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Single-Stranded Regions in Transforming Deoxyribonucleic Acid After Uptake by Competent *Haemophilus influenzae*

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About 15% of donor deoxyribonucleic acid (DNA) is single stranded immediately after uptake into competent *Haemophilus influenzae* wild-type cells, as judged by its sensitivity to S1 endonuclease. This amount decreases to 4 to 5% by 30 min after uptake. Mutants which are defective in the covalent association of recipient and donor DNA form little or no S1 endonuclease-sensitive donor. At 17 C donor DNA taken up by the wild type contains single-stranded regions although there is no observable association, either covalent or noncovalent. The single-stranded regions are at the ends of donor DNA molecules, as judged by the unchanged sedimentation velocity after S1 endonuclease digestion. The amount of single-stranded donor remains constant at 17 C for more than 60 min after uptake, suggesting that the decrease observed at 37 C is the result of association of single-stranded ends with single-stranded regions of recipient cell DNA. Three sequential steps necessary for the integration of donor DNA into recipient DNA are proposed: the synthesis of single-stranded regions in recipient DNA, the interaction of donor DNA with recipient DNA resulting in the production of single-stranded ends on donor DNA, and the stable pairing of homologous single-stranded regions.

During bacterial transformation single-stranded segments of donor deoxyribonucleic acid (DNA) are integrated into the recipient cell genome to form heteroduplex molecules of donor and recipient cell DNA (2, 7, 15). Double-stranded donor DNA is converted to a high-molecular-weight single-stranded form during uptake in both the *Diplococcus pneumoniae* (10, 11) and *Bacillus subtilis* (5, 6, 18) transformation systems, and is thus ready for pairing and integration into the recipient cell DNA. Such a single-stranded donor DNA intermediate has not been detected in *Haemophilus influenzae* (15), but 10 to 15% of the donor DNA after uptake is sensitive to S1 endonuclease (12), a nuclease which degrades only single-stranded DNA (1, 23). This conversion of donor DNA from completely double-stranded form to DNA with single-stranded regions is independent of the presence or absence of the adenosine 5'-triphosphate-dependent nuclease (12).

The recipient cell DNA in competent *H. influenzae* also contains single-stranded regions which are essential for interaction with donor DNA (12, 13). Single-stranded regions in the donor DNA may therefore pair with single-stranded regions in the competent cell DNA during recombination in *H. influenzae* transformation.

In the present work we have examined the sensitivity of donor DNA to S1 endonuclease at various times after uptake by competent cells of the wild-type and association-defective mutants of *H. influenzae*. The mutants do not produce single-stranded regions in the donor DNA. After uptake by the wild type at 17 C, at which temperature there is no evidence of donor-recipient DNA association, single-stranded regions are produced in the donor DNA. The single-stranded regions are apparently at the ends of the donor DNA molecules.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Rd* is the wild-type strain; strains *rec-1* (DB117), *rec-2*, and the KB mutants are defective in transformation (3, 12, 13, 20). The cells were grown in brain heart infusion liquid medium. Growth conditions have been described previously (20).

Preparation of competent cells. Exponentially growing cells at an optical density (OD) of 0.4 at 675 nm were washed and resuspended in the same volume of MIV medium (21). The cells were at the peak of competence after 100 min of incubation in MIV medium at 37 C with shaking.

Transformation. Transforming DNA was prepared according to Marmur (14). Tritium-labeled transforming DNA was isolated from a novobiocin-

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resistant *Rd* strain grown overnight in $M1_cCit$ medium (8) containing 12 μCi of [^{3}H]thymidine per ml. The specific activity of the DNA was 1.1×10^4 counts/min per μg. Competent cells were incubated with 0.82 μg of labeled DNA/ml at 37 C with shaking for 10 min. By this time 40 to 60% of the DNA was taken up by the cells. Fifty μg of deoxyribonuclease (DNase) per ml and 10 mM $MgSO_4$ were added, and the cells were incubated for 5 min. This stage in the experiment was considered as 0 min after uptake. The incubation at 37 C was continued, and samples were taken at various times. For uptake of transforming DNA at 17 C, the competent cells were transferred to 17 C for 3 min. They were then incubated with labeled transforming DNA for 30 min. Five minutes after the addition of DNase was considered as 0 min after uptake and the incubation was continued at 17 C.

Preparation of cell lysates. Cell samples were washed twice and concentrated 12 times in 10 mM tris(hydroxymethyl)aminomethane (Tris)-chloride, pH 7.6-1 mM ethylenediaminetetraacetic acid (EDTA) at 4 C. They were treated at the same temperature with 3 mg of lysozyme per ml for 10 min and then with 0.7% sodium lauryl sulfate for 30 min.

S1 endonuclease assay for single-stranded donor DNA after uptake by competent cells. A 0.4-ml cell lysate sample was added to 0.6 ml of the S1 endonuclease reaction mixture giving final concentrations of 30 mM sodium acetate buffer, pH 4.6, 5 mM $ZnSO_4$, 0.14 M NaCl, and 20 μg of sonically treated heat-denatured calf thymus DNA. The mixture was divided in half and 5 μliters of S1 endonuclease, prepared according to Sutton (23), was added to one-half. The samples were incubated at 50 C for 15 min at which time the degradation was at the maximum, and then placed on ice. The reaction kinetics have been described previously (13). A 0.3% amount of sodium lauryl sulfate in the final reaction mixture did not affect the degradation of single-stranded DNA by S1 endonuclease. Samples of 0.05 ml were spotted on Whatman 3MM filter paper disks for determination of the acid-insoluble radioactivity. Acid-soluble radioactivity was measured by mixing a 0.3-ml sample with 0.05 ml of bovine serum albumin (10 mg/ml) on ice and then 0.1 ml of 25% trichloroacetic acid was added. After 10 min the suspension was centrifuged and 0.15-ml samples of the supernatant fluid were mixed with 4 ml of scintillation fluid (10 parts of a toluene-permablend scintillator to 3 parts Triton X-100).

Association of donor and recipient DNA. To prelabel the recipient cell DNA, 0.2 μCi of [^{14}C]thymidine/ml was added to exponentially growing cells at OD_{675} 0.05. At OD_{675} 0.4 the cells were made competent and exposed to ^{3}H-labeled transforming DNA as described above. Samples were taken at 30 min after uptake of transforming DNA and placed on ice. The cells were washed and concentrated 10 times in 10 mM Tris-1 mM EDTA, pH 7.6. Twenty-microliter cell samples were lysed on the top of alkaline sucrose gradients in 0.1 ml of 0.5 N NaOH.

DNA pulse labeled in exponential and competent cells. [^{14}C]thymidine (1.3 μCi/ml) was added to exponentially growing cells at OD 0.05. At OD_{675} 0.4, the culture was washed and part was made competent and part was diluted in prewarmed brain heart infusion liquid medium to an OD of 0.17. The latter was incubated at 37 C to OD 0.35, and then pulse labeled with 200 μCi of [^{3}H]thymidine/ml for 10 min. The competent cells were pulse labeled with 17 μCi of [^{3}H]thymidine/ml for 10 min. A lower specific activity of [^{3}H]thymidine was added to the competent cells as they incorporate the label more rapidly during the pulse than do exponentially growing cells (13). The cells were washed and concentrated five times in 10 mM Tris-chloride, pH 7.6-1 mM EDTA. Twenty-microliter samples of exponential cells and 10-μl samples of competent cells were lysed on the top of alkaline sucrose gradients.

Alkaline sucrose gradient centrifugation. About 10^8 cells were added to 0.1 ml of 0.5 N NaOH on top of 5-ml 5 to 20% alkaline sucrose gradients containing 0.5 M NaCl, 0.2 N NaOH, and 1 mM EDTA. The gradients were kept in the dark for 25 min and then centrifuged at 30,000 rpm at 20 C in a SW50.1 rotor of a Beckman L-2 ultracentrifuge. Between 25 and 30 fractions were collected from each gradient on Whatman 17M paper strips and processed as described by Carrier and Setlow (4).

Neutral sucrose gradient centrifugation. Cells in 10 mM Tris-chloride (pH 7.6)-1 mM EDTA at 4 C were treated with 3 mg of lysozyme/ml for 10 min and then 0.7% Sarkosyl for 60 min. Lysates were frozen and thawed twice and 0.1 ml was placed on top of 5 to 20% neutral sucrose gradients containing 10 mM Tris-chloride (pH 7.6)-1 mM EDTA and 0.1 M NaCl. The gradients were centrifuged at 20,000 rpm at 20 C.

Assay of radioactivity. Filter paper disks and strips were washed once in 5% trichloroacetic acid, twice in 95% ethanol, and then dried. The strips, cut into fractions, and the disks were placed in Permablend-toluene scintillation fluid and counted in a Beckman scintillation counter. In sucrose gradient experiments where the peak of ^{3}H-labeled donor DNA had a maximum of only 50 to 100 counts/min, each sample was counted for 20 min.

RESULTS

Single-stranded regions in donor DNA. All but about 1% of heat-denatured ^{3}H-labeled transforming DNA was degraded by S1 endonuclease, but there was no observable degradation of the nondenatured DNA; thus this DNA did not contain a significant amount of single-stranded DNA before entering the competent cells. Competent cells were incubated with labeled transforming DNA for 10 min and then with DNase. Five minutes after the addition of DNase is referred to as 0 min after uptake. The lysates made from cells lysed at various times after uptake were added to the S1 endonuclease reaction mixture to determine the percentage of donor DNA which was single stranded. Immediately after uptake, 13 to 18% of the donor DNA taken up by competent wild-type cells was degradable by S1 endonuclease (Table 1 and Fig. 1) and was therefore single stranded. There

was little (0.2%) or no S1 nuclease degradation of recipient cell DNA labeled with [^{14}C]thymidine before the competence regime, as also found by LeClerc and Setlow (13). The amount of S1 endonuclease-sensitive donor DNA decreased to 4 to 5% 40 min after uptake (Fig. 1 and Table 1). Most of the single-stranded DNA must be either degraded or associated with the recipient cell DNA during this time.

At 0 min after uptake, 4.8% of the total donor DNA was detected as acid-soluble counts in the reaction mixture in the absence of S1 endonuclease. This amount decreased to 2.3% 40 min after uptake (Fig. 1). These acid-soluble counts could be due to degradation of donor DNA by cell nuclease (9, 15), in which case the acid-soluble counts might be expected to increase with time after uptake, unless the degraded donor DNA was lost from the cells or was reincorporated by DNA synthesis into the cell DNA.

Transformation-defective mutants. Wild-type cells, labeled before the competence regime, release some of the label into the medium on exposure to transforming DNA. The amount of label released is equivalent to the amount of donor DNA integrated into the recipient DNA (21). The *rec-2* and KB mutants show little or no release of label in this type of experiment, which suggests that they are defective in interaction of donor and recipient DNA (3). The lack of association has been demonstrated more directly by alkaline sucrose gradient sedimentation of cell DNA 30 min after uptake of donor DNA by KB1 and KB2 (Fig. 2) and by *rec-2* (12). The lack of association in KB6 has also been shown by the absence of donor DNA associated with recipient cell DNA in the pellet of digitonin cell lysates (17). Between 0.3 and 1.6% of the donor DNA taken up by these association-defective strains was sensitive to S1 endonuclease immediately after uptake (Table 1). We conclude that there is very little, if any, single-stranded donor DNA produced in these association-defective strains, compared with about 15% in the wild type immediately after uptake.

Association of donor and recipient DNA takes place in the *rec-1* mutant, although the transformation of this strain is 10^{-6} that of the wild type (12, 17). The *rec-1* mutant produced S1 endonuclease-sensitive donor DNA after uptake similar to the wild type (Table 1). The production of single-stranded regions in the donor DNA is therefore considered to be a feature of the interaction of donor and recipient DNA, as the association-defective mutants lack this property.

TABLE 1. *Percentage of donor DNA, taken up by the wild-type and transformation-defective mutants of H. influenzae at 37 and 17 C, which is sensitive to S1 endonuclease*

Temperature (C)	Strain	% DNA sensitive to S1 endonuclease			
		0[a]	15	30	60
37	*Rd*	14.8	6.7	3.5	
		13.4		4.8	
	rec-1	11.5	8.9	4.3	
		13.4		3.9	
	rec-2	1.4		0.8	
		0.7			
	KB1	1.1	0.8	0.4	
		0.4			
	KB2	0.3			
	KB3	0.8			
	KB4	1.0			
	KB5	0.6			
	KB6	0.7		1.2	
		0.4			
	KB8	1.6	1.3	1.1	
17	*Rd*	11.1			8.3
		9.5			10.6
	rec-2	0			
		0.7			

[a] Minutes after uptake.

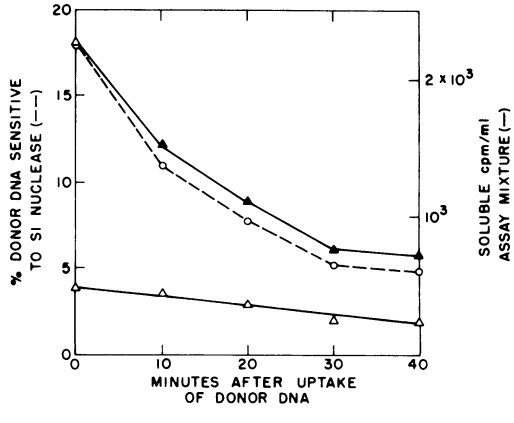

FIG. 1. *Sensitivity of donor DNA, after uptake by strain Rd, to S1 endonuclease. Cells lysed at various times after uptake were incubated with the S1 endonuclease reaction mix. The acid-soluble counts per minute (Δ, no S1 endonuclease in mix; ▲ with the enzyme) and the acid-insoluble counts per minute were determined, and the percentage of donor DNA which was sensitive to S1 endonuclease (O) was calculated from the net soluble counts per minute due to S1 endonuclease divided by the insoluble counts per minute.*

In wild-type cells, but not in *rec-2*, DNA pulse labeled at the peak of competence consists of more low-molecular-weight DNA on alkaline sucrose gradients than that pulse labeled in exponentially growing cells, and it contains single-stranded regions (12, 13). The defect in association of recipient and donor DNA in *rec-2* was therefore considered to result from the absence of possible pairing regions in the recipient DNA. The KB1 mutant synthesized more low-molecular-weight DNA in competent cells than in exponential cells similar to the wild type, and thus differs from *rec-2* in this respect (Fig. 3). (The other KB mutants have not been examined.)

Single-stranded regions in transforming DNA after uptake at 17 C. When transforming DNA is taken up by competent *H. influenzae* cells at 17 C, no recombinant DNA molecules are formed, as determined by reisolating the DNA and detecting recombinant molecules by transformation (9, 24). In the wild-type strain there is some degradation of donor DNA to mononucleotides after uptake at both 37 and 17 C (9). In the *rec-2* and KB6 mutants there is no degradation of donor DNA (17, 22), and there are no single-stranded regions produced in the donor (Table 1). Kooistra and Venema (9)

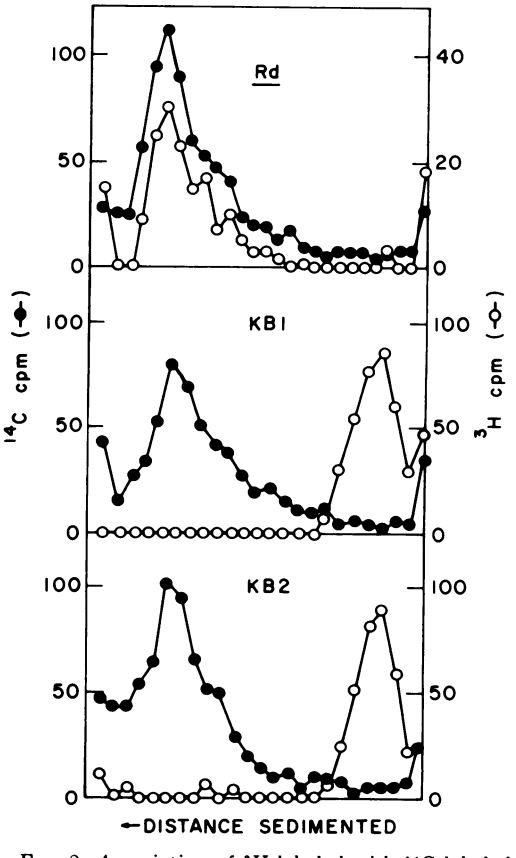

FIG. 2. *Association of ^{3}H-labeled with ^{14}C-labeled recipient DNA in Rd, KB1 and KB2 30 min after uptake. The cells were lysed on top of alkaline sucrose gradients and centrifuged at 30,000 rpm for 90 min at 20 C.*

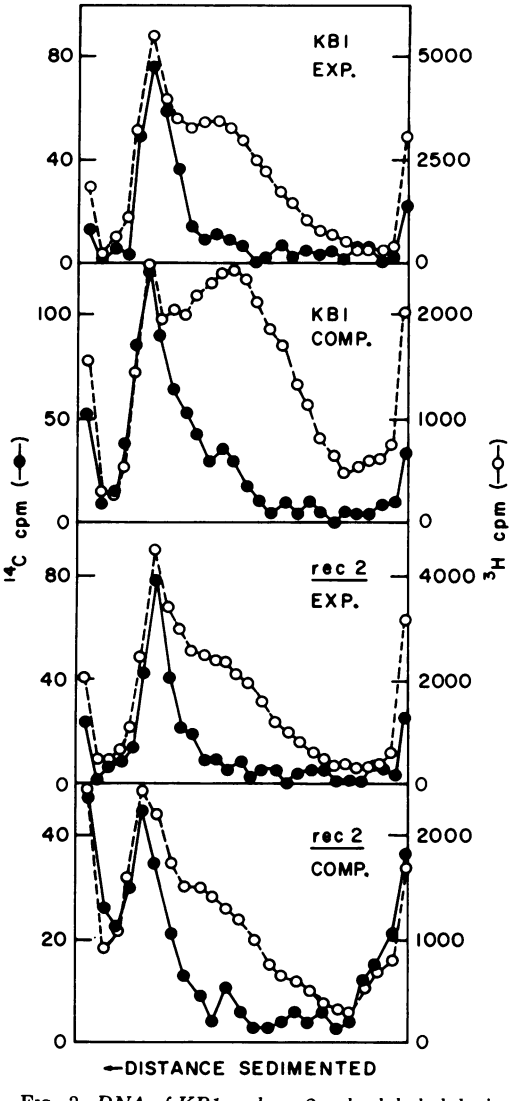

FIG. 3. *DNA of KB1 and rec-2 pulse labeled during exponential (EXP.) growth and at the peak of competence (COMP.). Cells prelabeled with [^{14}C]thymidine during exponential growth were pulse labeled with [^{3}H]thymidine during exponential growth or after being made competent. The cells were lysed on top of alkaline sucrose gradients and centrifuged at 30,000 rpm for 90 min at 20 C.*

suggested that the acid solubilization of the donor in the wild type was due to the production of single-stranded regions, which are unable to pair with the recipient cell DNA at 17 C. We have examined the S1 nuclease sensitivity of donor DNA after uptake by strain *Rd* at 17 C. At 0 and 60 min after uptake about 10% of the donor DNA was degraded by S1 nuclease and was therefore single stranded (Table 1).

The *rec-1* mutant produced single-stranded donor DNA after uptake at 37 C (Table 1) but forms very few active recombinant molecules (17). In these two characteristics the *rec-1* mutant at 37 C is similar to the wild-type strain at 17 C. In the *rec-1* mutant, however, although the donor and recipient DNAs become covalently associated, few transformants are produced (12, 17). We have examined the association of donor and recipient DNA at 17 C by velocity sedimentation to check whether there was any covalent or noncovalent association which did not result in the production of stable recombinant DNA molecules. No covalent association of donor and recipient DNAs 90 min after uptake at 17 C was detected by alkaline sucrose gradient sedimentation, and no noncovalent association at 0 and 60 min after uptake was detected by neutral sucrose gradient sedimentation (Fig. 4). The absence of association at 17 C may be because the pairing of homologous single-stranded regions is slowed down beyond a critical level, as suggested by Kooistra and Venema (9).

Single-stranded regions at the ends of donor DNA molecules. Two experiments were done to determine whether single-stranded regions were produced at the ends or along the length of the donor DNA molecules. Both experiments were performed after uptake at 17 C, so that the donor DNA could be examined in the absence of association with the recipient cell DNA. In the first experiment, donor DNA taken up by strains *Rd* and *rec-2* at 17 C was sedimented through alkaline sucrose gradients. If the single-stranded regions are produced along the length of the molecule, donor DNA in the wild type should have a lower average molecular weight than that in *rec-2* because single-stranded regions are not produced in the donor in *rec-2*. However, immediately after uptake there was little or no difference in the distance sedimented (Fig. 5). In the second experiment, the donor DNA taken up at 17 C by strain *Rd* was sedimented through sucrose gradients after treatment with S1 nuclease, which should decrease the molecular weight of the donor DNA if the single-stranded regions are along the length of the molecule by the formation of double-strand breaks. There was, however, no detectable change in the distance sedimented through neutral sucrose (Fig. 6) or alkaline sucrose (data not shown) as a result of the S1 nuclease treatment. We conclude from these experiments that the single-stranded regions are all or almost all at the ends of the donor DNA. This conclusion is in accord with the previous observation that unintegrated donor DNA extracted

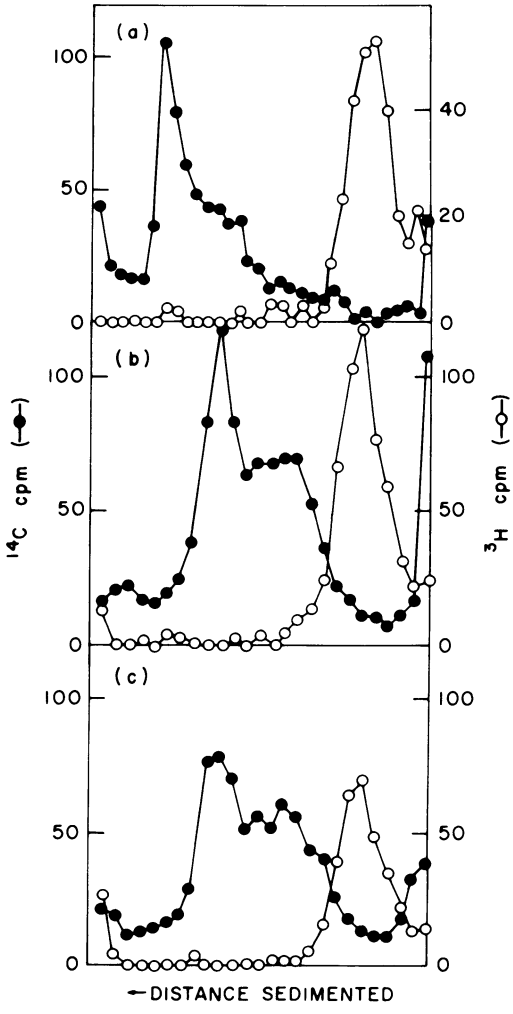

FIG. 4. *Lack of association of ^{3}H-labeled donor and ^{14}C-labeled recipient DNA in the wild-type strain at 17 C. Cells were lysed at 0 (b) and 60 min (c) after uptake and placed on neutral sucrose gradients; and at 90 min (a) after uptake on top of an alkaline sucrose gradient. The neutral gradients were centrifuged at 20,000 rpm for 180 min at 20 C and the alkaline gradient at 30,000 rpm for 90 min at 20 C.*

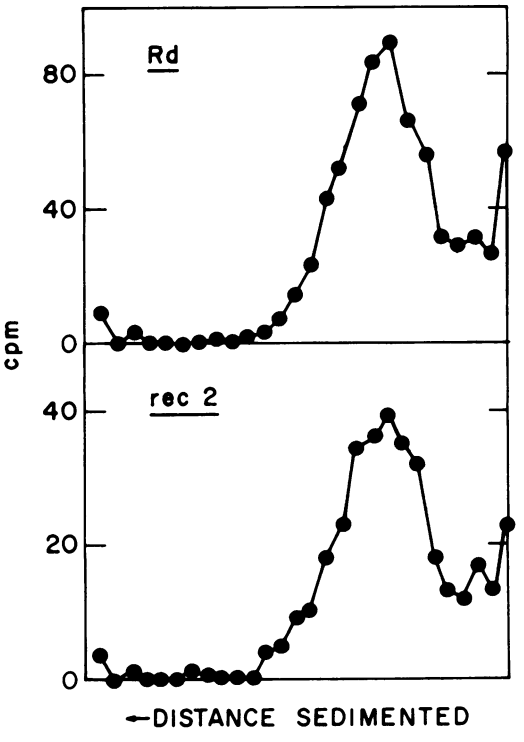

FIG. 5. *Alkaline sucrose gradient sedimentation of ^{3}H-labeled donor DNA immediately after uptake by strains Rd and rec-2 at 17 C. Cells were lysed on top of the gradients, which were centrifuged at 30,000 rpm for 180 min at 20 C.*

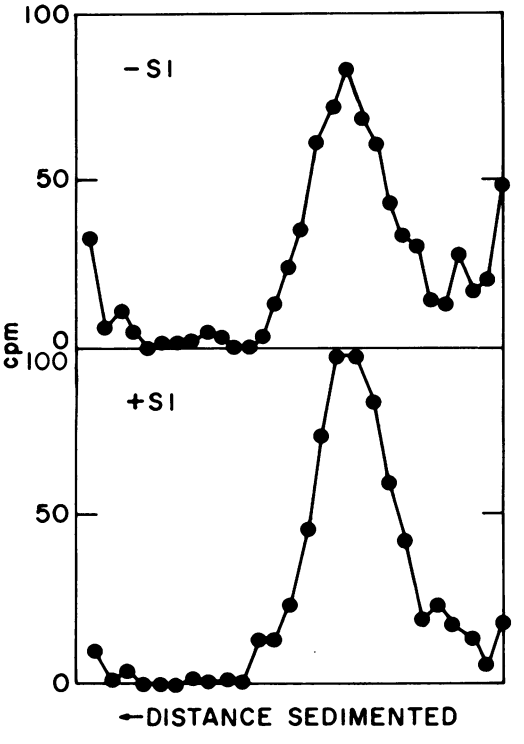

FIG. 6. *Sucrose gradient sedimentation of ^{3}H-labeled donor DNA treated with S1 nuclease after uptake by competent Rd cells at 17 C. Cells were lysed immediately after uptake and incubated with the S1 endonuclease reaction mixture with or without added enzyme. Samples were placed on top of neutral sucrose gradients and centrifuged at 20,000 rpm for 6 h at 20 C.*

from cells shows an increase in ability to transform after annealing (16).

DISCUSSION

Our data do not eliminate the possibility that the S1-sensitive material consists of oligonucleotides, which are small degradation products of the donor DNA. However, we consider this to be improbable for the following reason. Whereas the fraction of S1-degradable donor material declines markedly with time after uptake into the wild-type cell when the temperature is 37 C (Fig. 1) and there is covalent association (Fig. 2), there is little or no change at 17 C (Table 1) when there is no association, covalent or noncovalent (Fig. 4). These data suggest that the single-stranded donor material declines at 37 C because of stable pairing with recipient DNA, and thus is mostly in large pieces of DNA, some of which will become integrated. If the S1-degradable material were only in small degradation products, the temperature would not be expected to have such a large effect.

We have seen that two mutants deficient in covalent association between donor and recipient DNA do not form single-stranded regions in donor DNA. Single-strand regions in recipient DNA also appear to be required for such covalent association (12, 13). One interpretation of these phenomena is that single-strand regions in donor and recipient DNA are independently produced, and both are required for stable pairing and integration of transforming DNA. The mutant KB1 is defective only in the formation of single-stranded regions in donor DNA. However, *rec-2* fails to form both types of single-stranded regions. Since *rec-2* was isolated without treatment with a mutagen (19), we consider it unlikely that there is more than one step in recombination that is blocked in this mutant. Hence, we favor the idea that formation of single-stranded regions in donor DNA is dependent upon the presence of single-stranded regions in recipient DNA. We postulate that in

association-proficient strains there is interaction of donor DNA with competent cell DNA containing single-stranded regions that takes place at 17 C as well as at 37 C. This interaction stimulates the production of single-stranded ends on the donor DNA, but does not result in stable pairing at the lower temperature. The mutant *rec-2* does not produce single-stranded regions in donor DNA because it does not synthesize single-stranded regions in recipient cell DNA. KB1 must be deficient for a different reason. The two mutants differ in another important respect: *rec-2* is defective in vegetative phage recombination, whereas KB1 is not (16), indicating that the two mutants are blocked at entirely different steps in the interaction between DNAs. One possible explanation for the lack of interaction of transforming DNA with the KB1 recipient DNA, even though the latter is in a favorable configuration, is that the transforming DNA does not reach the recipient DNA. This would explain why competent KB1 cells are defective in transformation although normal in vegetative phage recombination, in which case the phage DNA is injected into the cell.

We have thus proposed three sequential events that must occur before there is integration of donor DNA into recipient cell DNA: the production of single-stranded regions in the recipient DNA, the interaction of the donor DNA with the recipient cell DNA resulting in the production of single-stranded ends on the donor DNA, and the stable pairing of homologous single-stranded regions of donor and recipient DNA.

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