Determinants of the position of a Flp-induced DNA bend

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Received December 15, 1997; Revised and Accepted February 2, 1998

ABSTRACT

The Flp site-specific recombinase from Saccharomyces cerevisiae induces DNA bending upon interaction with the Flp recognition target (FRT) site. The minimal FRT site is comprised of two inverted binding elements which flank a central core region. Binding of a single monomer of Flp to DNA induces a DNA bend of 60°. The position of this bend differed depending on whether the substrate contained a single binding element or a two-element FRT site. In the present work we tested and disproved a model in which a single Flp monomer interacts with both symmetry elements of a single FRT site. Likewise, we showed that a model in which a Flp monomer dissociates from a singly occupied FRT site and reassociates with the unbound element of another singly occupied FRT site during electrophoresis, does not account for the apparent shift in the position of the bend centre. It seems that the movement of a Flp monomer between the a and b elements of one FRT site during electrophoresis accounts for this anomaly. The position of the DNA bend resulting from the association of a Flp monomer with the FRT site is also influenced by the DNA sequences flanking the site. We conclude that attempts to measure the bend centre of a complex of one Flp molecule bound to a DNA containing two binding elements give misleading results. The position of the bend is more accurately measured in the presence of a single binding element.

INTRODUCTION

The Flp protein, which is a member of the integrase family of recombinases, is thought to play a role in amplification of the 2 µm plasmid. Flp-mediated site-specific recombination is thought to play a role in amplification of the 2 µm plasmid (1). Recombination is carried out between two Flp recognition target (FRT) sites that are contained within two 599 bp inverted repeats of the plasmid. The minimal FRT site (Fig. 1) contained a single symmetry element (a and b) which flank an 8 bp core region.

Flp interacts with the FRT site through two DNA binding domains that are present in the N-terminal 13 kDa region (P13) and in the C-terminal 32 kDa region (P32; 2,3). The N-terminal P13 domain interacts with the core-proximal 4 bp of the symmetry element, while the C-terminal P32 domain interacts with the core-distal 9 bp of the symmetry element (Fig. 2B, i; 4).

Binding of a single monomer of Flp to one symmetry element results in the formation of a Flp:DNA complex (complex I) and induction of a DNA bend of 60° (type I bend; 5). Further characterization of the type I bend showed that it was positioned at the core-distal end of the b element when the substrate contained a single symmetry element, but at the core-proximal end of the b element when the substrate contained a FRT site with two symmetry elements (6).

These results suggested an explanation for the difference in bend centres: that a single Flp monomer associates with the two symmetry elements of a single FRT site simultaneously (6; Fig. 2A, II). Here we considered this model as well as the possibility that a Flp monomer dissociates from a singly occupied FRT site and reassociates with the unbound element of another singly occupied FRT site during electrophoresis. We concluded that the apparent shift of the bend centre likely results from the movement of one Flp monomer between the a and b elements of one FRT site during electrophoresis. Furthermore the position of the DNA bend resulting from the association of a Flp monomer with the FRT site is apparently influenced by the DNA flanking the site.

MATERIALS AND METHODS

Oligonucleotide substrates

Unmodified oligonucleotides were synthesized at the Hospital for Sick Children/Pharmacia Biotechnology Service Centre, Banting Institute, University of Toronto. Oligonucleotides used in this study (symmetry element and core sequences of the wild-type FRT site are indicated in boldface and in italics respectively):

(HP-7) d(TGAAGTTCTATTCTCTAGAAAGTATAGGAACCT); (HP-31) d(GGTCCGAAGTTCTATACCTGCTCTAGAGAATAGGAACCTC); (KL-8) d(CCCTCGAAGTTCTATACCTGCTCTAGAAAGTATAGGAACCTC); (KL-9) d(ACCTTACCTTATACCTGCTCGAGG); (KL-18) d(CCCTCGAAGTTCTATTCTCTAGAAAGTATAGGAACCTC); (KL-19) d(TCCCTCTATTCTCTAGAAAGTATAGGAACCTC); (KL-22) d(CCCGAAGTTCTGACCTGCTAGGAACCTCAC); (KL-23) d(TCCCTCTATTCTCTAGAAAGTATAGGAACCTC); (KL-24) d(TCCCTCTATTCTCTAGAAAGTATAGGAACCTC). Oligonucleotides were labeled with [γ-32P]ATP (NEN DuPont) using T4 polynucleotide kinase (New England Biolabs). Following

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The reaction was incubated at 22°C for 30 min and 2.7 μl of dye mixture was added. Reactions were subjected to electrophoresis on a 5% polyacrylamide gel (1x TBE) for different lengths of time (as indicated in the figure legend).

Circular permutation assays were carried out and analyzed as described previously (6). The bend centre was estimated by extrapolating the linear portions of the curve, obtained by plotting the relative mobilities of Flp:DNA complexes versus the distance (bp) of the left-hand end of each substrate from the left-hand EcoRV site on the DNA fragment, to find the position at which the curve reached a minimum. Curves were plotted using Cricketgraph software.

**Quantitation**

Dried gels were scanned using a Molecular Dynamics Phosphorimager and analyzed using Imagequant software.

**RESULTS**

**Substrate-dependent position of the DNA bend in Flp:DNA complex I**

We have previously showed that the position of the DNA bend induced by the binding of a single monomer of Flp to DNA depended upon whether the substrate contained one or two symmetry elements (type I bend; 6). When the substrate contained a single symmetry element, the type I bend was positioned at the core-distal end of the b element (Fig. 1, bp position –17) but was positioned at the core-proximal end of the b element (Fig. 1, bp position –6) when the substrate contained a two-element FRT site. A model in which the P32 and P13 regions of a single molecule of Flp (2,3) bound to different symmetry elements of the FRT site was proposed (Fig. 2A, II). This would result in a shift in the bend centre observed when a single Flp monomer was bound to a two-element substrate (6).

To test whether a single Flp monomer may simultaneously interact with two symmetry elements, we examined the interference of methylated bases with the formation of Flp:DNA complex I. We used DMS to methylate bases of DNA substrates prior to incubation with Flp and a G > A specific cleavage of methylated bases to analyze DNA from isolated complex I.

Footprinting and cross-linking studies have shown that the C-terminal P32 domain interacts with the core-distal 9 bp of the symmetry element while the N-terminal P13 domain interacts with the core-proximal 4 bp of the symmetry element (Fig. 2B, i, shaded and dotted boxes; 4). To prevent formation of a mixed population of complex I in which Flp monomers were bound to either the a or the b element, substrates in which the P32-binding region of one symmetry element was absent were used. Thus, a DNA substrate contained a partial FRT site comprised of the b element, the core and the core-distal 7 bp (including the P13 binding region) of the a element (Fig. 2B, ii). Since a single Flp monomer can bind to a single symmetry element, two other substrates that contained the core-distal 9 bp (P32 binding region) of the b element, the core and P13 binding region of the a element (Fig. 2B, iii and iv) but lacked the P13-binding region of the b element were also used.

A two-element FRT site was used as a control for these experiments (Fig. 2B, i). These substrates would allow us to distinguish between two models of binding (Fig. 2A). i) Model I-Unipartite model: one molecule of Flp contacts a single symmetry element. ii) Model II-Bipartite model: one Flp molecule binds to two symmetry elements at once. In model I, the
Figure 2. Methylation interference in formation of complex I. (A) Models of the binding of one Flp molecule to the FRT site. The core region of the FRT site is indicated by an open rectangle and the a and b symmetry elements by horizontal arrows. The binding region of the P32 domain of Flp is indicated by a dark shaded box and the binding region of the P13 domain by a light shaded box. The vertical arrow indicates the location of the type I bend centre. (I) Unipartite model: a single molecule of Flp binds to one symmetry element. (II) Bipartite model: a single molecule of Flp binds to two symmetry elements. (B) The DNA substrates and summary of the interference data. Unmodified base pairs of the symmetry elements are indicated by solid lines and solid arrows. Base pairs of the b symmetry element which have been mutated are indicated by broken arrows. Modified guanine and adenine residues which interfered with the formation of complex I are indicated by solid circles. (C) Predicted interference data according to models I and II shown in (A). The sequences of Flp substrates designated as (i), (ii), (iii) or (iv) are shown in (B). Methylated bases (solid squares) that interfere with Flp binding to substrates (ii)–(iv), according to model I, are limited to a single symmetry element as shown to the left. Methylated bases that interfere with Flp binding, according to model II, are present in parts of two symmetry elements as shown to the right. (D) Flp substrates were prepared by 5'-end-labeling the top or bottom strand and annealing the following oligonucleotides: HP-7 and HP-31 (lanes 1–6), KL-18 and KL-19 (lanes 7–12), KL-8 and KL-9 (lanes 13–18), KL-22 and KL-23 (lanes 19–24). The DNA substrates were methylated with DMS and incubated with Flp for 30 min at 22°C. Complex I is isolated and the DNA was depurinated, cleaved with alkali and analyzed on a 15% denaturing polyacrylamide gel. The DNA sequences and nucleotide numbers are shown beside each panel. S, substrate in the absence of protein; U, isolated unbound substrate; cI, DNA isolated from complex I.
methylated bases (solid squares) that interfere with Flp binding would be confined to one symmetry element (Fig. 2C, left) whereas in model II, these methylated bases would be found in parts of two symmetry elements (Fig. 2C, right).

The results of methylation interference experiments designed to distinguish between the two models are shown in Figure 2. We found no interference by methylated residues in the formation of complex I on a two-element FRT site, consistent with the results of Beatty and Sadowski (10; Fig. 2D, i, lanes 3 versus 2 or 1 and lanes 6 versus 5 or 4). The explanation for this result is the following. When a methylated base of one symmetry element weakens the affinity of Flp for that element, a Flp monomer then preferentially binds to the unmodified element of a two-element FRT site and forms a complex I.

When a substrate lacking part of the a symmetry element but containing the entire b element, the core and core-proximal 7 bp of the a element, was used however, methylation of the –12A, –11G, –10G, –9A, –7A, –6A and –5G residues in the bottom strand of the b element, did interfere with the formation of complex I (Fig. 2D, ii, lanes 12 versus 11 or 10; Fig. 2B, ii, solid dots). As expected, the intensities of the bands were enhanced in the DNA from the unbound fraction as compared to the untreated substrate since Flp does not interact with DNA which is methylated at these positions (Fig. 2D, lanes 11 versus 10). Also methylation of the +5G and +7A residues in the top strand of the a element caused no interference with the formation of complex I (Fig. 2D, ii, lanes 9 versus 8 or 7). These results mean that the contacts required by Flp for the formation of complex I on this substrate are all contained within the b element. This confirms the predictions of model I and renders model II less likely (Fig. 2A).

It was possible that a single molecule of Flp might interact with parts of two symmetry elements only if the P13-binding region of one of the symmetry elements were mutated. To test this idea, the P13-binding region of the b element was mutated (from –8A/T, –7T/A, –6T/A, –5G/G to –8T/A, –7A/T, –6A/T, –5G/C); the substrate contained the P32-binding region of the b element, the core and the P13-binding region of the a element (Fig. 2B, ii). Using this substrate we found that methylation of the –12A, –11G, –10G and –9A residues interfered with the formation of complex I (Fig. 2D, iii, lanes 18 versus 17 or 16; solid dots) consistent with the observations obtained when a substrate comprised of the b element, the core and the core-proximal 7 bp of the a element, was used (Fig. 2B, ii). Unexpectedly, methylation of the –8A (bottom strand), –7A, –6A and –5G (top strand) residues of the mutated P13-binding region also interfered with the formation of complex I (Fig. 2D, iii, lanes 18 versus 17 or 16, lanes 15 versus 14 or 13; solid dots). This result suggests that there is some relaxed sequence specificity in the interaction of Flp with the P13 binding region, consistent with our previous findings that P13 has some non-specific DNA binding activity (3). Since these changes retained the same base pairs at each position, it was also possible that the P13 region of Flp could interact with G and A residues now located on the opposite strand.

In an attempt to abolish the interaction of P13 with the P13-binding region of the b element, the P13-binding region of the b element was mutated in a different way (changed –8A/T, –7T/A, –6T/A, –5C/G to –8G/C, –7G/T, –6G/C, –5G/C; Fig. 2B, iv). We again found that methylation of the –12A, –11G, –10G and –9A residues of the P32-binding region in the b element, interfered with the formation of complex I. However, methylation of residues in the mutated P13-binding region, the core and the core-proximal 7 bp of the a element, did not interfere with the formation of complex I (Fig. 2D, iv, lanes 21 versus 20 or 19, lanes 24 versus 23 or 22 and Fig. 2B, iv). We conclude that these experiments do not support model II but favour model I (Fig. 2A and C).

The substrate-dependent position of the type I bend centre may result from the association of a single Flp monomer with the a and b elements of one FRT site

The use of an FRT site with two symmetry elements as a substrate for Flp binding may cause an artifact in which a Flp monomer may dissociate during electrophoresis from a singly occupied FRT site and reassociate with the unoccupied symmetry element of a complex I on another DNA molecule to form a complex II. This reaction pathway was described for Tet repressor-tet operator binding, and results in the statistical disproportionation of the singly occupied complex in the gel (11). This gives rise to smearing and loss of the band corresponding to complex I under conditions of relatively low binding affinity or with increased electrophoresis time. Since circular permutation analysis involves measurement of the mobility of complexes, the smearing of the band corresponding to complex I may have introduced error in the determination of the position of the type I bend on a two-element FRT site. This may have accounted for the apparent shift in the bend centre observed when a single Flp monomer was bound to a two-element substrate.

To test whether disproportionation could be occurring in our experiments, we examined the stability of complex I formed on a one-element and a two-element site during electrophoresis times of 1.3, 3, 6, 9, 12 and 20 h (Fig. 3A). If disproportionation were occurring during electrophoresis, the band corresponding to complex I would have been expected to smear and then disappear as a function of time. Quantitation of the data shows that complex I formed on a two-element site was as stable as complex I formed on a one-element site during running times up to 20 h (Fig. 3B). Since there was no loss of complex I on a two-element site, the data suggest that it is unlikely that disproportionation of complex I was occurring in the gel.

The use of a two-element FRT site may give rise to another reaction pathway involving the dissociation of a Flp monomer from one symmetry element and its reassociation with the other symmetry element on the same DNA fragment (‘shuffling’; 11; note that the disproportionation phenomenon described above implies that a Flp molecule dissociates from one DNA molecule and reassociates with another DNA molecule). Shuffling may give rise to the merging of two bands with differing gel mobilities into one discrete band. Since binding of a Flp monomer to a two-element site gives rise to a single band, shuffling offers a plausible explanation for the apparent shift in the type I bend (6; Fig. 4A).

Tests of asymmetry in the FRT site as determinants of the asymmetrical position of the type I bend on a two-element FRT site

If shuffling of a Flp monomer between the a and b elements of one substrate molecule were occurring, the type I bend resulting from the association of a Flp monomer with a two-element FRT site would nevertheless have been expected to be positioned in the middle of the core region. The type I bend was, however, positioned at the core-proximal end of the b element (6). There are two possible sources of asymmetry in the FRT sequence that might contribute to
the asymmetrical position of the type I bend on a two-element site. There is a 1 bp difference in the sequences of the two symmetry elements and the 8 bp core region is not symmetrical. This discrepancy in the position of the type I bend might be explained by a lower binding affinity of Flp for the a element versus the b element. When a Flp monomer shuffles between the two-elements of one substrate molecule, the asymmetrical position of the type I bend could result from the fact that a single Flp monomer is associated with the b element more of the time than it is with the a element.

To test the possibility that a difference in the binding affinity of Flp for the a and b elements might influence the position of the type I bend, we determined the location of the type I bend centre using circular permutation substrates that contained FRT sites comprised of two b elements flanking a core region (Fig. 4, i). We found that the type I bend was positioned at the same location when the substrate contained a FRT site with two b elements (Fig. 1, bp position –7) as when the substrate contained a wild-type FRT site (Fig. 1, bp position –6). Thus, the position of the type I bend resulting from the association of a Flp monomer with a two-element FRT site did not arise from sequence differences between the a and b symmetry elements.

The asymmetrical position of the type I bend on a two-element FRT site may have been due to asymmetry in the core sequence of the FRT site. For example, an asymmetrically-positioned sequence-directed DNA bend in the core region, might influence the overall position of the Flp-induced type I bend formed due to the association of a Flp monomer with a two-element FRT site. To address this possibility, a completely symmetrical FRT site comprised of two b elements flanking a symmetrical core region was used to determine the location of the type I bend (Fig. 4, ii). If the asymmetrical position of the type I bend on a two-element FRT site arose due to asymmetry in the core sequence, then the type I bend would have been expected to be positioned in the middle of the FRT site when a symmetrical FRT site was used. The position of the type I bend was, however, unaffected by the use of substrates containing a symmetrical FRT site. The type I bend was positioned at the core-proximal end of the b element (Fig. 1, bp position –5) irrespective of whether the substrate contained a symmetrical FRT site or a wild-type FRT site (Fig. 1, bp position –6). Thus the asymmetrical positioning of the type I bend on a two-element FRT site does not arise due to asymmetries in the site.

**Figure 3.** Time course of electrophoresis of Flp:DNA complexes. (A). Gel mobility shift assay of Flp:DNA complexes electrophoresed for different lengths of time. Flp substrates containing either a two-element FRT site (152 bp; lanes 1–6) or the b symmetry element (131 bp; lanes 7–12) were isolated from the pBr2Flp(ba) and pBr2Flp(b) plasmids, (6) respectively, and 5′-end-labeled (*). DNA substrates (indicated at the bottom of the autoradiogram) were incubated with Flp for 30 min at 22℃ and loaded on a 5% native polyacrylamide gel at different times. Electrophoresis was carried out for 1.3 (lanes 1 and 7), 3 (lanes 2 and 8), 6 (lanes 3 and 9), 9 (lanes 4 and 10), 12 (lanes 5 and 11) or 20 h (lanes 6 and 12). In each of lanes 1–6, the bands with the slowest, intermediate or fastest mobilities represent complex II (cII), complex I (cI) or unbound substrate (S). In each of lanes 7–12, the bands with the slowest and fastest mobilities represent complex I (cI) or unbound substrate (S). In each of lanes 7–12, the bands with the slowest and fastest mobilities represent complex I and unbound substrate respectively. (B) Quantitation of the stability of Flp:DNA complexes as a function of the time of electrophoresis. The fraction of complex I was quantitated for reactions in which Flp was incubated with DNA substrates containing a single binding element or a two-element FRT site, respectively, as described in the Materials and Methods. Solid and hatched bars indicate % complex I retained on substrates containing a single binding element or a two-element FRT site after electrophoresis for different lengths of time.
Figure 4. Positions of bend centres induced by Flp bound to modified FRT sites with two \( b \) symmetry elements or to symmetrical FRT sites. (A) Gel mobility shift assays of circularly permuted substrates bound by Flp. DNA substrates (152 bp) were obtained by cleavage of the pB(b-b) plasmid, containing a FRT site with two \( b \) symmetry elements (i), or by cleavage of the pBsym plasmid, containing a symmetrical FRT site (ii), or by cleavage of the pB2Flp(ba) plasmid (iii), at the different restriction sites flanking each FRT site in tandem. Each circularly permuted substrate is named according to the restriction enzyme used to obtain it and is indicated below the autoradiograms. E, EcoRV; Sp, SpeI; N, NheI; Bg, BglII; M, MluI; B, BamHI; R, RsaI; S, SspI; St, StuI. 5′-end-labeled DNA substrates were incubated with Flp (indicated at the top) and complexes were separated on 5% native polyacrylamide gels. Flp-mediated recombination between two symmetrical FRT sites which have aligned in an antiparallel manner generates two products which are longer and shorter than the parental DNA molecules. Complexes which arise due to the association of Flp with the products of antiparallel recombination are indicated by solid dots. These products are not seen in (i) or (iii) because asymmetry of the core region blocks recombination between two FRT \( b-b \) sites or two FRT \( b-a \) sites that are aligned antiparallel to one another. Recombination between two sites aligned in parallel leads to recombination products that are identical to the substrate. S, unbound substrate; cI, complex I; cII, complex II; R, recombinant products (solid dot). (B) Determination of the bend centres in complex I (squares) and complex II (triangles). The position of the bend centres were estimated from the curves as described previously (6).

DISCUSSION

In this study the basis for a substrate-dependent anomaly in the position of the Flp-induced type I bend was investigated. We have used methylation interference studies to test a model whereby a single Flp monomer interacts with both symmetry elements of a single FRT site. These methylation interference studies did not support the interaction of a single Flp monomer with both symmetry elements of one site. Another explanation must account for the substrate-dependent position of the type I bend.

We then examined whether the apparent shift in the position of the type I bend centre arose due to ‘disproportionation’, a phenomenon in which a Flp monomer dissociates from a singly occupied FRT site and reassociates with the unbound element of another singly occupied FRT site during electrophoresis to form a complex II. No evidence for disproportionation was found. We have also excluded different affinities of Flp for the two symmetry elements and asymmetry in the core sequence as factors which influence the position of the type I bend on a two-element FRT site.

Therefore, we conclude that a Flp monomer may continuously dissociate from one symmetry element and reassociate with the other symmetry element on the same DNA fragment during electrophoresis. This reaction mechanism is supported by the finding that binding of a Flp monomer to a two-element FRT site results in one band. The observation that exonuclease stop points occur outside both of the \( a \) and \( b \) symmetry elements in the exonuclease footprint of a complex I formed on a two-element FRT site, also supports this reaction mechanism (10).

The circular permutation assay was devised by Wu and Crothers (15) as a method to determine the position of a bend in the DNA induced by the binding of a protein. The assay is based on the electrophoretic mobility of complexes formed by the binding of protein to a series of DNA fragments that differ from one another by the position of the protein binding site along the length of the fragment. The simplest interpretation of this assay is that the complex with the maximal relative migration contains a bend which is positioned near the end of the fragment, while a complex with the minimal relative migration contains a bend
which is positioned closest to the middle of the fragment. There are a number of factors other than DNA bending which may affect the mobility of the complexes. These include the aberrant shape of a protein or the extent of binding during the gel run (12–14). Thus, determination of the position of a protein-induced DNA bend using the circular permutation method may not be straightforward.

Two potential complications of interpreting circular permutation assays were observed. The observation of only one band when a single monomer of FLP associated with a two-element FRT site suggested that a FLP monomer shuffled between the a and b symmetry elements of a single FRT site during electrophoresis (6; Fig. 3A). The estimated position of the apparent bend would thus represent a composite of the positions of the DNA bends resulting from the association of FLP with each of the a and b elements. We conclude that the circular permutation assay cannot be used for the accurate determination of the location of a DNA bend induced by the binding of a single protein monomer to DNA when the DNA molecule contains more than one binding site.

Although the position of the type I bend on a two-element FRT site is apparently influenced by the continuous exchange of a FLP monomer between the a and b binding elements, this reaction does not account for the asymmetrical position of the type I bend on a two-element FRT site. We would expect the apparent position of a type I bend on a two-element FRT site to be in the middle of the core region rather than at the core-proximal end of the b binding element when both binding elements are the same. Since an FRT site with two identical binding elements or a completely symmetrical FRT site did not change the eccentric position of the type I bend, we conclude that the type I bend resulting from the association of a FLP monomer with a two-element FRT site is influenced by the flanking DNA. It is possible that the extent of DNA contacted by FLP in a supercoiled plasmid in vitro may exceed that defined on short linear substrates in vivo. Flanking DNA may also have contributed to the positioning of the type I bend at the extreme outside end of the b element (Fig. 1, bp position –17) when the substrate contained a single element (6). The position of the type I bend on a two-element FRT site is however also likely to be influenced by the continuous exchange of a FLP monomer between the a and b binding elements as was described.

There are several possible ways in which flanking DNA may influence the position of the FLP-induced type I bend. The centre of the bend induced by Tn5 transposase also maps to the extreme outside end of the consensus sequence for Tn5 transposase, a finding which has been attributed to additional upstream nucleotide contacts that are required for optimal binding (17). A second possibility is that the DNA flanking the site contains a sequence-directed bend which phases with the FLP-induced type I bend thus affecting the observed macroscopic position of the type I bend. A third possibility is that the DNA flanking the site may wrap around FLP forming non-specific contacts which may affect the position of the type I bend as in the case of the CAP and Fis proteins (18,19).

This work shows that a single molecule of FLP does not appear to contact more than one symmetry element of the FRT site. The data are fully consistent with the recently published X-ray structure of the closely related Cre recombinase (20). The N-terminus of Cre was found to contact the core-proximal region of the lox site in cis. The position of the type I bend with FLP using a substrate with a single symmetry element is consistent with that induced by the Cre protein in the symmetry element of the lox site.

ACKNOWLEDGEMENTS

We are grateful to Dr A. Becker and Dr B. Funnell for careful reading of the manuscript. We thank Donna Clary for preparing FLP protein. This work was supported by the Medical Research Council of Canada. K.H.L. held a University of Toronto Open Fellowship.

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