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An Analysis of the Arm-type Site Binding Domain of Bacteriophage λ Integrase

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The 356 amino acid long lambda integrase protein of bacteriophage λ contains two autonomous DNA binding domains with distinct sequence specificities. The amino terminal domain of integrase is implicated to bind to the arm-type sequences and the carboxyl domain interacts with the core-type sequences. As a first step to understand the molecular mechanism of the integrase-DNA interactions at the arm-type site, the int(am)94 gene carrying an amber mutation at the 94th codon of the *int* was cloned under the control of the P_{tac} promoter and the *lacI*^{q} gene. The Int(am)94 mutant protein of amino terminal 93 amino acid residues can be produced at high level from a suppressor free strain harboring the plasmid pInt(am)94. The arm-type binding activity of Int(am)94 were measured *in vivo* and *in vitro*. A comparison of the arm-type binding properties of the wild-type integrase and the truncated Int(am)94 mutant indicated that the truncated fragment containing 93 amino acid residues carry all the determinants for DNA binding at the arm-type sites.

Key words: bacteriophage λ, integrase, sequence-specific DNA binding, functional domain of protein

Sequence-specific DNA recognition by DNA-binding proteins is critical for the biological function of all cell types. The types of structures formed can range from simple protein-DNA complexes to elaborate higher-order complexes composed of several proteins and DNA. High-precision DNA transactions such as those involved in initiation of DNA replication and site-specific recombination are often carried out by complexes in which two or more proteins interact with specific DNA sites to form higher-order complexes (6). The site-specific recombination system encoded by bacteriophage λ provides a classic example of reactions controlled by a higher-order multiple protein-DNA complex (12).

Integrase (Int) is a viral coded protein which plays a central role during recombination. Int carries out the cleavage, strand exchange, and resealing of the attachment (*att*) site DNAs (4). It is a type I site-specific topoisomerase composed of 356 amino acid residues (9, 10). In addition Int is a sequence specific DNA binding protein that recognizes two distinct classes of DNA sequences (17, 18): "core-type" and "arm-type". The core-type has the consensus sequence CAACTTNNT and is present as two inverted repeats that flank the sites of DNA strand cleavage; Int bound to these sites promotes synapsis between partner att sites and carries out the cleavage and rejoining steps. The arm-type sites are characterized by a different consensus, C/AAGTCACTAT, which occurs five times in distal flanking regions of the core. Two sites, P1 and P2 are found in the P arm of the phage attachment site (*attP*) and three contiguous sites, P'1, P'2, and P'3 occur in the P' arm of *attP*. Int binding to these sites, in conjunction with accessary proteins encoded by both the phage and the host, is required to form higher-order nucleoprotein structures, called intasomes, that are necessary for both integrative and excisive recombination.

Recent biochemical studies on the role of integrase in synapsis, strand exchange and intasome formation have provided important insights into the mechanism of site-specific recombination (1, 7, 8, 10, 11, 21, 22). However, a few anlayses have been carried out to reveal the mechanism of Int recognition at the arm-type binding sites. Moitoso de Vargas *et al.* (16) have isolated proteolytic fragments of Int that were generated by cleavage between amino acid residues Leu-64 and Thr-65. They have shown that the N-terminal fragment binds to the arm-type sites and that the C-terminal fragment retains the core-type binding activity. Mutational analyses of the

P′ arm-type sites indicate that most base pairs in the conserved recognition sequence are involved in sequence-specific Int binding (13). Nothing is known about the arm-type sequence binding determinants of the Int protein. Int has no obvious helix-turn-helix motif or any other DNA-binding motifs that is characteristic of many DNA binding proteins (5, 20).

The long-term goal of this study is to elucidate the molecular mechanism of Int-DNA interactions at the arm-type binding sites. One of the strategies for studying specific Int and arm-type site interactions is to look for Int mutants with an altered binding specificity in such a way that the mutants recognize variant arm-type sites isolated previouly (13). By determining the amino acid substitutions in such mutants, it may be possible to determine the individual amino acids that contribute to the recognition of the arm-type sites. An arm-type binding domain, if available, which retains the arm-type binding ability rather than whole Int protein will be valuable for this genetic analysis to increase the chance to isolate the Int mutants with altered binding specificities. In addition, once the arm-type binding domain of Int protein is expressed and maintained stably in a cell, further structural studies such as an X-ray crystallography or a two-dimensional NMR analaysis can be persued to characterize specific amino acid-base interactions between Int and arm-type binding site. The work reported in this paper examined arm-type binding properties of two truncated amber fragments of Int and showed that Int(am)94 which carried 93 amino terminal amino acid residues was the one that is expressed at a high level in a suppressor free strain and at the same time that retains strong arm-type binding activity.

Materials and Methods

Bacterial strains

Derivatives of *E. coli* K-12 and *Salmonella typhimurium* LT2 were used in this study. *E. coli* DH1 (F$^-$ *thi-1 supE 44 hsdR17 endA1 recA1 gyr-96*) was used for DNA manipulation and LE292 (*arg*$^-$ *sup*^{0}) was used for preparation of crude extracts. *S. typhimurium* MS1868 (*leuA414 sup*^{0} *hsdSB* Fels$^-$) was the host strain for challenge phage infection.

Media, chemicals, and enzymes

Luria-Bertani (LB) medium and phage broth were used as rich medium and medium for phage P22 lysates, respectively, as described in Maloy (15). Antibiotics (Sigma Chemical Co.) were supplemented to medium when needed: ampicillin, 50 μg/ml for plasmid selection and 25 μg/ml for maintaining plasmids during challenge phage assays; kanamycin sulfate, 40 μg/ml. Isopropyl-β-D-thiogalactoside (IPTG) was supplemented to media as indicated. Restriction endonucleases, T4 DNA ligase, and T4 DNA polynucleotides were obtained from Bethesda Research Laboratories, or New England Biolabs. *Taq* DNA polymerase was purchased from Promega Biotec.

Plasmid construction

The *int*(am)94 gene, originally designated as *int*(am)29, was cloned from lambda B8006 (2). DNA of the phage B8006 was digested with *Xmn*I and *Hin*dIII. The *int*-bearing DNA fragment was purified from a 1% low melting point agarose gel, eluted, and retreated with *Sna*BI. The *Sna*BI-*Hin*dIII fragment was ligated with pCKR101 DNA that had been previoustly digested with *Sna*BI and *Hin*dIII. pCKR101 is a pBR322 derivative that contains the ampicillin resistance gene, the *lacI*^{q} gene, and the Ptac promoter with a unique downstram *Sna*BI site followed by M13mp8 polylinker (13). After transformation of DH1, a plasmid containing the *int*(am)94 gene was identifed by restriction mapping of miniprep DNA (19). One isolate, pInt(am)94 was saved for further study and transformed into *S. typhimurium* MS1868. Plasmid pSX1-2 (13) is a derivative of pCKR101 that contains the wild type lambda *int* gene cloned downstream of the Ptac promoter. Plasmid pInt(am)64 has the same construction as pSX1-2 except that it carries an amber mutation at amino acid position 64 and was supplied by Dr. MacWilliams (14).

Challenge phage assays

In vivo arm-type binding abilities of wild-type Int and truncated Int molecules were examined by using the bacteriophage P22-based challenge phage system (3). The assays were performed as described by Lee *et al.* (13). A *S. typhimurium* strain MS1868 containing one of the *int* plamids were grown to an A_{650} of 0.5 to 0.6 in LB medium supplemented with ampicillin. Cells were subcultured 1 : 4 into LB-ampicillin medium containing IPTG at various concentrations. After an additional hour of incubation at 37℃ to induce the expression of each *int* gene, the culture was infected with the P′1 or P′123 challenge phages at a multiplicity of infection (moi) of 20 to 25 and was incubated at room temperature for 1 hr to allow phage infection and phenotypic expression of the kanamycin-resistance gene carried on the phage. Dilutions of the adsorption mixtures were then plated on LB plates supplemented with ampicillin, kanamycin, and IPTG at the same concentration used in the previous induction. The number of viable cells was counted by plating IPTG-induced cultures on LB plated containing ampicillin and the same amount of IPTG. The fre-

quency of lysogenization (expressed as % lysogeny at a given IPTG concentration) was calculated as the number of lysogens obtained after each challenge phage infection divided by the number of viable colonies obtained before the phage infection multiplied by 100. As negative controls strain MS1868 carrying the plasmid pCKR101, the parent plasmid for the int clones used in this study, and a challenge phage that does not carry arm-type sites were also included in the assay. Both gave background levels of lysogeny ($<10^{-6}$%).

Preparation of cell extracts

A strain LE292 bearing *int* clones were grown at 37℃ to an A_{650} value of 0.1 in 300 ml of LB-ampicillin medium, followed by induction with IPTG at a concentration of 0.5 mM for 60 min. Cell pellets were collected after centrifugation at 4℃, resuspended in 300 μl of 50 mM Tris-HCl (pH 7.4), 10% (w/v) sucrose, and were frozen on solid CO_2 before being stored at −70℃. To lyse the cells, the pellets were thawed on ice prior to the addition of 25 μl of freshly made lysozyme (10 mg/ml) in 250 mM Tris-HCl (pH 7.4), followed by a 40 min incubation on ice. After a 30 min spin in a micro-centrifuge at 4℃, the supernatant was carefully removed and the remaining portions were frozen in liquid N_2 and stored at −70℃.

Gel mobility-shift assays

A 138 bp fragment containing the P'1 site and a 160 bp fragment with all three contiguous P' sites were synthesized using polymerase chain reaction on pPY190::P'1 and pPY190::P'123, respectively (13). Synthetic oligonucleotides (5'-CGGCATTTTGCTCATTCC and 5'-GATCATC TAGCCATGC) were used as primers for amplification. One of the primers was labeled prior to use with [γ-^{32}P]ATP and T4 polynucleotide kinase (19), and the amplified fragments were purified from a 5% polyacrylamide gel after electrophoresis.

Aliquots of labeled DNA fragments were incubated in binding buffer (44 mM Tris-HCl (pH 8.0), 66 mM KCl, 0.5 mg/ml Bovine Serum Albumin, 5 mM EDTA, 5 mM spermidine, 11 mM borate, 10% glycerol, 75 μg/ml calf thymus DNA) with various dilutions of Int crude extracts at room temperature for 30 min. The final reaction volume was 10 μl. The samples were run on 5% polyacrylamide gels at 15 V/cm. After electophoresis, the gels were dried and autoradiographed.

Results and Discussion

In vivo arm-type binding assays

Sequence-specific DNA binding of a protein can be assessed *in vivo* utilizing P22-based challenge phage system (3). This assay measures repression of the antirepressor (*ant*) gene that is transcribed from its promoter, P_{ant} (Fig. 1a). If sufficient antirepressor is synthesized during P22 infection, it inactivates the c2 repressor and the phage grows lytically to kill its host *S. typhimurium*. Alternatively, if antirepressor synthesis is repressed, the infecting phage undergoes lysogenic development and integrates its genome into the host chromosome. A challenge phage is a derivative of P22 in which the natural operator (O_{mnt}) that controls the ant gene expression is substituted by synthetic DNA that is a site recognized by a DNA binding protein (Fig. 1b). Challenge phages P'1(I) and P'1(II) contain the P'1 arm-type binding site of phage λ in both orientations relative to P_{ant} (13). The phage P'123(I) carries arm-type sites with an order of P_{ant}-P'1-P'2-P'3-*ant* and the phage P'123(II) contains the opposite orientation of the P' insert (13). If Int protein that is produced from the cloned plasmid binds to the synthetic arm-type site, P_{ant} is repressed and the infecting phage forms a lysogen. Since the phage carries a kanamycin resistance gene (Kan^r), lysogens are detected as Kan^r colonies. The frequency of lysogeny is thus a measure of the extent which Int protein binds to the arm-type site.

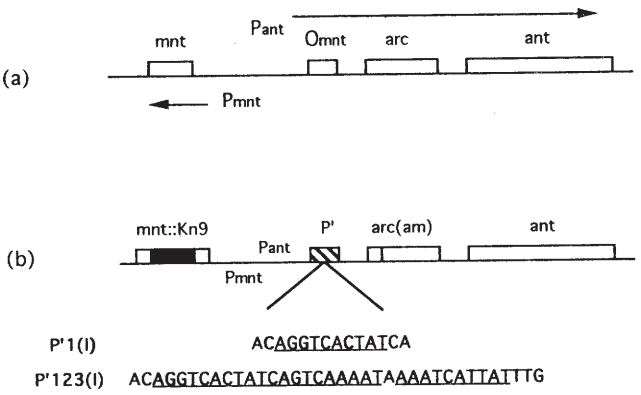

Fig. 1. Schematic diagrams of (a) the wild-type P22 *immI* region and (b) the challenge phage *immI* region. A double-strand synthetic oligodeoxynucleotide containing the P'1 site or the three contiguous P'1, P2 and P3 sites is cloned and substituted with O_{mnt} of the phage P22 to form P' challenge phages, the phage P'1 and the phage P123, respectively. The designations (I) and (II) indicate the orientation of the inserts containing the arm-type sites. Sequences of orientaion (I) in the P' challenge phages are shown in (b). Transcription from P_{ant} in the P' challenge phages is regulated by Int protein which can bind to the arm-type site. The Kn9 insertion confers Kanr phenotype on the lysogen.

The sources of Int protein for the challenge phage assays were plasmids that contain the *lacI*^{q} gene and an *int* gene cloned downstream of the P_{tac} promoter.

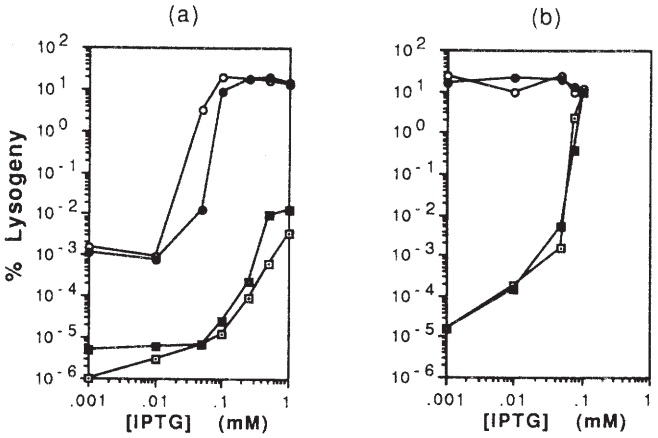

Fig. 2. Challenge phage assays with P'123 phages (orientation I [○] and orientation II [●]) and P1 phages (orientation I [⊡] and orientation II [■]). The curves show the percentage of Kan^r lysogens as a function of total cells infected by the phages at each IPTG concentration. The recipients were (a) MS1868/pInt(am)94 and (b) MS1868/pSX1-2.

Plasmid pSX1-2 carries the wild type *int* gene, and plasmid pInt(am)64 carries a mutant *int* gene with an amber mutation at amino acid position 64. The plasmid pInt(am) 64 produces a truncated Int protein consisting of 63 amino acid residues. The construction of plasmid pInt (am)94 which harbors another *int* amber mutation at amino acid position 94 is described in the Materials and Methods. The plasmids pInt(am)64 and pInt(am)94 differ from each other only in the location of an amber mutation. Plasmid pInt(am)64 produces an amino terminal fragment of 63 amino acids and pInt(am)94 produces a 93 amino acid-long truncated Int protein.

The challenge phage assays were performed on a *S. typhimurium* strain MS1868 carrying one of the Int-producing plasmids, pInt(am)64, pInt(am)94, or pSX1-2, that were incubated in the presence of IPTG at concentrations between 1 μM and 1 mM prior to infection. The frequencies of Kanr survivors at each IPTG concentration were measured. When *S. typhimurium* strain MS 1868 harboring pInt(am)64 was infected with the P' challenge phages, lysogenization frequency was below background level ($<10^{-6}$) even at high IPTG concentrations (0.1 mM or 1 mM).

The lysogenization frequencies at each IPTG concentration upon P' challenge phage infections on MS1868 /pInt(am)94 were plotted in Figure 2a. Both challenge phages, P'123(I) and P'123(II), lysogenized at low levels when IPTG concentrations were between 1 μM and 0.01 mM. As the IPTG concentration increased from 0.01 to 0.1 mM, the frequency of lysogenization of these phages increased upto 20%. At IPTG concentrations between 100 μM and 1 mM, no further increase in lysogenization occurred. The P'1(I) and P'1(II) phages lysogenized poorly at IPTG concentrations between 1 μM and 0.1 mM but the frequency increased as the IPTG concentration was raised and reached 0.01% at the highest concentration of IPTG.

Challenge phage assays performed on MS1868/pSX1-2 are shown in Figure 2b. Both P'123 phages lysogenized at a maximal frequency (approximately 10~20%) when IPTG concentrations changed from 1 μM to 0.1 mM. The P'1 phages showed a dramatic increase in the efficiency of lysogenization as the IPTG concentration increased, and a level of lysogenization equal to that of the P'123 phages was obtained at 0.1 mM IPTG.

It is important to note that the challenge phage assay reflects a subtle differences in protein concentrations in a cell. Although the lysogenization level of P'1 phages on MS1868/pSX1-2 dramatically increases over six orders of magnitude at IPTG concentrations ranging from 1 μM to 1 mM, differences in terms of Int protein amounts in the same range of IPTG concentrations are merely 3~4 fold (13). Therefore, it can be interpreted that the arm-type site binding properties of the Int(am) 94 are similar to that of the wild type Int (Fig. 2). Whereas, Int(am)64 did not form lysogens upon P'123 or P'1 challenge phage infections under the assay conditions used. This indicates either that the truncated Int protein with 63 amino acid residues does not stably maintained *in vivo* or that Int(am)64 does not bind the arm-type sequences strongly enough to repress the *ant* gene expression. In either case, Int(am)64 is proved to be inadequate within the scope of this study to search for the minimal region that is stably maintained and efficiently binds to the arm-type site, and it is not included in the following *in vitro* experiment.

In vitro arm-type binding assays

To determine if the truncated Int(am)94 protein binds to the arm-type sites *in vitro* as it does *in vivo*, gel mobility shift assays were performed using crude extracts from a suppressor free *E. coli* strain LE292 producing Int(am)94 or wild type Int protein. End-labeled DNA fragments carrying the three P'1, P'2 and P'3 sites or the P'1 site were prepared as described in the Materials and Methods and incubated with aliquots of different dilutions of cell extracts containing Int(am)94 or wild-type Int prior to gel electrophoresis. Autoradiographs of the gels are shown in Fig. 3.

Crude extracts of the parent strain LE292 failed to bind to any of the fragments using the same assay conditions (lanes 9 and 18). DNA fragments without specific arm-type sequences were not retarded by the cell extracts containing Int(am)94 nor wild-type Int proteins

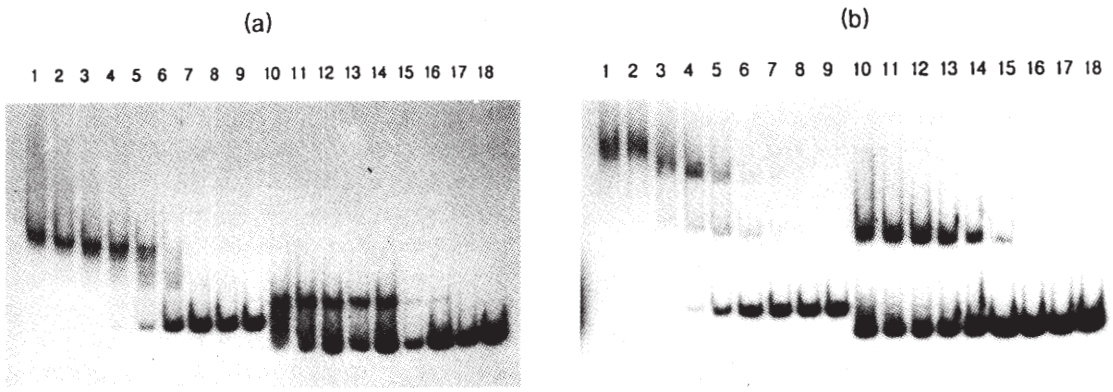

Fig. 3. Gel mobility shift assays of DNA fragments containing the arm-type sites. (a) Int(am)94 crude extracts. (b) Wild-type Int extracts. In lanes 1~8, end-labeled 160 bp DNA fragments carrying the contiguous P'1, P'2 and P'3 sites were incubated with various dilutions of crude extracts (1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64, 1 : 128 dilution, respectively). In lane 9, same 160 bp DNA fragment was treated with LE292 cell extracts which does not contain any of the *int* clones. Lanes 10~18 contain end-labeled 138 bp fragments incubated with same dilutions of crude extracts as lanes 1~9.

(data not shown). Retardation of the DNA fragments carrying the P'1 site (lanes 10~18) by the Int(am)94 extracts (Fig. 3a) and the wild-type Int extracts (Fig. 3b) was observed until the protein extracts were diluted to 1 : 64 (lanes 16). The DNA fragments with the three P' sites started to be retarded upon formation a protein-DNA complex with a single Int molecule in 1 : 64 dilution (lanes 7, Fig. 3a and b). The amount of the Int-DNA complex carrying a single Int molecule increased when 1 : 32 and 1 : 16 dilutions of cell extracts were added (lanes 6 and 5) and decreased when the Int protein level was further raised. As the band corresponding to the single Int binding complex disappeared, the bands at higher positions of the gel were newly formed and they represent the protein-DNA complexes of more than two molecules of Int. The arm-type DNA binding properties of the Int(am)94 protein and the wild-type Int appears to be same in general. Int(am)94, however, tends to form multiple Int binding complexes rather than a single Int binding complex (lanes 4~7, Fig. 3a), whereas the wild-type Int forms higher amount of a single Int-P'123 complex. In order to compare the exact binding affinity of the protein, it will be necessary to purify the Int(am)94 proteins further. Based upon these results, I can conclude that Int(am)94 protein can be produced at high amounts from a suppressor free strain containing the plasmid pInt(am)94 and the truncated Int protein retains strong arm-type binding activity when extracted *in vitro*.

The arm-type binding domain of the λ Int protein

The Int protein binds to the two types of recognition sites, characterized by distinct consensus sequences, using two different domains of the protein (16, 17, 18). Moitoso de Vargas *et al.* (16) isolated a mixture of amino-terminal chymotryptic fragments of Int that were probably 64 amino acids in length and showed that they protected arm-type sites in footprinting experiments, although binding affinity drops by more than a thousand fold. Thus, part if not all of the sequence-specific DNA recognition determinants for the arm-type sites resides in the amino-terminal 64 amino acids of the Int protein. To determine whether Int(am)64 retains sequence specific arm-type binding when it is produced *in vivo*, arm-type binding ability was measured using a challenge phage system. Under the conditions described above, *in vivo* binding activity of the Int(am)64 fragment to the arm-type sequences was not detected. This can be explained by the observation that the proteolytic fragments of 64 amino acids binds to the arm-type site *in vitro* but with a substantially lowered affinity (16). This may also reflect decreased stability of the Int(am)64 fragment *in vivo*.

Another truncated amino terminal fragment of Int, Int (am)94, was then tested for its arm-type binding activity. The Int(am)94 of 93 amino acids showed high level of specific arm-type binding both *in vivo* and *in vitro* (Fig. 2a and Fig. 3a). The 30 amino acid difference between the two amber fragments makes Int(am)94 binds to the arm-type sites much stronger than the shorter Int(am)64. This suggests that, even though the sequence-specific determinants of arm-type site resides within the first 64 amino acids as indicated in a previous study (16), up to 30 more amino acids are required to show maximum arm-type binding activity. These 30 amino acid residues may contribute to general or non-specific DNA binding at the arm-type sites.

In summary, this study shows that the arm-type binding domain of the λ integrase is subdivided into two regions and the Int(am)94 carries both parts: one that confers the sequence-specificity and the other that is required for maximum efficiency in arm-type site binding. Further structural analyses remain to be carried out to understand the structural and functional relationships of this multi-potent protein, integrase, and the int (am)94 will serve a good starting material to elucidate the molecular mechanism of Int-DNA interactions at the arm-type binding sites.

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