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I am submitting herewith a dissertation written by Joseph Eugene LeClerc entitled "On the Mechanism of Recombination during Haemophilus influenzae Transformation." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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5

ON THE MECHANISM OF RECOMBINATION DURING *HAEMOPHILUS INFLUENZAE* TRANSFORMATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Joseph Eugene LeClerc

August 1974

ACKNOWLEDGMENTS

Jane K. Setlow has helped me with major portions of this work and has advised me on numerous aspects of my training at Oak Ridge. I am extremely grateful. I also thank R. F. Kimball, S. Mitra and R. B. Setlow for serving on my dissertation committee; K. L. Beattie for numerous suggestions, M. E. Boling for technical assistance, B. LeClerc for help with some of the experiments and for typing various versions of this manuscript and N. G. Crowe for drawing various versions of the data.

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ABSTRACT

The purpose of this investigation was to determine if the DNA of competent Haemophilus influenzae cells has structural features that may promote recombination with donor DNA and to gain information on the mechanism of recombination during transformation. Mutations in H. influenzae strains that may affect recombination were utilized in order to assess the biological significance of observations made with the wild type strain and to determine the effect of these mutations on the recombination process.

Analysis of DNA from competent or exponentially growing cells labeled with radioactive and/or density isotopes indicates the following: (1) the DNA synthesized in competent wild type cells is predominently slow-sedimenting in alkaline sucrose, making the single-strand molecular weight of this DNA one-fourth to one-third the size of DNA synthesized in exponentially growing cells; (2) the DNA in competent cells synthesized before the competence regime has no detectable single strand breaks; (3) the DNA synthesized in competent cells has predominant single stranded character, unlike the DNA synthesized in exponentially growing cells; (4) DNA synthesis in competent cells is not predominently semiconservative synthesis; and (5) the DNA synthesized in competent cells, but not the DNA synthesized before the competence regime, is susceptible to a single-strand-specific nuclease. Evidence that the structural features of DNA synthesized in competent cells promotes

recombination include (1) the nucleoside inosine inhibits both competence development and the synthesis of DNA with these features and (2) competent cells of the rec2 mutant of H. influenzae, with a transformation frequency 10^{-7} of wild type, synthesize DNA without comparable single strand breaks nor single stranded character as the wild type strain.

To investigate the molecular basis of recombination during transformation, competent cells of the wild type and mutant strains labeled with [^{3}H]dThd were exposed to ^{32}P-labeled transforming DNA and the DNA analyzed by alkaline or neutral sucrose gradient sedimentation. The following information has been obtained on the interaction of donor DNA and recipient DNA in competent cells.

1. Donor DNA forms covalent bonds with recipient DNA in all strains except rec2.
2. When donor DNA interacts with recipient DNA, the strand labeled before the competence regime is broken, except in strain add1 which lacks the ATP-dependent nuclease.
3. There is an intermediate stage in the interaction of donor DNA and recipient DNA in which there is little covalent bonding between the DNAs as judged by the difference between sedimentation patterns in neutral and alkaline sucrose.
4. Donor DNA first becomes covalently linked with recipient DNA of intermediate single-strand length. The sealing of the DNA into high molecular weight DNA is more rapid when DNA synthesis is not occurring as determined by using strain dna9, a mutant temperature-sensitive for DNA synthesis.

5. In the recl mutant, which has a transformation frequency 10^{-6} that of wild type, there is extensive degradation of recipient DNA which may prevent the sealing of recombinant DNA into high molecular weight DNA.

TABLE OF CONTENTS

CHAPTER	PAGE
GENERAL INTRODUCTION	1
I. THE DNA OF COMPETENT CELLS	4
Introduction	4
Materials and Methods	5
Results	11
Discussion	18
II. INTERACTION OF DONOR DNA AND RECIPIENT DNA	26
Introduction	26
Materials and Methods	30
Results	32
Discussion	37
III. MODELS	45
LIST OF REFERENCES	53
APPENDIX	58
VITA	81

LIST OF TABLES

TABLE PAGE

1. Effect of Inosine on [^{3}H]dThd Incorporation in Competent Wild Type *H. influenzae* 59
2. S1 Nuclease Digestion of DNA Fractionated on BND-Cellulose . 60
3. Association of Donor DNA with Recipient DNA in *dna9* and *dna9rec1* 61
4. Summary of Transformation Events in *Haemophilus influenzae* Strains 62

LIST OF FIGURES

FIGURE PAGE

1. Incorporation of [^{3}H]dThd into Competent (-o-) and Exponentially Growing (-●-) Wild Type and Competent recl (-Δ-) and rec2 (-▲-) Cells 63
2. Alkaline Sucrose Gradient Sedimentation Patterns of DNA from Competent and Exponentially Growing Wild Type and Competent rec2 and rec2 Cells 64
3. Alkaline Sucrose Gradient Sedimentation Patterns of DNA from Competent Wild Type and rec2 Cells Pulse-Labeled for Short Times 65
4. Alkaline Sucrose Gradient Sedimentation Patterns of Competent Wild Type Cells Pulse-Labeled in M-IV Medium and Chased in M-IV Medium or MI_ccit Growth Medium 66
5. The Effect of Inosine in the M-IV Competence Medium on Competence, Transformation and Size of DNA Synthesized in the M-IV Competence Medium 67
6. BND-Cellulose Column Chromatography of DNA from Competent and Exponentially Growing Wild Type and Competent rec2 Cells 68
7. Effect of SI Nuclease on Native (-●-) and Heat-Denatured (-o-) H. influenzae DNA Labeled with [^{3}H]dThd 69

FIGURE PAGE

8. CsCl Density Gradient Sedimentation Patterns of BrdUrd-Substituted DNA from Competent and Exponentially Growing Wild Type Cells 70

9. Alkaline CsCl Gradient Sedimentation of BrdUrd-Substituted DNA from Competent and Exponentially Growing Wild Type Cells 71

10. Hypothetical Scheme for Strand Displacement Synthesis and Branch Migration 72

11. Release of Label from Recipient DNA of Competent Cells in Response to Transforming DNA 73

12. Alkaline Sucrose Gradient Sedimentation Patterns of DNA from Competent Wild Type, rec2 and rec1 Cells Exposed to Transforming DNA 74

13. Alkaline Sucrose Gradient Sedimentation Patterns of DNA from Competent dna9 Cells Exposed to Transforming DNA . . 75

14. Association of Donor DNA with Recipient DNA in Competent dna9 Cells at 36°C and 41°C 76

15. Alkaline Sucrose Gradient Sedimentation Patterns of DNA from Competent add1 Cells Exposed to Transforming DNA . . 77

16. Model for the Interaction of Donor DNA(—) and Recipient DNA (—) During H. influenzae Transformation 78

17. Models for Pairing of DNA During Recombination 79

18. Hypothetical Scheme for Single Strand Assimilation During Recombination 80

GENERAL INTRODUCTION

Recent models for recombination of DNA molecules feature the involvement of single strands of DNA that can participate in strand displacement and assimilation reactions (1, 2, 3). These models are largely extensions of the Whitehouse (4) and Holliday (5) models deduced from genetic exchange and cytological observations in eucaryotes, but are tied to known biochemical reactions mediated by DNA recognition proteins. DNA strand breaks, one precursor of single stranded regions, can arise from the action of nucleolytic enzymes, during processes of replication or repair, or by accident. The fate of strand breaks then depends on the action of polymerization or degradation enzymes, each conceivably giving rise to single stranded regions in DNA molecules that are immediately available for base pairing. Thus, at least for the model-builder, much of the mystery of DNA-DNA association during recombination is removed and it remains for the experimentalist to determine if strand breaks and single stranded regions in DNA are precursors of recombinant molecules.

Direct evidence for single stranded precursors in recombination reactions comes from molecular studies of the mechanism of recombination during transformation. The fate of donor DNA molecules interacting with cell genomes to produce recombinant molecules has been followed and at each stage of the interaction the biological activity of the intermediates has been determined. In the Diplococcus pneumoniae and Bacillus subtilis

transformation systems, an eclipse in biological activity of transforming DNA exposed to competent cells, measured as the loss of transforming activity for the donor marker (6, 7), has been correlated with the single stranded or degraded nature of these molecules (8, 9, 10). In the Haemophilus influenzae transformation system, however, the loss in biological activity of transforming DNA exposed to competent cells is only two-fold (11) and no single stranded DNA has been detected during transformation (12). Since the final product of recombination in all three systems is a heteroduplex molecule with donor and recipient DNA components (8, 12, 13) integration of donor DNA into the recipient genome must occur by the incorporation of a single strand of donor DNA. An interesting feature of the H. influenzae system therefore is how specific pairing and integration occur in the absence of detectable single stranded intermediates.

The research reported in this dissertation was undertaken to investigate the structure of resident DNA in competent H. influenzae cells (recipient DNA), in order to determine if detectable physical structures in this DNA may give clues to how specific pairing between donor DNA and recipient DNA may take place. The interaction between donor and recipient DNA in the competent cell has been investigated in order to determine intermediate steps between the pairing of these DNAs and final integration of donor DNA into recipient DNA. These studies have utilized mutations in H. influenzae that may affect the recombination process. In Chapter I, evidence is presented that the DNA synthesized at the peak of competence in wild type cells has single stranded

character, which is lacking in competent cells of a mutant defective in association of donor and recipient DNA. Thus, single stranded regions in recipient DNA of competent H. influenzae cells may be essential for the pairing reaction. In Chapter II, evidence is presented that during the interaction of donor and recipient DNA in strains of H. influenzae which associate these DNAs, single strand breaks occur in the DNA synthesized before competence development, except in competent cells of a mutant deficient in an ATP-dependent nuclease. Thus, single strand breaks in recipient DNA may play an important role in the normal interaction of donor and recipient DNA during recombination in H. influenzae. In Chapter III, a model is suggested for how recombination may take place during transformation in H. influenzae. This model is compared with recombination models based on evidence from other systems.

CHAPTER I

THE DNA OF COMPETENT CELLS

Introduction

Competence regimes have been devised to bring whole cultures of H. influenzae into the competent state, defined as the ability of cells to take up DNA irreversibly from the medium (14). Cells growing exponentially do not have the capacity to take up DNA, but have been made highly competent under conditions of reduced aeration in growth medium followed by a short period of aeration (14). Competence regimes (15, 16) have been developed which involve shifting exponentially growing cells to a slow growth phase in defined media with aeration, during which a peak of competence appears. Either prolonged cessation of growth or the reestablishment of exponential growth causes a decay of the competent state (15). Spencer and Herriott (15) conclude that competence is induced in culture conditions that permit protein synthesis but suppress duplication. In the M-IV medium (16) these conditions include high cell concentration, the presence of several amino acids and the absence of essential growth factors such as nicotinamide adenine dinucleotide (NAD), pantothenate and pyrimidine nucleosides. Several substances, probably affecting protein or nucleic acid synthesis, inhibit the development of competence, including chloramphenicol (15), NAD (17), inosine (17), uracil (17) and valine (15). Competence may be a precisely regulated

state requiring induction of particular proteins necessary for the development of several metabolic and structural changes (17). Such a regulated state is suggested by the finding that cyclic adenosine monophosphate induces exponentially growing cells to become partly competent (18).

Conditions that induce competence in H. influenzae suggest that DNA in competent cells is not being actively replicated (see above). Measurement of the DNA synthesized during the 100 minutes of the M-IV competence regime showed that total DNA increased by a factor of 1.8, closely paralleling the increase in cell titer (19). Thus the generation time is increased by a factor of about three, compared to the generation time for cells in growth medium.

In order to determine if the DNA synthesis observed in the slow growth phase of competent H. influenzae cells is similar to that seen in exponential phase growth, I have investigated DNA synthesis during the competence regime by pulse labeling of DNA with radioactive and/or density isotopes and analysis of the products of synthesis. DNA synthesis in competent cells of H. influenzae mutants that are recombination defective has also been studied.

Materials and Methods

Bacterial strains. H. influenzae strain Rd (wild type) was originally obtained from R. M. Herriott. The recombination-defective strains rec1 [(DB117)] and rec2 [Rd(DB117)$^{rec-}$] have been described (20, 21).

Media for cells. The liquid growth medium (22) contained 3.5% Brain Heart Infusion (BHI; Difco), was autoclaved 25-35 min, and was supplemented after cooling with 10 µg/ml hemin and 10 µg/ml of L-histidine (previously dissolved at 1 mg/ml in 4% triethanolamine), and 20 µg/ml of nicotinamide adenine dinucleotide (NAD). The solid growth medium contained 2.7% Brain Heart Infusion, 0.75% Eugonbroth (BBL), 1.2% agar (Difco), and was also supplemented with hemin, L-histidine and NAD. In some experiments the chemically defined MI_ccit (23) medium was used, in which inosine is the only nucleoside, other than in NAD, and in which H. influenzae cells grow rapidly.

Growth and storage of cells. Cells were grown at 37°C with aeration in a side-armed flask swirled in a gyratory shaker. Growth rate was monitored by measuring the optical density at 675 nm (OD_{675}). Cells in liquid growth medium supplemented with 15% glycerol were frozen and stored at -80°C.

Preparation of competent cells. Wild type or mutant cells were grown in BHI growth medium to an OD_{675} of 0.4. Cells were centrifuged, washed once with M-IV medium (16) and resuspended at a concentration of about 8×10^8/ml in 2 ml of M-IV medium. Cells were incubated for 1°0 min at 37°C with vigorous aeration and used immediately for experiments.

Labeling DNA with radioisotopes. For fully labeling DNA during exponential phase growth, cells in BHI growth medium at an OD_{675}

of about 0.05 were supplemented with 0.8 μCi of [^{14}C]deoxythymidine (dThd, 62 mCi/mmole) per ml and grown to an OD_{675} of 0.4, which represents a cell titer of about 8×10^8/ml. For pulse labeling DNA of competent cells, cells were washed and resuspended in M-IV medium (16) and incubated at 37°C with vigorous aeration for 100 min. The M-IV medium was then supplemented with 10 μCi of [^{3}H]dThd (17 Ci/mmole) per ml and incubated at 37°C with vigorous aeration for 10 min. Cells were then cooled to 0°C. For pulse labeling DNA of exponentially growing cells, cells were washed and resuspended in BHI growth medium at an OD_{675} of about 0.15 and grown to an OD_{675} of about 0.27. The growth medium was then supplemented with 30 μCi of [^{3}H]dThd (17 Ci/mmole) per ml and incubated at 37°C with aeration for 10 min. Cells were then cooled to 0°C. For pulse and chase experiments, samples labeled for 10 min with [^{3}H]dThd were washed twice in M9 salts (24), resuspended in either M-IV or MI_ccit growth medium and incubated further before cooling aliquots to 0°C. For measuring incorporation of [^{3}H]dThd, the same method as for pulse labeling was used, except that 15 ul aliquots were removed with time after addition of [^{3}H]dThd, spotted on Whatman 3MM paper discs, washed twice in ice-cold 5% TCA, twice in 95% ethanol, dried and counted. Counts were normalized to the [^{14}C]dThd prelabel.

Transformation assay. Transforming DNA conferring resistance to streptomycin (250 μg/ml) and purified according to the method of Marmur (25) was added to competent cells to a final concentration of 1 μg/ml.

After 30 min incubation at 37°C with aeration, cells were diluted and plated in 10 ml solid growth media. Plated cells were incubated at 37°C for 1.5 hr and overlayed with 10 ml solid growth medium with or without 250 μg/ml streptomycin sulfate. Plates were then incubated overnight at 37°C and totals and transformants counted the next day. Transformation frequency is defined as the number of transformants per ml/total number of cells per ml.

Measurement of DNA binding. Cells were subjected to the M-IV competence regime and an aliquot of 0.35 ml was mixed with approximately 0.01 μg [^{3}H]dThd-labeled DNA purified according to the method of Marmur (25). After incubation at 37°C for 15 min, 0.1 ml DNase (1 mg/ml, Worthington) was added and the cells were further incubated for 5 min. Cells were washed three times in M9 salts (24), resuspended in M9 salts and samples spotted on Whatman 3MM paper discs. Discs were processed and counted as above.

Preparation of sarkosyl lysates. A modification of the method of Iyer and Rupp (26) was used to prepare DNA for column chromatography and density-gradient sedimentation. Cells at 0°C were washed once in ice-cold 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA, resuspended in 10 mM Tris-HCl (pH 7.6) and 10 mM EDTA at three times the cell concentration, and lysozyme (Sigma) was added to approximately 3 mg/ml. After 10 min incubation at 0°C, the mixture was made approximately 0.8% with 4% Sarkosyl NL30 (Geigy Industrial Chemicals). Cells were left at 0°C until complete lysis.

Velocity sedimentation. Cells at 0°C were washed and resuspended in ice-cold 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA to a final concentration of 0.5 - 1.0 × 10^9 cells per ml. In a modification of the method of McGrath and Williams (27), samples of 0.1 ml were layered in 0.1 ml NaOH (0.5 N) on 5 to 20% sucrose gradients containing NaOH to pH 12.5 and 0.1 M NaCl. Tubes were centrifuged in a Beckman SW50.1 rotor in an L350 centrifuge at 30,000 rev/min for 100 min at 20°C.

Estimation of molecular weights. Molecular weights were estimated by a computer program with a calibration previously described (28). The program also corrected for background and spill-over in double-label experiments.

Density gradient sedimentation. A rapid equilibrium method (29) was used as follows. Two CsCL solutions in 10 mM Tris-HCl, pH 8.1 (for neutral CsCl) or in 0.15 N NaOH (for alkaline CsCl) were prepared with densities of 1.742 and 1.846 gm/cm^3, and 1.87 ml of the higher-density solution was placed in a 5-ml polyallomer tube. A sample diluted to 0.7 ml in 0.15 N NaCl plus 0.015 M trisodium citrate (for neutral CsCl) or 0.15 N NaOH (for alkaline CsCl) was mixed with 1.5 ml of the lower density solution and layered in the tube. One ml of mineral oil was added to the tube and the tube was centrifuged in an SW50.1 rotor of a Beckman centrifuge at 32,000 rev/min for 18 hr at 15°C.

Collection of fractions. About 30 10-drop fractions from sucrose gradients or 60 5-drop fractions from CsCl gradients were collected on

Whatman 17M paper strips and processed by the procedure of Carrier and Setlow (30).

Assay of radioactivity. Radioactivity on discs or strips was counted in a Packard liquid scintillation counter with approximately 5 ml of 0.3% Permablend I (Packard; 91% PPO and 9% dimethyl POPOP) in toluene.

Benzoylated naphthoylated DEAE-cellulose (BND-cellulose) column chromatography. BND-cellulose (Boeringher-Mannheim) was equilibrated with 0.3 M NaCl in 1 mM Tris-HCl (pH 8.1) and 1 mM EDTA (referred to as 0.3 M NTE) and packed in a 2 cc glass syringe on a 0.5 cm disc of Miracloth (Chicopee Mills, Inc., New York, N.Y.). A sample was slowly applied in 10 ml 0.3 M NTE and washed with 6 ml 0.3 M NTE. A gradient of 100 ml 0.3 M NTE to 1.0 M NTE and 2% caffeine (prewarmed to 37°C) was applied with a flow rate of approximately 0.5 ml per min and approximately 2-ml fractions were collected with a fraction collector. Samples of 0.2 ml were spotted on Whatman 17M filter paper strips and were processed and counted as above.

Labeling DNA with 5-bromodeoxyuridine (BrdUrd). DNA was fully labeled with [^{14}C]dThd as described above. For labeling DNA of competent cells with BrdUrd, competent cells in the M-IV medium were supplemented with a mixture of 25 µg cold BrdUrd per ml and 15 µCi [^{3}H]BrdUrd (15.3 Ci/mmole) per ml, incubated at 37°C with vigorous aeration, samples taken and placed at 0°C. For labeling DNA of exponentially growing cells, cells labeled with [^{14}C]dThd to an OD_{675} of 0.4 were washed and

resuspended in MI_ccit medium at an OD_{675} of about 0.15 and grown to an OD_{675} of about 0.27. The cells were then washed and resuspended in MI_ccit medium supplemented with the same level of BrdUrd as used for competent cells, incubated at 37°C with vigorous aeration, samples taken and placed at 0°C.

S1 nuclease purification and digestion. S1 nuclease from Aspergillus oryzae was purified from α-amylase (Sigma; crude Type IV-A) according to the method of Sutton (31). One-ml reaction mixtures contained a sample of DNA in 30 mM sodium acetate buffer (pH 4.5), 1 mM $ZnSO_4$, 0.2 M NaCl and 20 µg sonicated and heat-denatured calf thymus DNA (Worthington). Five µl of enzyme was added to the reaction mix at 0°C and the tube was transferred to a 50°C water bath. Aliquots were removed and spotted on Whatman 3MM filter discs, the discs were washed twice in 5% ice-cold TCA, twice in 95% ethanol, dried and counted.

Results

Incorporation of [^{3}H]dThd. Figure 1 (Appendix) shows the incorporation of [^{3}H]dThd by competent and exponentially growing H. influenzae wild type cells. In competent cells, the incorporation is rapid and short-lived, compared to cells in exponential phase growth. Furthermore, the incorporated counts remain in acid-precipitable form and do not decrease upon further incubation of the cells. Kinetics similar to wild type are shown in Figure 1 for competent cells of the rec1 and rec2 mutants of H. influenzae, although the total incorporation in competent rec2 cells is greater than in competent wild type cells.

The ribonucleoside inosine has been shown to enhance the incorporation of [^{3}H]dThd into exponentially growing H. influenzae cells (32), possibly by inhibiting the conversion of thymidine to thymine (33). Table 1 (Appendix) shows that at high concentrations inosine decreases the magnitude of incorporation into competent cells, but has little or no effect at levels used to enhance incorporation into exponentially growing cells (32).

Alkaline sucrose gradient sedimentation. To determine the size of the DNA synthesized in competent versus exponential phase cells, the method of McGrath and Williams (27) was used to sediment high molecular weight DNA. The DNA was labeled during two to three cell divisions before the competence regime with [^{14}C]dThd and pulse labeled at the peak of competence or in exponential growth with [^{3}H]dThd. Figure 2 (Appendix) shows sedimentation patterns in alkaline sucrose of DNA from lysed competent and exponential phase wild type, and competent rec2 and rec1 cells. In the competent wild type cells, the pulse label is mostly in smaller DNA whose molecular weight (weight average) in alkali is about 5×10^7. However the prelabeled DNA, with a molecular weight of about 2×10^8 in alkali, shows no peak at the position of the pulse-labeled DNA. The DNA of exponentially growing wild type cells also shows no peak comparable to the pulse label in competent cells. The DNA of competent rec2 cells does not contain a comparable amount of slow sedimenting, newly-synthesized DNA and the pulse-labeled DNA of rec1 cells, although containing more fast-sedimenting pulse label than wild type, does contain a predominant slow-sedimenting peak.

In order to verify the difference in the size of pulse-labeled DNA in competent wild type and rec2 cells, short pulses of [^{3}H]dThd were given to [^{14}C]dThd-prelabeled competent cells and the size of the DNA analyzed by alkaline sucrose gradient sedimentation. The results for 15 and 30 sec pulses are shown in Figure 3 (Appendix). It is seen that even at 15 seconds, pulse-labeled DNA in competent rec2 cells sediments with the bulk of the prelabeled DNA while little pulse-labeled DNA in competent wild type cells sediments with the prelabeled DNA. There are also clear differences in the proportion of fast sedimenting pulse-labeled DNA by 30 seconds in the two strains. It is further noted that in both strains a significant proportion of the pulse-labeled DNA is near the top of the gradient, sedimenting to a position representing about 2-4 $\times$ 10^{6} single strand molecular weight. This material was not observed in the 10 min pulse time (cf. Figure 2, Appendix).

Pulse-chase experiments have indicated another significant difference between the DNA synthesized in competent cells and in exponentially growing cells. The slow-sedimenting DNA synthesized in competent wild type cells does not rapidly chase into fast-sedimenting DNA upon incubation of the cells in nonradioactive M-IV medium. Figure 4 (Appendix) shows alkaline sucrose gradient profiles of DNA from competent wild type cells pulse-labeled in M-IV medium for 10 minutes, and chased in either nonradioactive M-IV medium (left panel) or MI_{c}cit growth medium (right panel) for 30 minutes and 45 minutes. It is seen that the pulse-labeled DNA slowly chases into faster-sedimenting DNA in the M-IV medium, but that the process is faster and may be more complete in the MI_{c}cit growth

medium (the 45 minute sample in MI_ccit medium cosediments with [^{14}C]dThd prelabeled DNA not shown in Figure 4, Appendix). DNA pulse-labeled in growth medium for 10 minutes cosediments with prelabeled DNA within 15 minutes incubation in nonradioactive growth medium (data not shown). Pulse-labeling of DNA throughout the 100 minutes of the M-IV competence regime shows that slow-sedimenting DNA is made initially, but only after 50-60 minutes does the pulse-labeled DNA not rapidly chase into fast-sedimenting DNA (data not shown).

To determine the size of the DNA made in noncompetent cells in M-IV medium, wild type cells were subjected to the M-IV competence regime in the presence of various amounts of inosine, shown by Miller and Huang (17) to inhibit competence development, pulsed for 10 minutes with [^{3}H]dThd and the DNA sedimented in alkaline sucrose. Even at levels of inosine that do not affect the incorporation of [^{3}H]dThd, there is a decrease in the fraction of slow-sedimenting DNA with increasing concentrations of inosine. Figure 5 (Appendix) summarizes these results and compares the fraction of slow-sedimenting DNA with the level of transformation of cells exposed to the same concentrations of inosine during the competence regime. There is a dose dependent decrease in both the amount of slow-sedimenting DNA made during the labeling period and the level of transformation in the presence of inosine. The inset of Figure 5 compares the relative amount of radioactive DNA bound to cells and the relative transformation frequency of cells subjected to the competence regime with increasing concentrations of inosine. The data indicate that inosine inhibits the development of competence as defined by irreversible

binding of DNA, besides decreasing the level of transformation and the formation of slow-sedimenting DNA characteristic of competent cells.

Benzoylated naphthoylated DEAE (BND) cellulose column chromatography. BND-cellulose column chromatography permits the separation of double stranded DNA containing single stranded regions from completely double stranded DNA (26). To determine if the DNA synthesized in competent and exponentially-growing wild type cells and competent rec2 cells contains single stranded regions, lysates of cells prelabeled with [^{14}C]dThd before the competence regime and pulse-labeled for 10 minutes at the peak of competence were chromatographed. The top panel of Figure 6 (Appendix) shows that about 75% of the pulse-labeled DNA and 30% of the prelabeled DNA from competent cells elutes from the column at a position indicating single stranded regions in the DNA. Two observations are made from elution profiles of labeled DNA from competent rec2 cells (middle panel, Figure 6) and exponentially growing wild type cells (bottom panel, Figure 6): (1) the pulse-labeled DNA has less single stranded character, mostly eluting at the position of fully double stranded DNA; and (2) both the prelabeled and pulse-labeled DNA elute with a similar pattern.

S1 nuclease digestion. S1 nuclease from Aspergillus oryzae (34) degrades denatured DNA and single stranded regions in double stranded DNA, but has little effect on fully double stranded DNA (31). Figure 7 (Appendix) shows a control experiment in which the enzyme has no detectable effect on native H. influenzae DNA isolated from stationary phase

cells, but degrades approximately 97% of the same DNA which has been heat-denatured. Eluted material from BND-cellulose has been extensively dialyzed against 10 mM NaCl and treated with this enzyme. Table 2 (Appendix) shows the results of S1-nuclease digestion of this DNA. Neither the [^{14}C]dThd prelabel nor the [^{3}H]dThd pulse label in DNA eluted from the completely double stranded position on BND-cellulose is significantly affected by the enzyme, as expected. Of the DNA eluted from the partially single stranded position on BND-cellulose, approximately 15% of [^{3}H]dThd in pulse-labeled DNA is released by the enzyme, whereas there is no effect on the [^{14}C]dThd prelabel. This indicates that only the pulse-labeled DNA synthesized in competent cells is exposed to the single-strand-specific enzyme as a single stranded region in the DNA of competent cells.

CsCl density gradient sedimentation. In order to determine if the DNA synthesis seen in competent cells is a replicative-type or a repair-type synthesis, fully [^{14}C]dThd labeled competent wild type cells were labeled with the density isotope [3H]5-bromodeoxyuridine (BrdUrd), sarkosyl lysates made and the DNA sedimented in CsCl density gradients. For comparison, [^{14}C]dThd labeled cells from the same preparation but not subjected to the competence regime were labeled with BrdUrd at the same concentration, and the DNA sedimented in CsCl. The results are shown in Figure 8 (Appendix). The following observations are made: (1) both competent and exponentially growing cells incorporate more BrdUrd with time of incubation; (2) the newly synthesized DNA in

competent cells has a buoyant density slightly greater than the prelabeled, "light" DNA, and the density of this DNA does not increase despite more incorporation of BrdUrd with time of incubation; (3) the newly-synthesized DNA in exponentially growing cells is heterogeneous in buoyant density initially, but with time forms DNA of hybrid density; and (4) the prelabeled DNA in exponentially growing cells shifts to the hybrid density with time of incubation, unlike that in competent cells. Alkaline sucrose gradient sedimentation of the same samples shows that in the presence of BrdUrd, slow sedimenting DNA is synthesized in competent cells similar to that shown in Figure 2 (Appendix) (data not shown). Also, control experiments have shown that the buoyant density of prelabeled DNA in cells exposed to BrdUrd at the peak of competence (at the same level as in the experiment of Figure 8, Appendix) is the same as in competent cells not exposed to BrdUrd (data not shown). Figure 9 (Appendix) shows 20-min sample profiles on alkaline CsCl density gradients. The denatured DNA from competent cells substituted with BrdUrd does not shift to a fully dense position as does the DNA from exponentially growing cells and shows a heterogeneous buoyant density. Further, no detectable prelabeled DNA from competent cells shifts to a denser position than expected for denaturation alone, indicating that BrdUrd is incorporated only into a strand of DNA synthesized during the competence regime.

Discussion

Summary of results. The results of an investigation of the DNA synthesized in competent H. influenzae wild-type cells are summarized as follows. The strand of DNA synthesized during the competence regime is synthesized in pieces sedimenting in alkaline sucrose gradients with an average size one-fourth that of the template DNA, as judged by alkaline sucrose gradient sedimentation. The major differences between this type of synthesis and the synthesis observed in exponentially growing cells are (1) the DNA synthesized in competent cells is smaller, and (2) the lower-molecular weight DNA synthesized in competent cells does not rapidly chase into high-molecular weight DNA. No evidence has been obtained for detectable breaks in the template DNA (the prelabeled DNA in these experiments) made before subjecting cells to the competence regime. The results of BND-cellulose chromatography of DNA from competent wild type cells show that some or all of the DNA with single strand breaks or alkali-labile bonds seen in alkaline sucrose contain single stranded regions, since there is correspondence between the fraction of DNA containing breaks and the fraction with single stranded character. The results of nuclease digestion suggest that single stranded regions expose the newly-synthesized DNA made during the competence regime. Finally, incorporation of the density isotope 5-bromodeoxyuridine into the DNA of competent cells shows that (1) this analog is incorporated only into a strand of DNA synthesized during the competence regime, and (2) the incorporation does not represent mainly semiconservative DNA synthesis since DNA of hybrid density is not made.

Incorporation of [^{3}H]dThd in competent cells. The aberrant incorporation of [^{3}H]dThd observed in competent cells remains unexplained. The phenomenon is not easily studied, since the use of pyrimidine nucleosides affects the competent state (17). Three explanations for the rapid and short-lived kinetics of incorporation are suggested. First, conversion of thymidine to thymine (33) may be very efficient, especially at the high cell concentration of competent cultures. [^{3}H]thymine is not incorporated into the DNA of competent H. influenzae cells (35). The depression of incorporation of [^{3}H]dThd into competent cells in the presence of high concentrations of inosine (at a level present in the MI_ccit medium) does not favor this interpretation, since inosine has been shown to inhibit the conversion of thymidine to thymine in exponentially-growing cells (32). Inosine, which is not required for growth of H. influenzae in a chemically defined medium (23), is essential during growth in defined medium in order for cells to become fully competent during the slow growth phase in M-IV medium (36). Nevertheless, the presence of inosine in the M-IV medium inhibits competence development (17). Thus the effect of this nucleoside on competence development remains complex. Second, as suggested by R. M. Herriott (37), competent cells starved for pyrimidine nucleosides may rapidly incorporate low levels of exogenous nucleosides at gaps in nonreplicating chromosomes. Third, [^{3}H]dThd may be incorporated at several, halted replication forks in competent cells.

DNA synthesis in competent cells. Since DNA is synthesized as short single stranded pieces in M-IV medium even in noncompetent cells,

factors necessary for rapidly sealing this DNA into high-molecular weight DNA must be absent from the medium. This is consistent with the absence of essential growth factors such as NAD and pyrimidine nucleosides in the M-IV medium (16). However, only as the cells in M-IV medium become competent do the lower-molecular weight pieces not chase rapidly into high molecular weight DNA, suggesting that either (1) low levels of essential growth factors present at early times inside the cells are depleted throughout the competence regime or (2) the induction of competence also involves the induction of factors regulating the type of DNA synthesis observed in competent cells.

The presence of single stranded regions in the strand of DNA synthesized during the competence regime could be simply attributed to exonucleolytic degradation at the site of single strand nicks, enlarging the nicks into gaps and thus exposing the prelabeled template strand as single stranded DNA. However, the use of an enzymatic probe selective for single stranded DNA has shown that the pulse-labeled DNA is more susceptible to degradation, indicating that the newly synthesized pieces of DNA are exposed as single stranded regions. One interpretation of this result is that the labeled structure is the product of a single strand displacement reaction at the site of nicks in the newly synthesized DNA, such that as demonstrated *in vitro* for *E. coli* DNA polymerase I by Masamune and Richardson (38). In this reaction, diagrammed in Figure 10 (Appendix), a nick in duplex DNA serves as a primer template for the polymerizing enzyme (a). New nucleotides are added covalently on the 3' terminus at the site of the nick while degradation takes place

at the 5' terminus of the nick (b), thus translating the nick. After the addition of approximately 50 nucleotides, the 5' end of the primer strand is displaced rather than being degraded by the 5' → 3' exonucleolytic activity of the polymerase (c). Masamune and Richardson (38) found that the newly synthesized DNA is susceptible to hydrolysis by exonuclease I, a single-strand specific enzyme that degrades from a 3' terminus. They proposed that the originally displaced strand could replace the newly synthesized strand by a branch migration process (d) and that structures (c) and (d) are in equilibrium. Evidence for a branch migration process comes from the work of Lee et al. (39). They found that single stranded branches could migrate, breaking and reforming hydrogen bonds while displacing a single strand on the opposite side of a nick and resulting in a single stranded branch of the opposite polarity. Why a polymerase switches from a nick translation mode of synthesis to one of strand displacement remains unexplained. One possibility, again suggested by Masamune and Richardson (38), is that gaps are created ahead of the polymerizing enzyme, giving rise to a metastable DNA duplex that allows the polymerase to invade the duplex at the growing point. Conceptually, this type of "repair" synthesis is not too different from the displacement synthesis at the chromosomal growing point first suggested by Mitra et al. (40), except that one product of this unidirectional synthesis is single stranded.

As a rationale for the results presented in Chapter I, I propose that most of the DNA synthesis represented by incorporation of [^{3}H]dThd in competent H. influenzae cells is a product of strand displacement

synthesis. Nicks or gaps may be present in the DNA synthesized in competent cells, due to the absence of essential growth factors in the M-IV competence medium. Replication point-like regions may arise at these nicks or gaps and polymerization result in structures similar to those shown in Figure 10 (Appendix). Progression of the replication fork would be halted either because of the absence of essential precursors for continued synthesis or because of degradation of the single stranded ends and eventual resealing into the DNA duplex.

Several lines of evidence may be interpreted in terms of this model proposed for DNA synthesis in competent cells.

1. The large amount of incorporation of [^{3}H]dThd in competent cells (Figure 1, Appendix) may result from several such replicating points on the chromosome. Similar incorporation is seen in competent rec2 cells (Figure 1) and in noncompetent cells in the M-IV medium (Table 1, Appendix), both of which synthesize DNA of higher-molecular weight than competent wild type cells. Thus, there may be a similar number of replicating points in competent rec2 cells and noncompetent cells in M-IV, with faster sealing of newly synthesized DNA into DNA of high molecular weight.
2. Alkaline sucrose gradient sedimentation of DNA pulsed for short times in competent cells (Figure 3, Appendix) shows a significant proportion of single stranded DNA sedimenting in a position similar to the newly synthesized fragments described by Okazaki et al. (71). Such single stranded

pieces would not be expected to be synthesized during normal repair replication (42) and may be products of limited synthesis at new replicating points.

3. The results of CsCl density gradient sedimentation of BrdUrd-containing DNA indicate that DNA synthesis in competent cells is not semiconservative in that hybrid DNA is not formed. However, the density of the newly synthesized DNA is similar to that seen at a very early time in semiconservative DNA synthesis (Figures 8 and 9, Appendix), however, and may represent limited incorporation of the density isotope at halted replication points.
4. The product of new synthesis in competent wild type cells is partly exposed as single stranded DNA to a single-strand specific enzyme.

The possibility that the prelabeled DNA in competent cells (synthesized during exponential phase growth) is also exposed as a single stranded DNA is not excluded. The absence of significant degradation by the S1 nuclease may be due to the low specific activity of the [^{14}C]dThd label in this DNA. Indeed, earlier experiments (not shown) using a different DNA preparation showed release of this label that may have been due to differences in labeling conditions.

Possible involvement of DNA synthesis in transformation. Several lines of evidence, albeit circumstantial, suggest that single stranded regions in DNA synthesized during the competence regime play a role in recombination reactions.

1. No comparable single stranded character is present in the DNA of exponentially growing cells, which do not bind DNA and become transformed.
2. As cells become competent during the M-IV competence regime, low-molecular weight DNA does not chase rapidly into high-molecular weight DNA.
3. The pyrimidine nucleoside inosine, at levels that do not affect the incorporation of [^{3}H]dThd, inhibits both the development of competence and synthesis of low-molecular weight DNA.

The most direct evidence that single stranded regions in the DNA of competent cells comes from experiments with recombination-defective mutants of H. influenzae. The rec2 and rec1 mutants, with respective recombination frequencies of 10^{-7} and 10^{-6} relative to wild type, can be made fully competent as judged by irreversible binding of DNA. However only the competent rec1 cells synthesize DNA with features similar to that of wild type. The pulse-labeled DNA of competent rec2 cells does not contain comparable single strand breaks as judged by alkaline sucrose nor comparable single stranded character as judged by BND-cellulose chromatography. Notani et al. (44) have shown that in strain rec2 there is little or no association between donor and recipient DNA during transformation, whereas donor DNA molecules become covalently linked to recipient DNA in the wild type and rec1 strains. It is therefore possible that single stranded regions in the DNA of competent cells are required for association of donor and recipient DNA in an early step in recombination, such as providing pairing sites for association with

incoming donor DNA. This interpretation implies an active role for DNA synthesis in transformation, in providing a single strand precursor for the recombination reaction. Such a role does not conflict with the relatively low amount of net DNA synthesis taking place during the competence regime (19), since DNA synthesis, proposed to take place at induced replication points, is limited and not like semiconservative DNA synthesis.

CHAPTER II

INTERACTION OF DONOR DNA AND RECIPIENT DNA

Introduction

Several investigations provide information on the fate of donor DNA upon uptake and its interaction with recipient DNA in competent H. influenzae. Features relevant to this dissertation are reviewed as follows.

Donor transforming DNA is taken up in competent H. influenzae cells as a double stranded molecule, as determined by (1) lack of an eclipse phase of donor DNA after entrance into the cell (11, 45), (2) lack of detection of single stranded DNA by equilibrium centrifugation in CsCl of unintegrated donor DNA extracted from cells (12, 11) and (3) analysis of specific biological activity of unintegrated donor DNA in supernatants of digitonin treated transforming cells (44). Donor DNA pairs with the recipient DNA by an unknown mechanism. Somewhat more than half of the biological activity of donor DNA exposed to competent wild type cells is lost either before or during integration into recipient DNA (11, 46). Covalent linkage with recipient DNA is established, since recombinant DNA consisting of heavy donor and light recipient DNA atoms does not shift to a fully dense position upon denaturation and equilibrium centrifugation (12, 44). The KB6 (47) and rec2 (21) mutants of H. influenzae are blocked at or before the association step, since little or no association between heavy donor and light recipient DNA is revealed by

equilibrium sedimentation (44, 35). The following lines of evidence indicate that the association reactions involve the transfer of one donor strand to the recipient DNA: (1) the position of recombinant molecules on CsCl gradients of light recipient and heavy transforming DNA remains the same upon one replication (12); and (2) upon denaturation, hybrid DNA shifts to a denser position than expected for denaturation alone (12). Notani and Goodgal (12) estimated the size of this single tract of donor DNA to be 6×10^6 daltons, although the material appears to be quite heterogeneous. By following the transforming activity of the recipient strand associated with the donor DNA as a function of growth of the transforming cells, Goodgal and Notani (18) showed that either of the donor DNA strands in duplex donor DNA strands in duplex donor DNA is active in transformation.

The donor DNA not integrated into the recipient genome is degraded (12, 45, 49) which may account for the loss of approximately half of the biological activity of donor DNA after uptake (11, 46). The fate of degraded donor DNA may depend upon transformation conditions. Notani and Goodgal (12) concluded that degraded donor DNA is reincorporated into recipient DNA under conditions of growth while Stuy (45) and Kooistra and Venema (49) showed degraded donor DNA is released into the medium under conditions where the cell is not synthesizing an appreciable amount of DNA. Steinhart and Herriott (50) showed H. influenzae recipient label is also degraded and released into the medium as a result of exposure of labeled competent cells to unlabeled transforming DNA. Since the released resident DNA approximates the amount of donor DNA integrated, resident DNA may be displaced when a single stranded piece

of donor DNA is inserted into the recipient genome. The recl mutant of H. influenzae (20) shows the same release of resident label, with or without exposure to transforming DNA (51) although there is covalent linkage between donor and recipient DNA (44); thus specific release of resident DNA may be a late stage in the recombinant reactions.

Voll and Goodgal (11) found a lag of two to three generations after DNA uptake before the number of transformants in the recipient population began to increase. However, assay of lysed samples of the transformed recipient culture on a strain sensitive for markers initially in transforming DNA or recipient cells showed that by 30 minutes after uptake the donor marker was increasing at the same rate as recipient markers (11). They attributed this lag in the increase of transformants to joined daughter cells, giving rise to only one clone, and to multiple nuclei per cell, only one of which integrates transforming markers.

Several studies show little involvement of DNA synthesis in integration. Voll and Goodgal (11) concluded that integration could occur with less than 15% synthesis of total DNA, by transforming cultures in saline and assaying the increase of a recipient marker. Assaying by the incorporation of [^{3}H]dThd, Notani et al. (52) measured increases of 3% and 0.3% in recipient DNA for transformations done in saline and saline-fluorodeoxyuridine, respectively. Muhammed and Setlow (53), using the temperature-sensitive DNA synthesis mutant dna9, observed integration at the restrictive temperature, although uptake was allowed under permissive conditions. Integration has also been measured in the presence of an inhibitor of DNA replication, 6-(p-hydroxyphenylazo)-

uracil, and found to be normal (54). Finally, thymidine deprivation in a thymidine-requiring mutant of H. influenzae has little effect on transformation frequency (55). None of these authors have ruled out a requirement for a repair-type synthesis for pairing or integration of donor DNA.

Few mutants of H. influenzae have been characterized to the extent that specific biochemical lesions are known. Mutants deficient in the ATP-dependent nuclease have been isolated however, and found to be only slightly (56) or highly (57) recombination deficient. Only the recombination-proficient addl mutant isolated by Wilcox and Smith (56) has been transformed into a wild type background however, so that the single mutation probably has little effect on transformation or phage recombination frequencies.

In Chapter I of this dissertation, I have presented evidence suggesting that single stranded regions in the DNA synthesized in competent H. influenzae cells are important for an early step in recombination. Since it is possible that donor transforming DNA may pair with recipient DNA at these single stranded sites, it becomes important to study the interaction of donor DNA with the recipient DNA. Thus I have studied this interaction in competent wild type H. influenzae and have further utilized H. influenzae strains with mutations that may affect this interaction.

Materials and Methods

Bacterial strains. In addition to the strains used in Chapter I, the following strains were used. Strain dna9 has a temperature-sensitive DNA synthesis mutation from the strain ts9, isolated by K. L. Beattie and J. E. LeClerc. The double mutant dna9rec1 was constructed by transformation of strain dna9 with a DNA lysate made from a streptomycin-resistant strain rec1 and selection for streptomycin resistence. Its isolation and characteristics have been described (58). Strain add1 (KW31) lacks an ATP-dependent nuclease (56) and was kindly provided by K. W. Wilcox. All mutations have been transformed into the wild-type strain free of the mutagenized background.

Media for cells, Growth of cells, Preparation of competent cells, Velocity sedimentation in alkaline sucrose, Collection and assay of fractions, Estimation of molecular weight, Transformation assay, Preparation of sarkosyl lysates and S1 nuclease digestion. These materials and methods are described in Chapter I.

Release of recipient DNA. A modification of the method of Steinhart and Herriott (50) was used. H. influenzae cells were grown in the presence of 0.8 μCi (wild type and rec2) or 1.6 μCi (rec1) of [^{14}C]dThd (62 mCi/mmole) per ml, made competent in M-IV medium and pulsed for 10 min with 30 μCi of [^{3}H]dThd (17 Ci/mmole). At the same time cells were treated with approximately 45 μg/ml of transforming DNA or the same volume of 0.067 M phosphate buffer (pH 7) used as a diluent for the DNA (experimental and control samples, respectively). After a 10-min

incubation at 37°C with [^{3}H]dThd and with or without transforming DNA, the cells were washed three times with M9 salts (24) and resuspended in M-IV medium with approximately 45 µg/ml transforming DNA or the same volume of 0.062 M phosphate buffer (experimental and control samples, respectively). After various incubation times, aliquots were taken and diluted two-fold with phosphate buffer, put through 0.45 µm Millipore filters and the radioactivity in 0.3 ml of filtrate was counted in a scintillation counter after addition of dioxane-napthalene-2, 5-bis-[2-(5-tert-butylbenzoxazolyl)] thiophene scintillation fluid. The total incorporated label was determined as acid-insoluble radioactivity of the mixture before filtration and used to calculate the fraction of incorporated label released from the cell DNA into the medium.

Preparation of ^{32}P-labeled DNA. Wild type H. influenzae cells were grown in a low phosphate MI_ccit medium (23) and supplemented with 20 uCi $^{32}PO_4$ per ml. After overnight growth, cells were washed three times with 0.15 M NaCl plus 0.015 M trisodium citrate (SSC) and lysed at 37°C with 0.5% sodium lauryl sulphate. The lysate was made 2 M with NaCl and shaken with an equal volume of chloroform-amyl alcohol (24:1) for 1 hr, as described by Marmur (25). After separation by low-speed centrifugation, the aqueous layer was removed and treated with ribonuclease at 37°C for 30 min. After another treatment with chloroform-amyl alcohol and separation, the preparation was passed through a Sephadex G-100 column, with a bed volume of approximately 60 ml, and eluted with SSC. The excluded volume containing most of the radioactivity was used in the experiments.

Transformation of [^{3}H]dThd-labeled cells with ^{32}P-labeled DNA. Cells were grown in BHI growth medium supplemented with 8 μCi [^{3}H]dThd per ml and made competent in nonradioactive M-IV medium as described above. At 100 min, 0.3 ml transforming DNA labeled with ^{32}P was added to two ml competent cells. After 10 min incubation at 37°C with aeration, DNase (Worthington) was added at 50 μg/ml final concentration and the cells were further incubated. Time 0 means after 10 min of uptake and 2 min of DNase treatment. At specified times, the cells were placed at 0°C.

In experiments utilizing cells with the dna9 mutation, the same method was used, except that following uptake at 36°C the cells were exposed to DNase for 2 min at either 36°C or at 41°C. Incubation was then carried out for specified times at either 36°C or 41°C.

Velocity sedimentation in neutral sucrose. Two-tenths ml of sarkosyl lysates was gently layered on 5-20% sucrose gradients containing 0.1 M NaCl. Tubes were centrifuged in a Beckman SW50.1 rotor in an L350 centrifuge at 20,000 rev/min for 180 min at 20°C. Collection and assay of fractions and estimation of molecular weights are described above.

Results

Release of recipient DNA. To determine if transforming DNA enters only one or both strands of recipient DNA, the release of recipient DNA label was measured from cells labeled during exponential growth with

[^{14}C]dThd, pulse-labeled with [^{3}H]dThd at the peak of competence and exposed to unlabeled transforming DNA. Assuming that the specific release of label in response to transforming DNA represents displacement of recipient DNA as a result of the interaction with transforming DNA, then release will be either from the [^{3}H]-labeled or the [^{14}C]-labeled strand, depending upon which strand the transforming DNA enters. The results for wild type, rec1 and rec2 are seen in Figure 11 (Appendix), plotted in terms of the fraction of total acid-insoluble counts at the start of the experiment, after the pulse of [^{3}H]dThd, against time of incubation of the cells. In the wild type there is a slow, nonspecific release of label from cells not exposed to transforming DNA, and considerably more release of both labels in response to the transforming DNA. In the two mutants, the release is the same with or without the exposure to transforming DNA. In rec2 the release of both labels is similar to those observed from wild type cells not exposed to transforming DNA. However, the release of label from rec1 cells, whether or not transforming DNA has entered the cell, is greater than even the specific release from wild type cells. In a similar experiment with the ATP-dependent nuclease deficient add1 mutant (data not shown) there is little specific release of either label relative to wild type cells.

Transformation of [^{3}H]dThd-labeled cells with ^{32}P-labeled transforming DNA. For further information on the interaction of transforming DNA and recipient DNA in competent H. influenzae, experiments of the following type have been performed on mutants and wild type. Transforming DNA

labeled with ^{32}P was taken up by competent cells, labeled with [^{3}H]dThd during exponential growth. After various times of incubation of cells with transforming DNA followed by DNase treatment, the cells were either lysed on top of an alkaline sucrose gradient or lysed with sarkosyl before neutral sucrose gradient sedimentation. Figure 12 (Appendix) shows alkaline sucrose profiles for wild type, rec2 and rec1 for two incubation times following uptake of transforming DNA. In wild type, breaks are apparent in the strand labeled before competence as a result of interaction with transforming DNA. The prelabeled strand contains little or no material sedimenting at an intermediate position in the gradient when the cells have not been exposed to transforming DNA (cf. profile of ^{14}C-labeled DNA in top left panel of Figure 2, Appendix). After 30 minutes most of the transforming DNA label is covalently associated with recipient DNA and sediments in alkaline sucrose as single strand pieces in a position similar to that of pulse-labeled DNA (cf. profile of ^{3}H-labeled DNA in top left panel of Figure 2). In rec2 there is no interaction between transforming DNA and recipient DNA, and no breakage of the prelabeled recipient DNA. In rec1 there is little interaction between the transforming DNA and recipient DNA at 0 time, but by 30 minutes some of the prelabeled strands are broken; the transforming DNA has formed covalent bonds with the recipient DNA and sediments mostly in an intermediate position similar to wild type.

Similar experiments have been performed with strain dna9, a mutant temperature-sensitive for DNA synthesis. In this strain, incorporation of [^{3}H]dThd shuts off within two to three minutes when the temperature

is raised to 41°C and resumes at the normal rate when the temperature is shifted back to 36°C, provided that the time at 41°C has been no more than about 90 minutes. After uptake of transforming DNA at 36°C, the cells were incubated either at the permissive or the restrictive temperature for DNA synthesis. Alkaline sucrose profiles are shown in Figure 13 (Appendix) from which three observations are made:

1. Covalent association of transforming DNA with recipient DNA takes place at the temperature restrictive for DNA synthesis as well as at the permissive temperature.
2. At both temperatures breaks appear in the DNA strand labeled before competence.
3. At the restrictive temperature the transforming DNA goes into high-molecular weight DNA and breaks in the prelabeled strand are sealed much more rapidly than at the permissive temperature.

Thus, after 30 minutes at 36°C there is approximately 30% of transforming DNA covalently associated with recipient DNA of intermediate molecular weight, but at the restrictive temperature almost all the associated transforming DNA is in high-molecular weight DNA by 30 minutes. That this was not solely a temperature effect was shown by performing a similar experiment with wild type cells at 36°C and 41°C in which breaks in the prelabeled strand were not sealed by 30 minutes and associated transforming DNA was of intermediate-molecular weight. That less transforming DNA is associated with recipient DNA in dna9 at 36°C than wild type (Figure 12, Appendix) may be attributed to the level of competence of the cells in this experiment.

A summary of the alkaline and neutral gradient information for dna9 is shown in Figure 14 (Appendix) in which the percent associated donor DNA is plotted against time at the permissive or restrictive temperatures. There is more association seen in the neutral gradients than in the alkaline gradients suggesting that some of the donor DNA is held in place only by hydrogen bonds. At 30 minutes about 90% of the associated donor DNA is covalently bonded, as judged by the ratio of association seen in alkaline and neutral gradients. Substantially more noncovalent association is seen in dna9 for early times at the permissive temperature than at the restrictive temperature for DNA synthesis. Results very similar to those shown for dna9 at 36°C were obtained for wild type and rec1 at early times. No association in neutral sucrose was seen between donor DNA and recipient DNA in rec2.

Similar experiments with the double mutant dna9rec1 have been done with incubation of the double mutant at 36°C or 41°C following uptake of transforming DNA at 36°C. Table 3 (Appendix) shows some of the results, in comparison with results of a similar experiment with the single mutant dna9. In neither strain is there appreciable covalent association of donor label at time 0 (see Figure 13, Appendix, for dna9 at this time). However, in both strains at 30 minutes there is covalent association of donor and recipient DNA at both the permissive and restrictive temperatures for DNA synthesis. Two significant differences are noted in the behavior of the dna9rec1 double mutant:

1. At neither the permissive nor the restrictive temperature is there appreciable donor DNA covalently associated with high

molecular weight DNA, unlike the situation in the single mutant dna9.

2. From the weight average molecular weight in neutral sucrose, double strand breaks are evident in the prelabeled recipient DNA of the double mutant transformed at the restrictive temperature for DNA synthesis.

Double strand breaks are a result of interaction with donor DNA, since in a control experiment, double strand breaks were not seen in the recipient DNA of dna9rec1 at 41°C without exposure to transforming DNA.

Figure 15 (Appendix) shows the interaction between donor DNA and recipient DNA in the ATP-dependent nuclease deficient mutant add1. Alkaline sucrose profiles reveal that donor DNA is associated with recipient DNA nearly as well in add1 as in wild type, but two differences between add1 and wild type are noted:

1. The prelabeled DNA synthesized during exponential growth does not contain comparable strand breaks after exposure to transforming DNA as is seen in wild type (cf. Figure 12, Appendix).
2. A greater proportion of donor DNA is associated with recipient DNA of high molecular weight than in wild type.

Discussion

Release of recipient DNA. The specific release of both prelabeled DNA and pulse-labeled DNA as a result of exposure of competent wild type cells to transforming DNA suggests that donor DNA can be integrated into either strand of recipient DNA. Quantitative interpretation of this

experiment is difficult, since the bulk of the DNA synthesized during the competence regime is probably unlabeled, including postulated pairing sites. Because the kinetics of release and fraction of specific release are similar for the two labels, one cannot postulate that integration is favored in one strand. The lack of specific release of either label in competent rec2 cells exposed to transforming DNA is consistent with lack of association of donor DNA with recipient DNA shown by Notani et al. (44) and with the absence of pairing sites suggested in this dissertation. That the release of pulse label from competent rec1 cells, whether or not transforming DNA has entered the cells, is faster than the release from the prelabeled strand argues that there is degradation preferentially at the site of single stranded regions in this strand.

Covalent association of donor DNA and recipient DNA. The results of alkaline sucrose gradient sedimentation of DNAs from transforming wild type cells indicate covalent association between donor DNA and recipient DNA by 12 minutes after addition of transforming DNA (0 time). That the associated donor DNA sediments in a position similar to the pulse-labeled DNA in competent cells suggests, but does not prove, that some covalent association occurs with the DNA pieces made during the competence regime. Breakage of the prelabeled strand as a result of exposure of competent cells to transforming DNA has been shown. In conjunction with the results of the specific release experiments with wild type cells, covalent association of donor DNA may occur with both strands of DNA and intermediate size molecular weight pieces of

DNA are the products of this recombination reaction. This hypothesis could explain in part, the lag of two to three generations after DNA uptake before the number of transformants in a recipient population begins to increase, despite the formation of recombinant molecules immediately after uptake (11). The intermediates, identified in alkaline sucrose gradient sedimentation, would be expected to have transforming activity when purified and assayed in a recipient culture, but repair reactions may be required in the transformed cell before those intermediates are sealed into recipient DNA and replicated.

Further evidence that breakage of the prelabeled strand of DNA is a result of interaction between donor DNA and recipient DNA is that in competent rec2 cells, where there is no interaction between donor DNA and recipient DNA (44 and Figure 12, Appendix), there is no breakage of the prelabeled recipient DNA. In competent rec1 cells, blocked at a later stage of recombination (44), there is such an interaction.

Noncovalent association of donor DNA and recipient DNA. Comparison of alkaline and neutral sucrose gradient data suggests that donor DNA is initially associated with recipient DNA by hydrogen bonds. That the major portion of DNA to be associated is bound noncovalently by 0 time (12 minutes after uptake) indicates that the pairing reaction is rapid. That less noncovalent association of donor and recipient DNA is seen at early times in the dna9 mutant at the restrictive temperature remains unexplained, but suggests involvement of DNA synthesis in the initial pairing reaction. In agreement with the results of CsCl equilibrium

sedimentation (44), the rec2 mutant shows no detectable noncovalent association of donor and recipient DNA.

The role of DNA synthesis in the interaction of donor DNA and recipient DNA. In agreement with results obtained by different methods (11, 52, 53, 54), experiments with the dna9 mutant of H. influenzae show that DNA synthesis is not required for association of donor DNA with recipient DNA. Indeed, the associated DNAs are sealed into high-molecular weight DNA more rapidly in the absence of DNA synthesis. One interpretation of this result is that when DNA synthesis is taking place in competent cells, sealing may be retarded because of strand displacement synthesis and the necessity for subsequent nucleolytic degradation. Alternatively, there may be competition for polymerases if repair synthesis is prerequisite to sealing.

Effect of the rec1 mutation on the interaction of donor DNA and recipient DNA. Notani et al. (44) demonstrated that donor DNA atoms are transferred to recipient DNA during recombination in rec1, but could not detect biological activity in recombinant DNA. These authors offered several interpretations to explain their data: (1) incorporation of donor label that is nonmacromolecular, a product of degradation and resynthesis; (2) integration of small donor fragments devoid of biological activity; or (3) accumulation of branched intermediates devoid of biological activity. Experiments with the double mutant dna9rec1 at least eliminate the first possibility, since there is covalent association of donor label with recipient DNA in the absence of DNA synthesis. The

DNA of competent rec1 cells is prone to degradation as shown by the specific release data (Figure 11, Appendix) and the detection of double strand breaks in the recipient DNA of the dna9rec1 mutant exposed to transforming DNA at the restrictive temperature for DNA synthesis (Table 3, Appendix). Thus, degradation may occur at the site of association of transforming DNA with recipient DNA in rec1, preventing the associated transforming DNA from sealing into high-molecular weight DNA (Table 2, Appendix). This degradation, in the absence of DNA synthesis, together with the breaks made in the prelabeled strand as a result of exposure to transforming DNA, may cause double strand breaks in the recipient DNA. In the presence of DNA synthesis, the degradation of recipient DNA may eliminate biological activity in the associated DNA.

The role of the ATP-dependent nuclease in the interaction of donor DNA and recipient DNA. Experiments with an ATP-dependent nuclease-deficient mutant of H. influenzae provide the following information: (1) competent cells synthesize DNA with single stranded regions similar to the wild type strain; (2) recipient DNA label is released from competent cells, but to an extent much less than the wild type strain in response to donor DNA; (3) breaks are not induced by donor DNA in the prelabeled strand of DNA synthesized in exponentially growing cells; and (4) donor DNA associates with recipient DNA of high molecular weight to an extent greater than the wild type strain. These results indicate that the ATP-dependent nuclease may act in late stages of recombination and suggest that the mutant, with a transformation frequency of 0.5-1.0

relative to wild type (56), may recombine DNA by a pathway different from wild type. The possibility of a large proportion of inviable cells in the culture (56) make interpretation of some results difficult. For instance, the low level of release of recipient DNA label in response to donor DNA may result from a competence level too low to show specific release. The data are clear, however, that detectable breaks are not induced in prelabeled DNA in response to donor DNA despite the association of donor and recipient DNAs. Thus the enzyme may display endonucleolytic activity at recombination sites. Friedman and Smith (59) have demonstrated that the enzyme from H. influenzae has ATP-dependent endonucleolytic activity on single stranded phage fd circular DNA but not double stranded circular SV-40 DNA with single stranded scissions. Endonucleolytic activity on joint molecules that may be intermediates in recombination has not been tested (60). Possible roles for the enzyme include (1) unwinding of helices during pairing, (2) strand assimilation of donor DNA by exonucleolytic degradation of recipient DNA, (3) endonucleolytic cleavage of recipient DNA to permit integration into an intact strand and/or (4) degradation of excess DNA at pairing sites to permit final integration and sealing of donor DNA.

Summary of transformation events in H. influenzae strains. Table 4 (Appendix) summarizes some of the steps identified in the transformation process, and indicates some of the defects in mutant strains utilized in this study. Clearly, mutants are lacking that are defective in each stage identified in transformation, but the complexity of the process

may make such mutant isolation unachievable. In the case of the rec2 and rec1 mutations, defects can be identified during the process that eliminate later stages, resulting in a barely detectable transformation frequency. Mutations that only slightly depress transformation frequencies, like the add1 mutation, are also useful however since they may provide evidence for multiple pathways in recombination during transformation.

Comparison with other transformation systems. A primary difference between the H. influenzae transformation system and the D. pneumoniae and B. subtilis systems is the fate of donor DNA upon uptake into competent cells (see General Introduction). In H. influenzae no single stranded donor DNA has been detected during transformation (12) whereas a single stranded phase has been observed in the other well-studied systems. Thus the uptake process is most probably different in these systems. Harris and Barr (61) have reported that the DNA of competent B. subtilis has a higher buoyant density in CsCl than the DNA of growing cells; these authors speculated that recipient DNA has single stranded regions. Thus the possibility exists that single stranded regions in recipient DNA is a general feature of transformation systems.

Later stages in H. influenzae transformation reported in this dissertation have been observed in other systems. In B. subtilis, donor DNA is released upon treatment of associated donor and recipient DNAs with alkaline treatment (62, 65) and the recombinant and donor marker activity in extracted DNA of transforming cells increases upon incubation

with polynucleotide ligase (62). Thus, a donor-recipient intermediate in B. subtilis transformation is noncovalently bonded and may contain nicks. Covalent linkage between donor and recipient DNAs has been demonstrated in D. pneumoniae (8, 63) and B. subtilis (64, 65).

The role of DNA synthesis and the ATP-dependent nuclease in transformation has been studied in other systems. Dubnau and Cirigliano (66) found no effect on the formation of recombinant DNA by specific inhibition of DNA polymerase III of B. subtilis and concluded that normal DNA replication is not required for integration. Laipis and Ganesan (67) found that a DNA polymerase I-deficient mutant of B. subtilis is transformed normally, although the role of another repair polymerase during transformation is not excluded. Vovis and Buttin (68) have studied the role of the ATP-dependent nuclease in transformation of D. pneumoniae using a mutant lacking the enzyme. That the mutant forms recombinant DNA normally but has a transformation frequency approximately six times lower than wild type suggests that the enzyme operates at a late stage of recombination (68, 69). Vovis (69) has speculated that the enzyme may be required to trim excess DNA from donor-recipient complexes before integration of the final product.

CHAPTER III

MODELS

To the experimentalist, models are most useful for placing a myriad of results into a conceptual framework and suggesting further experiments that will each most likely require modification of the model. In that perspective, a "working model" for recombination during transformation of wild type Haemophilus influenzae is presented in Figure 16 (Appendix), based partly on results presented in this dissertation.

The recipient DNA in competent cells, indicated by light lines, is shown in I-a as having single stranded branches, a product of strand displacement synthesis hypothesized in Chapter I (Figure 10, Appendix). The rec2$^+$ gene must be present for the formation of this structure. The donor DNA, indicated by heavy lines in I-b of Figure 16 (Appendix) is shown with single strand ends. This structure is suggested by the following lines of evidence: (1) donor DNA shows some inactivation and breakdown in the absence of integration (49); (2) radioactivity in the unintegrated donor DNA in supernatants of digitonin-treated cells has a slightly higher buoyant density in CsCl than donor biological activity (44); and (3) donor DNA is rendered 10-15% degradable by single strand specific S1 nuclease after it enters the competent cell (35). The presumed single stranded ends of donor DNA are shown pairing with single stranded branches in II. The association between donor DNA and recipient DNA depicted in II is noncovalent, although covalent bonding is quickly

established (cf. Figure 14, Appendix). In III and IV, single stranded branches of recipient DNA paired with donor DNA are assimilated into the recipient duplex. A possible mechanism for assimilation of donor DNA strands into either recipient strand is indicated in III-a and III-b. In III-a, the donor strand covalently bonded to the single stranded branch is assimilated into the same strand with concomitant degradation of the noncovalently bonded donor strand. In III-b, the donor strand hydrogen-bonded to the single stranded branch is assimilated into the opposite recipient strand after becoming covalently bonded at the site of a nick in this strand (indicated by the arrow). The donor strand not assimilated is released and/or degraded. Breakage of the intact recipient strand is seen specifically in response to interaction with donor DNA (cf. Figure 12, Appendix) and is not detectable in the ATP-dependant nuclease deficient addl mutant after interaction with donor DNA (cf. Figure 15, Appendix). This enzyme may play a role in the integration of donor DNA into the intact strand, either by incising the ungapped strand or by mediating the strand assimilation process that places the donor DNA into the intact strand. Further, the energy of ATP-hydrolysis may effect unwinding of the duplex required for the process. Exonucleolytic degradation of recipient DNA is also indicated in III and IV, required for single strand assimilation of donor DNA (cf. Figure 11, Appendix). Degradation of recipient DNA would be terminated at the nick resulting from completed assimilation of the paired strand, thus providing a mechanism for the insertion of discrete pieces of donor DNA into the recipient duplex. The structure depicted in IV-b has breaks in both

recipient strands and is held intact by hydrogen bonding. However the kinetics of these rapid reactions may make unnecessary this speculation. Sealing of donor and recipient DNA into DNA of high molecular weight is represented in V. Sealing requires the *rec1*$^{+}$ gene (cf. Table 3, Appendix) and may be absent in the *rec1* strain because of extensive degradation at the site of association between donor and recipient DNA. Further, since donor DNA is sealed into recipient DNA of high molecular weight sooner relative to wild type in the *add1* mutant (cf. Figure 15, Appendix), which is postulated to integrate donor DNA only into the strand with single stranded branches, sealing of this strand may be sooner than the originally intact strand. Sealing is more rapid in the absence of DNA replication (cf. Figure 13, Appendix), but the possibility that repair synthesis is required is not excluded. The final products of the recombination reactions depicted in V are heteroduplex molecules with single donor DNA strands inserted into (a) the strand synthesized during competence development or (b) the strand synthesized during exponential growth. The model does not exclude the possibility that donor DNA strands may be inserted into both recipient strands.

Numerous models for recombination have been proposed (cf. 70), although few were designed to depict recombination during transformation. Despite attempts to present a "general theory" of recombination (cf. 2), there is little reason to believe that all recombination systems are similar. For instance despite the demonstrated involvement of exonuclease V (the *recBC* nuclease) in recombination of phage and during conjugation in *E. coli* (cf. 71), the same enzyme drastically limits transformation

in this system (72). Recognizing the different requirements of the systems for which recombination models have been devised, the following discussion compares various aspects of these models with that proposed for H. influenzae recombination (Figure 16, Appendix).

The classical recombination models of Whitehouse (4) and Holliday (5), described for eucaryotic recombination, are debated ten years after their proposal. The Whitehouse model (4) features exchange initiated by staggered breaks in parental strands of opposite polarity, forming a hybrid overlap that is resolved into a recombinant duplex molecule by more breaks in nonparticipating strands. The Holliday model (5) differs in that exchange is initiated by breaks in parental strands of the same polarity, free single strands pair with unbroken strands of the other parent, and breaks follow that give either a recombinant or an insertion heteroduplex. These models are not easily applied to recombination during transformation, since they are aimed at describing reciprocal exchange while reciprocity in transformation is precluded by degradation of one donor strand. The Whitehouse and Holliday models are instructive however in classifying the pairing reaction that initiates exchange in the proposed model for H. influenzae recombination (Figure 16, Appendix). Figure 17 (Appendix) compares initial pairing reactions in the Whitehouse (a) and Holliday (c) models and pairing of donor DNA at a single stranded branch (b) in recipient DNA, slightly modified from the H. influenzae model for recombination shown in Figure 16. It is seen that pairing between a single stranded end of donor DNA and a single stranded branch in recipient DNA (b) resembles the Whitehouse structure (a) in that

participating strands are of opposite chemical polarity. Resolution of the paired strands at a single stranded branch (b) during transformation is different from resolution of the Whitehouse structure (a), however, as the product in the Whitehouse model (4) is a recombinant heteroduplex unlike the insertion-type product of recombination during transformation. Resolution of the Holliday structure may result in an insertion heterozygote depending on how pairing strands of DNA in the intermediate (c) are cut. Fox (63) proposed such a model of recombination during transformation in pneumococcus, without specifying how single stranded regions arise on the participating DNA partners. Such a model is possible for recombination during transformation in H. influenzae, especially since the possibility of single stranded gaps in the DNA of competent cells is not excluded (see Discussion, Chapter I).

The models of Thomas (1) and Radding (3), for T-even phages and phage λ, respectively, are most useful in providing an enzymatic basis for postulated recombination reactions. The "chop and nibble" model of Thomas (1) invokes an exonuclease I-like enzyme to chop DNA chains at recognition sites and an exonuclease III-like enzyme to nibble the broken ends; exposed single stranded ends then pair. Nuclease digestion also corrects imprecise pairing of strands and a DNA polymerase repairs left over gaps. Radding (3) emphasizes the role of a DNA polymerase [suggested earlier by Thomas (1)] and λ exonuclease-like enzyme to enzymatically drive strand exchange as shown in Figure 18 [Appendix; after Radding (3)]. Polymerase action (P) effects strand displacement while exonucleolytic action (N) effects strand assimilation in a concerted exchange reaction.

Strand exchange is completed either by cutting the exchanging strand or by termination of branch migration at a nick in one of the participating strands. Hotchkiss (2) suggests that an advantage of such enzymatically driven exchange: the partners enter the confrontation unequally, so that return to the status quo is disfavored during recombination reactions. Although enzyme systems are not specified in model reactions in Figures 10 and 16 (Appendix), the concepts proposed by Thomas (1) and Radding (3) play a major role in the reactions proposed for H. influenzae transformation. Specifically, polymerization to form extra strands that participate in branch migration and degradation by a λ exonuclease-like enzyme during strand assimilation of branched molecules are enzymatically driven reactions hypothesized for recombination during H. influenzae transformation.

Boon and Zinder (73) proposed a model for recombination in the circular DNA of phage f1 and suggested that it could be extended to describe recombination in bacterial transformation. In summary, the model visualizes a Whitehouse-like pairing structure (Figure 17-a, Appendix) as equivalent to a normal replication fork, synthesis proceeding at a gap unidirectionally. A recombinant molecule is resolved by a second set of breaks and joining of strands. Since the model requires extensive DNA synthesis--not of the repair type--the model probably cannot apply to H. influenzae transformation. A role for DNA synthesis is not specified in the model of Figure 16, but synthesis of the repair type may be involved in filling gaps at pairing sites.

The ability of the ATP-dependent nuclease from H. influenzae to cleave DNA into fragments with long single stranded tails that can pair suggested to Friedman and Smith (74) that the DNA fragments may be intermediate in a "break and rejoin" mechanism for recombination. The recombination proficiency of the ATP-dependent nuclease-deficient mutant of H. influenzae (56) and the near-normal degradation of donor DNA in the mutant strain (56) make such a mechanism unlikely. The role for the enzyme as proposed in Figure 16 (Appendix)--that of cutting an intact strand during assimilation of the opposite strand at a recombination site--is more like the role proposed by Clark (75) for the ATP-dependent (recBC) nuclease from E. coli. Clark (75) visualizes the enzyme processively digesting one strand, then switching to digest an intact strand, thus opening up DNA duplexes to participate in recombination reactions. Low (referred to in 76) has shown that the ATP-dependent nuclease deficiency in recBC mutants in E. coli can be corrected by the λ red system, which includes the λ exonuclease. Thus, the ATP-dependent nuclease may operate in a single strand assimilation during recombination, similar to λ exonuclease (76). Cassuto and Radding (76) have speculated a similar mechanism for bacterial transformation.

The complexity of devising realistic recombination models derives not only from fulfilling the requirements of different systems, but also from the realization of multiple pathways operating in the same system (cf. 71). It is likely that the pathway utilized by a cell to recombine its genes depends upon the complex interactions of replication, repair and recombination, and until the precise biochemistry of these pathways

is understood, no model will be realistic. Although no single model of recombination may suffice to unify recombining systems, there may be unity in the biochemistry of individual recombination reactions.

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APPENDIX

Table 1. Effect of inosine on [^{3}H]dThd incorporation in competent wild type H. influenzae

Conc (μg/ml)	Relative incorporation
0	1.00
20	1.04
200	1.07
1000	0.53
2000	0.35

Table 2. S1 nuclease digestion of DNA fractionated on BND-cellulose.

	Fractions	Time	Acid-precipitable DNA			
			^{14}C-cnts/min	%	^{3}H-cnts/min	%
Exp. 1	Completely DS	0	1010		2330	
		15	975	97	2260	97
	Partially SS	0	955		3090	
		15	960	100	2620	84
Exp. 2	Completely DS	0	1179		4601	
		15	1169	99	4490	98
	Partially SS	0	1292		6744	
		15	1244	96	5854	87

Table 3. Association of donor DNA with recipient DNA in *dna9* and *dna9rec1*.*

Strain	Temp.	% donor DNA associated with recipient DNA		M_w ($\times 10^{-8}$) of recipient DNA in neutral sucrose
		HMW	IMW	
dna9	36°C	10	31	3.5
	41°C	31	18	3.8
dna9rec1	36°C	4	20	3.5
	41°C	2	20	1.5

*Calculated from sucrose gradient data for 30 min incubation. HMW and IMW indicate DNA sedimenting in positions representing high and intermediate molecular weight, respectively. M_w represents weight average molecular weight.

Table 4. Summary of transformation events in Haemophilus influenzae strains.*

	DNA binding	SS regions in DNA	Noncovalent association of donor DNA	Covalent association of donor DNA	SS breaks induced by donor DNA	Specific release of recipient DNA	Donor-recipient complex forms HMW DNA	Relative Transformation frequency
rec2	+	-	-	-	-	-	0	10^{-7} (21)
rec1	+	+	+	+	+	-	-	10^{-6} (21)
add1	+	+	+	+	-	±	+	0.5-1.0 (56)
dna9								
36°C	+	+	+	+	+		+	1 (19)
41°C	± (19)		±	+	+		++	1 (19)
wild type	+	+	+	+	+	+	+	1

*Ability of each strain represented by: + normal relative to wild type; ++ greater than wild type; ± less than wild type; - lacking; and 0 not applicable; blanks represent no data. Abbreviations used: SS - single stranded; HMW - high molecular weight. References given in parentheses, otherwise data presented in dissertation.

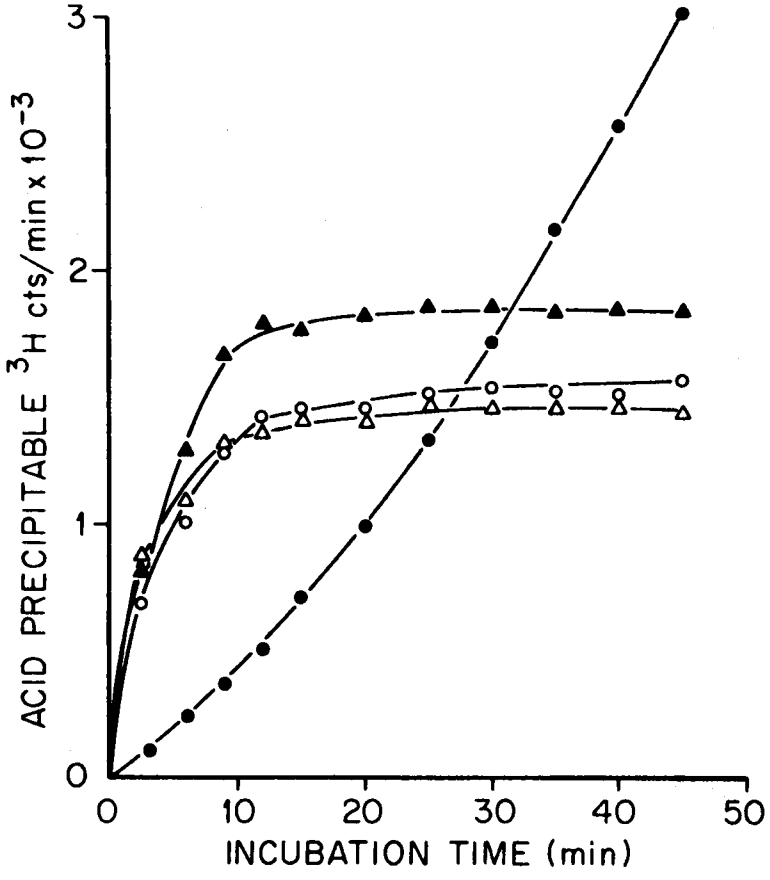

Figure 1. Incorporation of [^{3}H]dThd into competent (-o-) and exponentially growing (-●-) wild type and competent recl (-Δ-) and rec2 (-▲-) cells.

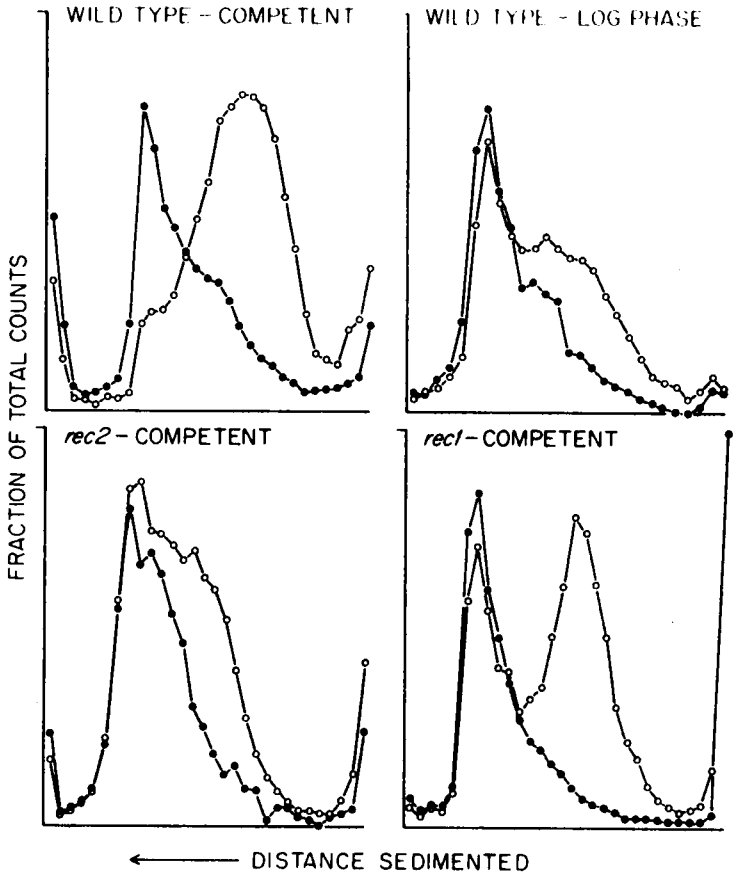

Figure 2. Alkaline sucrose gradient sedimentation patterns of DNA from competent and exponentially growing wild type and competent rec1 and rec2 cells. Cells were prelabeled with [^{14}C]dThd (-●-) and pulse-labeled with [^{3}H]dThd (-o-) for 10 min before sedimentation. The height of the ordinate represents 10% of the total acid precipitable counts.

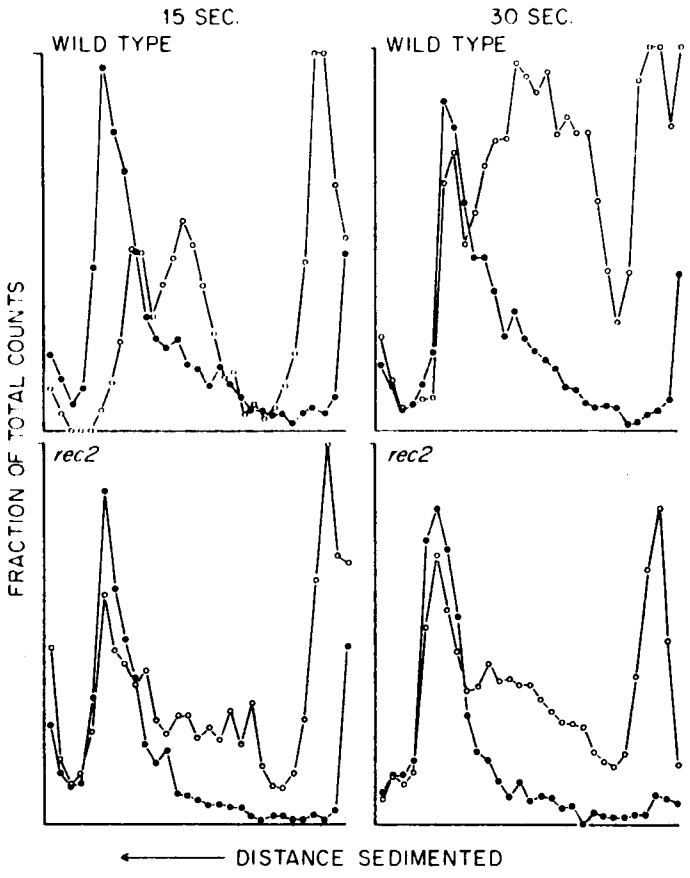

Figure 3. Alkaline sucrose gradient sedimentation patterns of DNA from competent wild type and rec2 cells pulse-labeled for short times. Cells were prelabeled with [^{14}C]dThd (-●-), made competent and pulse-labeled with [^{3}H]dThd (-o-) for the times shown before sedimentation. The height of the ordinate represents 20% of the total acid precipitable ^{14}C counts and 5% (wild type, 30 sec) or 10% (wild type, 15 sec; rec2, 15 and 30 sec) of the total acid precipitable ^{3}H counts.

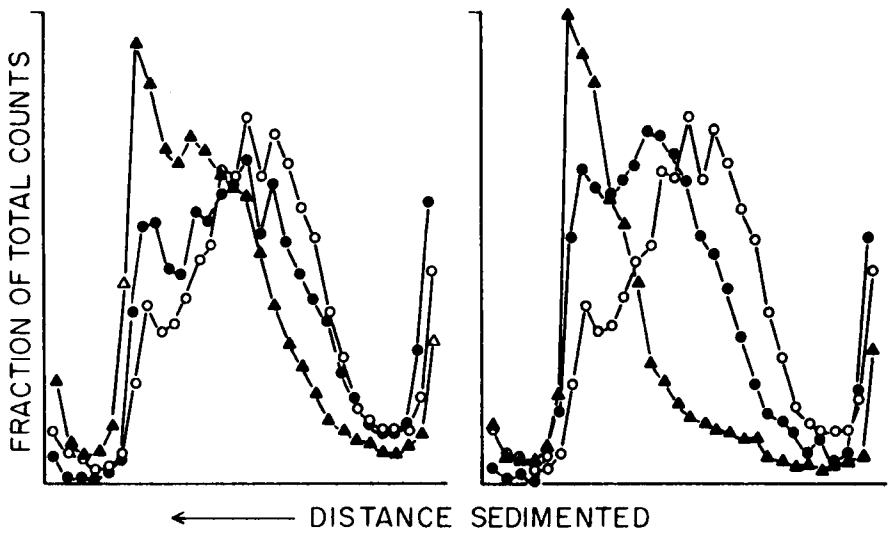

Figure 4. Alkaline sucrose gradient sedimentation patterns of competent wild type cells pulse-labeled in M-IV medium and chased in M-IV medium or MI_ccit growth medium. Cells were prelabeled with [^{14}C]dThd, made competent and pulse-labeled with [^{3}H]dThd for 10 min, washed and resuspended in M-IV medium (left) or MI_ccit growth medium (right). Samples were taken at 0 min (-o-), 30 min (-●-) and 45 min (-▲-) before sedimentation. Sedimentation patterns of ^{14}C-labeled DNA (not shown) were used to indicate the position of high molecular weight DNA. The height of the ordinate represents 10% of the total acid precipitable counts.

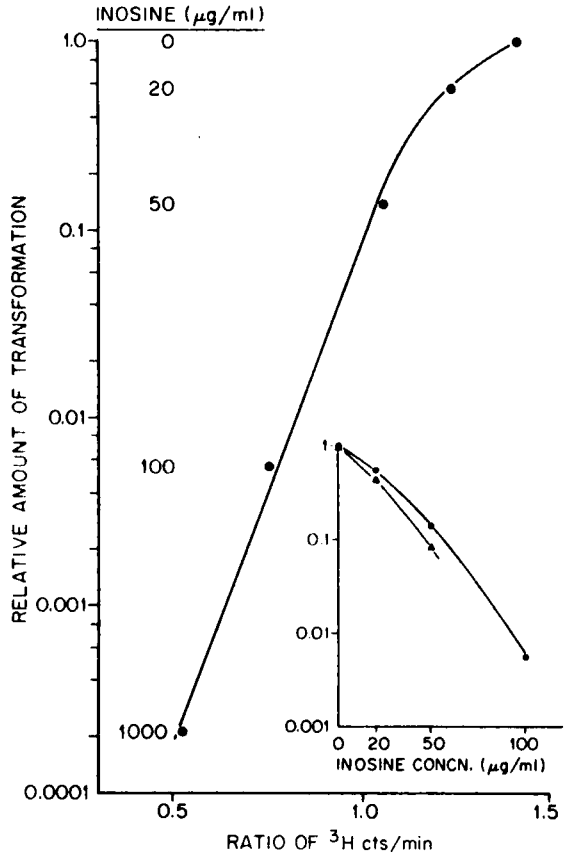

Figure 5. The effect of inosine in the M-IV competence medium on competence, transformation and size of DNA synthesized in the M-IV competence medium. Cells were prelabeled with [^{14}C]dThd, subjected to the competence regime in M-IV medium containing the amounts of inosine shown, pulse-labeled with [^{3}H]dThd for 10 min and sedimented in alkaline sucrose. Transformation frequencies were assayed by exposing the same sets of cells to transforming DNA. ^{3}H counts in the high- or intermediate-molecular-weight positions on the gradients, determined with reference to the position of the ^{14}C-prelabeled DNA, were added to determine the ratio of ^{3}H cnts/min in the intermediate-molecular-weight position to ^{3}H cnts/min in the high-molecular-weight position. The inset shows the relative level of transformation (-●-) and amount of radioactive DNA bound irreversibly to cells (-▲-) as a function of inosine concentration.

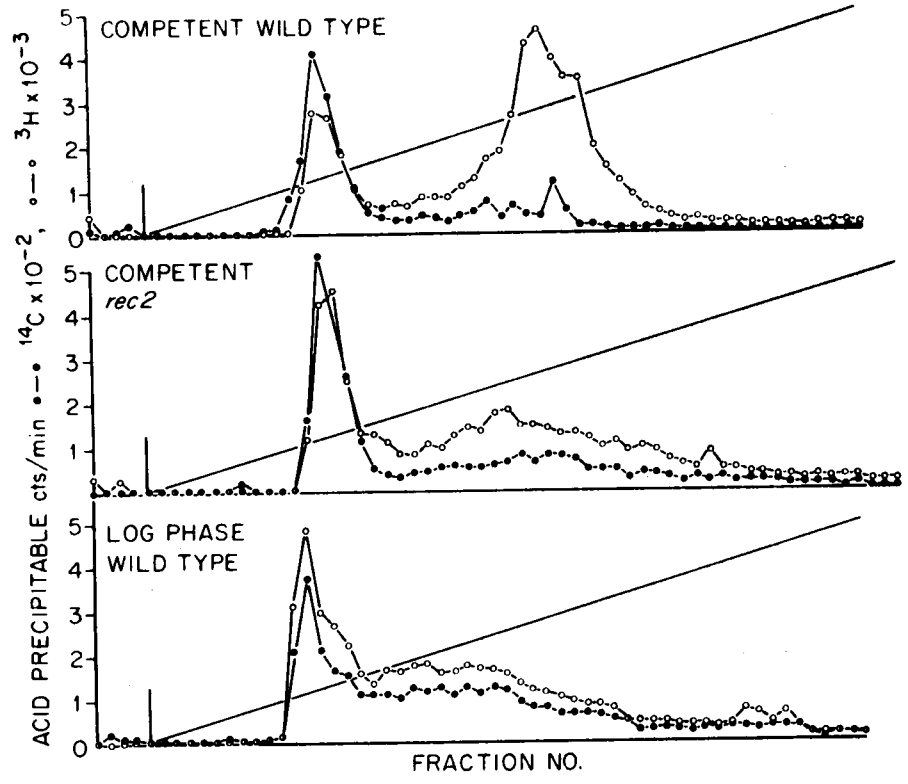

Figure 6. BND-cellulose column chromatography of DNA from competent and exponentially growing wild type and competent rec2 cells. The gradient buffer was 0.3M - 1.0 M NaCl-Tris-EDTA and 0 - 2% caffeine. An aliquot of 0.2 ml of each eluted fraction was acid precipitated and counted.

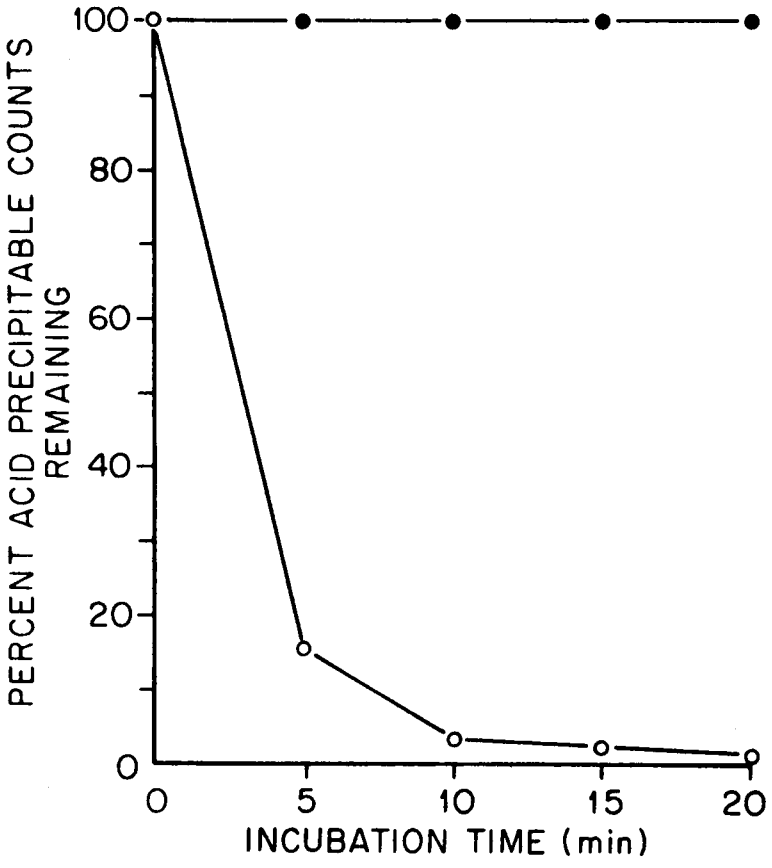

Figure 7. Effect of SI nuclease on native (-●-) and heat-denatured (-o-) H. influenzae DNA labeled with [^{3}H]dThd. The samples before degradation contained approximately 9000 counts/min of acid precipitable material.

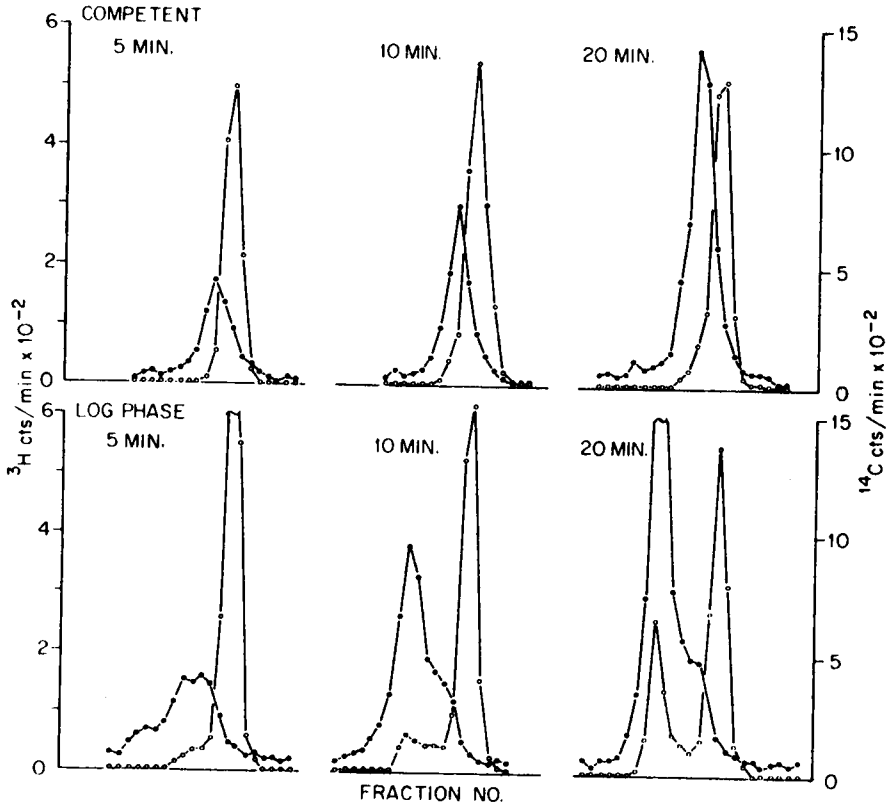

Figure 8. CsCl density gradient sedimentation patterns of BrdUrd-substituted DNA from competent and exponentially growing wild type cells. Cells were prelabeled with [^{14}C]dThd (-o-) and pulse-labeled with [^{3}H]BrdUrd (-•-) for the times shown. Sedimentation was from right to left.

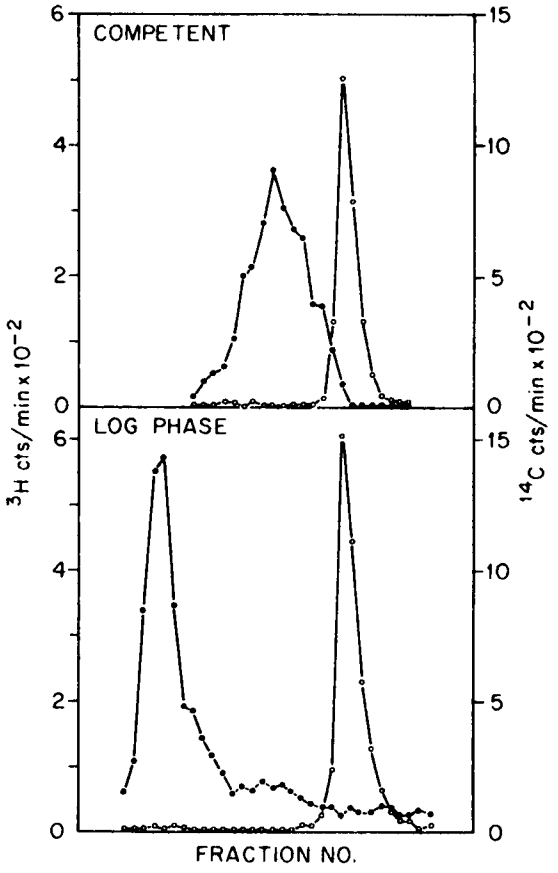

Figure 9. Alkaline CsCl gradient sedimentation of BrdUrd-substituted DNA from competent and exponentially growing wild type cells. Cells were prelabeled with [^{14}C]dThd (-o-) and pulse-labeled with [^{3}H]BrdUrd (-●-) for 20 min. Sedimentation was from right to left.

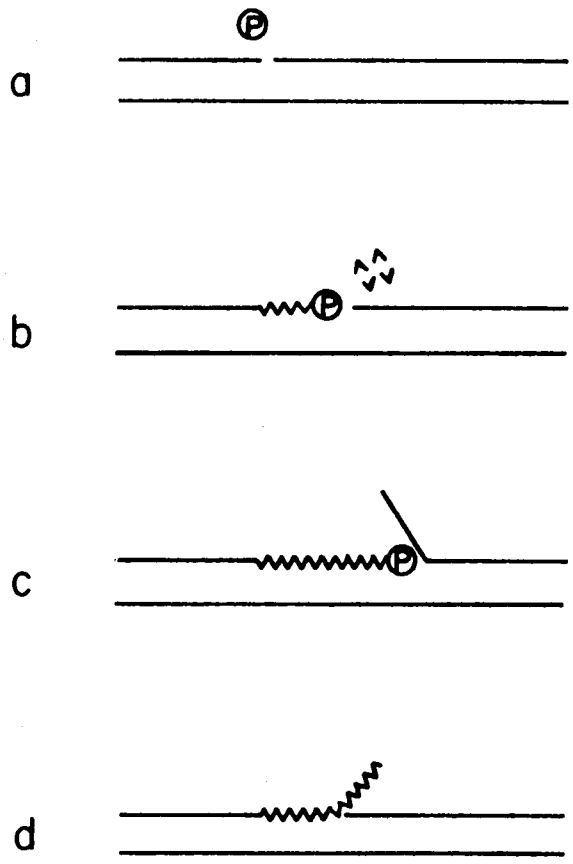

Figure 10. Hypothetical scheme for strand displacement synthesis and branch migration. See Discussion (Chapter I) for details.

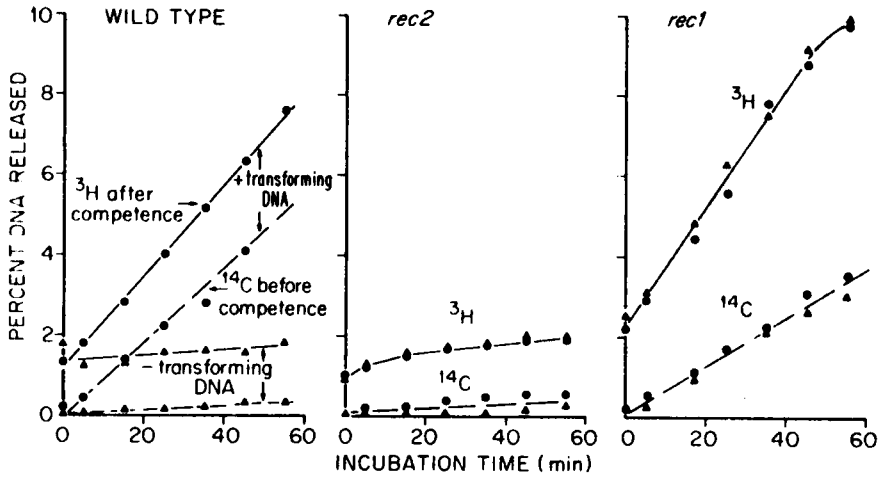

Figure 11. Release of label from recipient DNA of competent cells in response to transforming DNA. Cells were prelabeled with [^{14}C]dThd (----), made competent and pulse-labeled with [^{3}H]dThd (——) at the same time as the transforming DNA (-●-) or M/15 phosphate buffer (-▲-) was added. Percent DNA released was calculated from the number of counts in filtrates of cells withdrawn with time and the number of acid precipitable counts at 0 time.

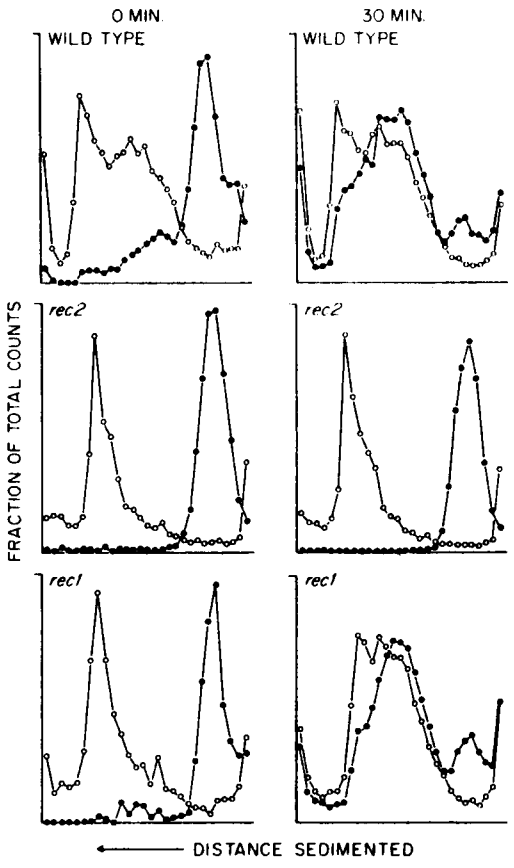

Figure 12. Alkaline sucrose gradient sedimentation patterns of DNA from competent wild type, rec2 and rec1 cells exposed to transforming DNA. Cells were prelabeled with [^{3}H]dThd (-o-), made competent, exposed to ^{32}P-labeled transforming DNA (-●-) and incubated for the times shown before sedimentation. The height of the ordinate represents 10% of the total acid precipitable counts.

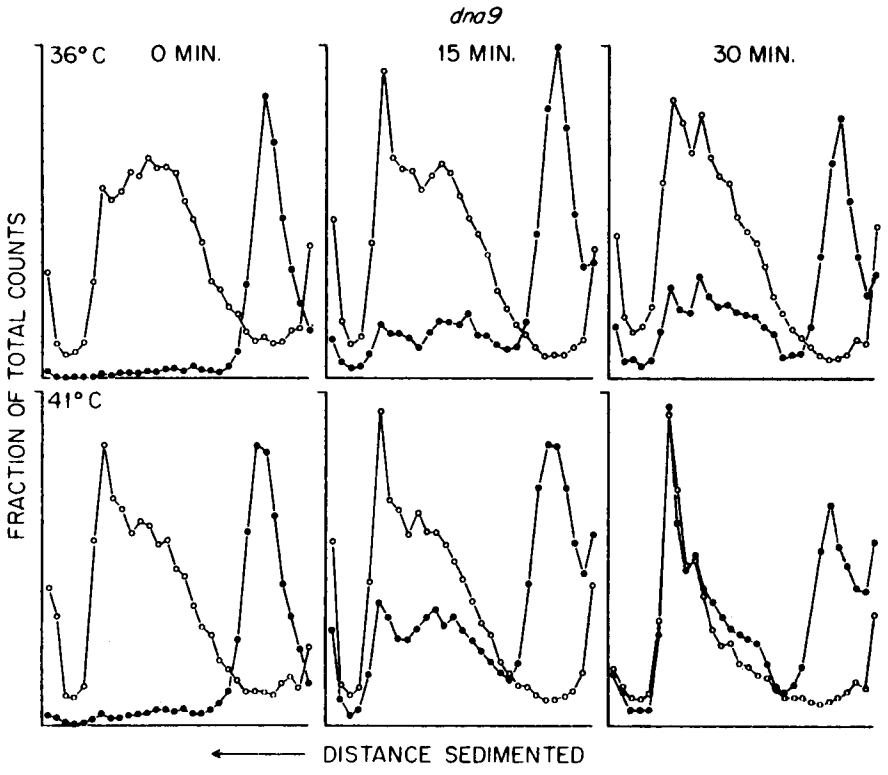

Figure 13. Alkaline sucrose gradient sedimentation patterns of DNA from competent dna9 cells exposed to transforming DNA. Cells were pre-labeled with [^{3}H]dThd (-o-), made competent, exposed to ^{32}P-labeled transforming DNA (-●-) and incubated at 36°C or 41°C for the time shown before sedimentation. The height of the ordinate represents 10% of the total acid precipitable counts.

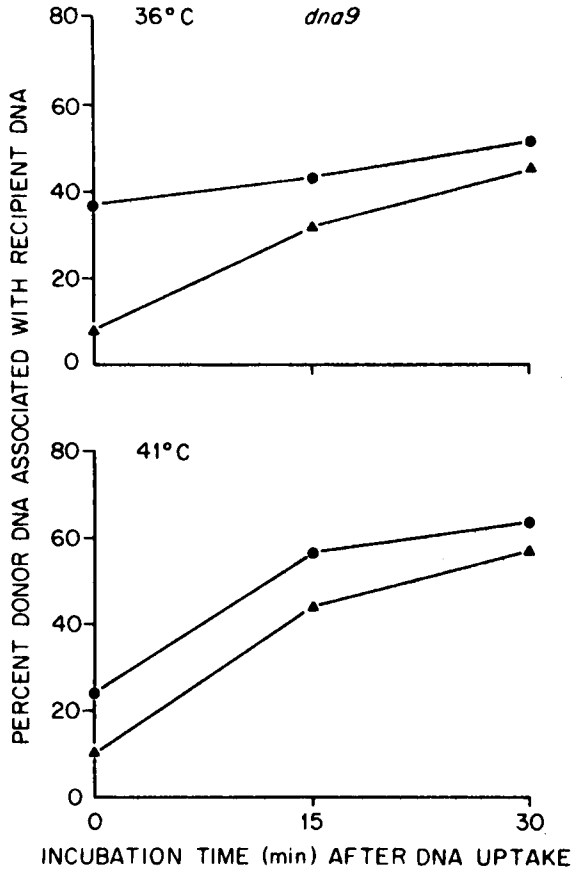

Figure 14. Association of donor DNA with recipient DNA in competent dna9 cells at 36°C and 41°C. Percent association was calculated by adding the ^{32}P-counts/min in the ^{32}P-labeled transforming DNA associated with ^{3}H-counts/min in recipient DNA on alkaline (-▲-) and neutral (-●-) sucrose gradients, and dividing by the total ^{32}P-counts/min on the gradient.

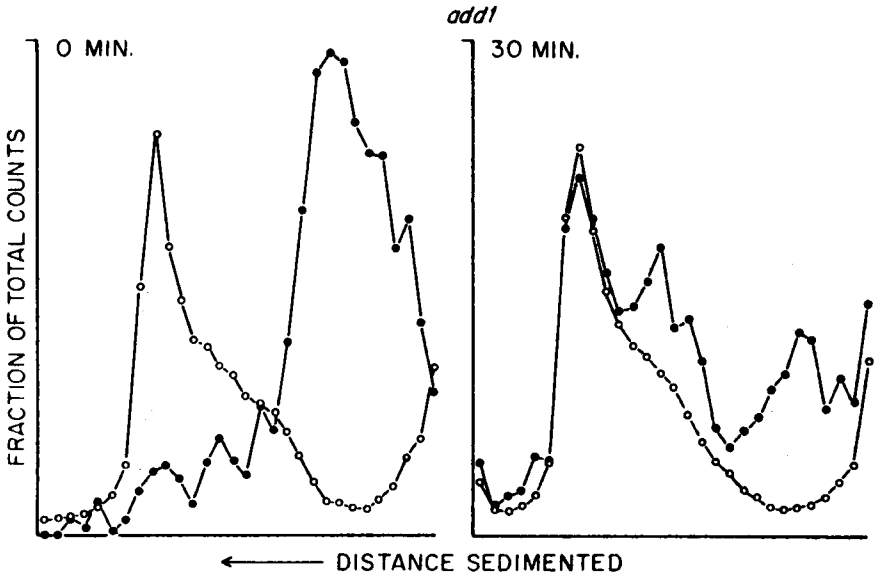

Figure 15. Alkaline sucrose gradient sedimentation patterns of DNA from competent add1 cells exposed to transforming DNA. Cells were prelabeled with [^{14}C]dThd (-o-), made competent, exposed to ^{32}P-labeled transforming DNA (-●-) and incubated for the time shown before sedimentation. The height of the ordinate represents 10% of the total acid precipitable counts.

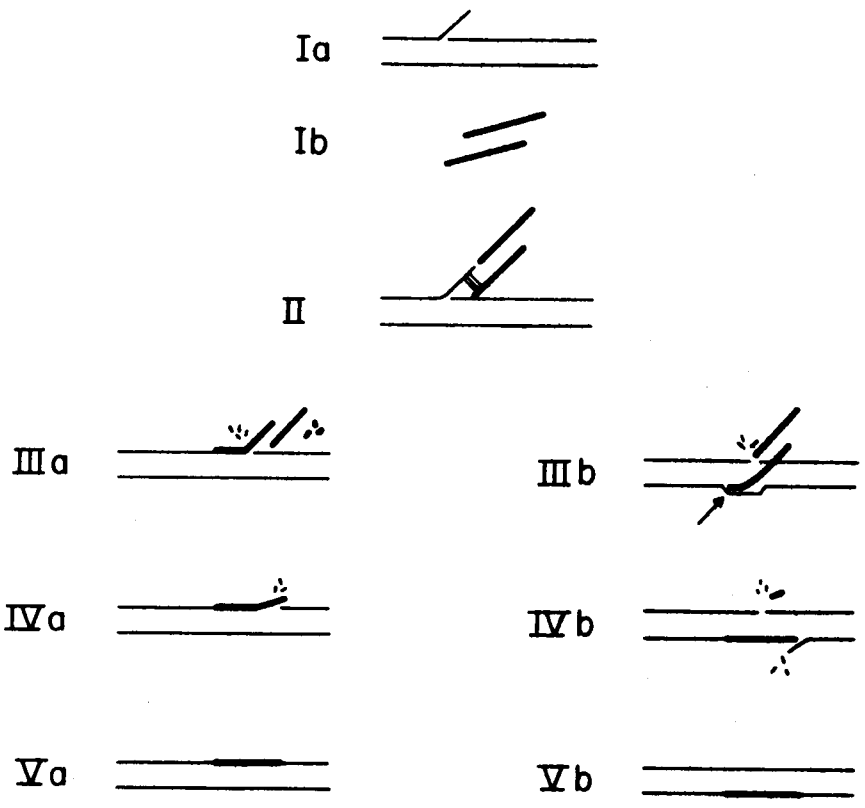

Figure 16. Model for the interaction of donor DNA (—) and recipient DNA (—) during H. influenzae transformation. See Chapter III for details and discussions.

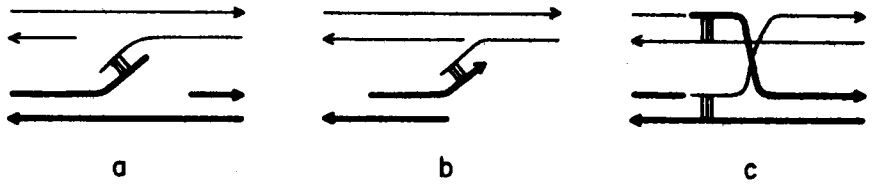

Figure 17. Models for pairing of DNA during recombination. See Chapter III fro discussion.

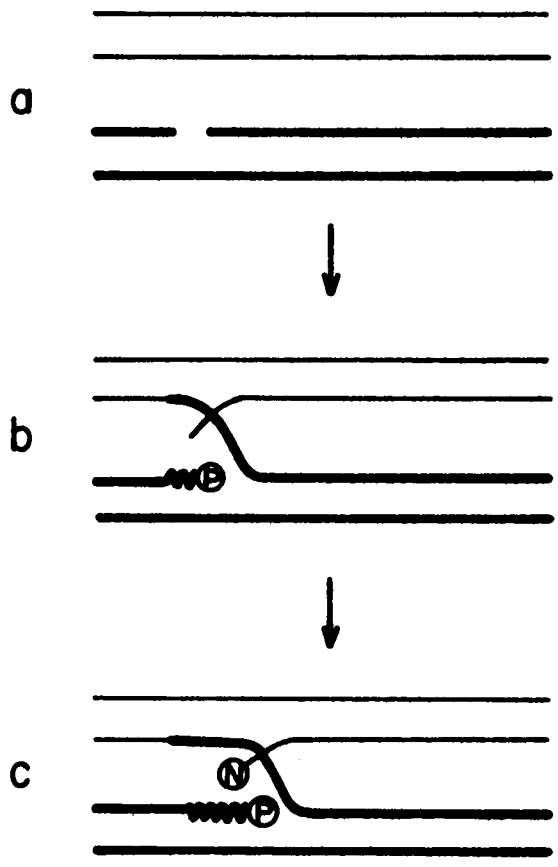

Figure 18. Hypothetical scheme for single strand assimilation during recombination [after Radding (3)]. See Chapter III for discussion.

VITA

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