which allows cells to rapidly respond to the onset of near-UV stress. It would be advantageous to the cell if an adaptive response which induces protection from near-UV would be triggered via s⁴U. If this were the case, mutants which lack s⁴U would be more sensitive to near-UV.

We have isolated a *nuv* mutant in *S. typhimurium* and have found it to be more sensitive to killing by a long exposure to a low-intensity, broad-band, near-UV light source, a stress designed to be environmentally relevant. Since the *nuv* mutant lacks s⁴U, this base may be involved in triggering a response which leads to protection from near-UV. Since ppGpp has been implicated in triggering growth delay, we considered it possible that ppGpp has a role in protection from near-UV killing. This possibility is supported by the observation that *relA* mutants were found to be more sensitive to killing by near-UV.

We have characterized the mechanism of this s⁴U- and ppGpp-dependent protection from killing by near-UV. Growth delay alone could not be responsible for this protection because the length of time that entry into growth delay is postponed in longer exposures of the mutant strains did not correlate with the degree of near-UV sensitivity; i.e., the *nuv* and *relA* mutants had the same growth response to near-UV (data not shown), but the *relA* mutants were more sensitive than the *nuv* mutants to killing by near-UV (Fig. 3). Furthermore, this protective response has been found to involve induction of oxidative defense pathways (23), which are not induced by the stringent response (Fig. 4). Bacteria respond to a number of environmental stresses by synthesizing discrete subsets of cellular proteins (30). Near-UV induced at least 57 proteins in wild-type cells, of which 48 had altered induction in the *nuv* mutant. Of these 48 proteins, 17 were not induced by the stringent response alone. Thus, near-UV induced a set of proteins whose induction was dependent on s⁴U but independent of the stringent response (ppGpp itself). The delayed induction of these

proteins may account for the observed sensitivity of the *nuv* mutant to near-UV killing. Among these proteins were six which are also induced by H₂O₂, including both subunits of alkyl hydroperoxide reductase (C22 and F52a), an enzyme shown to be involved in protecting cells from killing by near-UV (23). Three heat-inducible proteins were also induced by near-UV. These results were consistent with the observation of overlapping protein induction among various stresses in *S. typhimurium* (30) and with a report of near-UV induced proteins in *E. coli* (36). Other proteins which may be included in this set of s⁴U-dependent proteins are tRNA repair enzymes, responsible for the repair of photo-cross-linked tRNA after near-UV treatment (2, 19). The induction of such a repair enzyme would allow the cells to resume growth after cessation of near-UV exposure. We also observed a set of near-UV-induced proteins in the *nuv* mutant which were not induced in wild type. These proteins may have been induced by damage which is otherwise protected against by the s⁴U-dependent mechanism. We were also unable to detect any synthesis of catalase HP-I (H₂O₂ inducible) after near-UV exposure of wild-type or mutant cells. This observation is consistent with earlier reports of H₂O₂ not being an important photoproduct under these conditions (23).

One possible mechanism through which s⁴U could mediate the induction of this set of stringency-independent proteins in response to near-UV is via adenylylated nucleotides. The accumulation of these dinucleotides after oxidative stress in *S. typhimurium* (4, 26, 27) has been suggested to be due to the oxidation of modified bases in tRNA (26). It has been proposed that dinucleotides act as alarmones or second messengers which induce a response to stress (4). Our data support a model in which photo-cross-linked s⁴U induces resistance to near-UV killing by both the induction of ppGpp via the stringent response and the initiation of dinucleotide synthesis via altered interaction with aminoacyl-tRNA synthetases which, in turn, leads to increased synthesis of ApppGpp. Synthesis of dinucleotides by tRNA-aminoacyl synthetases has been shown to be altered by certain tRNA base modifications (20), and s⁴U has been reported to be a contact point between at least one tRNA and the corresponding aminoacyl synthetase (42). Consistent with this model is the observation that mutants lacking s⁴U do not accumulate ApppGpp after exposure to near-UV, whereas in wild-type cells this dinucleotide is induced over 100-fold under these conditions. This lack of ApppGpp accumulation in the *nuv* mutant appears to be due to a specific defect in synthesis rather than to limitation of the substrate, ppGpp, and correlates with an inability to induce the synthesis of specific proteins which are induced by near-UV in wild-type cells. Taken together, our data are consistent with a model in which damage to a specific tRNA-modified base serves to stimulate dinucleotide synthesis via interaction with aminoacyl-tRNA synthetases. These dinucleotides then participate in the induction of a specific set of proteins that act to enhance cell survival.

This model, however, does not preclude the additional involvement of other factors in response to near-UV. Since the *relA* mutant is more sensitive to near-UV than the *nuv* mutant, it seems likely that ppGpp itself may play a role in protection from near-UV toxicity in addition to serving as a precursor of ApppGpp. The induction of stringency-dependent proteins by ppGpp could be involved in this protective response. The delayed induction of many stringency-dependent proteins and ppGpp in the *nuv* mutant by near-UV is consistent with this hypothesis. The response of cells to

near-UV may therefore be a complex phenomenon involving induction of both oxidative defense proteins via ApppGpp and stringency-dependent proteins via ppGpp. The differential sensitivity of these strains to near-UV could also be explained by differences in ApppGpp induction. Reduced but significant ppGpp induction has been observed in the *nuv* mutant, which could allow limited ApppGpp induction in response to near-UV-induced cell damage. The reduced induction of ApppGpp in this strain may provide partial protection from near-UV as compared with that in the *relA* mutant, which would be totally unable to induce ApppGpp.

Other investigators have reported induction of stress proteins in the absence of dinucleotide accumulation (37, 49). These data are not necessarily inconsistent with our model for near-UV protein induction by dinucleotides. In the case of heat shock protein induction in the absence of dinucleotide accumulation (49), dinucleotides were assayed by a method which allowed neither the measurement of basal levels nor the detection of the four- to eightfold increases in AppppN that we observe with nonlethal doses of H_2O_2 or near-UV (G. Kramer, J. Baker, and B. Ames, unpublished data). Although other workers have reported that changes in AppppN levels are not necessary for the induction of oxidative stress protein synthesis by H_2O_2 (37), basal levels reported in these experiments were higher than the induced levels which we observed in *E. coli* after treatment with a lethal dose of H_2O_2 (data not shown), suggesting differences in experimental systems which may have allowed the stimulation of oxidative stress protein synthesis in the absence of additional increases in dinucleotide levels. Furthermore, it has been demonstrated that near-UV toxicity under our conditions does not involve the generation of H_2O_2 (23). Although it has been suggested that both heat shock and near-UV are oxidative stresses (4, 23), it is neither necessary nor likely that the mechanisms for inducing protective systems within the cell are identical in detail for each of these stresses.

Although damage to s^4U by near-UV is closely correlated with the accumulation of ApppGpp, near-UV-induced protein synthesis, and cell survival, a causal relationship has not been demonstrated. Other models besides the dinucleotide hypothesis could be set forth to explain the s^4U-dependent induction of proteins in response to near-UV. For instance, the damaged (photo-cross-linked) tRNA could react directly with a regulatory system which operates at the level of transcription, either through direct interaction with regulatory sequences of DNA or through interaction with regulatory proteins (25). It is also possible that dinucleotides act to prolong or modulate the initial response during long-term stress. This hypothesis is consistent with the suggestion that the DnaK protein, believed to be involved in turning off the heat shock response, is regulated by ApppppA (3).

ACKNOWLEDGMENTS

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