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Site-specific DNA condensation and pairing mediated by the int protein of bacteriophage λ

(integrative recombination/DNA–protein interaction/nucleosome/electron microscopy)

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ABSTRACT **The int protein of bacteriophage λ catalyzes the site-specific integrative recombination that inserts λ DNA into the host chromosome. The attachment site region of λ DNA required for this reaction spans 230 base pairs and includes four separable binding sites for int protein. We have used the electron microscope to determine the functional consequences of the interaction of int with its multiple binding sites. We find that int condenses a 230-base pair segment of DNA into a compact structure about 14 nm in diameter; the condensed region includes all of the four binding sites for int. Condensed segments will form paired structures between attachment sites. We suggest that a sequential cooperative interaction between bound int molecules provides for a distinct reactive DNA conformation and for pairing between substrate sites.**

The genome of bacteriophage λ associates with the bacterial chromosome by the recombinational mechanism suggested by Campbell (1–3). Insertion and excision of λ DNA are site-specific recombination events, involving at least two phage-coded proteins, int and xis (4–6). Although formally a reversible DNA breakage and reunion, the integration–excision reaction is complex. Each recombining substrate site is distinct, and the forward and reverse reactions have different catalysis—int protein for both directions and xis for excision only (4–6). Based on recombinational experiments *in vivo* the reaction pathways for integration–excision can be described as follows:

$$\underset{\text{POP'}}{\textit{att}\text{P}} + \underset{\text{BOB'}}{\textit{att}\text{B}} \underset{\text{int/xis}}{\overset{\text{int}}{\rightleftharpoons}} \underset{\text{BOP'}}{\textit{att}\text{L}} + \underset{\text{POB'}}{\textit{att}\text{R}}$$

The substrate sites are the phage (*att*P), host (*att*B), and recombinant prophage attachment sites (*att*L and *attR*) (3). The integration–excision reaction also requires two host-coded proteins, designated himA and himD (or hip) (7–9). Much of the complexity probably results from the need of the virus to control the direction of the reaction, toward integration in lysogenization after infection or toward excision during induction from the prophage state (10, 11).

Recent experiments on the integration–excision reaction *in vitro* have more clearly defined the reactive sites and components, but not yet clarified the mechanism. Integrative recombination *in vitro* requires int protein and the host component, integration host factor (IHF) (3, 12, 13); IHF is a dimer of himA and another protein, probably himD (8, 9). As judged by nitrocellulose filter assays, int is a specific DNA-binding protein, forming a tight complex with DNA carrying the OP' segment and a lower affinity complex with DNA containing the P segment (12, 14–16). Specific binding has also been shown by an electron microscope study (17). Based on sequence determination and DNase protection experiments, int binds to four separable sites in the phage attachment site—two in the left (P) region, one in the right (P'), and the common core of homology between phage and host attachment sites (O) (18–20). The DNA strand exchange occurs in the core region, producing a staggered cleavage 7 base pairs (bp) apart (21).

In the work reported here, we have used the electron microscope to examine the interaction of int with its multiple binding sites in the phage attachment site. We find that int condenses a 230-bp segment of DNA including all of the binding sites identified by DNase digestion. We have also observed int-mediated paired structures between attachment sites.

MATERIALS AND METHODS

Proteins. int protein was purified as described by Kotewicz *et al.* (15) from bacteria carrying a plasmid with the *int* gene under the *lac* promoter (unpublished work). Purified int protein (28 μg/ml) was stored in 10 mM potassium phosphate, pH 6.4/1 mM EDTA/600 mM KCl/10% glycerol/1 mM dithiothreitol at −70°C. IHF, purified as described by Nash and Robertson (13), was a gift from Howard Nash. Restriction endonucleases *Bam*HI and *Hin*dIII were purchased from Bethesda Research Laboratories.

DNA. The *att*P-containing plasmid, pWG1, was constructed by inserting a *Hin*dIII to *Bam*HI fragment of λ DNA (493 bp) into the *Hin*dIII and *Bam*HI sites of pBR322. The *att*B-containing plasmid, a gift from S. Brenner, was constructed by cloning an *Eco*RI to *Bam*HI fragment from *λgalbio* (1,600 bp) into the *Eco*RI and *Bam*HI sites of plasmid RSF1050 (22). The *att*L-(pPH201) and *att*R- (pPH202) containing plasmids were constructed by Hsu *et al.* (20); pPH201 and pPH202 contain 1,300- and 800-bp inserts in pBR322, respectively. Plasmid DNA was purified as described by Clewell and Helinski (23); monomer supercoils were further purified by electrophoresis in agarose (24).

Binding of int Protein to DNA and Visualization by Electron Microscopy. The binding reaction was conducted in either of two buffers: buffer A (10 mM potassium phosphate buffer, pH 7.2/70 mM KCl/2 mM sodium EDTA) or buffer B (50 mM Tris, pH 7.8/70 mM KCl/1 mM sodium EDTA/10 mM $MgCl_2$). In most experiments, the reaction mixture (0.02 ml) contained buffer solution supplemented with DNA at 10 μg/ml and int protein at 1–7 μg/ml. After incubation of DNA with int protein, glutaraldehyde (Polysciences) was added to 0.1%, and samples were incubated at 37°C for 15 min. The DNA was then diluted to 0.5 μg/ml; adsorption of specimen to polylysine-coated electron microscope films and rotary shadowing were carried out as described (25, 26).

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Abbreviations: bp, base pair(s); IHF, integration host factor.

RESULTS

Specific Binding and DNA Condensation by int. To study the functional interaction of int protein with its substrate DNA, we used a supercoiled plasmid carrying *att*P because the recombination reaction occurs efficiently only with a supercoiled *att*P DNA (27). The interaction of int and *att*P is shown in Fig. 1. The complex formed at a low int-to-DNA ratio, which we believe to be the active reaction intermediate, is shown in Fig. 1*a*. This "simple complex" between int protein and DNA forms readily with supercoiled DNA (but not linear DNA) at a relatively low int-to-DNA ratio (about 10 int monomers per *att*P DNA). The plasmid shown in Fig. 1 carries the *att*P segment of λ DNA inserted into pBR322. Supercoiled pBR322 lacking *att*P does not form a complex with int protein that is detectable by electron microscopy under similar reaction conditions.

As the concentration of int protein is increased relative to the *att*P DNA, the nature of the int–DNA interaction changes. A 5-fold increase in int results in the complete covering of some supercoiled DNA molecules with protein; intermediate concentrations of int cover a variable fraction of the DNA molecule (Fig. 1 *b–d*). Several features suggest that this int–DNA interaction is cooperative. (*i*) the complete covering of *att*P DNA occurs at only 5 times the concentration of int at which the localized simple complex is formed; (*ii*) the covering of DNA with int protein initiates at nonrandom locations on the supercoiled plasmid (i.e., molecules containing two or more int–DNA complexes on the same DNA molecule are not seen); (*iii*) samples that contain DNA molecules almost completely covered with int protein also have a small number of molecules devoid of associated protein. A previous electron microscope study has also demonstrated a specific complex with *att*P (17); in that study, which utilized a much higher concentration of int, mainly the more highly covered complexes were seen.

Although int protein can bind over a large region of DNA at high concentration, the complex of int with supercoiled *att*P plasmid shown in Fig. 1*a* is probably sufficient to catalyze the recombination reaction between *att*P and *att*B. Samples of DNA were taken from an *in vitro* recombination reaction, under the conditions described by Nash and Robertson (13), and found to yield only the simple complex between *att*P DNA and int.

To demonstrate that the simple complex between int and the *att*P plasmid forms specifically at the *att*P site, we have located the binding site with respect to cleavage sites for restriction enzymes. Supercoiled molecules with bound int protein (fixed with glutaraldehyde) have been cleaved near the *att*P site with either *Hin*dIII or *Bam*HI, and the linearized DNA has been examined by electron microscopy (Fig. 1 *e* and *f*). All DNA molecules associated with int protein exhibit the complex near one end of the cleaved plasmid DNA. After digestion with either enzyme, the average molecular distance from the short free end of the plasmid to the center of the protein complex is <5% of the length of the plasmid; the cloned fragment of λ DNA bounded by the *Hin*dIII and *Bam*HI sites comprises 12% of the plasmid length. This observation indicates that the int protein is bound specifically to the *att*P region.

To examine more closely the region of int binding, supercoiled molecules with bound int protein have been cleaved with both *Hin*dIII and *Bam*HI. The resultant *att*P fragment has a novel structure (Fig. 2); the binding of int results in the condensation of *att*P DNA, as judged by a marked shortening of the restriction fragment compared to its length free of int protein (Fig. 2*d*). Another interesting feature of the int–*att*P complex is a characteristic asymmetry; the *Hin*dIII and *Bam*HI ends of the fragment always extend from the same side of the protein complex, a structural feature found also for a nucleosome (28). This asymmetry of the DNA extending from the complex with int is also evident after cleavage with only one restriction enzyme (Fig. 1 *e* and *f*). Possible explanations for such an orientation are either (*i*) the DNA is wrapped around an interacting protein core or (*ii*) int molecules initially bound to two regions within *att*P (for example, the P and P′ regions) condense the DNA by a protein–protein interaction that joins distant segments (or both).

The extent of DNA condensation that results from int binding to *att*P has been determined by comparing the measured lengths of 75 *Hin*dIII to *Bam*HI fragments with bound int to 23 similar fragments without int. The two free tails extending from the DNA–protein complex have different lengths; by comparing the lengths of these tails with the length of the short tails

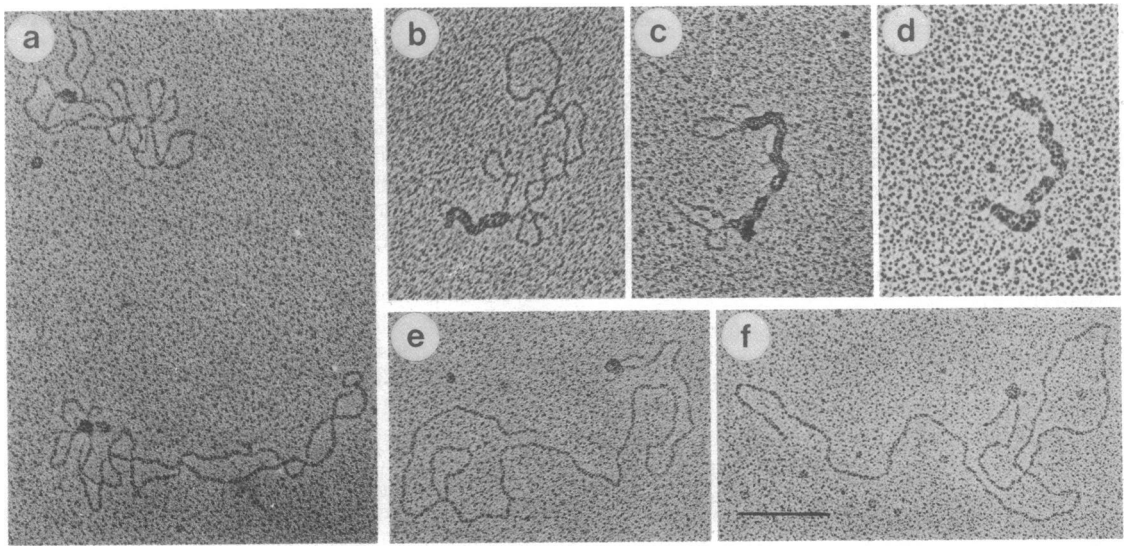

FIG. 1. Electron micrographs of int protein bound to *att*P DNA. (*a*) The simple complex of int protein (1.4 μg/ml) and supercoiled plasmid DNA (10 μg/ml). (*b–d*) Larger complexes with higher int concentrations: 2.8 μg/ml (*b*), 5.6 μg/ml (*c*), and 7 μg/ml (*d*). (*e* and *f*) int protein associated with *att*P DNA after cleavage by restriction enzymes *Hin*dIII (*e*) and *Bam*HI (*f*). For *e* and *f*, the standard reaction mixture (int protein at 1.4 μg/ml and DNA at 10 μg/ml) was incubated with glutaraldehyde, diluted 1:20 into buffer containing 0.002 unit of restriction enzyme, and incubated for 30 min at 37°C. Restricted samples were added directly to grids for electron microscopy. The bar represents 0.1 μm.

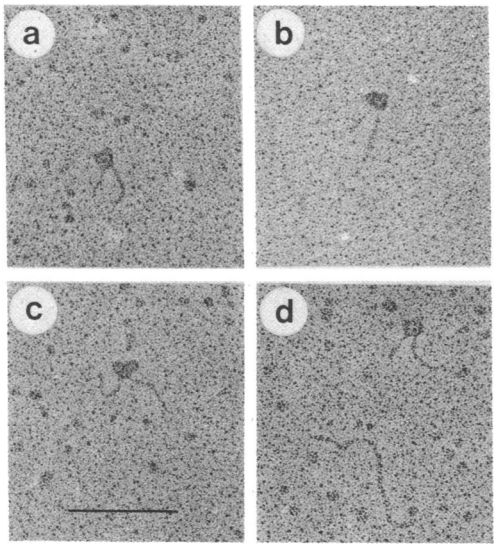

FIG. 2. Electron micrographs of int protein bound to the *Hin*dIII to *Bam*HI fragment containing *att*P (493 bp). int protein was added to supercoiled plasmid DNA, followed by cleavage with *Hin*dIII and *Bam*HI. *a–c* each show one fragment with associated protein; *d* shows one fragment with associated protein and one fragment without int protein. The bar represents 0.1 μm. Identical results were obtained with either buffer A or buffer B.

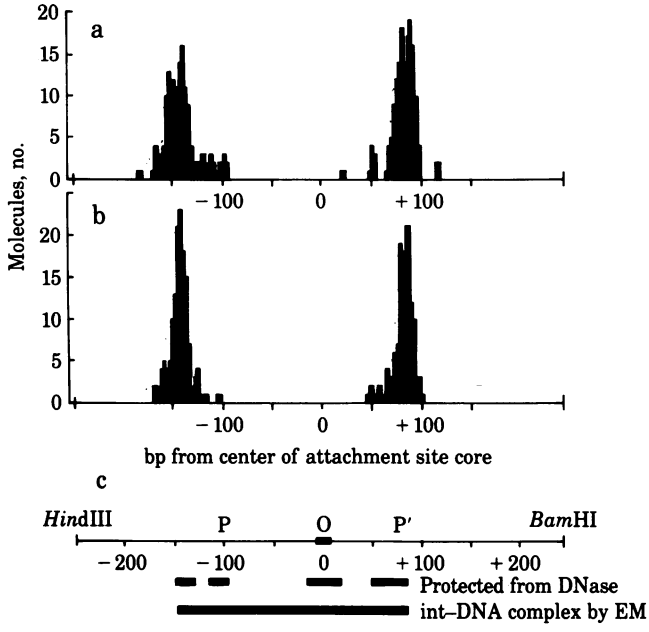

FIG. 3. Length distribution of the DNA segments extending from the int–DNA complex. int protein was bound to supercoiled *att*P DNA, followed by cleavage with *Hin*dIII and *Bam*HI. Molecules similar to those depicted in Fig. 2 were photographed, and the lengths of the DNA tails were measured at a magnification of ×180,000. The shorter DNA tails extend from the int–DNA complex to the *Hin*dIII end, whereas the longer tails extend to the *Bam*HI end. The distances from these free ends to the center of the int–DNA complex are plotted relative to distance along the 493-bp fragment. Zero represents the position of the center of the core region; positive numbers are the number of bp from 0 toward the P′ arm, whereas negative numbers are the bp from 0 toward the P arm. (*a*) int protein bound to *att*P DNA. (*b*) int protein and IHF bound to *att*P DNA. (*c*) A comparison of the DNA region condensed in the int–DNA complex with those regions of *att*P DNA protected from DNase I digestion (19, 20). EM, electron microscopy.

found after cleavage with *Hin*dIII or *Bam*HI alone, we have located the condensed region on *att*P DNA. Fig. 3*a* shows the distribution of lengths for the free ends of the DNA fragment with bound int protein. Assuming that the DNA length per bp is constant across the 493-bp fragment, ≈230 bp are condensed by int protein. The orientation of condensed DNA on the *Hin*dIII to *Bam*HI fragment is shown in Fig. 3*c* together with those regions of *att*P known to be protected by int protein from DNase digestion. The average diameter of the condensed int–*att*P complex is 14 nm.

int-Mediated Paired Structures. In an effort to determine the role of the condensation complex in integrative recombination, we have sought structures in which two attachment sites are joined. Integrative recombination *in vitro* requires the host protein IHF and magnesium or spermidine (13). We have found that addition of magnesium to the int–*att*P reaction described above shifts about 50% of the supercoiled population into paired structures (Fig. 4*a* and Table 1). Paired molecules are joined via int protein at their *att* sites because cleavage to the *att*P segment with *Hin*dIII and *Bam*HI yields the four-stranded product shown in Fig. 4*b*. Experiments with IHF are described below.

Binding of int to *att*B, *att*L, and *att*R DNA. We have also studied the association of int protein with plasmid DNA containing *att*B, *att*L, and *att*R. Using an int concentration sufficient to form a complex with about half of the *att*P molecules, we have determined the percentage of DNA molecules of each *att* site that forms a simple complex (Table 2). Although all four classes of supercoiled plasmid DNA form an observable complex with int, the functional interaction is clearly different for *att*B and *att*R compared to *att*P and *att*L. The complexes with *att*B and *att*R are fewer in number, and only the complexes with *att*P and *att*L are stable after linearization of the plasmids with restriction endonucleases. Because int protein was not found associated with linearized *att*B or *att*R plasmid DNA, the specificity of the interaction with int protein in these cases could not be determined. We suspect that the association of int with DNA found for *att*B and *att*R is at least primarily at the *att* site because similar complexes were not found for pBR322 alone. However, the interaction appears to be considerably less stable than that found for *att*P and *att*L.

We have shown that the binding of int protein to the *att*L

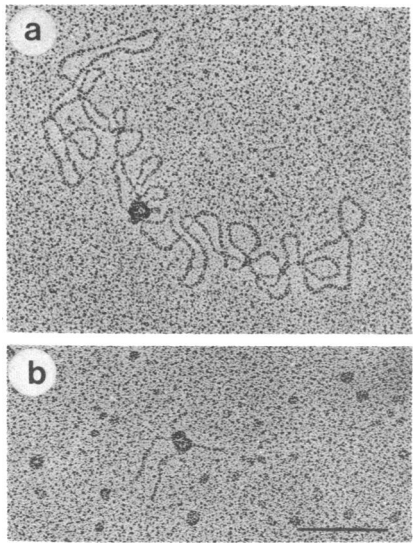

FIG. 4. Paired molecules of *att*P DNA formed by int protein. int was incubated with *att*P DNA in buffer B. (*a*) Two supercoiled *att*P molecules joined by int protein. (*b*) The four-stranded structure after cleavage with *Hin*dIII and *Bam*HI. The bar represents 0.1 μm.

Table 1. int-mediated paired structures

int protein, μg/ml	DNA molecules in int complex, no.				
	0	1	2	3	>3
1.4	170	58	30	11	9
2.5	5	98	67	12	3

Headings: 0, the number of molecules seen on the grids without associated int protein; 1, int–DNA complexes similar to those of Fig. 1*a*; 2, two DNA paired structures of the type shown in Fig. 4*a*; 3, three DNA molecules joined via int protein; >3, structures with more than three *att*P molecules joined by int protein.

plasmid DNA is specific for the *att* region by the analysis described for the characterization of int binding to *att*P. Interaction of int and *att*L resembles that with *att*P in two respects: (*i*) int protein condenses a region of *att*L DNA, although in this case only about 50 bp are involved; (*ii*) paired structures of *att*L with *att*L are found (data not shown). The average diameter of the int–DNA complex with *att*L appears to be smaller than that with *att*P, about 10 nm.

Effect of IHF on int Binding to *att*P DNA. The integration reaction catalyzed by int protein is dependent upon the host protein, IHF, although the role of IHF in recombination is unknown. IHF is a DNA-binding protein with specificity for *att*P. Sites on *att*P protected from DNase by IHF have been localized adjacent to those regions protected by int protein (ref. 3; N. Craig and H. A. Nash, personal communication). We reasoned that the inclusion of IHF in the reaction mixture with plasmid DNA and int might alter the association of int with DNA and thus help elucidate the role of IHF in the recombination reaction. IHF is a small protein of M_r 20,000 and is thus too small to be visualized alone by the method of microscopy used in these studies.

We have examined the effect of IHF on int binding to supercoiled *att*P DNA and to the restriction fragment of *att*P. The concentration of IHF used was sufficient to saturate an *in vitro* recombination reaction with supercoiled *att*P and linear *att*B substrates. The binding of int to supercoiled *att*P is indistinguishable from that in the absence of IHF. DNA condensation by int and IHF is diagrammed in Fig. 3*b*. There is no difference in the average amount of condensed DNA, as determined by the lengths of the free tails of the *Hin*dIII to *Bam*HI DNA fragment; however, there is less scatter in the data for molecules containing bound int and IHF. This suggests that IHF might act to confer a more precise conformation to the int–*att*P complex, possibly facilitating the recombination reaction.

Attempts to Find *att*P/*att*B Pairing. Although recombination occurs between *att*P sites *in vivo* and *in vitro*, the most important form of integrative recombination is *att*P with *att*B. We have therefore sought paired structures between *att*P and *att*B. Using reaction conditions that allow *in vitro* recombination to proceed (13), we incubated int protein with supercoiled plasmid DNA carrying *att*P and linearized plasmid DNA containing *att*B. The approximate position of the *att*B site on the linear molecule is known; we thus have looked for the appropriate structures that contain a supercoiled molecule joined to *att*B via int protein. Molecules with the expected structure are found only very rarely; they account for <0.1% of the total DNA molecules (data not shown). The addition of IHF to the reaction mixture does not result in the accumulation of additional joint molecules, although the dimer products of recombination are then seen.

Table 2. int–DNA complexes with *att*P, *att*B, *att*L, and *att*R

Attachment site	Molecules, no.		Percent in int–DNA complex
	Without int	With int	
*att*P	93	69	42
*att*B	128	12	8
*att*L	74	106	58
*att*R	109	31	22

The reaction mixture consisted of int protein at 1.4 μg/ml and DNA at 10 μg/ml. At this low concentration of int, not all *att*P molecules were in the int–DNA complex. The number of molecules that had associated int protein were counted at random from the electron microscope grids. The int–DNA complex for *att*B or *att*R was not stable to the cleavage procedure for restriction enzymes; thus we do not know if the binding of int protein with these plasmids is specific for *att* sites.

DISCUSSION

We have characterized the binding of int protein to its substrate DNA by electron microscopy. Most strikingly, the binding of int protein to the phage attachment site *att*P induces the condensation of ≈230 bp of DNA into a compact structure resembling a nucleosome. Such a possibility has been noted previously from anomalies in the DNase cleavage pattern of the int–*att*P complex (16, 20). The DNA segments that interact with int to form the condensed complex include all four regions protected by int protein from DNase digestion. The condensed region is also the same as the minimal *att*P segment determined by DNA-trimming experiments (20, 21, 29). Thus, all the functional sites of *att*P are involved in a coordinate interaction with int to generate a distinct structure; we believe that this structure is the activated form of *att*P DNA required for recombination.

The extent of int binding to *att*P DNA over a range of int concentrations indicates that int binds cooperatively. Cooperative interaction of int protein can also explain how as large a segment as 230 bp of DNA (roughly 75 nm in length) is condensed by protein. Because the highest affinity binding site of int protein to *att*P is probably at P′ (12, 15, 16, 19, 20), binding at this site by one or two int monomers might stabilize a lower affinity binding of int at the O and P region sites; by a sequential interaction between int molecules, the entire *att*P region might assume a condensed conformation that is favorable for the recombination reaction (perhaps unwinding the core region). IHF might be involved in the stabilization of the reactive complex.

A comparison of the int–DNA complex with rotary shadowed proteins of known molecular weight gives a rough idea of the number of int monomers involved in complex formation. The int–DNA complex is intermediate in size between core RNA polymerase (M_r = 400,000) and aspartate transcarbamylase (M_r = 300,000). Allowing for the DNA content of the condensed region, the molecular weight of the protein component is probably about 200,000–300,000. Thus we estimate that the number of int monomers (M_r = 40,000) involved in complex formation is four to eight. Although the estimate suffers from the uncertainty surrounding the nature of the protein–DNA structure, we can conclude that the int–DNA complex involves a relatively small number of int monomers.

The formation of a paired structure between *att* sites during recombination is a necessary intermediate in the recombination reaction. We have found a high frequency of paired structures between two *att*P or two *att*L molecules, although only rare junctions of *att*P and *att*B.

From the data presented here, we postulate that integrative recombination occurs through the sequential series of events shown diagramatically in Fig. 5. int forms a high affinity P′ complex and lower affinity P and O complexes. The P′ complex serves as an initiation point for a series of cooperative interactions with int at O and P, resulting in a tightly wrapped "intasome" structure. A second level of interaction between int protein molecules produces the synaptic structure designated intasome II. int catalyzes the breaking and joining in the core

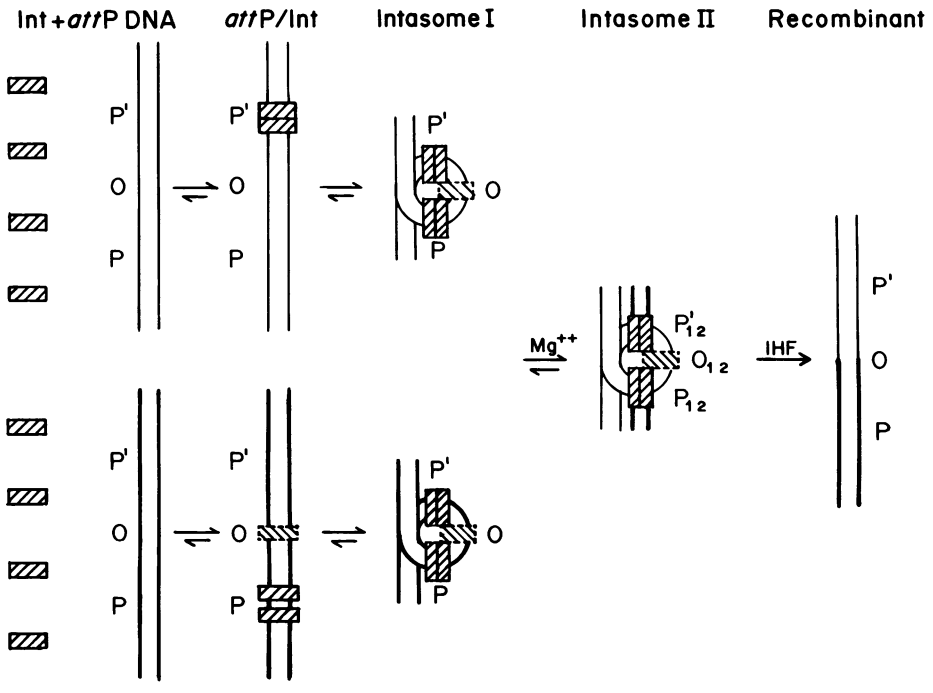

FIG. 5. A possible mechanism for int-mediated site-specific recombination. The sequence of interactions depicted is inferred from the electron microscopic observations presented in the text and from other data about integrative recombination (3).

regions (O_{12}) to produce the recombinant structure shown at the right. For an *att*P by *att*B reaction, we presume that the *att*P intasome I forms as in Fig. 5 with its complete complement of int bound at P', P, and O; however, a less stable intasome II ensues because *att*B probably binds int only at O (20, 21, 29). A weaker synaptic structure may explain why the *att*P by *att*B reaction is highly temperature sensitive (30). For excisive recombination (*att*L by *att*R), we must assume that in the absence of xis protein either the synaptic intasome (*i*) forms but is poorly reactive or (*ii*) does not form effectively (e.g., because *att*R lacks a P' site, binding at P by int might not help and might even hinder binding at O). xis might influence the reaction by converting the *att*R structure into a more reactive one at either level.

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