Unstable Association In Vitro Between Donor and Recipient DNA in *Bacillus subtilis*

JAN VAN RANDEN,* KOOS WIERSMA, AND GERARD VENEMA

Department of Genetics, Biology Center, 9751 NN Haren, The Netherlands

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In addition to stable donor-recipient DNA complexes, unstable complexes between donor and recipient DNA were formed in vitro with *Bacillus subtilis*. Whereas the stable complexes survived CsCl gradient centrifugation at pH 11.2 and phenol plus sodium *p*-aminosalicylate extraction with 0.17 M NaCl, the unstable complexes dissociated during these manipulations. The donor moiety from the unstable complexes remained associated with the recipient DNA during phenol plus sodium *p*-aminosalicylate treatment at 0.85 M NaCl. The unstable complexes could be stabilized artificially by cross-linking with 4,5',8-trimethyl-psoralen. Dissociation of the complexes during CsCl gradient centrifugation could be prevented by centrifuging at pH 10. Heterologous DNA fragments derived from phage H1 DNA appeared to be unable to form complexes with the recipient *B. subtilis* DNA. Unstable complexes were also formed with *Escherichia coli* DNA, although under all conditions tested, more complex was detectable by using homologous *B. subtilis* DNA.

An intriguing question is by what mechanism recombination during bacterial transformation is initiated. Base pairing is probably involved in the early steps of recombination. In *Bacillus subtilis*, the entering donor DNA molecules are single stranded (11, 15). The double-stranded nature of the recipient DNA seems to limit base-pairing events with the donor molecule. However, the recipient DNA might expose bases for pairing with the entered DNA through the presence of single-stranded (16), or superhelical regions (13, 22), which behave as if they were transiently single-stranded (3). In addition, proteins may be involved in the opening of the double helix and in stabilizing the open regions.

The latter possibility is attractive, particularly since recent investigations have shown that purified *recA* protein of *Escherichia coli* promotes pairing between homologous DNA molecules of various structures. For instance, *recA* protein promotes the pairing of linear single-stranded DNA to homologous regions in duplex DNA to form a D-loop (9, 14, 20). Homologous pairing between two duplex DNA molecules is also promoted if one of the molecules contains at least one short single-stranded region (6, 8). A free end is not essential for the promotion of homologous pairing by *recA* protein, since it has been observed that specific complexes were formed between superhelical phage G4 DNA and single-stranded circular M13 DNA which carried an insertion of 274 bases from phage G4. This indicates that *recA* protein can promote

side-by-side homologous pairing of a single strand with duplex DNA, in which the newly paired strand is not interwound with its complement (10).

In a previous paper we have described an in vitro association system that uses nucleoid-containing lysates of *B. subtilis* and single-stranded homologous donor DNA molecules (22). At physiological temperatures, it appeared that superhelicity of the recipient nucleoids was not required to form donor-recipient DNA complexes. However, a requirement for recipient strand separation and homology was clearly established for the formation of at least the majority of the complexes detected. In the course of these experiments, it was observed that complexes were lost during the experimental procedure (22). Because these unstable complexes are probably precursors of the complexes previously described and therefore might represent very early intermediates in recombination, it was of interest to investigate the nature of their instability.

The results presented in this report provide evidence that the unstable complexes are base paired to some extent and that their dissociation is caused by treatments interfering with DNA helix stability.

MATERIALS AND METHODS

Strains. *B. subtilis* 168 strain 1G33 (*trpC2*) or strain 8G5, an auxotrophic strain (essential markers, *ade*, *trpC2*, and *tyr-1* [4]), were used for the preparation of competent cultures. Tritiated, ^{15}N , and ^2H density-

labeled donor DNAs were isolated from *B. subtilis* 168 strain 2G8 (*tyr-1 thy*) and *Escherichia coli* K-12 Hfr *thy*. Unlabeled donor DNA was isolated from *B. subtilis* 168 strain 1G21 (*tyr-1*). Bacteriophage H1 has been described by Arwert and Venema (2).

Media and chemicals. Unless stated otherwise, all chemicals were obtained from BDH, Poole, England.

The medium for *B. subtilis* consisted of Spizizen minimal salts (21) plus 0.5% glucose, 0.02% casein hydrolysate (Difco Laboratories, Detroit, Mich.), and growth factors (20 μ g of each per ml). The minimal medium for *E. coli* consisted of M-9 glucose minimal medium (1) supplemented with 0.02% casein hydrolysate, 20 μ g of thymidine per ml, and 1 μ g of thiamine per ml. Starvation medium was composed of Spizizen minimal salts plus 0.5% glucose.

Preparation of competent cells. Competent cells were prepared as previously described (22).

Preparation of transforming DNA. Heavy radioactive DNA containing [*methyl-³H*]thymidine and the density isotopes ¹⁵N and ²H was isolated from cultures of *B. subtilis tyr-1 thy* and cultures of *E. coli* K-12 *thy* as previously described (22).

Unlabeled transforming DNA was isolated in essentially the same way, by using cultures of *B. subtilis tyr-1* grown overnight in minimal medium.

Preparation of ³H-labeled phage H1 DNA. *B. subtilis* 8G5 was grown at 37°C with vigorous aeration in minimal medium to an absorbance at 450 nm of 1.0 as measured in a Zeiss PMQII spectrophotometer. The cells were washed once with minimal medium by centrifugation and suspended in the original volume of prewarmed minimal medium supplemented with essential growth factors except for adenine, and incubation was continued for 1 h at 37°C. Deoxyadenosine (0.6 μ g/ml) and deoxy-³H]adenosine (20 μ Ci/ml, specific activity 12 Ci/mmol; Radiochemical Centre, Amersham, England) were added, and the cells were infected with bacteriophage H1 with a multiplicity of approximately 1. Lysis occurred after 2 to 3 h of further incubation. DNase I (10 μ g/ml), pancreatic RNase (10 μ g/ml; E. Merck, Darmstadt, Germany), and 0.01 M MgCl₂ were added to the lysate, and incubation was continued for 30 min at 37°C. Phage lysates were purified by two subsequent low-speed centrifugation runs (6,000 rpm, 10 min, 20°C; MSE high speed 18 centrifuge); thereafter, phage particles were pelleted and washed three times with standard saline citrate (0.15 M NaCl plus 0.015 M sodium citrate) by high-speed centrifugation (35,000 rpm, 15 min, 20°C; SW41 rotor, Beckman ultracentrifuge). Then ³H-labeled H1 DNA was isolated as described (2). The specific activity of the DNA was approximately 10⁶ cpm/ μ g.

Thermal inactivation of transforming DNA. A DNA solution of 100 μ g/ml in micro-test tubes (Eppendorf) was slowly heated in a water bath. The desired temperatures were kept constant for at least 5 min, and 50- μ l samples were taken and mixed immediately with 450 μ l of ice-cold dilute saline citrate (0.015 M NaCl plus 0.0015 M sodium citrate). The samples were dialyzed twice for 10 h against dilute saline citrate at 4°C, and the biological activity of the DNA was tested by transformation of competent *B. subtilis* cells. In some experiments, the physical state of the DNA was also investigated by isopycnic centrifugation in CsCl at pH 11.2.

Phenol preparation. Phenol was washed once with a saturated tetraborate solution, once with 0.01 M Tris-hydrochloride (pH 7.4)–1 mM EDTA, and twice with 0.01 M Tris-hydrochloride (pH 7.4). Washed phenol was stored refrigerated at –20°C until use.

Transformation. The biological activity of transforming DNA was measured by transformation of competent *B. subtilis* 8G5 cells. A stock of competent cells had been frozen at –80°C in the presence of 10% glycerol in small samples. After quickly thawing, the cells were transformed with 1.5 μ g of DNA per ml during 45 min at 34°C. Further uptake of DNA was prevented by the addition of DNase I (40 μ g/ml) and 0.01 M MgCl₂. Transformants were scored on appropriately supplemented minimal agar plates.

Donor DNA preparation for in vitro complex formation. Sonicated, denatured donor DNA molecules were prepared as described previously (22). In some experiments, the DNA molecules carried 4,5',8-trimethylpsoralen (Paul B. Elder Co.) monoadducts. In the experiments presented, unmodified and monoadducted DNA behaved identically.

Lysates. Lysates containing nucleoids of competent *B. subtilis* and donor-recipient DNA complex formation in vitro were essentially the same as described previously (22).

All glassware and centrifugation tubes used in these experiments had been immersed previously for 30 s in a solution containing 0.2% Aquasil (Pierce Chemical Co.).

Sucrose gradient centrifugation. Sucrose gradient centrifugation and subsequent fractionation were as described previously (22). In some experiments, the dialyzed fractions were cross-linked with trimethylpsoralen just before being subjected to isopycnic centrifugation in CsCl.

Cross-linking by 4,5',8-trimethylpsoralen. One part of a saturated trimethylpsoralen solution in absolute ethanol was added to 50 parts of the DNA solution to be cross-linked. The mixtures were transferred into Aquasil-treated scintillation vials and irradiated for 3 h at 4°C with a long-wave UV lamp (Chromatovue transilluminator model C-60; Ultraviolet Products Inc., San Gabriel, Calif.). To eliminate short-wave components of the spectrum, we used a filter consisting of a potassium biphtalate solution (0.245 M, optical path 1 cm; Merck) together with a Plexiglas filter (optical path, 0.4 cm). The incident light intensity corresponded to 0.4 mW/cm².

Isopycnic centrifugation. The volume of the samples was adjusted to 7.3 ml with 1 mM EDTA–0.15 M NaCl (pH 8.5). Then, 0.9 ml of 0.4 M K₂HPO₄ solution of pH 10.35 or pH 11.35 was added to obtain a final pH of 10 or 11.2, respectively. Solid CsCl (extra pure; Merck) was added to adjust the refractive index to 1.4020 (25°C). Centrifugation was carried out in polyallomer tubes in the 40 fixed-angle rotor of a Beckman ultracentrifuge at 30,000 rpm and 20°C for 40 or 60 h. Gradients were fractionated from the top. Fractions (ca. 0.4 ml) were collected in micro-test tubes (Eppendorf); 0.15-ml samples of the fractions were used to determine trichloroacetic acid-precipitable radioactivity.

Pronase treatment. Samples to be treated with pronase were dialyzed overnight against 0.15 M NaCl–1 mM EDTA (pH 8.5) at 4°C. Pronase (400 μ g/ml, B-grade, previously autolyzed during 1 h at 45°C,

Calbiochem, La Jolla, Calif.) was then added, and the mixtures were incubated at 37°C for 4 h.

Nuclease S1 treatment. Nuclease S1 (Boehringer Mannheim Corp., New York) was dissolved in 0.1 M sodium acetate (pH 4.5)–0.1 mM ZnSO₄–50% glycerol to a concentration of 10,000 U/ml and stored at –20°C. Samples to be treated with S1 nuclease were dialyzed twice against dilute saline citrate at 4°C. Then, 0.4 ml of the samples was mixed with 0.05 ml of a buffer containing 0.3 M sodium acetate, 1 mM ZnSO₄, and 0.67 M NaCl, adjusted to pH 4.5 with acetic acid. Heat-denatured low-polymer calf thymus DNA was added to a final concentration of 20 µg/ml, and the volume was adjusted to 475 µl with dilute saline citrate. After addition of 25 µl of S1 nuclease solution the mixtures were incubated at 50°C for 30 min in Eppendorf test tubes. Control samples were incubated identically without nuclease S1; 0.15-ml amounts of the samples were used to determine trichloroacetic acid-precipitable radioactivity.

When carried out under these conditions, nuclease S1 did not degrade double-stranded DNA, whereas single-stranded DNA was almost completely degraded.

Determination of radioactivity. Radioactivity was determined with a Nuclear Chicago Mark II or with a Packard 460 CD liquid scintillation counter. Samples to be measured were transferred onto Whatman 3MM filter paper disks. The filters were dried, washed once with 10% trichloroacetic acid, once with 5% trichloroacetic acid, and twice with 96% ethanol, dried again, and placed into vials filled with 5 ml of Xylofluor (J. T. Baker Chemicals, Deventer, The Netherlands). ³H counts were corrected for crossing over of ¹⁴C counts in the ³H channel.

RESULTS

Loss of donor-recipient DNA complexes formed in vitro. A nucleoid lysate, prepared from competent *B. subtilis* cells, was incubated with single-stranded donor DNA molecules under various conditions at 37°C. After phenol extraction, the mixtures were sedimented on sucrose gradients, and the fractions containing the bulk of the recipient DNA were collected and subjected to CsCl density gradient centrifugation at pH 11.2 (Fig. 1). It is clear that after complex formation a fraction of the donor DNA cosedimented with the recipient DNA, indicating that complexes had been formed in vitro between the donor and the recipient molecules. These results are identical to those reported previously (22). However, when the collected sucrose gradient fractions were centrifuged in CsCl density gradients, only a relatively small amount of the donor material originally cosedimenting with the recipient DNA appeared to be of the same density as the recipient DNA. The majority of the donor molecules banded at higher densities.

One possible explanation for the apparent dissociation of the donor and recipient moieties was that the cosedimenting donor material was largely composed of uncomplexed donor DNA molecules of size ranges equal to the recipient

DNA segments. However, this explanation was made improbable by the observation that the sonicated donor DNA preparation did not contain DNA molecules with sedimentation values as high as the recipient DNA (data not shown). The major part of the donor-recipient DNA complexes formed was therefore unstable and was lost in a step following sucrose gradient centrifugation.

This conclusion was supported by the results of an experiment in which after in vitro complex formation, the mixture was diluted with lysis buffer to a concentration of 0.17 M NaCl before the addition of sodium *p*-aminosalicylate and phenol extraction. Figure 2B shows the sedimentation profile of the DNA in the lysate thus treated, whereas the sedimentation profile in Figure 2A was obtained from a sample which had been extracted by phenol under the standard conditions (in the presence of 0.85 M NaCl). It is clear that as a consequence of phenol extraction at the lower NaCl concentration much less donor DNA was cosedimenting with the recipient DNA. The loss of cosedimenting donor DNA was accompanied by an increase of uncomplexed donor material at the top of the gradient. This indicates that the loss of donor-recipient

FIG. 1. (A) Sucrose gradient sedimentation profile of ¹⁴C-labeled recipient (○) and ³H¹⁵N-labeled homologous donor (●) DNA after in vitro complex formation. Sedimentation was from right to left. The fractions indicated by the bar were pooled and used for isopycnic CsCl centrifugation. (B) Density profile of ¹⁴C-labeled recipient (○) and ³H¹⁵N-labeled homologous donor (●) DNA in CsCl gradients (pH 11.2) of the pooled sucrose gradient fractions. Density increased from left to right.

FIG. 2. Sucrose gradient sedimentation profiles of ^{14}C -labeled recipient (O) and $^3\text{H}^{15}\text{N}$ -labeled homologous donor (●) DNA after complex formation *in vitro*. Phenol extraction before dialysis, concentration, and subsequent sucrose gradient centrifugation was at 0.85 M NaCl (A) or at 0.17 M NaCl (B). Sedimentation was from right to left.

DNA complexes was due to dissociation of the donor moiety. Apparently, the unstable complexes were protected from dissociation during phenol extraction by the high salt concentration.

DNA denaturation in the presence of phenol and sodium *p*-aminosalicylate. Because increasing ionic strength of the solute results in an increased stability of the DNA double helix (12) and because the unstable complexes apparently survived dialysis at low ionic strength (dialysis was against dilute saline citrate, 0.015 M NaCl–0.015 M sodium citrate) (22) before the sucrose gradient centrifugation, the observations reported above indicate that the dissociation of the unstable complexes, if they are base paired, might be due to helix-destabilizing interactions of phenol and *p*-aminosalicylate during phenol extraction at 0.17 M NaCl. For that reason, we have tested whether phenol–sodium *p*-aminosalicylate treatment lowered the melting temperature of transforming DNA. A solution of transforming DNA (100 $\mu\text{g}/\text{ml}$ of $0.8\times$ standard saline citrate) was slowly heated in a water bath in the presence or absence of 6% sodium *p*-aminosalicylate and 10% phenol or in the presence of 6% sodium *p*-aminosalicylate alone. In the control, sodium *p*-aminosalicylate was replaced by NaCl

to obtain the same Na^+ concentration in both mixtures. Samples were taken, and after dialysis, the biological activity of the DNA was tested by transformation (Fig. 3). In the presence of sodium *p*-aminosalicylate, the transforming DNA was inactivated at a temperature several degrees lower than that required to inactivate the transforming DNA in the control mixture. In the presence of phenol–sodium *p*-aminosalicylate, the temperature at which the transforming activity of the DNA was lost had decreased even more, indicating that both agents inactivated transforming DNA at temperatures below the normal melting temperature of DNA. Figure 4 shows that the loss of transforming activity was mainly if not entirely due to an enhanced denaturation of the transforming DNA in the presence of both agents, and not, for instance, to the introduction of single-strand or double-strand breaks. These density profiles were obtained from a [^3H , ^2H , ^{15}N]DNA solution incubated at 90°C for 5 min in the presence and absence of phenol and sodium *p*-aminosalicylate as described above. Most of the DNA heated in the presence of phenol and sodium *p*-aminosalicylate banded at the position of denatured heavy DNA. In contrast, almost all DNA heated in the absence of these substances still banded at the native position. Thus, the DNA double helix was apparently destabilized by interaction with phenol and *p*-aminosalicylate. Consequently, unstable complexes may have been lost as the result of helix destabilization by phenol and sodium *p*-aminosalicylate in the presence of 0.17

FIG. 3. Effect of sodium *p*-aminosalicylate and phenol on the terminal inactivation of transforming DNA. Transforming DNA was slowly heated in the presence of 6% (wt/vol) sodium *p*-aminosalicylate (▽), 6% sodium *p*-aminosalicylate plus 10% phenol (○), or in the absence of both agents (●). Samples were taken at the temperatures indicated, and after dialysis overnight, biological activity was determined by transformation.

FIG. 4. Density profiles in CsCl gradients (pH 11.2) of $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled transforming DNA heated for 5 min at 90°C in the presence (●), or absence (○) of 6% sodium *p*-aminosalicylate and 10% phenol. Arrows indicate the positions of heavy native (HN) and heavy denatured (HD) reference *B. subtilis* DNA.

M NaCl. Low salt concentration alone is insufficient to dissociate the complex, because it survives dialysis at 0.015 M NaCl.

Complex stabilization by cross-linking with 4,5',8-trimethylpsoralen. We stabilized the unstable complexes artificially by an additional 4,5',8-trimethylpsoralen cross-linking period just before CsCl centrifugation. Donor-recipient DNA complexes were allowed to form as described previously (22). After sucrose gradient centrifugation, the pooled fractions containing the donor-recipient DNA complex were dialyzed and divided in two equal parts. One part remained untreated, and the other part was irradiated with long-wave UV light in the presence of trimethylpsoralen. Both parts were then subjected to isopycnic centrifugation at pH 11.2 (Fig. 5). The amount of heavy donor DNA cobanding with the light recipient DNA increased sharply, whereas the amount of free heavy donor DNA correspondingly decreased as the consequence of the psoralen treatment. Since 4,5',8-trimethylpsoralen in conjunction with long-wave UV irradiation forms preferentially interstrand cross-links in double-stranded DNA (7), this observation suggests that the unstable complexes were base paired. The possibility that the donor and recipient DNA moieties were connected through protein was made improbable by an experiment in which the pooled sucrose gradient fractions containing the donor-recipient DNA complex had been treated with pronase before stabilization by cross-linking with 4,5',8-trimethylpsoralen (Fig. 6). Both samples, the pronase-treated and the untreated one, showed similar increases in the amount of donor-recipient DNA complex as a consequence

of treatment with psoralen. Apparently digestion with pronase did not result in dissociation of the unstable complexes.

Unstable complexes detected at pH 10 in the absence of psoralen cross-linking. Since helix destabilization might be responsible for the dissociation of the unstable complexes (see above) we tested whether dissociation of the complexes could be prevented by centrifugation in CsCl gradients at a lower pH value. After sucrose gradient centrifugation, the pooled fractions containing the unstable complexes were divided in four equal parts. Two parts were irradiated with long-wave UV light in the presence of trimethylpsoralen, whereas the other parts remained untreated. Both an irradiated and an untreated part were subjected to CsCl density gradient centrifugation at pH 11.2 and at pH 10 (Fig. 7). As before, a substantial increase in detectable complexes at pH 11.2 could be seen as the result of artificial stabilization by psoralen cross-linking (compare Fig. 7A and B). An even larger increase was observed without psoralen cross-linking when the sample was subjected to CsCl density gradient centrifugation at pH 10 (compare Fig. 7A and C). That the unstable complexes, stabilizable by psoralen cross-linking, were the same as those detectable without cross-linking at pH 10 was evidenced by the results presented in Fig. 7C and D; no additional increase could be observed at pH 10 after psoralen cross-linking.

Apparently the donor-recipient DNA complexes, being unstable at pH 11.2, were stable at pH 10 in CsCl density gradients. This instability

FIG. 5. Cross-linking with 4,5',8-trimethylpsoralen on the stability of complexes at pH 11.2. Density profiles in CsCl gradients (pH 11.2) of $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled donor DNA (●) and ^{14}C -labeled recipient DNA (○) after in vitro complex formation. The dialyzed sucrose gradient fractions containing the donor-recipient DNA complex remained untreated (A) or were cross-linked with 4,5',8-trimethylpsoralen (B) before being subjected to isopycnic centrifugation. Density increased from left to right. Details of the experiment are given in the text.

FIG. 6. Effect of treatment with pronase on the stability of donor-recipient DNA complexes. Density profiles in CsCl gradients (pH 11.2) of $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled donor DNA (●) and ^{14}C -labeled recipient DNA (○) after in vitro complex formation. The dialyzed sucrose gradient fractions were incubated for 4 h at 37°C (A and B) in the absence or in the presence (C and D) of pronase. After that, the mixtures remained untreated (A and C) or were cross-linked with 4,5',8-trimethylpsoralen (B and D) before being subjected to centrifugation in CsCl gradients at pH 11.2. Density increased from left to right.

might suggest that the complexes would be sensitive to nuclease S1 treatment. The donor moiety of the stable complexes exhibited a resistance of 50% to nuclease S1 treatment, whereas only approximately 20% of that of the fraction of unstable complexes was resistant to the enzyme (data not shown). This indicates that the unstable complexes contained more unpaired donor DNA regions. (The fraction of unstable complexes was obtained by correcting the amount of donor-recipient DNA complexes detected at pH 10 for the amount of complexes detected at pH 11.2.)

Specificity of the unstable complexes. One might argue that the unstable complexes described in this paper were not formed by the complex-forming mechanism active at 37°C (22), but were the result of reannealing occurring during the manipulations between phenol extraction and sucrose gradient centrifugation (dialysis and concentration). This idea was ruled out by an experiment in which a fivefold excess of unlabeled single-stranded donor DNA (relative to radioactive donor DNA) was added to the complex-forming mixture immediately after phenol extraction. If the unstable complexes formed during dialysis and concentration, we would expect the unstable complex peak to decrease drastically. The results presented in Fig. 8 show

that the addition of unlabeled donor DNA molecules had no appreciable effect on the amount of the stable and unstable complexes. Thus, both kinds of complexes were already present in the complex-forming mixture before phenol extraction. The introduction of 4,5',8-trimethylpsoralen diadducts in the double-stranded recipient DNA before the addition of donor DNA fragments inhibited the formation of both stable (22) and unstable complexes (data not shown), suggesting that strand separation of the recipient DNA was needed for the formation of both kinds of donor-recipient DNA complexes.

We also tested the formation of unstable complexes between *B. subtilis* recipient nucleoids and *E. coli* or bacteriophage H1 donor DNA fragments (Fig. 9). The results show that, in contrast to bacteriophage H1 DNA, *E. coli* DNA is capable of associating in an unstable way with the recipient DNA, although to a lesser extent than the homologous combination.

DISCUSSION

In this paper we have described a donor-recipient DNA complex in which the donor DNA was associated with the recipient DNA in an unstable way. The results presented in Fig. 8 showed that the complexes were formed in the

FIG. 7. Density profiles in CsCl gradients of $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled donor DNA (●) and ^{14}C -labeled recipient DNA (○) after in vitro complex formation. The dialyzed sucrose gradient fractions containing the donor recipient DNA complex remained untreated (A and C), or were cross-linked with 4,5',8-trimethylpsoralen (B and D) before being subjected to centrifugation in CsCl gradients at pH 11.2 (A and B) or at pH 10 (C and D). Density increased from left to right. Further experimental details are given in the text.

FIG. 8. Effect of the addition of unlabeled donor DNA to the complex-forming mixture. Density profiles in CsCl gradients of $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled donor DNA (●) and ^{14}C -labeled recipient DNA (○) after in vitro complex formation. The phenol-extracted complex-forming mixtures were dialyzed, concentrated, and subjected to sucrose gradient centrifugation without (A, B, and C) or after the addition (D, E, and F) of a fivefold excess of unlabeled donor DNA. The collected and dialyzed sucrose gradient fractions containing the donor-recipient DNA complex were cross-linked with 4,5',8-trimethylpsoralen before being subjected to CsCl gradient centrifugation at pH 11.2 (B and E) or remained untreated and were subjected to CsCl gradient centrifugation at pH 11.2 (A and D) or at pH 10 (C and F). Density increased from left to right.

complex-forming mixture before phenol extraction and not during subsequent manipulations.

Several possibilities may be envisaged to explain the instability of the complexes. In a previous paper (22) it was hypothesized that small paired regions between chromosomal and donor DNA might have been stabilized by a protein which resisted extraction by phenol at

0.85 M NaCl but was removed from the DNA during CsCl centrifugation. This hypothesis is unlikely since once formed, the complexes survived extensive pronase treatment (Fig. 6). Also, complexes dissociated with phenol extraction at 0.17 M NaCl, and they remained intact during CsCl centrifugation at pH 10. In addition, the protein patterns obtained by sodium dodecyl

FIG. 9. Effect of heterology on complex formation in vitro. Density profiles in CsCl gradients (pH 10) of ^3H -labeled donor DNA (●) and ^{14}C -labeled *B. subtilis* recipient DNA (○) after in vitro complex formation. $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled *B. subtilis* DNA (A), $^3\text{H}^2\text{H}^{15}\text{N}$ -labeled *E. coli* DNA (B), and ^3H -labeled phage H1 DNA (C) were used as donor DNA sources. Density increased from left to right.

sulfate-polyacrylamide gel electrophoresis of the phenol-extracted mixtures showed no specific proteins remaining attached to the DNA during phenol extraction at 0.85 M NaCl (J. van Randen and G. Venema, unpublished data).

Two observations suggest that dissociation of the unstable complexes was due to DNA helix destabilization. First, unstable complexes were lost upon phenol extraction at 0.17 M NaCl but not at the high salt concentration normally used. Second, unstable complexes dissociated during CsCl gradient centrifugation at the relative high pH (11.2) routinely used to separate donor-recipient DNA complex from uncomplexed donor DNA (5, 11, 17), but not during CsCl gradient centrifugation at pH 10. Since it is unlikely that the association of donor and recipient DNA is stabilized by protein-DNA interactions (see above), these observations suggest that the donor and recipient DNA moieties are associated through base-pairing interactions. This suggestion is supported by two further observations. First, the introduction of 4,5',8-trimethylpsoralen diadducts in the double-stranded recipient DNA before the addition of donor DNA fragments inhibited the formation of the complexes, suggesting that recipient DNA strand separation is a prerequisite for complex formation. Second, after formation, the unstable complexes could be stabilized artificially by cross-linking with 4,5',8-trimethylpsoralen. Treatment with psoralen plus long-wave UV light stabilizes specific secondary structures in single-stranded bacteriophage fd DNA both in vitro and in situ (18, 19). This observation favors the idea that the unstable donor-recipient DNA association described in this paper is mediated by base-pairing interactions. The dissociation of these complexes under helix-destabilizing conditions may be attributed to base-pairing over short distances.

Apparently the unstable complexes, artificially stabilizable by psoralen cross-linking after phenol extraction, had not been stabilized by psoralen cross-linking in the initial reaction mixture of nucleoids and donor DNA (22). This was probably due to the longer duration of the second cross-linking period in combination with the finding that trimethylpsoralen cross-links were introduced more efficiently at lower NaCl concentrations (Van Randen and Venema, unpublished data).

In the present context, it is of importance to consider the question of whether the stability of the complexes, detectable by isopycnic centrifugation at pH 11.2, is an intrinsic property of the complexes or whether the stability is artificially caused by cross-linking during the first cross-linking period, which is included in the complex-formation procedure (see above). However, omitting this cross-linking step from the com-

plex-forming procedure did not have a substantial effect on the yield of complex observed in CsCl gradients at pH 11.2 (data not shown). We conclude, therefore, that these complexes are stable without artificial stabilization.

Unstable complexes between recipient *B. subtilis* nucleoids and single-stranded donor DNA fragments were also formed with *E. coli* donor DNA in rather large amounts, but not with bacteriophage H1 DNA. To explain this difference, we hypothesize that *E. coli* DNA carries base sequences which are sufficiently homologous to those of *B. subtilis* to allow the formation of unstable complexes. In contrast to the formation of the unstable complexes, extensive homology is apparently required for the formation of stable complexes, for hardly any stable complex was observed when heterologous *E. coli* donor DNA fragments were used (22).

It is relevant to note that we have obtained evidence that shortly transformed *B. subtilis* cells contain early donor-recipient DNA complexes with similar properties as the unstable complexes described in this paper (manuscript in preparation). They were also formed with *E. coli* DNA, indicating that very limited homology is sufficient for donor-recipient DNA complex formation during transformation of *B. subtilis*. This observation seems to indicate that certain steps of the recombination process acting in vivo can be accomplished by the in vitro system.

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