A Protein Dissociation Step Limits Turnover in FLP Recombinase-mediated Site-specific Recombination*

Leslie L. Waite and Michael M. Cox‡

From the Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

When two ongoing FLP-mediated recombination reactions are mixed, formation of cross-products is subject to a lag of several minutes, and the subsequent rate of cross-product formation is greatly reduced relative to normal reaction progress curves. The lag reflects the formation of a stable complex containing multiple FLP monomers and two FLP recombination target-containing DNA recombination products, a process completed within 5-10 min after addition of FLP recombinase to a reaction mixture. The reaction products are sequestered within this complex for an extended period of time, unavailable for further reaction. The length of the lag increases with increasing FLP protein concentration and is not affected by the introduction of unreacted (non FLP-bound) substrate. The results provide evidence that disassembly of FLP complexes from products occurs in a minimum of two steps. At least one FLP protein monomer is released from reaction complexes in a discrete step that leaves the reaction products sequestered. The recombination products are released in a form free to react with other FLP recombination target-containing DNA molecules only after at least one additional disassembly step. One or both of these disassembly steps are rate limiting for reaction turnover under conditions often used to monitor FLP-mediated recombination in vitro.

The FLP recombinase (M<sub>n</sub> 48,794) is encoded by the 2-micron plasmid of the yeast Saccharomyces cerevisiae, and promotes a site-specific recombination reaction at sequences within the same plasmid (1, 2). FLP is a member of the integrase family of recombinases, which includes the Cre recombinase of bacteriophage P1 and the Int recombinase of bacteriophage λ, among others (3, 4).

The site at which FLP recombinase acts is called the FLP recombination target (FRT). The minimal FRT consists of 2 inverted repeats of 13 base pairs, each flanking an 8-base pair spacer. The 13-base pair repeats serve as FLP protein binding sites; thus, there are two protomer binding sites per minimal FRT or 4 per recombination reaction. The sequence of the wild type FRT spacer is asymmetric, and alignment of two spacer sequences is one of the factors that determines the course of an FLP-mediated recombination reaction. If the spacer is replaced with a symmetric (palindromic) sequence, two reacting FRTs can align in either of two orientations, resulting in a new but predictable set of reaction products. This feature of the symmetric spacer is useful for in vitro analysis of FLP protein-catalyzed reactions.

The FLP reaction involves four DNA cleavage and rejoicing reactions, occurring sequentially in pairs. A Holliday structure is formed after the first reciprocal set of cleavage and religation events. After an isomerization step, a new Holliday intermediate is resolved to products via the second set of cleavage and ligation steps. Much of the chemistry and an outline of reaction steps in these reactions has been elucidated (5).

The interaction between FLP protomers in the protein-DNA complex has recently come under close scrutiny. Each active site for FLP-mediated DNA cleavage and strand exchange includes amino acid residues contributed by two different FLP monomers. The nucleophile tyrosine involved in a given cleavage reaction is derived from a monomer distinct from that bound on the adjacent 13-base pair repeat (trans cleavage) (6, 7). Schwartz and Sadowski (8, 9) have observed that FLP recombinase induces sharp bends in its FRT substrate, which require strong protein-protein interactions, and that this bending is required for FLP protein catalysis. When FLP protein is incubated with FRT half-sites (FRTs that have been cleaved in the spacer to generate a partial site with only one FLP binding site), dimeric and trimeric protein complexes are formed with bound DNA that are held together only by noncovalent interactions and have a half-life of at least 1–2 h (10). Recent experiments suggest that a pair of cleavage and strand exchange reactions may be carried out by a complex containing three tightly bound FLP monomers.²

These strong protein-protein interactions led us to ask about the pathway for disassembly of an FLP-FRT complex. Since the disassembly step(s) should be the reverse of the assembly steps at the beginning of a conservative site-specific recombination reaction, examining disassembly might provide clues about assembly as well. There are several possible pathways for complex disassembly, and two are illustrated in Fig. 1. In the first pathway, FLP protein binds to an FRT; two protein-bound FRTs are brought together, react to form products, and then separate into two protein-DNA complexes. The separated complexes continue on to another recombination event with a new FLP-FRT partner. This model provides for multiple recombination events without complete dissociation of FLP protein. A second possibility would involve the release of FLP protomers from the protein-DNA complex after an FLP recombinase-catalyzed recombination reaction as a prerequisite to further reaction. In option one, the action of FLP protein is processive in that multiple recombination events may occur before the FLP protein dissociates from the FRT. There are many versions of

* This work was supported by National Institutes of Health Grant GM37835. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 608-262-1181; Fax: 608-256-2603; E-mail: COXLAB@MACC.WISC.EDU.

1 The abbreviations used are: FRT, FLP recombination target; MOPS, 3-(N-morpholino)propanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; GdnHCl, guanidine hydrochloride.

2 Qian, X.-H., and Cox, M. M. (1995) Genes & Dev., in press.
option two; FLP dissociation could occur in a single step or as multiple steps of one or more monomers at several points in the reaction. Our experiments were designed to shed light on the disassembly pathway. We report here that a discrete step involving dissociation of one or more protein monomers from the FLP-product complex occurs prior to the step in which product FRTs are released in a form free to react with new FRT partners.

**Fig. 1.** Potential dissociation pathways for the FLP-FRT protein-DNA complex. Two types of dissociation pathways are shown. In both cases, FLP protein binds its DNA target and catalyzes a reaction to products. On the left, these products dissociate from the reactive complex as protein-DNA complexes ready to react with other protein-DNA complexes. According to this model, protein dissociation from the FRT is not required for reactions with new FRT partners to occur. On the right, FLP protein must dissociate from the reactive complex to free the FRT products for new rounds of reactions. This dissociation may be concerted or may occur in a stepwise manner.

**MATERIALS AND METHODS**

Enzymes and Reagents—FLP protein was purified according to a published procedure (12), as modified by Iype (13),3 and stored at −70 °C in a solution of 25 mM MOPS (pH 7.0 at 4 °C), 15% glycerol, and 1 M NaCl. This FLP protein was at least 90% pure as determined by densitometric scanning of SDS-polyacrylamide gels. FLP protein concentration was determined by UV absorption using an extinction coefficient for FLP protein of ε280 = 1.4 A280 mg⁻¹ ml⁻¹ (determined as described above). FLP protein was diluted as needed into a dilution buffer containing 25 mM TAPS (22% anion, pH 7.9, at 25°C), 1 mM Na2EDTA, 2.5 mg/ml bovine serum albumin, 20% glycerol (v/v), 10% polyethylene glycol (8000), 200 mM NaCl, and FLP protein and DNA substrates as indicated in the Fig. legends and the text. The final pH of a solution containing all reaction components was 7.90 at 25°C.

Mixing Experiments to Monitor Complex Disassembly—DNA substrates derived from plasmid pFS39 were radiolabeled with 32P at the 5′-end of each strand. Reactions were started by adding FLP protein to a solution containing all other reaction components. Two portions of a single aliquot of FLP protein were used to start two separate prereactions (a and b, 75 μl final volume each) employing substrates generated by cutting plasmid pFS39 DNA with different restriction enzymes (Fig. 3). The remainder of the FLP protein aliquot was then stored at 30°C for 30 min while prereactions a and b incubated. FLP protein from the aliquot incubated at 30°C was then used to start a control reaction (c) containing both substrate DNAs. Reactions a and b were then mixed exactly 30 s before reaction c was initiated. The 30-min incubation of FLP protein at 30°C was used to control for any loss of FLP protein activity that might occur in prereactions a and b prior to mixing. Final reaction volumes of reactions a + b (after mixing) or c were typically 150 μl. The control reaction (c) and the mixed reaction (a + b) were tracked simultaneously. Reactions were stopped by removal of 5-μl aliquots to tubes containing 3 μl of 10% SDS at time points ranging from 0 to 120 min. A 2-μl addition of GED (12.5% glycerol, 0.0125% bromphenol blue, and 12.5 mM EDTA) was made to all aliquots, which were then loaded onto either a horizontal 0.8% agarose gel or a vertical 2.0% agarose gel. After electrophoresis, gels were dried, and DNA bands were visualized using a PhosphorImager 425e (Molecular Dynamics).

In a few cases, experiments were carried out to see if unbound DNA substrates could react with pre-formed complexes of FLP protein on another substrate. Reactions were carried out as described above, except that 2 aliquots of FLP protein were added to reaction a. After a 30-min incubation at 30°C, reaction mixture b (without FLP protein) was added to reaction a, and product formation was monitored as described above. Control reaction c was carried out as for the standard experiments.

Variation of FLP Protein Prereaction Times to Monitor Substrate Sequestration—Reactions were performed as above, except that the length of time during which prereactions a and b were allowed to proceed before mixing was altered. Reactions were mixed 2, 5, 10, 20, and 30 min after the prereactions were initiated. Control reaction c was performed in each case as described above, with the FLP protein preincubated for 30 min prior to mixing.

---

3 Copies of this procedure are available from this laboratory on request.

4 Marrione, P. E., and Cox, M. M. (1995) Biochemistry 34, 9809–9818.
Disassembly of FLP Complexes

Experimental Design—The FLP-mediated recombination reaction is relatively slow, even when the FLP recombinase is present in stoichiometric excess relative to available FRT sites. The turnover number has previously been estimated to be about 0.1 min⁻¹ (20). To provide a measure of the disassembly of FLP-FRT complexes following recombination, we designed an experiment to determine the rate at which recombination products were made available for subsequent reactions. Two ongoing FLP-mediated recombination reactions, each employing a different DNA substrate, are mixed. Each separate reaction produces a characteristic set of two products. The substrate FRT sites in the two reactions are compatible such that a number of unique cross-products (distinguishable by size from the products formed in the individual reactions) are formed when the substrates are mixed. If the DNA substrates or products in an ongoing reaction are sequestered (e.g. if the disassembly of FLP-FRT complexes is slow), a lag in the appearance of cross-products should be observed. The lag can be taken as a measure of the rate at which reaction products are made available for further reaction.

We generated two substrates for FLP protein by cutting the plasmid pJFS39 with either of two restriction enzymes, Sty I or Eag I (Fig. 3). The two linear DNA substrates generated are identical in length and both contain a single FRT. They differ only in the placement of the FRT relative to the ends of the DNA. The FRT in pJFS39 has a symmetric spacer sequence. Reactions involving one of the substrates can have two different outcomes. If the substrates are aligned so that the sequences flanking the FRT are parallel, the subsequent reaction will generate products that are indistinguishable from substrates. If the substrates are aligned so that sequences flanking the FRT are antiparallel, two distinct products will arise from a recombination reaction, one larger and one smaller than the substrate. Because the FRT is positioned differently in the two different substrates, the sizes of the products generated from each are distinct (P1 to P4). Since they contain identical FRT DNA sequences, the two substrates can also react with each other. The various possible alignments of the two substrates with each other give rise to four additional cross-products (CP1 to CP4). The substrates have been designed so that all of the possible products and cross-products are distinguishable on an agarose gel. The expected products of reactions involving one or both substrates are illustrated in Fig. 3.

The lag in cross-product formation seen after mixing of reactions a and b was not due to loss of FLP recombinase activity. The control reaction shows that a 30-min incubation of FLP recombinase prior to reaction does not result in a loss of activity. The rate of the reaction is essentially identical to a reaction initiated by FLP protein that had not been incubated at 30 °C. In some reactions (including that in Fig. 4), the FLP incubation prior to the control reaction c was carried out in FLP storage buffer. In others, the FLP recombinase was incubated under standard reaction conditions, and reaction c was initiated by addition of the DNA substrates. There was no significant difference in the FLP-mediated reaction observed with these varied protocols (data not shown).

The lag in cross-product formation seen after mixing of reactions a and b was due to loss of FLP recombinase activity. The control reaction shows that a 30-min incubation of FLP recombinase prior to reaction does not result in a loss of activity. The rate of the reaction is essentially identical to a reaction initiated by FLP protein that had not been incubated at 30 °C. In some reactions (including that in Fig. 4), the FLP incubation prior to the control reaction c was carried out in FLP storage buffer. In others, the FLP recombinase was incubated under standard reaction conditions, and reaction c was initiated by addition of the DNA substrates. There was no significant difference in the FLP-mediated reaction observed with these varied protocols (data not shown).

The lag in cross-product formation exhibits a dependence on FLP Protein Concentration—We next investigated whether or not protein concentration affected the observed lag in cross-product formation. Reactions were carried out as described above, but with several different protein concentrations ranging from 60 to 300 nM FLP recombinase. As seen in Fig. 5, the lag in product formation increased with increasing protein concentration. Following the lag, the rate of cross-product formation was also reduced relative to the control reaction.

Since FLP protein was in excess in all reactions, the simplest explanation of this result is that a longer lag in product forma-
in many reactions at relatively late times (11). Bands labeled above the product bands are primarily Holliday structures (11). Small 
120 min after addition of FLP protein in addition of FLP protein. Timepoints are 0, 2, 5, 7, 10, 15, 30, 60, 90, and reaction in which the 
Products and cross-products are labeled as in Fig. 3.

We attribute the lag information of P2 and P3 to an nonspecific binding of the excess FLP recombinase to DNA sequences re- 
 sist from the FRT. Similar lags in product formation when FLP is 
mediated recombination after mixing, yielding the products P2 
and P3 efficiently after an 8–10-min lag. This is presumably 
due to the presence of excess FLP recombinase in reaction a. 
We attribute the lag in formation of P2 and P3 to a nonspecific 
binding of the excess FLP recombinase to DNA sequences re- 
 mot from the FRT. Similar lags in product formation when FLP is 
inhibition of an FLP protein dissociation step.

The Presence of Unbound FRT Substrate Does Not Relievethe 
Lag in Cross-product Formation—Since all of the FRTs in the 
previous experiments were incubated with FLP recombinase 
before being mixed, a possible explanation of the lag in cross-

product formation is that FLP protein forms a complex on one 
target substrate and then requires a second, unbound FRT 
substrate to react. If this were true, the observed lag in cross-

product formation would not be due to a direct requirement for 
complete FLP protein dissociation from all reaction products 
but rather to a requirement for one unbound DNA substrate in 
the FLP recombination pathway. To investigate this possibil-
it was performed to determine if the presence of unbound substrate affects the lag in cross-product formation.

Recombination experiments were repeated as above, with 
the exception that only one of the FRT substrate prereaction 
mixtures (a) included FLP protein. After 30 min, a second 
reaction mixture (b) containing unreacted FRT DNA was mixed 
with the first, and product formation was monitored as before 
(Fig. 7). These reactions show the same lag in cross-product 
formation as reactions where both FRTs were prereacted with 
FLP protein. As seen in Fig. 7, the unbound FRT substrate 
introduced with reaction mixture b does participate in FLP-
m ediated recombination after mixing, yielding the products P2 
and P3 efficiently after an 8–10-min lag. This is presumably 
due to the presence of excess FLP recombinase in reaction a.
**FIG. 7. Effect of prereacting only one of two FRT-containing DNA substrates.** Reactions were carried out as described in the text. Final FLP protein and DNA concentrations were 300 and 10.7 nM, respectively. Individual reactions are \( \Delta \rightarrow \Delta \) and \( \triangledown \rightarrow \triangledown \), plots of CP1/S and P2/S, respectively, for a single control reaction in which the Eagl-cut and StyI-cut substrates were mixed prior to addition of FLP protein (no prereaction); \( \Delta \rightarrow - \), time course (CP1/S) after mixing for a reaction in which both of the FRT-containing DNA substrates were prereacted with FLP protein for 30 min; \( \triangledown \rightarrow \), time course (CP1/S) after mixing for a reaction in which the Eagl-cut substrate was prereacted with FLP protein for 30 min but the StyI-cut substrate was prereacted without FLP protein; \( - \rightarrow \), a plot of P2/S time course after mixing for the reaction in which the Eagl-cut substrate was prereacted with FLP protein for 30 min but the StyI-cut substrate was prereacted without FLP protein. Note that P2 is derived from the StyI-cut DNA substrate, which is not prereacted with FLP protein in this last reaction.

preincubated with nonspecific DNA.\(^5\)

This experiment was done in two ways. In one case (plotted on the graph), reaction a contained 10.7 nM FRT sites and 600 nM FLP protein. This reaction was incubated for 30 min at 30°C, at which point a second reaction mixture of equal volume (75 \( \mu \)l) containing the other FRT site at 10.7 nM but no FLP protein was mixed with it, and the subsequent reaction followed. In this reaction the FLP concentration was diluted by 50% upon mixing with reaction mixture b, while the FRT substrate concentration remained constant throughout the experiment. In the second version of this experiment, reaction a contained 300 nM FLP protein and 5.4 nM FRT substrate in 150 ml. A small aliquot of a concentrated solution of the second FRT was added to this reaction mix after 30 min, bringing the final FRT concentration up to 10.7 nM. In this case, the FRT concentration increased from 5.4 to 10.7 nM at the mixing step, while FLP protein concentration remained constant at 300 nM. Both reactions produced similar cross-product formation lags (data not shown).

**DISCUSSION**

FLP protein undergoes a slow dissociation from a stable FLP protein-FRT complex before reaction products are free to react with new FRT partners. The dissociation occurs in at least two steps (Fig. 8). A first step involves disassembly of part of the complex. This is shown as dissociation of one monomer in Fig. 8, although the actual number of monomers released may be different. The product of disassembly step 1 is a complex in which product DNAs are still held together, unavailable for further reaction. The existence of this step is based on the observation that higher FLP protein concentrations delay the release of products to react again. The illustrated step would be affected by mass action in a way that would affect the partitioning of intermediate generated by disassembly step 1. Under the conditions used here and in many other studies, the rate-limiting process for catalytic turnover in FLP-mediated recombination is one or both of the disassembly steps shown in Fig. 8.

These conclusions are derived from several observations.

\(^5\) L. L. Waite, unpublished results.

When two ongoing FLP protein reactions are mixed, there is a lag in cross-product formation. The simplest explanation for the lag is that products in a reaction that is ongoing are sequestered, unable to react with new DNA partners. This idea is further supported by experiments which demonstrate that the degree to which cross-product formation is suppressed depends on how long the two separate FRTs are allowed to react before being mixed. This apparently reflects the formation of a stable FLP-FRT complex during the prereaction that occurs before mixing. The delay in cross-product formation also exhibits a mass action effect. Higher concentrations of FLP protein increase the length of the lag and decrease the rate of subsequent cross-product formation. Since FLP protein is in excess in all the reactions described here, only reaction steps that involve a change in the number of bound FLP monomers (i.e. a binding or dissociation step) in the reactive complex should be affected by changes in FLP protein concentration. Since we see a decrease in system turnover, we believe the delay in cross-product formation is due to an inhibited dissociation step. If the dissociation step producing the lag resulted in release of FRT reaction products to a substrate pool available for further reaction, no dependence of the lag in cross-product formation on protein concentration would be evident. Hence, the product of the dissociation step affected by protein concentration must be an FLP-FRT complex in which the FRT DNAs are still sequestered.

As indicated above, changes in the concentration of FLP protein will affect the partitioning of the intermediate produced by disassembly step 1. The breakdown of this intermediate to free products will be governed by a first order rate constant and depend on the concentration of the intermediate. The rate of the reverse reaction generating a fully populated complex by adding FLP protein to the intermediate will depend on the concentrations of both the intermediate and free FLP protein and be governed by a second order rate constant. Free FLP protein will produce a larger effect on the partitioning if the breakdown of the intermediate to free products is slow. The
strong suppression of cross-product formation observed in this study suggests that the breakdown of intermediate to free products is at least partially rate limiting under normal reaction conditions.

We considered the possibility that FLP protein binds to a substrate and that this complex then binds to a second, unbound FRT substrate to initiate a reaction. Our experiments argue against this pathway for FLP-mediated reactions, since experiments in which unbound substrate is introduced into an ongoing reaction show the same lag in cross-product formation as experiments where two ongoing reactions (both with FLP protein present) are mixed (Fig. 7).

Based on binding studies done by Beatty and Sadowski (21), one could envision a reaction path in which FLP protein binds to one FRT target, two such protein-DNA molecules come together to react, and the products of this reaction separate as two new FLP-FRT complexes, which go on to find new reactive partners. The experiments presented here argue against this reaction sequence and for a pathway in which most or all of the FLP monomers must dissociate before the FRT products are free to react with new partners.

The time course for the suppression of cross-product formation in the mixing reactions is similar to the rate of product formation in the control reactions (Fig. 6), indicating that the stable complexes are predominantly bound to products. This suggests that the chemical steps in a recombination reaction are fast relative to stable complex formation so that assembly and disassembly of complexes are both slow processes. Alternatively, FLP protein could bind rapidly to substrate DNA but form a stable complex only after recombination had occurred. In the latter case, the recombination pathway would involve conformation changes in the FLP monomers in the complex so that the products were bound differently (and more tightly) than the substrates. This would lead to a "one-way enzyme" (17) in which release of products would be rate limiting.

Jayaram and colleagues (6, 7) have shown that domains from two different FLP monomers must come together to form a single active site for DNA cleavage and strand exchange. Qian et al. (10) have demonstrated that protein-protein interactions across the spacer region of the FRT are strong enough to form complexes with a half-life of 1 to 2 h. Schwartz and Sadowski (8, 9) have shown that FLP protein promotes a sharp bending of the FRT substrate in the course of catalyzing site-specific recombination. These results, together with the data presented here, reveal a protein-DNA complex that is held together very tightly and cooperatively. The stepwise dissociation pathway outlined in this work suggests further that at least one of the FLP monomers in a presumably tetrameric complex is held less tightly than the rest. Recent work indicating that three FLP monomers may be necessary and sufficient to carry out a set of DNA cleavage and strand exchange reactions 2 leads to the suggestion in Fig. 8 that the "loose" component is a single monomer, and it seems reasonable to hypothesize that three monomers would be sufficient to continue the sequestration of the reaction products. Although we emphasize that this proposal is not uniquely consistent with the data in the current study, we presume that the only alternative is that two FLP monomers remain after disassembly step 1, which would seem to be the minimum required to sequester two FRT sites in the manner demonstrated.

Acknowledgments—We thank Lisa Iype for providing the purified FLP recombinase used in these experiments. We also thank Alberto I. Roca for help with data analysis programs.

REFERENCES
1. Sadowski, P. (1986) J. Bacteriol. 165, 341–347
2. Cox, M. M. (1988) In Genetic Recombination (Kucherlapati, R., and Smith, G. R., eds) Vol. 1, pp. 429–443. American Society for Microbiology: Wash, D. C.
3. Argos, P., Landy, A., Abremkedi, K., Egan, J. B., Haggard, L. E., Hoess, R. H., Kahn, M. L., Kallinis, B., Narayana, S. V., Pirozz, L. S., Sternberg, N., and Leong, J. M. (1980) EMBO J. 6, 433–440
4. Craig, N. L. (1986) Annu. Rev. Genet. 20, 77–105
5. Jayaram, M. and Beatty, P. D. (1989) Trends Biochem. 12, 78–82
6. Beatty, L. G., and Sadowski, P. (1988) J. Biol. Chem. 263, 14147–14152
7. Chen, J.-W., Lee, L., and Jayaram, M. (1992) Cell 69, 647–658
8. Schwartz, C. J. E., and Sadowski, P. D. (1990) J. Mol. Biol. 216, 289–298
9. Schwartz, C. J., and Sadowski, P. D. (1989) J. Mol. Biol. 205, 647–658
10. Qian, X.-H., Inman, R. B., and Cox, M. M. (1990) J. Biol. Chem. 265, 21779–21788
11. Meyer-Leon, L., Huang, L.-C., Umilau, S. W., Cox, M. M., and Inman, R. B. (1991) Mol. Cell Biol. 13, 289–298
12. Pan, G., Luoee, K., and Sadowski, P. D. (1991) Mol. Cell Biol. 13, 3167–3175
13. Iype, L. E. (1989) J. Biol. Chem. 264, 6582–6588
14. Bittman, L., and Sadowski, P. (1988) J. Biol. Chem. 263, 7380–7386
15. Lohman, T. M., Leong, J. M., and Cox, M. M. (1988) J. Biol. Chem. 263, 10139–10147
16. Kucherlapatil, R., and Smith, G. R., eds) Vol. 1, pp. 429–443, American Society for Microbiology: Madison, Madison, WI
17. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1988) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY