Site-specificity of abnormal excision: the mechanism of formation of a specialized transducing bacteriophage λ plac5*

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ABSTRACT

Molecular mechanism of the specialized transducing bacteriophage λ plac5 formation has been studied. Phage-bacterial DNA junctions in λ plac5 DNA are localized and primary structure of regions of the abnormal excisional recombination leading to the phage formation is elucidated; the crossover region proved to be comparable with the central part of *attP* and *attB* sites (the core and the adjacent tetranucleotide) in length and degree of homology. Bacterial insert in λ plac5 DNA is shown to end immediately after Z-Y spacer, the DNA not containing *lacY* gene segments. The data obtained led to the conclusion of site-specific (homologous) character of abnormal excision upon formation of lambda transducing bacteriophages. Possible mechanisms of the excision are discussed.

INTRODUCTION

Molecular bases of genetic recombination became approachable mainly due to the rapid progress in structural study of nucleic acids. Besides exchange between homologous chromosomes (general recombination) (3) known are many examples of heterologous segments exchange, which used to be referred to as illegitimate recombinations (4,5). Since many of these recombinations proved to be associated with specific loci of genome, they were classified as site-specific (6); structural studies showed them to include interaction of homologous sequences, though much shorter than in general recombination (7,8). The term of illegitimate recombination, as recent sequencing data show (*e.g.* (9,10)), is to be left (so far) for those proceeding with participation of non- or only slightly homologous sequences. Site-specific recombinations involve replicative or conservative mechanisms (11), the former providing for mobility of IS sequences, transposons, some bacteriophages (12), whereas integration and excision of various temperate bacteriophages are typical examples of conservative site-specific recombination. Breakage and reunion of DNA strands, playing central role in these processes, have been best studied in case of λ and *E.coli* DNA interaction (13). This area has been evolving at a great pace, with many spectacular results obtained (*e.g.* (14-20)).

Considerably worse studied are much rarer recombinational events, namely, abnormal (aberrant, faulty) prophage excisions, which lead to transducing bacteriophages (5). Unknown are not only their molecular mechanism but even degree (if any) of structural specificity of the abnormal excision. At the same time formation of transducing bacteriophages is of special interest as one of few efficient methods of recombinant DNA construction *in vivo* which provided for specific isolation and thorough investigation of genetic material at the B.G. (before genetic engineering) era. We have been studying mechanism of the excision which had yielded a wide-known transducing bacteriophage λ p_{lac}5 carrying a fragment of *E.coli lac* operon (1,21,22).

MATERIALS AND METHODS

Bacterial and bacteriophage strains are listed below (all bacterial strains are derivatives of *E.coli* K12).

C 600	F ⁻ , <i>supE44</i> , <i>thi-1</i> , <i>thr-1</i> , <i>leuB6</i> , <i>lacY1</i> , <i>tonA21</i> , λ^-	(23)
MM 294	F ⁻ , <i>endA1</i> , <i>hsdR17</i> (r_K^- , m_K^+), <i>supE44</i> , <i>thi-1</i> , λ^-	(24)
HB 101	F ⁻ , <i>supE44</i> , <i>hsdS20</i> (r_B^- , m_B^-), <i>ara-14</i> , <i>galK-2</i> , <i>lacY1</i> , <i>proA2</i> , <i>rpsL20</i> (<i>str</i> ^r), <i>xyL-5</i> , <i>mtl-1</i> , <i>recA13</i> , λ^-	(25)
71-18	Δ (<i>lac pro</i>), λ^- /F' <i>lacI^qlacZΔM15pro</i> ⁺	(26)
λ	<i>lacIts857</i> Sam7	(27)
λ p _{lac} 5	<i>lac</i> p _{lac} 5 <i>cIts857</i>	(21,22)
λ p _{lac} 5-2	<i>lac</i> p _{lac} 5 <i>cIts857 sRIλ3 sRIλ4 sRIλ5</i>	(28,29)

Chemicals: 2-mercaptoethanol (ME), 1,4-dithiothreitol (DTT), ethidium bromide, chloramphenicol, dNTPs (Calbiochem), [γ -³²P]ATP, [α -³²P]dNTPs (2000-3000 Ci/mole, Amersham), Sephadex G-50 superfine (Pharmacia), hydroxylapatite, agarose, Bio-Gels (BioRad), tris, acrylamide (Merck), methylene-N,N'-bisacrylamide (bis), EDTA, TEMED, SDS, heparin (Serva), 4-chloro-5-bromo-3-hydroxyindolyl- β -D-galactopyranoside (Xgal), isopropylthio- β -D-galactopyranoside (IPTG), polyethyleneglycol, sucrose, boric acid (Sigma), yeast extract, tripton, agar (Difco).

Enzymes. Restriction endonucleases *Ban*I (30), *Cfr*I, *Cfr*6I (*Pvu*II isoschisomer) (31), *Cfr*9I (*Xma*I) (31), *Cfr*10I (32), *Cfr*13I (*Asu*I) (31), *Eco*RI, *Hind*III, *Mva*I (*Bst*NI), *Msp*I, *Sau*3A and T4 DNA ligase were kindly given by A.A.Janulaitis (Vilnius), *Hind*II, DNA polymerase I (holoenzyme and Klenow's fragment), lysozyme were purchased from Boehringer, *Hha*I was from P-L Biochemicals, RNase A from Reanal. Endonuclease *Hae*III was isolated by the modified method (33), T4 polynucleotide kinase and *E.coli* alkaline phosphatase according to (34,35). Endonuclease *Kpn*I was isolated from *K.pneumoniae* OK8 crude extracts by sequential chromatography on Bio-Gel A-0.5m, HAP, and aminoheyl- or heparin-agarose

(18000 u from 5 g cells); the absence of phosphodi- and phosphomonoesterases was tested by incubating 1 pmole d³²pAATCAAGCTT with 5 u *Kpn*I.

Preparation of DNA. Bacterial strains were grown in YT or M9 media (36). Ampicillin, tetracycline, Xgal, and IPTG were used in concentrations 100, 20, 40, and 100 µg/ml, resp. Competent *E. coli* cells prepared and stored according to (37) were transformed with recombinant DNA by the modified technique (38). *E. coli* HB 101 (pBR322mpt3) culture was prepared by transforming the bacteria with the corresponding plasmid (39). Cells harbouring plasmids were grown at 37°C till A₆₀₀ 0.5-0.7, chloramphenicol was added to the final concentration 170 µg/ml, and aeration continued ca. 15 hr. Plasmids were isolated by buoyant density centrifugation in CsCl - ethidium bromide (40) or by the selective alkaline denaturation method (41) adjusted to larger amount of DNA, phage DNAs were prepared by phenol or phenol-SDS extraction of purified phages (42).

Ligation reactions were carried out in 60 mM tris-HCl, pH 7.6, 10 mM MgCl₂, 1 mM DTT, 0.1 mM ATP (12 to 16 hr at 4-6°C) with 25-50 µg/ml DNA and 300-500 u/ml T4 DNA ligase (for the activity unit see (43)). Reaction mixtures were used to directly transform competent *E. coli* HB 101 or MM 294 cells in rich media or 71-18 cells in minimal media (ca. 1 µg DNA per 10⁸ cells). After standard treatment the transformants were grown on an agarized medium.

Restriction endonuclease hydrolyses (1 µg DNA and 1 u enzyme in 10 µl) were run for 1-3 hr at 37°C (with *Taq*I at 65°C) in the buffers: 40 mM tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl (*Eco*RI, *Hind*III); 10 mM tris-HCl, pH 7.8, 5 mM MgCl₂, 1 mM DTT, 20 mM NaCl (*Ban*I, *Cfr*6I, *Cfr*10I, *Cfr*13I, *Eco*RII, *Hind*II, *Mva*I); 6 mM tris-HCl, pH 7.8, 6 mM MgCl₂, 2 mM DTT (*Cfr*9I, *Hae*III, *Hha*I, *Kpn*I, *Msp*I, *Taq*I, *Xma*I); 10 mM tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 20 mM KCl (*Sma*I); 75 mM tris-HCl, pH 8.6, 5 mM MgCl₂, 2 mM DTT, 10 mM NaCl (*Cfr*I).

Other techniques. DNA fragments with 5'-protruding ends were labelled by filling in 3'-ends with DNA polymerase I (50 u/ml), dNTP, and [α-³²P]dNTP (0.2-1 µM, 1-10 µCi) in 30 mM tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM NaCl, 1 mM DTT, for 20 min at 20°C. All enzymes used (except *Taq*I) were inactivated by 10 min incubation at 70°C with 50 mM EDTA. Electrophoreses were run in 1% agarose gel (ca. 10 mA per tube) in 40 mM tris-acetate, 20 mM NaOAc, 20 mM NaCl, 1 mM EDTA, pH 8.1, or in polyacrylamide slabs at 25 V/cm in 50 mM tris-borate, 1 mM EDTA, pH 8.3. Labelled substances localized by radioautography were eluted and ethanol-precipitated (44) or electroeluted and chromatographed on Sephadex G-50. Chemical modifications in the Maxam-Gilbert DNA sequencing (44) were carried out at room temperature with Me₂SO₄ (G), 66% HCOOH or 5% CF₃COOH (G+A), N₂H₄·H₂O (T+C), and 3 M NaCl + 0.25 M NaOH in N₂H₄·H₂O (C). Sequencing gels were 8-20% PAG (acrylamide-bis 30:1) in 7 M urea, 0.04 cm thick and 40-75 cm long.

Figure 1. Construction of plasmids carrying some λ plac5-2 DNA fragments. Fragmented loci are marked with apostrophes. *Pvu*II sites are indicated only for pGVS64 (47), *Bcn*I and *Cfr*I sites only for *Eco*RI, *Kpn*I fragment. Abbreviations: A (*Alu*I), B (*Bam*HI), C (*Cfr*I), D (*Hind*II), E (*Eco*RI), F (*Cfr*10I), H (*Hind*III), K (*Kpn*I), M (*Msp*I), N (*Bcn*I), P (*Pvu*II, *Cfr* 6I), R (*Eco*RII), S (*Sma*I), T (*Taq*I), U (*Sau*3A), X (*Hha*I), Y (*Tha*I), Z (*Hae*III); s.a. Figures 2 and 4.

RESULTS

Primary structure of λ plac5 DNA in the abnormal excision region.

The λ plac5 phage formation was mainly the result of two independent events: a deletion which fused bacterial *lacI* gene with the phage DNA, and an abnormal prophage excision which led to another fusion of both DNAs (21). We have elucidated the primary structure of the λ plac5 DNA segment which includes the latter fusion. To this aim we used a derivative of the phage, λ plac5-2, whose DNA contains only two out of five original *Eco*RI sites (28,29). The segment between these two sites comprises most part of the bacterial insert (Fig.1), only distal end of *lacZ* gene (62 bp (45,46)) being on the other side of an *Eco*RI site, in

Figure 4. Restriction map and sequencing scheme of the λ pLac5-2 DNA *Pvu*II, *Kpn*I segment (1280 bp; closed rectangle represents the crossover region); of *Msp*I, *Hha*I, and *Tha*I sites only those used in structural analysis are given. Most fragments were labelled with DNA polymerase (straight arrows, 3' $\rightarrow$ 5' direction); the curly arrow depicts a fragment labelled with T4 polynucleotide kinase (5' $\rightarrow$ 3' direction). The sequence of *Pvu*II, *Eco*RII (P-R) segment elucidated mainly at both chains (thick horizontal lines) is presented at Figure 5.

proximal end (45). It showed that the bacterial and phage DNA junction in λ pLac5-2 is situated between a *Bon*I and a *Cfr*I site (63 and 139 bp from the *Eco*RI site, respectively) (Fig.2). Then, basing on the λ DNA restriction map (50), we localized and excised the corresponding segment of λ DNA as a *Sma*I, *Cfr*I subfragment (65 bp) of the *Sma*I, *Kpn*I fragment. The sequencing data (Fig. 3) in combination with the bacterial DNA sequence (45) gave the primary structure of the region of the crossover which had led to bacteriophage λ pLac5. Figure 4 presents a detailed restriction map of the λ pLac5-2 DNA *Pvu*II, *Kpn*I segment comprising the crossover area, whereas Figure 5 depicts the sequence of its *Pvu*II, *Eco*RII fragment.

λ pLac5 DNA does not contain any *lacY* gene segment.

Data presented in Figure 5 show the bacterial insert into λ pLac5 DNA to end immediately after Z-Y spacer, just before the initiating triplet ATG of *lacY* gene, with two parental DNAs being fused without any distortion of sequences. Original papers on the λ pLac5 phage construction (21) and use (1) pointed out that λ pLac5 DNA contained at least a part of *lacY* gene, which further used to be taken for granted (e.g. (55,56)). Our results prove that λ pLac5 DNA does not include *lacY* gene segments at all. The *lac* operon DNA described in (1) contained therefore, besides a *lacI* gene fragment and regulatory segments *lacO* and *lacP*, only *lacZ* gene and Z-Y spacer cut off exactly before *lacY* gene.

Structure of λ pLac5 phage DNA.

The above data in comparison with (21), the detailed genetic map of λ DNA (57), and its complete primary structure (58) show that the excisional recomb-

Figure 5. Sequence of the λ*p**lac*5-2 DNA *Pvu*II, *Eco*RI segment (645 bp, nucleotides 19189 through 19833 of the λ*p**lac*5 genome; *Pvu*II, *Eco*RI fragment was isolated from plasmid pGV864, *Eco*RI, *Kpn*I fragment containing *Eco*RI, *Eco*RI sequence was from plasmid pGV8 (Fig.1)). At the crossover region (upper-case letters) the λ DNA sequence (*Sma*I, *Cfr*I fragment) elucidated by us and the corresponding segment of *E. coli* DNA (45) are also shown, one strand in each case, viz. the sense strand of the bacterial DNA and homologous strands of the phage DNAs, being given. Underlined are parts of the parental DNAs fused in λ*p**lac*5. AT links of the bacterial DNA (a part of the *lacY* initiating triplet) cut out in the recombination (see below, Fig.7) are in low-case letters. At the top, numerated are some triplets of *lacZ* gene mentioned in the text (51). A segment of the complementary strand of λ*p**lac*5-2 DNA is presented containing triplets TGA, TAA, and TAG which correspond to terminating codons in *orf208a'* (see Fig.6 and the text).

Figure 6. Formation of λ plac5 bacteriophage (bacterial DNA hatched, homologous sequences (out of scale) shaded, fragmented loci marked with apostrophes). A - λ lysogen of the *E. coli* HX-196 strain. (a): *lac* operon, *lacI* gene of which after HX-196 deletion (21) is fused with the phage locus *Ea47*; (b): λ DNA inserted, after repeated lysogenation (21), at the hybride *att* site of *E. coli* HX-196. B - abnormal excision of the prophage as a result of the site-specific recombination. C - the excision product, λ plac5.

nation which yielded λ plac5 phage (see Fig.6) occurred at the phage locus *orf206a* (*lom*, see (59)). As a result, out of the total 206 triplets coding for the hypothetical Orf206a polypeptide, λ plac5 DNA retains first 137 followed by 17 triplets from *z-y* spacer and by a terminating triplet TGA in the same reading frame (see Fig.5). Hence, a new hybride LOM' protein (in case it is produced by λ plac5) must contain 154 amino acid residues. Since λ plac5 does not radically differ from the original λ phage, either no protein possibly corresponds to *orf206a* locus, or such a protein (or else its C-end) is not indispensable for the normal reproduction of λ phage. Similar is true for the phage locus *Ea47*, within which we localized second junction of the bacterial and phage DNAs in λ plac5; it is to that junction that deletion HX-196 led in course of the transducing phage construction (60). The deletion as can be seen from our data proved to be more long-stretched than it had been considered (21) and goes far beyond (ca. 4300 bp) *J* gene. We showed that the deletion eliminated almost half of the phage *b2* region, so that left are about 4500 bp (cf. (22)).

DISCUSSION

Site-specificity of the λ prophage abnormal excision.

As already mentioned, λ DNA integration and excision are examples of conservative site-specific recombination (13). On the other hand, abnormal excision, resulting in transducing phages, used to be considered as a random event proceeding at a wide variety of sites (5). Our data, however, show that at least in terms of sequence homology of interacting regions, the λ prophage ab-

normal excision is similar to the normal λ excision. In fact, the homology region in the structurally elucidated area of the λ prophage abnormal excision (Fig.5) is 19 bp long (without two extra links of bacterial DNA), with two breaks. In *attB* and *attP* sites (7) the homology region can also be expanded from the 15-membered *att*-core to the 19-bp segment, with one nucleotide varying in both sequences; the size is in accordance with data on the length of the functionally active and Int-protected segments in *attB* site (13,62,63). This allows to consider the λ prophage abnormal excision as a site-specific recombination; with such length of almost uninterrupted homology, probability of a comparable structural situation elsewhere within the interacting DNAs is very low. Of course, evidence of site-specificity of the abnormal excision offering by the present study could be considerably substantiated by sequencing a variety of transducing phages, first of all, the rest of ten λ p*lac* phages mentioned in the paper (21).

Hypothetical mechanism of the λ p*lac*5 phage formation.

At the initial stage of the recombination (λ prophage abnormal excision) synapsis of the bacterial and phage DNA segments in the crossover region (Fig. 7-I) can be described in terms of the four-stranded-complex conception (19,64-67), with B-P' and P-B' strand interactions playing the main role (Fig.7-II); these combinations of strands will operationally be termed heteroduplexes. In each of them a site of non-homology is provided by two extra nucleotides AT of bacterial DNA. We assume it to be an initiation signal for the recombination, which determines a point of the specific endonucleolytic breakage and therefore the first point of crossover (cf. Fig5). The breakage is followed by the exonucleolytic elimination of the two extra links AT, thus completing formation of the first nick and demonstrating on the sequence level the heteroduplex adjustment in course of a recombination (Fig.7-II,III).

Position of second point of the crossover is ascribed by analogy with some λ mediated site-specific recombinations, proceeding via staggered cuts in complementary strands (14,20). We suppose the second cut to yield the longest protruding ends within AATCCATT segment (shift to the left would lead, after crossover, to a hybriide DNA containing non-complementary nucleotides). Corresponding combinations of nicks in the four interacting strands are presented in structures IV and V in Fig.7(second and third nick stages).

Structures VIa and VIb depict the strand exchange leading to the recombination product (VII), *i.e.* to λ p*lac*5 DNA. Both structures, VIIa and VIIb, are identical, differing only in the origin (phage or bacterial) of strands of the central octanucleotide; a circumstantial evidence in favour of VIIb lies with

Fig.7. Scheme of excisional recombination leading to λ plac5 phage (*three-nick mechanism*).

I - *E. coli* DNA (B and B' strands) and λ DNA (P and P' strands) segments of the HX-196 prophage (cf. Fig.6A) in the excisional recombination region. Two additional nucleotides to the right of the crossover region are shown, simplifying differentiation of strands at later stages. Phage DNA links corresponding to non-homology sites in subsequent heteroduplexes are marked with asterisks.

II - Heteroduplexes at the initial stage of the recombination (dinucleotides not forming Watson-Crick bonds in heteroduplex structures are parenthesized). Arrows (s.a. below) indicate positions of nicks.

III - Nicked heteroduplexes containing dangling dinucleotide ends (first nick stage).

IV - Four-strand complex after cutting off the dangling dinucleotide ends and prior to second nick formation. Complexes IVa-VIa and IVb-VIb correspond to two variants of the recombinational event and differ in positions of subsequent nicks (s.a. below).

V - The same complex prior to the third nick.

VI - Third nick and exchange between homologous strands of phage and bacterial DNAs.

VII - λ plac5 DNA duplex as a result of the excision.

Figure 8. A hypothetical heteroduplex intermediate, corresponding to Fig.7-II (left), in the λ p $lac5$ prophage excision (girl-skier-with-pigtails model). Open arrows indicate positions of primary nicks.

the molecular mechanism of the λ phage normal integration/excision (14).

Secondary structure of the recombination complex.

As an attractive hypothesis, we suggest a hairpin structure in bacterial DNA (Fig.8), similar to that postulated by Büchel *et al.* (45) for the *lacY* mRNA. It is particularly noteworthy that the recombination region localized by us (Fig.5) lies entirely within the hairpin loop, *i.e.* is ultimately exposed. A structure of the kind presented in Fig.8 may depict one of intermediates of the abnormal excision, particularly if it proceeds along with replication of the corresponding segment of bacterial genome when duplex structure of DNA in

that region is not yet sufficiently stabilized (cf. (68)). A similar loop in bacterial DNA (and the corresponding complex with phage DNA) could also arise within a local cruciform structure (69,70).

The plausibility of a long-stretched hairpin as an important recombinational intermediate is supported by our finding the previously elucidated (7) primary structure of the λ phage *attP* site to also contain two rather long, though imperfect, inverted repeats (nucleotides 27645 to 27713 and 27748 to 27812) capable of a hairpin formation (a loop 34 nucleotides long, with the core comprising nucleotides 27724 to 27738). Both hairpins are located in A•T-rich areas, which must favour their formation by promoting local DNA denaturation. Being in the very middle of a loop and therefore extremely exposed, the core of *attP* site in this respect is similar to the λ abnormal excision area (Fig.8). Increase of recombination efficiency by superhelicity of phage DNA (71) may be due (at least, partially) to the fact that superhelicity promotes hairpin formation (72). The structures considered in this subsection might be connected with some general principles of genetic material organization in site-specific recombinations, *e.g.* with intasome formation (cf. (15)).

Enzymes possibly involved in the abnormal excision.

The reported abnormal excision independence of *recA* and *int,xis* systems (5, 73) leaves unclear which enzymes (if any) are involved in the process. Structural resemblance of the normal and abnormal excision regions suggests that both kinds of recombination might be enzymatically related. At the crossover region we found a potential binding site of bacterial IHF protein: the endonuclease breakage (Fig.7-II) would lead to the structure 5' -A-T-T^{T-A}G-T-A- similar to the standard IHF-specific sequence ATTGATA (13). Concerning phage proteins, the crossover region and the adjacent stretches (Fig.5) do not contain sequences considered as *Int* binding sites, *viz.* CAGTCACTAT (*Int* arm-type sequence)(74) or CAACTTNNT (*Int* junction-type sequence)(16). A deficient *Int* protein can, however, be postulated which is modified strongly enough to prevent the normal excision (and therefore a standard test of the protein) but retains ability of bringing about the low-efficient abnormal excision. Indeed, a deficient (virtually inactive) *Int* protein is known to form "simple" complexes with DNA (75); formation of complete intasome structures (15) in case of a low-efficient excision might be not obligatory. As for *Xis* protein, it can partially make up for the *Int* inadequacy through relatively efficient pairing of interacting DNA segments.

Another process to which the λ abnormal excision is structurally (and, perhaps, mechanistically) related is *RecA* partially independent spontaneous dele-

tions occurring at short repeating sequences (76). However, much larger dimension of the excised DNA in the λ plac5 formation as compared with the above deletions suggests that slipped mispairing replication discussed as a possible mechanism of the deletions (76) can hardly be reconciled with the λ abnormal excision. For the latter, *E.coli recBC* system may play an important role, e.g. via specific interaction with χ sites or χ -like structures; it is to be noted that λ plac5 DNA, between the crossover region and the nearest *CfrI* site (Fig. 5), contains a GTCGGTGG segment (see the complementary strand) very similar to the ubiquitous component of χ sites, GCTGGTGG (77), as well as a sequence GAGTGGC which present also in the χ^+ B121 site (78). The hypothesis concerning *recBC* system may be of value for long-stretched deletions in general, including the deletion which led to HX-196 λ lysogen (Fig.6A) and the deletions described in the paper (76).

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**Dedicated to the 15th anniversary of "Isolation of pure lac operon DNA" (1) as a token of admiration for the first genetic engineering study, which preceded the era of recombinant DNA in vitro and was performed without restriction endonucleases and molecular cloning. It is in that paper that λ plac5 phage was first described.*

[§]Preliminary note see (2). A part of the present work was reported at the All-Union Conference on Recombinant DNA (Pushchino, 1982).

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