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P. Kreiss · B. Cameron · A. M. Darquet
D. Scherman · J. Crouzet

Production of a new DNA vehicle for gene transfer using site-specific recombination

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Abstract Supercoiled DNA molecules, minicircles, were produced by in vivo site-specific recombination. They contained exclusively the desired excisable fragment. Recombination was driven by bacteriophage λ integrase from a plasmid substrate containing the *att*P and *att*B recombination sites in the same orientation. Conditions for minicircle production within the lysogen *Escherichia coli* D1210HP were optimised. Up to 1.5 mg minicircles could be produced per litre bacterial culture, and the remaining, unrecombined plasmid comprised less than about 15% of the minicircle produced. However minicircle multimers were also produced, and comprised up to 30% of all minicircles synthesised. The *parABCDE′* locus from plasmid RK2 was introduced into the minicircle fragment, resulting in minicircle dimers being reduced to less than 2% of all minicircles. The *parA* gene encodes a resolvase that catalyses recombination at the multimer resolution site in the *parABCDE′* locus. Minicircle multimers were also resolved when *parA* was introduced downstream from the integrase gene of the λp_L transcript in D1210HP together with a multimer resolution site carried by the minicircle fragment.

Introduction

Recent studies have demonstrated the value of gene therapy not only for the treatment of genetic diseases but also for that of cancers (Cooper 1996) and cardiovascular (Feldman and Steg 1996), metabolic (Kay and Woo 1994) and neurogenetic (Müller and Graeber 1996) disorders. DNA transfer techniques are also a promising tool for novel approaches to vaccination. However, both in vitro and in vivo studies show that vector optimisation is the limiting factor in gene therapy. Most clinical trials in gene therapy have involved the use of viral vectors. However, integration of viral DNA into the host cell genome is a major limitation for retroviruses, and immune reactions to treatment mean that adenoviral vectors cannot be used at high doses or administered repeatedly (Crystal et al. 1994; Schulick et al. 1997). Moreover, replication-competent viruses can cause adverse effects in patients.

Non-viral methods for gene transfer have also been developed, using plasmid DNA, either naked (Wolff et al. 1990) or complexed with formulating agents. However the efficiency of non-viral gene delivery must be improved for in vivo applications (Lee and Huang 1997).

The aim of this work was to develop safer genetic material for non-viral gene therapy. Minicircles lack an origin of replication and an antibiotic selection marker. They cannot replicate or confer antibiotic resistance to other micro-organisms or cells. In a recent study (Darquet et al. 1997) minicircles were shown to transfect cells and express the transgene with higher efficiency than larger plasmids. Minicircles are thus a promising alternative to plasmid DNA for non-viral gene therapy in terms of biosafety, improved gene transfer and potential bioavailability because of their small size (Hodgson 1995; Klinman et al. 1996).

Minicircles are produced by in vivo excision of the desired cassette by site-specific recombination, driven by the lambda bacteriophage integrase at the directly oriented *att*P and *att*B recombination sites. The use of plasmids containing the *att*P and *att*B recombination sites in opposite orientations has been described (Podhajska et al. 1985). Integrase-based recombination in these plasmids efficiently inverts a promoter between

P. Kreiss · A. M. Darquet · D. Scherman
UMR 133 CNRS/Rhône-Poulenc Rorer,
Centre de Recherche de Vitry-Alfortville,
Vitry-sur-Seine, France

B. Cameron · J. Crouzet (✉)
Rhône-Poulenc Rorer Gencell,
Centre de Recherche de Vitry-Alfortville,
13, Quai Jules Guesde,
94403 Vitry- sur-Seine, France
Tel.: +33 1 55 71 38 96
Fax: +33 1 55 71 37 96

*att*P and *att*B, leading to expression of the corresponding gene (Hasan and Szybalski 1987). Plasmids derived from those described by Hasan and Szybalski (1987) were initially used for DNA minicircle production (Darquet et al. 1997). However, only small quantities of minicircles were recovered because of suboptimal plasmid copy number, incomplete recombination and the presence of multimeric forms.

The *par* genes are part of a very efficient partitioning system that stabilises plasmids in many gram-negative bacteria (Gerlitz et al. 1990; Davis et al. 1992). For example, no loss of pBR322 into which the *par* locus had been introduced is detected even after 200 generations of growth under non-selective conditions (Gerlitz et al. 1990). The stabilisation mechanism involves the *parCBA* operon, which encodes a multimer resolution function and the *parDE* operon responsible for a post-segregational killing mechanism (Jovanovic et al. 1994; Roberts et al. 1994; Johnson et al. 1996). The *parA* gene encodes a 24-kDa resolvase belonging to the Tn*3* resolvase family (Gerlitz et al. 1990; Roberts and Helinski 1992). This resolvase catalyses site-specific intramolecular recombination between two multimer resolution sites (MRS) in the same orientation. The *par* resolution system was used to resolve minicircle multimeric forms.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli D1210 [*supE44 ara14 galK2* Δ(*gpt-proA*)*62 rpsL20 xyl5 mtl1 recA13* Δ(*mcrC-mrr*) *hsdS lacI*^{q}] was obtained from Stratagene. D1210HP was obtained by lysogenisation of D1210 with the lambda bacteriophage (λ[*c*I857 *xis*$^{-}$ *kil*$^{-}$]) as described by Hasan and Szybalski (1987). *E. coli* W3101λ is a λ1 (λ[*c*I857, *xis*1, *P80*, *red114*]) lysogenised W3110 (Bliska and Cozzarelli 1987). *E. coli* TG1 and HB101 cells (New England Biolabs) were used for plasmid construction. All bacterial strains were propagated in Luria-Bertani (LB) medium containing antibiotics purchased from Sigma Chemical as needed.

Standard molecular biology procedures and reagents

Plasmid isolation and construction, bacterial transformation or electroporation, and DNA analysis were performed as described by Sambrook et al. (1989). Southern blots were carried out using radioactive probes labelled with the rediprime kit (Amersham Life Science). DNA sequencing was performed with −21 and reverse M13 fluorescent primers, using a 377 DNA sequencer (Applied Biosystems). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the manufacturer. Shrimp alkaline phosphatase and [γ-^{32}P]dCTP were obtained from Amersham Life Science.

Plasmid construction

Plasmid pXL2675 was constructed as described by Wils and Ollivier (1996; patent reference FR 96 03519). This plasmid contains the ColE1 origin of replication from pBluescriptII SK+ (Stratagene), the Km^{R} gene from the Tn*5* transposon and a polylinker containing the following restriction sites: *Sac*I, *Not*I, *Xba*I, *Bam*HI, *Eco*RI, *Pst*I, *Xho*I and *Hin*dIII. The *att*B site was obtained by cloning the hybridised oligonucleotides 6194 (5′-ACTAGTGGCCATGCATCCGCTCAAGTTAGTATAAAAAAGCAGGCTTCAG-3′) and 6195 (5′-AGCTCTGAAGCCTGCTTTTTTATACTAACTTGAGCGGATGCATGGCCACTAGTAGCT-3′) as a *Hin*dIII-*Sac*I insert into pXL2675 resulting in pXL2675-*att*B. The lambda *att*P site (position 27480–27860 in the published λ sequence; Sanger et al. 1982) was obtained from pXL2649 (Darquet et al. 1998) by polymerase chain reaction (PCR) amplification using the primer pair 6190 (5′-GCGTCTAGAACAGTATCGTGATGACAGAG-3′) and 6191 (5′-GCCAAGCTTAGCTTTGCACTGGATTGCGA-3′). Amplification was performed using 30 cycles (94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min) with 2 mM $MgCl_2$ on a Perkin Elmer GeneAmp PCR system 2400 apparatus. The resulting 380-bp product was digested using *Xba*I and *Hin*dIII and ligated to M13mpEH (Benes et al. 1993). The sequences of the cloned *att*B and *att*P were checked. The *att*P site was then prepared as a doubled-stranded *Xba*I-*Nsi*I fragment and cloned into pXL2675-*att*B previously cut with *Spe*I and *Nsi*I, giving pXL2776 (Fig. 1A).

A 2.1-kb spectinomycin-resistance gene (Ω interposon, Genbank accession number M60473) linked to the 1.9-kb *Bacillus subtilis sacB* gene from pSL302 (Gay et al. 1983) was isolated as a 4-kb *Nsi*I-*Eco*RV fragment and ligated into pXL2776 giving pXL2777.

The *parABCDE′* locus from plasmid pGMA60 (Gerlitz et al. 1990; Eberl et al. 1992) was isolated as a 2.4-kb *Bam*HI fragment and ligated into pXL2777, replacing the Ω interposon and generating pXL2960.

A 450-bp *Xho*I-*Eco*RI fragment containing the ParA resolution site MRS was extracted from pCK155 (Eberl et al. 1994) and ligated into pMTL22 (Chambers et al. 1988) previously digested with *Xho*I and *Eco*RI. The recombinant vector was digested with *Eco*RV

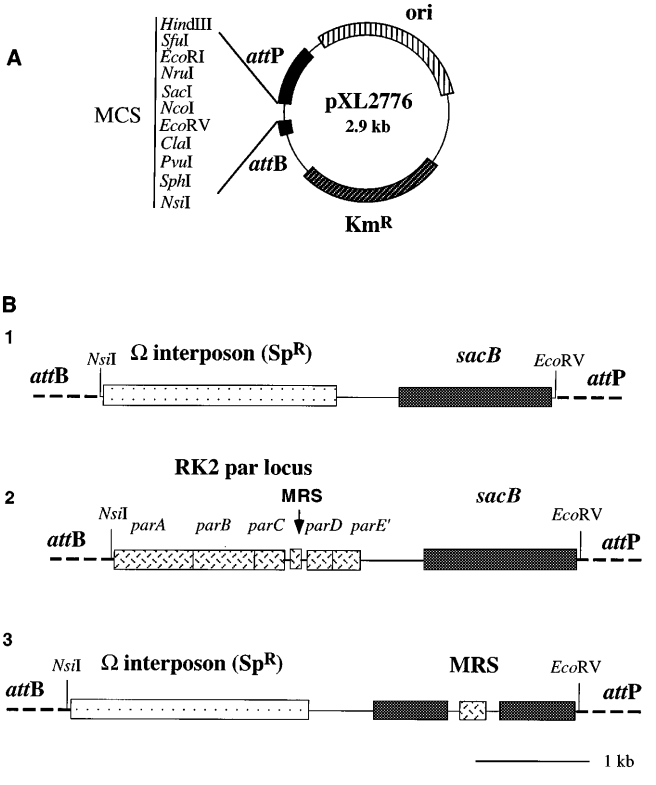

Fig. 1A, B Plasmids for minicircle production. **A** The minimal plasmid pXL2776. **B** Linear representation of the minicircle parts of the plasmids used in this work: *1* pXL2777, *2* pXL2960, *3* pXL2980. These fragments become minicircles after integrase-mediated recombination. *Shaded boxes*, *sacB* sequences; *dotted lines*, *att*P and *att*B sites

and *Nru*I and the MRS was ligated to pXL2777 cut with *Stu*I and *Sna*BI, resulting in plasmid pXL2980.

The suicide vector pXL3225, used for double homologous recombination and integration of *parA* downstream from *int* in D1210HP, contains the conditional origin of replication γ (oriγ) from plasmid R6K (Metcalf et al. 1994), the Km^R gene from transposon Tn*5*, the *B. subtilis sacB* gene, and the integration cassette for genetic recombination (Fig. 2). This cassette is composed of the *parA* gene with the strong transcription terminator, *t*fd, from bacteriophage fd, flanked by a 500-bp region from the 3′ end of the integrase gene and a 500-bp region from the *E. coli ybhC* gene, located between *att*B and the galactose operon. The integration cassette was designed to allow the expression of *parA* by translational coupling to λ *int* after recombination. The *parA* gene was obtained by PCR amplification on pGMA60 DNA using the primer pair 8101 (5′-GAGATATACATATGGCGACGCGAGAGCAA-3′) and 8102 (5′-TCTGCTAGCTTATTTTTGCTGCTGCGCGT-3′). The transcription terminator *t*fd was obtained by hybridising oligonucleotides 8108 (5′-ATCTCCAAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGTATCGGTTTAG-3′) and 8109 (5′-CTAGCTAAACCGATACAATTAAAGGCTCCTTTTGGAGCCTTTTTTTTTTGGAGAT-3′). The integrase fragment (position 27810–28290 on the λ published sequence; Sanger et al. 1982) involved in homologous recombination was obtained by PCR amplification using primers 8099 (5′-AGTTCTAGATTGGCTCAGACTTGCAATG-3′) and 8100 (5′-CATATGTATATCTCCTTCTTATTTGATTTCAATTTTGTCC-3′). The *ybhC* fragment (Genbank accession number U39938) was obtained by PCR amplification using primers 8103 (5′-CTAGCTAGCAAGATATCTATTTCATAGCCCGGAGCAAC-3′) and 8104 (5′-ACTGGATCCACGTCGTGACGCCAGTCGG-3′). All DNA fragments used for plasmid construction were sequenced by the dideoxy-DNA method. Plasmid pXL3225 is shown in Fig. 2.

Construction of D1210HPintpar

We used a modified version of the method developed by Metcalf et al. (1994). Plasmid pXL3225 was introduced into D1210HP by a standard electroporation procedure. The first homologous recombination event, resulting in complete integration of pXL3225 into the D1210HP genome, was isolated by selecting recombinant bacteria on LB medium containing 50 μg/ml kanamycin. The second homologous recombination event, causing replacement of the *att*B region by the *parA* gene and resulting in D1210Hpintpar, was obtained by reisolation of recombinant clones on LB medium containing 5% sucrose. We checked that the *parA* gene was correctly integrated into the p_L transcript by PCR analysis using primers 8099 and 8104.

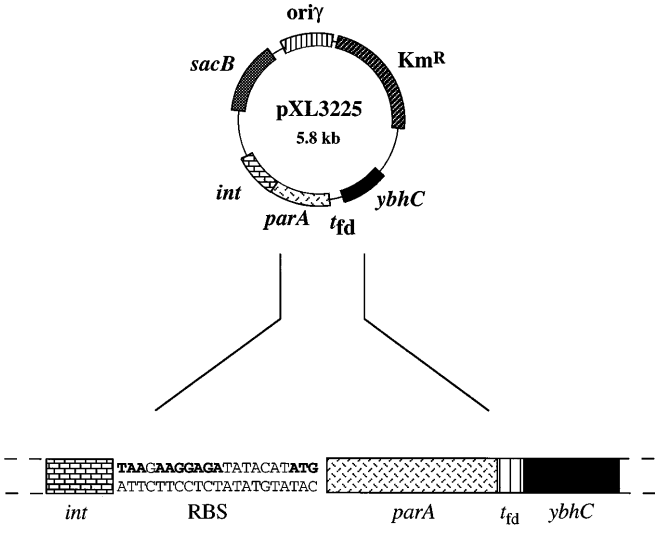

Fig. 2 Plasmid pXL3225 used for *parA* integration into D1210HP. The *int* stop codon, *parA* translation initiation codon, and consensus ribosome-binding site (RBS) upstream from *parA* are shown in bold characters. The genes and elements on the linear map are not drawn to scale

Induction of minicircle production

Cultures grown overnight at 30 °C in LB medium containing 50 μg/ml spectinomycin (pXL2777, pXL2960) or 50 μg/ml kanamycin (pXL2960) were diluted 1/100 in LB containing kanamycin and incubated at 30 °C, with shaking at 200 rpm, until the culture reached an A_{600} between 0.3 and 3. The culture was subjected to heat shock at 42 °C, 200 rpm, for 15 min in a water-bath agitator (Infors AG) and then incubated at 30 °C for various periods of time.

DNA analysis and quantification

DNA prepared from heat-induced cultures was analysed on 0.7% agarose gel by electrophoresis. Quantification was done using the ImageMaster apparatus and appropriate software provided by Pharmacia Biotech. Results were only considered significant when the correlation coefficient was greater than 0.93.

Results

Tight regulation of minicircle formation

Expression of λ integrase is controlled in the two λ lysogenised strains D1210HP and W3101λ by the thermolabile *c*I857 repressor. When the temperature is increased to 42 °C, the *c*I repressor becomes inactivated and integrase is synthesised. Lambda integrase and the cellular protein, IHF (integration host factor), form a complex at the *att*P recombination site causing recombination with the *att*B site, which is in the same orientation as *att*P in the plasmid. The products of recombination are two circular DNA molecules, the miniplasmid and the minicircle. Plasmid pXL3210 contains an origin of replication and a kanamycin-resistance gene (Km^R), both in the 4.8-kb miniplasmid part of the plasmid. The *sacB* gene of *Bacillus subtilis* responsible for sucrose sensitivity (Suc^S) is on the 5.2-kb minicircle part of the plasmid (Fig. 3A).

The integrity of pXL3210 was studied at 30 °C, in the absence of integrase induction, in W3101λ and D1210HP and their non-lysogenic parents, W3110 and D1210. The products of integrase-induced recombination events in pXL3210 were a Km^R miniplasmid and a *sacB*-containing minicircle lost after cell division because it did not contain an origin of replication. Thus, in vivo integrase-induced recombination in pXL3210 gives rise to a Km^R and Suc^R clone (Fig. 3A). In the absence of thermal induction of integrase expression, integrase-mediated recombination at 30 °C occurs at a frequency of approximately 3.5×10^{-6}/generation in D1210HP and 2.8×10^{-4} per generation in W3101λ (Fig. 3B), assuming that recombined bacteria have the same generation time as the parent strain. Therefore, minicircle

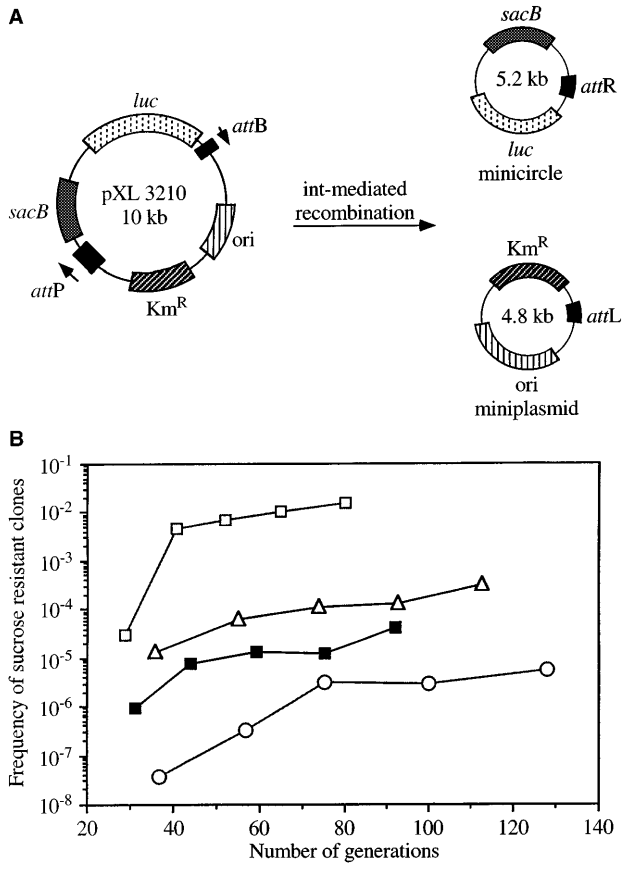

Fig. 3A, B Integrase-mediated recombination in plasmid pXL3210. **A** Reaction scheme. **B** Culture dilutions of the four strains (W3110: ○, D1210: ■, W3101λ: □, D1210HP: △) were plated on Luria-Bertani agar containing kanamycin or kanamycin and 5% sucrose. The frequency of sucrose-resistant clones is shown as a function of the number of cell divisions

formation at 30 °C was more tightly regulated in D1210HP than in W3101λ. Moreover, preliminary experiments showed that D1210HP had higher recombination efficiency than W3101λ, so the D1210HP genetic background was further studied to optimise minicircle production.

Optimisation of culture conditions for minicircle production

Minicircles were produced upon thermal shift of D1210HP cultures, which caused integrase-mediated recombination at the *att*P and *att*B sites in the plasmid. Plasmids used to improve minicircle production were all derived from plasmid pXL2776. Plasmid pXL2776 is very small, 2.9 kb, and contains the pBluescript origin of replication for high plasmid copy number, a Km^R gene derived from the Tn*5* transposon and a multi-cloning site flanked by the 380-bp *att*P site and the 33-bp *att*B site in the same orientation (Fig. 1A). Plasmid pXL2777, which contains a 4.3-kb minicircle part, was derived from pXL2776 by the addition of a 2.1-kb spectinomycin resistance (Sp^R) gene and the *sacB* gene (Fig. 1B). Heat shock (15 min at 42 °C) was performed for cultures at various absorbances densities (A_{600}). Cultures were then incubated for various times at 30 °C. Plasmid DNA was isolated and analysed by agarose gel electrophoresis (Fig. 4). The efficiency of integrase-mediated recombination was measured by dividing the amount of minicircle by the amount of minicircle and unrecombined plasmid. Efficiency was calculated for D1210HP pXL2777 at various growth phases. Recombination occurred with similar efficiency (80%–90%), with an average of 10%–20% plasmid substrate remaining, when the culture was subjected to heat shock at A_{600} of 0.3–3. However, no integrase-mediated recombination occurred in a culture of D1210HP pXL2777 grown overnight. This shows that the physiological state of the bacteria is critical for lambda integrase-mediated recombination.

The kinetics of integrase-mediated recombination was studied. Integrase was induced when the culture reached an A_{600} of 2. The recombination rate reached a plateau after 1 h at 30 °C (data not shown), whereas longer incubation times (from 3 h to 12 h) led to accumulation of high-molecular-mass DNA (Fig. 4).

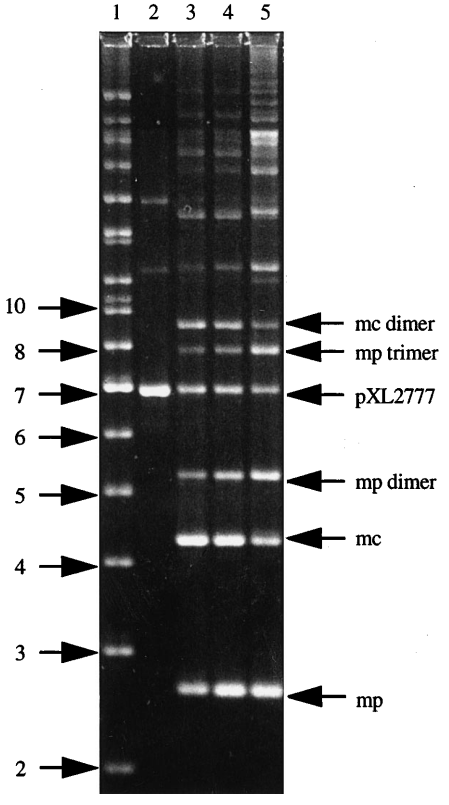

Fig. 4 Kinetics of minicircle excision in D1210HP pXL2777. *1* Supercoiled DNA ladder (size in kb indicated on the *left*); *2* uninduced culture; *3–5* DNA obtained after heat induction and after 30 min (*3*), 1 h (*4*), and 12 h (*5*) further culture at 30 °C. *mc* minicircle, *mp* miniplasmid. The positions of minicircle dimers, and miniplasmid dimers and trimers were determined from the supercoiled DNA ladder and probe-specific hybridisation

Up to 1.5 mg minicircle DNA was produced per litre D1210HP pXL2777 culture after heat induction at an A_{600} of 3 and incubation for 1 h at 30 °C.

Despite efficient minicircle production, integrase-mediated recombination also gave rise to additional DNA molecules (Fig. 4, lanes 3–5). DNA bands with molecular masses corresponding to those of minicircle dimers and miniplasmid dimers were detected. They comprised between 20% and 30% of the total minicircle and miniplasmid DNA. Restriction analysis and hybridisation to ^{32}P-labelled probes specific for minicircle or miniplasmid DNA confirmed the presence of these dimers. The molecules were relaxed by *E. coli* topoisomerase I, indicating that they were supercoiled (data not shown). Other higher-molecular-mass DNA molecules did not have the characteristics of minicircle or miniplasmid multimers. Analysis by restriction and hybridisation showed these forms were relaxed forms of miniplasmid, minicircle and unrecombined plasmid.

Minicircle multimer resolution by introduction of *parABCDE′* into the minicircle

The *parABCDE* locus from plasmid RK2 encodes a multimer resolution system (Gerlitz et al. 1990). The *parABCDE′* locus (Eberl et al. 1992), slightly modified from *parABCDE,* was introduced into pXL2777 between *att*P and *att*B, giving pXL2960 (Fig. 1B). Analysis of the products of integrase-mediated recombination within D1210HP pXL2960 showed that the multimeric minicircle forms were resolved (Fig. 5). Minicircle dimers, which represented up to 30% of the minicircles present with pXL2777, were no longer detectable after 30 min at 30 °C, even after specific hybridisation of an α-^{32}P-labelled *par* fragment to the minicircle (Fig. 5B). The detection limit of the ImageMaster apparatus was about 4 ng in our experimental conditions. Thus minicircle dimers comprised less than 2% of all minicircles present after introduction of the *parABCDE′* locus. Moreover, less high-molecular-mass DNA was detected than with pXL2777. A low-intensity band, with a molecular mass corresponding to that of a 8.5-kb supercoiled plasmid, was shown to hybridise to the fragment specific for the minicircle (Fig. 5B, lanes 10–12). It is thought to be the relaxed form of the minicircle.

Minicircle multimer resolution by ParA resolvase

The *E. coli* strain, D1210HPintpar, was constructed by double homologous recombination using the suicide ori γ plasmid pXL3225 described in Materials and methods. D1210HPintpar was designed such that the *parA* gene was expressed under the control of the very strong, tightly regulated p_L promoter (Deuschle et al. 1986; Knaus and Bujard 1988) in translational coupling with the integrase gene.

Plasmid pXL2777 was introduced into D1210HPintpar and plasmid stability was analysed under conditions similar to those described in Fig. 3B. Minicircle production was as tightly regulated in D1210HPintpar as it was in D1210HP (data not shown).

Plasmid pXL2980 was constructed to improve minicircle production in *E. coli* D1210HPintpar. This plasmid corresponds to pXL2777 with the multimer resolution site from the *par* locus inserted into the minicircle. Integrase-mediated recombination was induced in *E. coli* D1210HPintpar pXL2980. Minicircle multimer resolution was as efficient as that observed in D1210HP pXL2960, in which the whole *par* locus is

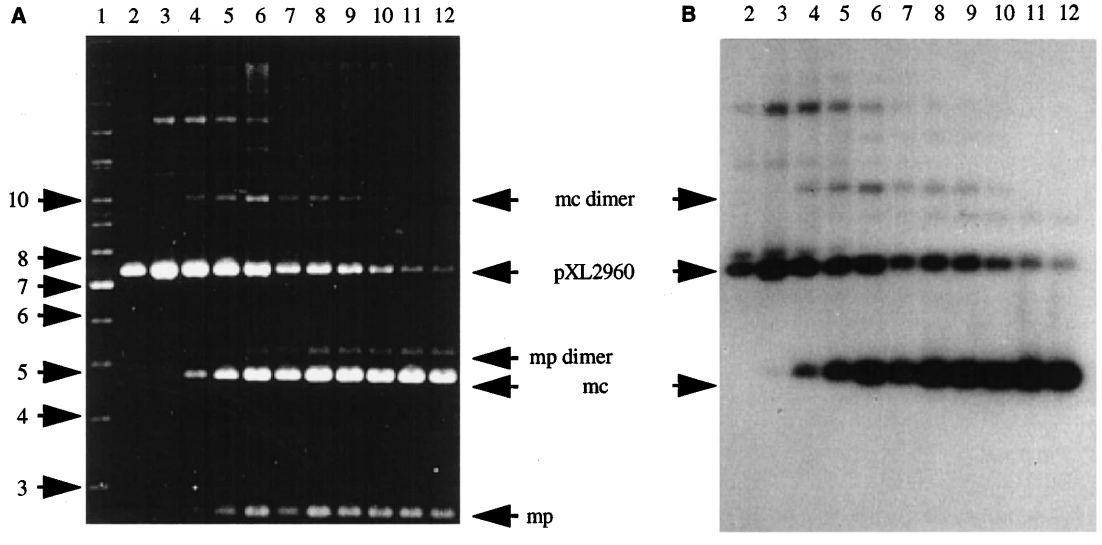

Fig. 5A, B Kinetics of the recombination reaction in D1210HP pXL2960. **A** *1* Supercoiled DNA ladder (size in kb indicated on the *left*); *2–12* DNA obtained after 0, 2, 4, 6, 8, 10, 12, 16, 32, and 64 min expression at 30 °C. **B** The fragments from the agarose gel shown in **A** were transferred to a nylon membrane and probed with a ^{32}P-labelled DNA fragment specific for the minicircle fragment

present on the minicircle. Thus, D1210HPintpar could be of great value for minicircle production. Multimer resolution was optimal at expression temperatures of 25–37 °C (Fig. 6).

Finally, the use of the *par* site-specific recombination system not only eliminated recombination reaction biproducts (multimers) but also increased the production of minicircle monomers by converting multimeric minicircles into monomers. Up to 38% more minicircle monomers were indeed produced using D1210HPintpar pXL2980 than when D1210HP pXL2777 was used.

Discussion

Site-specific recombination, involving DNA sequence exchange or excision, is becoming increasingly important in biotechnology. Site-specific recombination has been used for diverse purposes, including protein expression (Hasan and Szybalski 1987), gene deletion (Lewandoski et al. 1997) and chromosomal exchange (Dale and Ow 1990) in both prokaryote and eukaryote organisms. Many animal and plant model systems have been described that have either a recombinase gene or a DNA substrate flanked by specific recombination sites (Kilby et al. 1993).

The site-specific recombination system based on lambda integrase in D1210HP has two major advantages: the high efficiency of recombination catalysed by the integrase and the very tight regulation of recombination (Hasan and Szybalski 1987). We made use of these advantages of the integrase recombination system for high-level and stringently controlled DNA minicircle production. The use of minicircles containing a luciferase reporter gene, with appropriate expression signals, was recently described by Darquet et al. (1997). About 0.13 mg luciferase minicircles was produced per litre bacterial culture with a recombination efficiency of 60%. Optimisation of the plasmid vector, the bacterial strain and the induction conditions led to the production of 1.5 mg DNA minicircles/l bacterial culture with an efficiency of recombination of up to 90%.

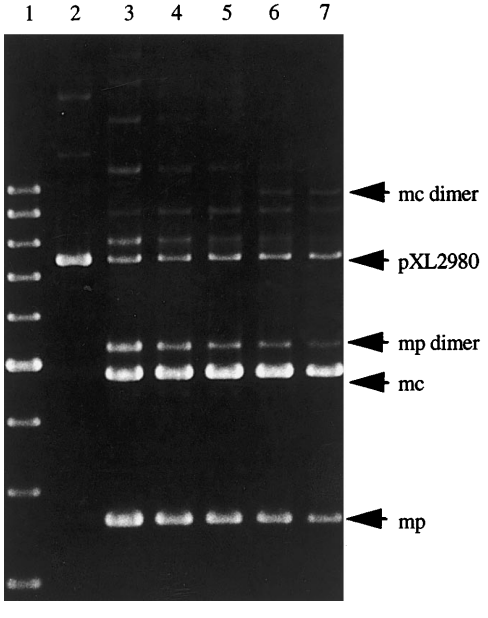

Fig. 6 Resolution of minicircle dimers as a function of temperature in D1210HPintpar pXL2980. *1* Supercoiled DNA ladder, from 2 kb to 10 kb; *2* uninduced pXL2980; *3–7* pXL2980 DNA after heat induction and expression for 2 h at 37 °C (*3*), 30 °C (*4*), 25 °C (*5*), 20 °C (*6*), 15 °C (*7*). Minicircle dimers are visible in *6* and *7*

Recombination efficiency may be determined by the Xis^- phenotype of D1210HP and W3101λ, because the formation of *att*P and *att*B from *att*R and *att*L does not occur in the absence of Xis (Weisberg and Landy 1983; Landy 1989). The very tight regulation of recombination is also important because only small levels of uninduced integrase-mediated recombination would lead to the loss of DNA minicircle molecules after a few generations.

The presence of minicircle and miniplasmid multimers can be explained by integrase-mediated recombination between two different plasmids (intermolecular recombination). The presence of high-molecular-mass weight DNA, corresponding to relaxed forms of miniplasmid, minicircle or unrecombined plasmid may be due to the type I topoisomerase activity of λ integrase (Kikuchi and Nash 1979).

Several approaches were used to resolve multimers. The insertion of the *cer* site from plasmid ColE1 (Summer and Sherrat 1984) into the minicircle did not result in efficient multimer resolution (data not shown). MRS-containing minicircle multimers were also not resolved when the *parABCDE′* locus was inserted into the *uidA* gene of D1210HP by double homologous recombination (data not shown). Expression of *parA* from the ParA down-regulated p_{CBA} promoter may be insufficient when *parABCDE′* is present only in one copy in the D1210HP genome. The ParA resolvase is probably synthesised at a much higher level when *parABCDE′* is present as hundreds of copies on the minicircle fragment of pXL2960, or when expressed as a translational coupling with the *int* gene downstream from the p_L transcript of D1210HPintpar. This hypothesis is consistent with the fact that MRS-containing minicircle multimers were resolved when *parA* was inserted into a plasmid and expressed from the *ptac* promoter (data not shown).

D1210HPintpar is of value because it provides efficient expression of the genomic *parA* gene, allowing the *par* multimer resolution system to be restricted to its minimal form, the MRS, on the minicircle fragment. Recent studies have demonstrated the value of vectors of minimal size and minimal import of foreign DNA sequences for non-viral gene therapy (Hartikka et al. 1996; Klinman et al. 1996). Minicircles contain only the 700-bp sequence of *att*R and MRS in addition to the transgene. Moreover, the *att*B site has been deleted in D1210HPintpar, resulting in an *E. coli* that is unable to produce lambda bacteriophage and thus conforms to the biosafety constraints for DNA production. Minicircle production can also be compared to that of ColEI-de-

rived plasmids, which were produced under the same conditions with only a three- to five-times better yield. The main limitation to minicircle development as a gene therapy vector is presently its purification from unresolved plasmid and miniplasmid. A new chromatography method, based on the formation of triple-helix interactions between a specific sequence on the plasmid and an immobilised oligonucleotide (Wils et al. 1997), could prove useful to overcome this problem, which is under investigation at present.

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