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Localization of Low-Molecular-Weight Basic Proteins in *Bacillus megaterium* Spores by Cross-Linking with Ultraviolet Light

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Two low-molecular-weight basic proteins, termed A and B proteins, comprise about 15% of the protein of dormant spores of *Bacillus megaterium*. Irradiation of intact dormant spores with ultraviolet light results in covalent cross-linking of the A and B proteins to other spore macromolecules. The cross-linked A and B proteins are precipitated by ethanol and can be solubilized by treatment with deoxyribonuclease (75%) or ribonuclease (25%). Irradiation of complexes formed in vitro between deoxyribonucleic acid (DNA) or ribonucleic acid and a mixture of the low-molecular-weight basic proteins from spores also resulted in cross-linking of A and B proteins to nucleic acids. The dose-response curves for formation of covalent cross-links were similar for irradiation of both a protein-DNA complex in vitro and intact spores. However, if irradiation was carried out in vitro under conditions where DNA-protein complexes were disrupted, no covalent cross-links were formed. These data suggest that significant amounts of the low-molecular-weight basic proteins unique to bacterial spores are associated with spore DNA in vivo.

Approximately 20% of the protein of dormant spores of *Bacillus megaterium* is a group of low-molecular-weight basic proteins, termed proteins A, B, C, D, E, F, and G (15, 18). Similar proteins have also been found in spores of other bacterial species (15, 18, 22). In *B. megaterium* spores the total level of two of these proteins, proteins A and B, is three times the level of the other five combined (18). All seven proteins are found only in developing and dormant spores, and all are degraded in the first minutes of spore germination (15, 18). The amino acids produced by this proteolysis support much of the protein synthesis early in spore germination (20).

Proteins A, B, and C are located in the central region of the spore, the spore core, but they have not been localized further (15). Their specific location within the spore core would be of interest, because it has been proposed that these proteins are involved in the high resistance of spore DNA in vivo to UV light (16). Dipicolinic acid, a molecule which absorbs UV light and is present at high levels in bacterial spores, is not involved in the resistance of the spores to UV light since dipicolinic acid does not accumulate in spores until after they have become UV light resistant (15). In contrast, proteins A through C (as well as D, F, and G) appear during sporulation at the time the developing spore acquires resistance to UV light, and proteins A through G bind to spore DNA in vitro (15, 16, 18). However, proof that these proteins are bound to spore DNA in vivo is lacking. Attempts have been made to isolate proteins A through C complexed to DNA after gentle spore lysis; despite some evidence to this effect, these experiments have not been conclusive (9, 15). However, there is evidence that in vitro the binding between proteins A, B, and C and DNA is weak (16). Consequently, it is possible that spore disruption, with the attendant dilution and changes of state, could disrupt DNA protein complexes might which exist in vivo.

We reexamined this problem with the aim of cross-linking proteins A through G to neighboring molecules in intact spores and identifying these neighboring molecules. Since chemical cross-linking agents probably do not penetrate into the spore core (4), we used UV light as a cross-linking agent. Many studies have demonstrated that UV irradiation of nucleic acid-protein complexes can cause the formation of covalent cross-links between protein and either DNA or RNA (11).

MATERIALS AND METHODS

Spores, proteins, and enzymes. Spores of *B. megaterium* QM B1551 (originally obtained from Hillel S. Levinson, U. S. Army Research Laboratories, Natick, Mass.) were prepared by growth in supple-

mented nutrient broth, harvested, washed, and stored as previously described (19). Spores with [^{3}H]thymidine-labeled DNA were prepared similarly (14). The fraction containing proteins A through G was isolated from *B. megaterium* spores by direct HCl extraction, dialyzed at 4°C in acetylated tubing against 1% acetic acid, and lyophilized (16, 18). This preparation is referred to as fraction P. Fraction P contains a small amount of spore mucopeptide (1 to 3%, wt/wt) but no nucleic acid. Proteins A, B, C, D, E, F, and G, as well as the spore protease specific for the A, B, and C proteins, were purified from *B. megaterium* spores (16–18). Electrophoretically purified DNase I was obtained from Sigma Chemical Co.; pancreatic RNase and T-1 RNase were obtained from Worthington Biochemicals Corp. Goat anti-rabbit γ-globulin serum was obtained from Miles Laboratories. The crude γ-globulin fraction was isolated from this serum by precipitation three times at room temperature with 33% ammonium sulfate. The final solution was dialyzed at 4°C against 25 mM KPO_4 (pH 7.4)–0.1 M NaCl and stored frozen at one-half the concentration in the original serum. Total RNA was purified from spores of *B. megaterium* as previously described (21). DNA was purified from vegetative cells of *B. megaterium*, and contaminating RNA was removed by digestion with pancreatic and T-1 RNases (8).

Formation of nucleic acid-fraction P complexes. Complexes between fraction P and nucleic acids were formed by the gradient dialysis technique of Shih and Bonner, using acetylated tubing as previously described (16, 23). DNA or RNA was 0.5 to 1 mg/ml, and the ratio of protein to DNA was 3:1 (wt/wt). Complexes with ratios of protein to DNA of up to 5:1 could be made, but significant precipitation of nucleic acid took place. The final dialysis was against 1 mM Tris-hydrochloride (pH 7.4)–0.2 mM EDTA, and the complexes were stored frozen. Analysis of fraction P-DNA complexes by gel filtration on Sephadex G-75 showed that at least 50% of the A antigen and 65% of the B antigen were associated with the DNA. This is a minimum estimate, since Sephadex chromatography would promote dissociation of the complex. Previous work has shown that proteins A, B, and C bind to DNA-cellulose columns, albeit rather weakly (16).

Irradiation of spores or complexes and subsequent purification. Irradiation was carried out at 4°C in 1 mM Tris-hydrochloride (pH 7.4)–0.2 mM EDTA by using a short-wavelength UV lamp (UVS, Ultraviolet Products, Inc.; maximum output at 254 nm) about 2 cm above the liquid. The solution (5 ml) was in a glass petri dish (10-cm diameter) which was swirled throughout irradiation. Volumes were kept constant by additions of water. Spores were irradiated at 10 mg/ml; a small number of spores with [^{3}H]-thymidine-labeled DNA were present to monitor DNA recovery. Nucleic acid-protein complexes were irradiated at a nucleic acid concentration of 0.2 to 0.3 mg/ml.

Soluble protein plus DNA and RNA were extracted from spores as described previously (15). Spores (50 mg) were incubated in a solution containing 2.5 ml of 8 M urea, 1.5% sodium dodecyl sulfate, 0.1 M 2-mercaptoethanol, and Tris-hydrochloride (pH 8.0) for 45 min at 37°C. The spores were washed three times with 5 ml of 4 M urea and three times with 5 ml of 0.1 M Tris-hydrochloride (pH 8.0)–25 mM EDTA (buffer A) and suspended in 3 ml of buffer A. Lysozyme (100 μl of a 20-mg/ml solution) was added, and the suspension was incubated for 15 min at 37°C, cooled, and centrifuged at 78,000 × *g* for 30 min. The supernatant fluid was saved, and the pellet was homogenized with 3 ml of cold buffer A. After another centrifugation at 78,000 × *g* for 30 min, the two supernatant fluids were pooled. Upon extraction of unirradiated spores this fraction contained soluble proteins including more than 95% of the A and B antigens as well as about 90% of spore DNA and RNA (15).

After samples were saved for subsequent analysis, the pooled supernatant fluid from spores extracted as described above and fraction P-nucleic acid complexes were further purified to remove A and B antigens not covalently bound to some macromolecule. Purification took advantage of the fact that A and B antigens alone were not precipitated by ethanol from guanidine hydrochloride, whereas antigen cross-linked to some macromolecule was insoluble under these conditions. Solutions were made 4 M in guanidine hydrochloride by the addition of solid guanidine hydrochloride, and 2 volumes of cold ethanol was added. After 15 min at 4°C, the suspension was centrifuged (10 min, 15,000 × *g*), dissolved in 4 ml of 4 M guanidine hydrochloride–50 mM Tris-hydrochloride (pH 7.4), and precipitated again with ethanol. The pellet was dissolved in 2 ml of 0.1 M Tris-hydrochloride (pH 7.4)–0.15 M NaCl (occasionally sonication was required) and precipitated again with 2 volumes of cold ethanol. The final pellet was air dried to remove ethanol and dissolved in buffer. In some heavily irradiated samples the pellet had to be dissolved in 10 mM NaOH. Approximately 75% of the nucleic acid present in the original sample was recovered in the final pellets. All values given for antigen cross-linked to nucleic acid have been corrected for loss of nucleic acid during purification. This correction assumes that all nucleic acid in our preparations contained the same amount of cross-linked antigen as our final purified material. Although we have no evidence for this assumption, the fact that our dose-response curves are linear (see below) suggests that this assumption is not unreasonable.

In a few cases samples were purified by precipitation with H_2SO_4. Supernatant fluids from spores or nucleic acid-fraction P complexes were made 0.4 M in NaCl and 0.1 M in H_2SO_4. After 15 min at 4°C the mixture was centrifuged (10 min, 10,000 × *g*), and the pellet was washed two times with 3% acetic acid in 50 mM Tris-hydrochloride (pH 7.4)–0.4 M NaCl and dissolved. Both purification procedures gave identical results where tested.

Nuclease digestion of cross-linked A or B antigen. Cross-linked A or B antigen was purified as described above and incubated in 300 μl of 0.05 M Tris-hydrochloride (pH 7.4). Each incubation contained 0.6 to 1.5 mg of nucleic acid. DNase (200 Kunitz units) or RNase (2 μg) was added as noted. Incubations with DNase were made 5 mM in $MgSO_4$. After 2 h at 37°C, samples were taken for assay of A and B antigens, and the remainder was purified by reprecipitation with ethanol from 4 M guanidine hydrochloride

as described above. Incubation for longer than 2 h gave no further digestion.

Preparation of antisera. Because immunization with native A or B protein raised no detectable antibodies, we resorted to using A and B proteins which had been cross-linked to themselves with glutaraldehyde. A protein or B protein (5 mg/ml) was incubated in 50 mM KPO_4 (pH 7.0) with 7 mM glutaraldehyde. After 2 h at 24°C, 0.5 ml of 50 mM lysine was added and incubation was continued for 1 h. The proteins were dialyzed overnight in acetylated tubing against 1 mM Tris-hydrochloride (pH 7.0) and stored frozen.

New Zealand white female rabbits (weight, 6 pounds [ca. 2.7 kg]) were injected intradermally in five sites on the back with a 1:1 mixture of cross-linked protein and Freund complete adjuvant. Rabbits received 1.2 mg of A protein or 2 mg of B protein. After 30 days each rabbit was boosted intravenously with 0.5 mg of cross-linked A protein or 0.7 mg of cross-linked B protein, and rabbits were bled after 6, 8, and 10 days. The boosting procedure was repeated after another 30 days; however, sera from all bleedings gave similar titers. Sera were allowed to clot overnight at 4°C and centrifuged (30 min, 25,000 × *g*), and the γ-globulin fraction was isolated, dialyzed, and stored frozen at the concentration in the original serum. The γ-globulin fraction from unimmunized rabbits was a gift from Michael Connors, University of Connecticut Health Center.

Radioimmunoassay for A or B protein. Tests of all sera by Ouchterlony double diffusion gave no precipitin lines with A or B protein. Consequently, we prepared iodinated antigen in order to set up a radioimmunoassay. Fortunately, both A and B proteins contain single tyrosine residues (16). The tyrosyl residues in both proteins have normal pK values (P. Setlow, unpublished data), suggesting that they are on the outside of the molecule. Consequently, we iodinated the A and B proteins by using the chloramine T procedure (5). A protein or B protein (500 μg) was incubated in 1 ml of a solution containing 0.25 M KPO_4 (pH 7.5) and 200 μg of chloramine T per ml. Carrier-free ^{125}I (0.25 mCi; New England Nuclear Corp.) was added, and after 3 min at 4°C the reaction was halted by the addition of 150 μl of sodium metabisulfite (10 mg/ml). This solution was passed through a Sephadex G-10 column (1 by 12 cm) equilibrated at room temperature in 0.1 M NaCl–50 mM KPO_4 (pH 7.4). Protein fractions were pooled, dialyzed in acetylated tubing against four 1-liter changes of 0.1 M NaCl–50 mM KPO_4 (pH 7.4), and frozen in small samples. The yield was about 80 μCi of iodinated A or B protein.

Radioimmunoassays were carried out in 200 μl of a solution containing 0.1 M NaCl, 25 mM KPO_4 (pH 7.4), and 2 mg of bovine serum albumin per ml with 10 μl of a 1:50 dilution of unimmunized rabbit γ-globulin and 10 ng of iodinated A or B protein. With this amount of antigen in the assay, 10 μl of a 1:75 dilution of anti-A γ-globulin or 5 μl of undiluted anti-B γ-globulin gave optimal results and precipitated about 70% of the labeled antigen. After incubation for 1 h at 37°C the mixture was incubated overnight at 4°C. Antibody-antigen complexes were then precipitated by the addition of goat anti-rabbit γ-globulin serum. Optimum precipitation was obtained with 15 μl in the anti-A assay and 100 μl in the anti-B assay. After incubation for an additional 2 h at 4°C, the mixture was centrifuged (25 min, 10,000 × *g*), the supernatant fluid was removed by aspiration, and the pellet was counted in a γ-counter. Determination of unknown amounts of A or B antigen was by reference to calibration curves constructed by using pure A or B protein.

Other methods. Protein was determined by the procedure of Lowry et al., and DNA and RNA were deteined with diphenylamine and orcinol, respectively (7, 12). Dipicolinic acid was determined as described by Rotman and Fields (10). The output of our UV lamp was determined with a ferric oxalate actinometer (13). These determinations were carried out under the exact conditions of irradiation of complexes or spores and were also done in the presence of appropriate amounts of nucleic acid or spores to correct as well as possible for absorption and scatter.

RESULTS

Development of radioimmunoassay. Preliminary experiments in which a [^3H]valine-labeled fraction P-DNA complex was irradiated suggested that some of the ^3H-labeled protein was cross-linked to the DNA. However, analysis of the species bound was difficult, because the only method for assay of proteins A through G was gel electrophoresis. Consequently, analysis of proteins which were cross-linked to DNA by irradiation of intact spores required a new sensitive assay. The assay we developed was a radioimmunoassay.

Antisera were raised in rabbits against both the A and B proteins. Although the titers of these sera were not high, they were sufficient for a sensitive radioimmunoassay using ^{125}I-labeled antigen. The assay with the anti-A protein serum gave a smooth standard curve and could detect 2 ng of A protein (Fig. 1). Similar data were obtained with the anti-B protein serum (data not shown). The sera were reasonably specific, with proteins D through G cross-react-

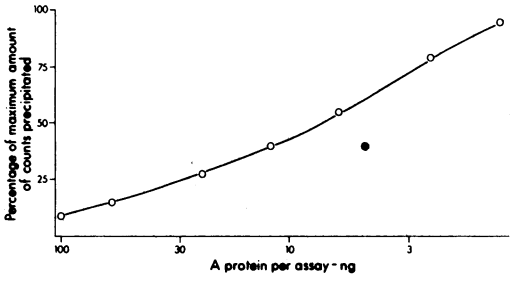

FIG. 1. *Calibration curve for radioimmunoassay of A protein. The radioimmunoassay was carried out with anti-A protein serum, ^{125}I-labeled A protein, and varying amounts of unlabeled A protein as described in the text.*

ing poorly with both anti-A and anti-B protein sera (Table 1). However, there was a small but significant cross-reaction between the A and C proteins and the anti-B protein serum, and between the B protein and the anti-A protein serum (Table 1). The C protein reacted almost as well as the A protein with anti-A protein serum. Strikingly, the antigenicity of both the A and B proteins was decreased an order of magnitude by treatment with the spore protease (Table 1). This endoprotease cleaves only one bond in the A protein and two in the B protein (17) (P. Setlow, unpublished data). The antigenicity of these proteins was not affected by prior incubation in NaOH, HCOOH, 4 M guanidine hydrochloride, or H_2SO_4. Similarly prolonged UV irradiation had no significant effect on the majority of the A and B antigens in fraction P (Table 1).

TABLE 1. *Cross-reaction of various proteins with anti-A protein serum or anti-B protein serum*

Protein	Cross-reaction[a]	
	Anti-A protein serum	Anti-B protein serum
A	1.0	0.06
B	0.09	1.0
C	0.39	0.04
D	<0.007	<0.003
E	0.01	0.005
F	<0.001	<0.001
G	<0.001	<0.001
A, dissolved in 0.1 M NaOH,[b] 88% HCOOH, 0.1 M H_2SO_4, or 4 M guanidine hydrochloride[b]	0.89–1.05	—
B, dissolved in 0.1 M NaOH,[b] 88% HCOOH, 0.1 M H_2SO_4, or 4 M guanidine hydrochloride[b]	0.09–0.13	0.9–1.1
A, spore protease treated[c]	0.09	—
B, spore protease treated[c]	0.01	0.06
Fraction P	0.5	0.3
Fraction P irradiated[d]	0.55	0.25

[a] All proteins were assayed for inhibition in the radioimmunoassay as described in the text. For each protein tested data were calculated by using a calibration curve constructed with either pure A or B protein (see Fig. 1). The cross-reaction is given as milligrams of A (or B) antigen per milliliter, as determined in the radioimmunoassay, divided by the actual protein concentration determined chemically.

[b] Proteins were dissolved in the indicated solution and incubated at 4°C for 2 h. Samples were then diluted into immunoassay buffer and analyzed.

[c] Samples of A or B protein were digested with spore protease as previously described and then made 10 mM in EDTA to inactivate the spore protease (17).

[d] Fraction P (1 mg/ml) was irradiated with 12×10^4 ergs/mm^2 as described in the text.

UV irradiation of fraction P-DNA or fraction P-RNA complexes in vitro. With the availability of a sensitive, specific assay for the A and B proteins, we could now examine the cross-linking of these species to nucleic acids upon irradiation with UV light. We chose to irradiate fraction P-nucleic acid complexes rather than complexes of A or B protein with nucleic acid for two reasons: (i) this was much more sparing of the homogeneous proteins; and (ii) it was possible that there might be interactions among various proteins in fraction P which migh facilitate complex formation with nucleic acid. Indeed, interaction among various histones is extremely important in their appropriate binding to DNA (6). In one experiment, we did find that irradiation of a complex of pure A protein with DNA resulted in cross-linking of the A protein to the DNA with an efficiency about 40% of that for cross-linking between fraction P and DNA (data not shown).

Irradiation of a fraction P-DNA complex in vitro resulted in significant cross-linking of A antigen to DNA (Table 2). The dose-response curve was linear, and at the highest dose used 1.3% of the A antigen was cross-linked. We believe that the antigen became covalently cross-linked to the DNA, since bound antigen was not removed from the DNA by further washing with H_2SO_4 or guanidine hydrochloride (Table 2). However, if DNA was not present during the irradiation or if there was no irradiation, then almost no antigen behaved as if it were linked to a macromolecule (Table 2). In addition, if irradiation of this complex was carried out in the presence of high salt or urea or both, added before or after complex formation, then the amount of antigen remaining with the DNA was reduced to background levels (Table 2).

Both A and B antigens were cross-linked to DNA and RNA by UV irradiation (Fig. 2a and b). Although the dose-response curves were similar for cross-linking of A or B antigen to a given nucleic acid, approximately twice as much energy was required to cross-link protein to RNA as to DNA (Fig. 2a and b).

Irradiation of intact spores. Since proteins A and B became covalently bound to nucleic acids upon UV irradiation in vitro, it was worthwhile to examine the effect on dormant spores. Irradiation of dormant spores resulted in significant cross-linking of A and B antigens to some spore macromolecules (Fig. 3a). Both A and B antigens were cross-linked to spore macromolecules with equal efficiency, and the dose-response curve was similar to that for irradiation of a fraction P-DNA complex in vitro (Fig. 2a

TABLE 2. *Irradiation experiments*[a]

Sample	Irradiation	Purification procedure	% of A antigen bound
Expt 1[b]			
Fraction P	Yes or no	Guanidine or H_2SO_4	<0.01
DNA-fraction P complex	No	Guanidine or H_2SO_4	0.01
DNA-fraction P complex	Yes	H_2SO_4	0.65 (0.64)[c]
DNA-fraction P complex	Yes	Guanidine	0.63 (0.61)[d]
Expt 2			
DNA-fraction P complex	Yes	Guanidine	0.81
DNA-fraction P complex + 2 M $(NH_4)_2SO_4$	Yes	Guanidine	0.04
DNA-fraction P complex + 1 M NaCl + 4 M urea	Yes	Guanidine	0.2
DNA-fraction P complex	No	Guanidine	0.02
Expt 3[e]			
DNA + fraction P	Yes	Guanidine	(0.81)[f]
DNA + fraction P + 1 M NaCl	Yes	Guanidine	0.025
DNA + fraction P + 4 M urea	Yes	Guanidine	0.08
DNA + fraction P + 4 M urea + 1 M NaCl	Yes	Guanidine	0.01

[a] All irradiations were with 12×10^4 ergs/mm^2. Irradiation, purification, and analyses were as described in the text.

[b] In this experiment, the purification procedure used only one precipitation with H_2SO_4 or one precipitation from guanidine.

[c] Value in parentheses was determined after two additional precipitations by H_2SO_4.

[d] Value in parentheses was determined after an additional precipitation from guanidine.

[e] In this experiment, fraction P and DNA were mixed together at a 3:1 ratio with additions as noted just before irradiation.

[f] Mixing DNA and fraction P together without salt or urea present resulted in precipitation of most of the DNA. Consequently, we could not test this sample experimentally. However, irradiation of a DNA-fraction P complex at the concentration used in this experiment gave the value in parentheses.

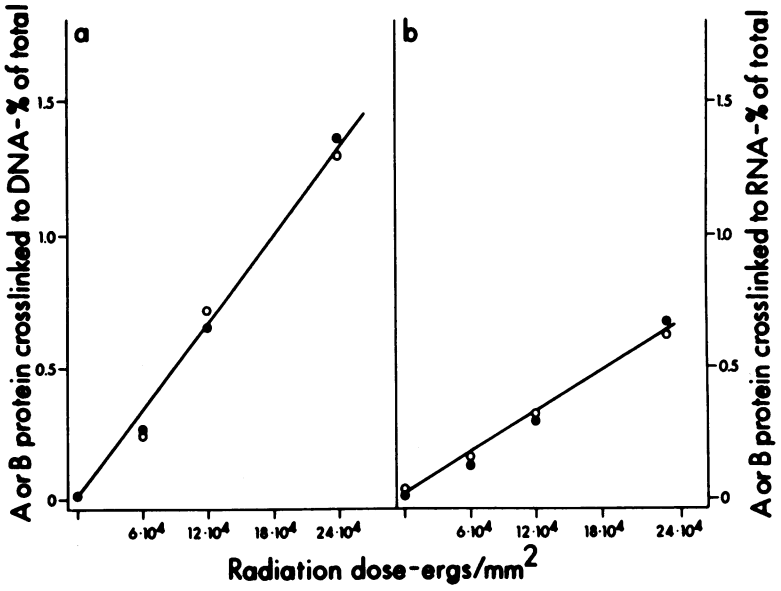

FIG. 2. *Cross-linking of A and B protein to DNA (a) or RNA (b). DNA-fraction P complexes (a) or RNA-fraction P complexes (b) were irradiated, purified, and assayed and the data were calculated as described in the text. Symbols: ○, A antigen; ●, B antigen.*

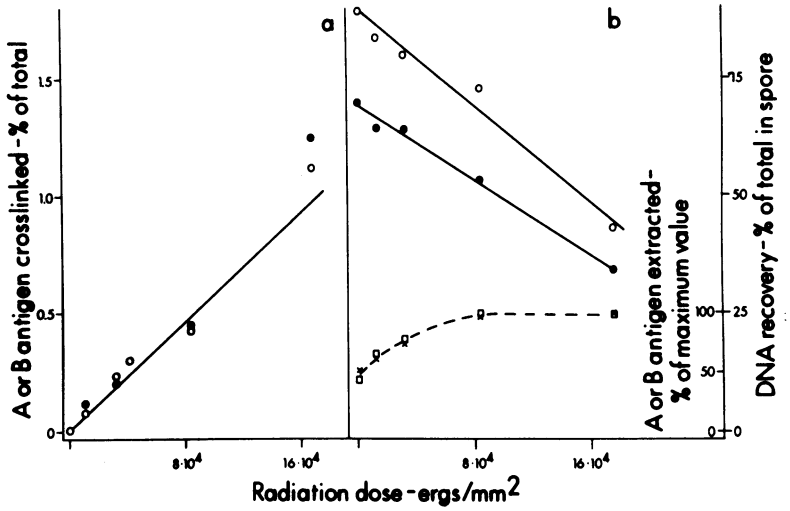

FIG. 3. *Effect of irradiation of intact spores on (a) cross-linking of A and B antigens to spore macromolecules and (b) recoveries of DNA and yield of A and B antigens. Intact dormant spores with some [^{3}H]-thymidine-labeled spores were irradiated, extracted, purified, and analyzed as described in the text. DNA recoveries were determined by counting samples of various solutions. Because subsequent experiments (Table 3) indicated that the majority of the A and B antigens were cross-linked to DNA, the data in (a) has been corrected for the recovery of DNA in the final samples. (a) Symbols: ○, A antigen; ●, B antigen. (b) Symbols: ○, DNA extracted from spores; ●, DNA recovered after purification by precipitation from guanidine hydrochloride, □, A antigen; ×, B antigen.*

and 3a). Despite the significant cross-linking of A and B antigens at high radiation doses, there was no effect on the molecular weight of the A and B antigens not cross-linked (>98% of the total), as shown by chromatography on Sephadex G-75 (data not shown).

Irradiation of spores also significantly increased the total amount of A and B antigens extracted from spores (Fig. 3b). This was not due to more efficient extraction from heavily irradiated spores, but rather to significant cleavage of A and B antigens by spore protease during lysis of untreated and lightly irradiated spores, as shown previously (15). Presumably the spore protease is inactivated at high radiation doses. When untreated spores were broken and extracted under conditions precluding spore protease action (15), yields of A and B antigens were identical to those from heavily irradiated spores (data not shown).

In contrast to the effect of irradiation on the yield of the A and B antigens, irradiation decreased the yield of spore DNA (Fig. 3b). Possibly, some spore DNA became cross-linked to membrane proteins, or it may be that protein-protein cross-links were introduced into the spore coat, making DNA extraction difficult. However, even at the highest dose given intact spores, they remained bright in a phase-contrast microscope, and none (<5%) of their dipicolinic acid was lost. The spores were killed (>90%) by a dose of 10^4 ergs/mm^2 (data not shown).

Nuclease digestion of cross-linked A and B antigens. The results with intact spores described above made it imperative to determine the macromolecule(s) to which the A and B antigens became cross-linked. Treatment of the cross-linked antigen from spores with DNase resulted in a twofold drop in its antigenicity, even without further purification (Table 3). This suggests that A or B antigen covalently linked to nucleic acid is a better antigen than the proteins alone. This is not wholly surprising, since the original immunogens were cross-linked with glutaraldehyde.

Since there appears to be a difference in the antigenicity of the A and B proteins depending on whether they are cross-linked, it is not possible to correlate directly the amount of A or B antigen cross-linked with the amount of A or B protein cross-linked. Purification of the cross-linked antigen by reprecipitation showed that DNase treatment had removed about 75% of the A and B antigens (Table 3). DNase treatment of fraction P protein cross-linked to DNA gave similar results, but even more antigen was removed (Table 3). DNase treatment had no effect on the A and B antigens when they were not cross-linked to DNA; similarly, DNA and RNA alone had no effect on the radioimmunoassay (data not shown). RNase treatment of fraction P cross-linked to RNA solubilized about 95% of

TABLE 3. *Nuclease treatment of cross-linked A and B antigens*[a]

Preparation	% of A antigen remaining after the following treatments:		
	None	DNase	RNase
Fraction P cross-linked to DNA			
After incubation	96	40 (45)[b]	
After reprecipitation	91	9 (6)	
Fraction P cross-linked to RNA			
After incubation	101		43 (69)
After reprecipitation	87		12 (11)
Antigen cross-linked to spore macromolecules[c]			
After incubation	98	45 (49)	90 (88)
After reprecipitation	85	22 (20)	65 (62)

[a] Samples were incubated with enzymes present as noted, precipitated, and analyzed as described in the text. The samples used were the cross-linked material purified from a DNA-protein complex, an RNA-protein complex, and spores; these samples were irradiated with 12×10^4, 12×10^4, and 8.3×10^4 ergs/mm^2, respectively.

[b] Values in parentheses are for the B antigen determined with anti-B serum.

[c] Data similar to these were also obtained with spores irradiated with 3×10^4 ergs/mm^2.

the antigen, but similar treatment solubilized only about 25% of the cross-linked A and B antigens from spores (Table 3).

DISCUSSION

UV irradiation of complexes of nucleic acid with a mixture of the A through G proteins results in significant covalent cross-linking of the proteins to the nucleic acid. The dosages required for cross-linking A or B protein to nucleic acid in vitro were similar to those others have used (11). The B protein in fraction P accounts for 85% of the antigen with anti-B protein serum, whereas the A plus C proteins account for 95% of the antigen with anti-A serum (calculated from the amounts of proteins A through G in spores [18] and the data in Table 1). Consequently, the similar dose-response curves for cross-linking of A and B antigens suggest that both proteins become linked to nucleic acid with equal efficiency. Because of the cross-reaction of the C protein with anti-A serum, it is possible that only protein C and not protein A is cross-linked to nucleic acid by UV light. However, we feel that this is extremely unlikely for two reasons: (i) the C protein comprises only about 10% of the antigen in fraction P with anti-A serum (calculated from the amounts of proteins A through G in spores [18] and the data in Table 1); and (ii) proteins A and C contain the same spectrum of amino acids (18). Consequently, it seems more likely (although we have no direct evidence for it) that proteins A and C are also cross-linked to nucleic acid with equal efficiency.

Our experiments on irradiation of fraction P-nucleic acid complexes in vitro were meant to provide a comparison for results with intact spores. Clearly, there are large differences between the two cases. For instance, in vivo the ratio of proteins A through G to DNA is 9:1 and to RNA is 1.5:1 (15, 18); our complexes formed in vitro had ratios of protein to nucleic acid of only 3:1. Unfortunately, at higher ratios of protein to nucleic acid in vitro much of the nucleic acid precipitated, with complete precipitation taking place at ratios between 5:1 and 6:1 (B. Setlow and P. Setlow, unpublished data). Second, the conditions under which we irradiated the complexes in vitro must be different from conditions in vivo with regard to parameters such as protein and nucleic acid concentration. Ionic strength, pH, and free divalent ion concentration also probably differ substantially, and these might affect the efficiency of irradiation-induced protein-nucleic acid cross-linking. However, with regard to the latter parameters, it is not known what their values are within intact spores. Consequently, it was difficult to decide what conditions to use in vitro. Therefore, we chose those which would maximize protein-nucleic acid interactions in hopes of maximizing cross-link formation in vitro. Indeed, when protein-nucleic acid interactions were abolished with high salt or urea or both, protein-nucleic acid cross-linking was also abolished.

Keeping in mind the proviso that conditions within the spore may affect the efficiency of UV-induced nucleic acid-protein cross-linking, it is striking that the dose-response curve for the cross-linking of A and B antigens in intact spores is similar to the curve for a DNA-fraction P complex in vitro. Since more than 90% of the cross-linked antigen is solubilized by nuclease treatment, these data suggest that the majority of the A and B (as well as C and, by inference, D through G) proteins in spores are associated with nucleic acid. The finding that 75 and 25% of the cross-linked antigen from spores is solubilized by DNase and RNase, respectively, further suggests that 60 and 40% of the A and B (and possibly C through G) proteins are associated with DNA and RNA, respectively. (These values were calculated by correcting for the relative efficiency of formation of protein-DNA and protein-RNA cross-links, as shown in Fig. 2.) There are, of course, alternative explanations for our data. However, we feel that they are unlikely. These other explanations and our reasons for discarding them are given below. (i) Only a

small amount of A and B proteins, perhaps 10%, is associated with nucleic acid in vivo, but the efficiency of UV-induced cross-link formation in dormant spores is 10-fold higher than in vitro. We feel that this is a less likely explanation because (a) it would then be fortuitous that our dose-response curves are similar in vivo and in vitro, and (b) there is no evidence that the efficiency of cross-linking is different in vivo and in vitro. (ii) Proteins A through G are not normally associated with nucleic acid in vivo, but irradiation destroys some barrier within the spores. Destruction of this barrier then allows protein-nucleic acid association and eventual cross-link formation. We feel this is unlikely, in part because no dipicolinic acid leaks out of the spores during irradiation. It is difficult to imagine irradiation destroying a barrier to protein movement without allowing movement of small molecules out of the spore. In addition, movement of charged molecules within the spore is thought to be slow (2). (iii) Proteins A through G are not associated with nucleic acid. However, upon irradiation of spore nucleic acid some activated intermediate(s) is formed which can then react with proteins A through G in a second-order process. Our reasons for discarding this hypothesis are twofold: (a) movement of charged molecules within dormant bacterial spores is thought to be slow, much slower than in free solution (2); and (b) under conditions where nucleic acid-fraction P complexes were disrupted, irradiation generated no covalent cross-links in vitro.

If the conclusion we feel is most appropriate from our data is correct (i.e., that a significant amount of proteins A through G is associated with spore DNA in vivo), then this lends further support to a suggestion made previously that proteins A through C as well as D through G may be involved in the high resistance of spores to UV light (16). These proteins appear during sporulation at the time the developing spores become resistant to UV light. During this period there is also a change in the appearance of spore DNA in the electron microscope (1). From the results given in this report we suggest that some of these changes in the DNA of the developing spore are due to the binding of large amounts of proteins A through G.

It is clear, however, that the presence of proteins A through G within spores is of itself not sufficient for resistance to UV light. Spore protease mutants of *B. megaterium* which show slowed degradation of proteins A, B, and C during spore germination lose UV light resistance at their normal rapid rate (9). Therefore, proteins A through G can only be necessary for UV light resistance, not sufficient. Possibly, there is some other crucial change within the developing spore at the time of acquisition of UV light resistance. This may be an accumulation of some small molecule or a change in the environment within the spore. This change, together with the binding of proteins A through G to spore DNA, may then facilitate the conformational change in spore DNA which results in resistance to UV light (3, 24).

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