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Minicircle Patents: A Short IP Overview of Optimizing Nonviral DNA Vectors

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The use of nonviral vectors for gene and cell therapy and especially for vaccination started with the observation of Wolff *et al.* [1] that the direct application of plasmid DNA containing the expression cassette for a protein into animal muscle led to the expression of this and – subsequently – to the appearance of antibodies against this protein – the idea of a DNA vaccine. While this was initially done with standard cloning or gene expression plasmids typically driven by a CMV promoter, its use in pharmaceutical context required the improvement of the structure of the plasmid with respect to the coding sequence (e.g., codon usage) and also concerning the total molecule: starting from the removal of abundant sequences (e.g., multiple cloning site residues) and the replacement of the antibiotic resistance gene *bla* (for ampicillin resistance) by a kanamycin resistance up to the removal of CpG motifs from the coding and backbone sequence [2]. Also, the physical structure of plasmid vectors was modulated by using process technology to obtain exclusively ccc-supercoiled DNA through specific cultivation technology [3] or purification processes [4], resulting in the depletion of toxic bacterial chromosomal DNA (with CpG motifs) as recently published [5].

The first major improvement was the removal of *any* resistance marker sequence from the plasmid (resulting in so-called miniplasmids); many are described in this book (see Chapters 6–13). However, a selection marker was still present on the plasmid, and also the large sequence element responsible for the plasmid replication (bacterial origin of replication – ori [6]) was still there.

The major improvements to further reducing the size and – by the way – removing the nonintended backbone sequences, *including* the ori, were made by approaches to reducing the DNA molecules carrying the pharmaceutically required expression unit to (mainly) circular structures with almost no other sequence than the sequence of interest, the so-called minicircles.

The first minicircle patent application to be filed was an international application by the US Department of Health with Adhya and Choy as inventors, priority date October 16, 1992, and published as WO 94/09127. The application was subsequently withdrawn in November 1994, and no patents were granted. Claim 1

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referred to a DNA construct comprising attB and attP sites with a multiple cloning site (in a later application, by Bigger *et al*. in 2001 (US application 11/249929), also called "multicloning site sequence") and a transcription terminator in between. This DNA was to be introduced into a host cell expressing the lambda Int protein, leading to site-specific recombination and excision of a circular construct. Since the construct thus formed was not supposed to contain a resistance gene or an origin of replication, it can be regarded as a minicircle, and the very term was in fact coined in this application. The intention of the inventors was, however, quite different from the gene vector and therapeutic approaches that have characterized later minicircle applications. In fact, the aim of these first constructs was to study the kinetics of promoters, to which end a construct containing only a single promoter with a reporter gene was needed.

It then took several years until the potentially superior properties of minicircles as vectors for gene transfer and therapeutic approaches were exploited in the field of patents. A further approach was submitted by Seeber and Krüger, with priority date August 11, 1994, and published as WO 96/05297. The application led to the grant of patents in Europe (EP 0775203) and the United States (US Patent 6,573,100), which are still in force and directed to the use of minicircles in therapy. The inventors intended to remove the resistance gene *bla* from a circular plasmid vector by site-specific recombinase (SSR) systems by dividing the circular plasmid into two circles – one containing the gene cassette and the other the residual portion including *bla*. The growth of the plasmid was performed under selective pressure and the two circles were separated by chromatography. The recombination system proposed was, for example, FLP/FRT. The major field of intended application was the gene therapy of cystic fibrosis.

The first patents to minicircles as such were obtained by the CNRS in France, who had filed an international application published as WO 96/26270 with priority date February 23, 1995, and Cameron *et al*. as inventors. The application resulted in granted patents in Europe (EP 0815214), the United States (US Patents 6,143,530 and 6,492,164), and Canada (CA 2211427), which are still in force. Claim 1 referred to a double-stranded DNA molecule characterized in that (a) it is circular and supercoiled; (b) it contains an expression cassette under control of a mammalian promoter; (c) it does not contain an origin of replication; (d) it does not contain a marker gene; and (e) it contains a region resulting from the site-specific recombination between two sequences, which is not present in the expression cassette. The introduction of therapeutic genes and the use of minicircles in gene therapy were expressly stated points of the application. The patent emphasizes that the absence of marker and resistance genes and other prokaryotic sequences (e.g., the origin of replication) affords a high genetic purity and low risk of transmission of undesired sequences and proliferation of antibiotics resistance.

Within these patents, methods for the production of such constructs were also provided. In particular, a preferred method involved the generation of minicircles from a precursor plasmid with two recombination sites, which are to be recombined by the coexpression of a recombinase. Recombinases from the lambda integrase

family and from the Tn3 family were suggested. The presence of the recombinase gene on the precursor plasmid itself is also contemplated. For the purification of the resulting constructs, several methods are suggested. In particular, specific affinity binding of a ligand to a recognition sequence in the miniplasmid is mentioned and illustrated by the example of triple-helix formation with a specific binding oligonucleotide. This interaction was also used to immobilize minicircles on a chromatography column during purification.

The next minicircle application that has left rights outstanding is US application 11/249929 by Bigger *et al.*, priority date April 10, 2001. The corresponding European application has been abandoned, but the US case is still pending. The novel aspect of this invention is the use of modified recombination sequences for minicircle production. In each of the two sequences that are to be recombined, one half-site is mutated so that the affinity of the recombinase is decreased. While a site consisting of one mutated and one wild-type half-site is still capable of binding the recombinase and being recombined, the resulting new site consisting of two mutated half-sites is no longer active. In this way, unidirectionality can be imparted to the recombination process. If the mutated half-sites are designed to lie on the minicircle after recombination, the yield of minicircle can be increased. Furthermore, the application also discloses the use of a restriction endonuclease to digest specifically the miniplasmid or nonrecombined parental plasmid after recombination. In addition, an additional treatment with an exonuclease for the removal of restriction fragments deriving from miniplasmid or nonrecombined parental plasmid is disclosed there. With this strategy, the yield of minicircles can be increased.

A further minicircle patent has been granted in the United States as US Patent 7,897,380 to Kay and Chen based on an application with priority date August 29, 2002. The corresponding European application (EP 03749280.8) has been refused and is currently under appeal. The claims are directed to minicircles that provide for persistent and high expression levels when present in the cell. The minicircles of the disclosed embodiments are produced by recombination with ΦC31 integrase and subsequent restriction of the miniplasmid, as disclosed already in 2001 by Bigger *et al.* (US application 11/249929).

In Europe, a patent has been granted to Mayrhofer *et al.* as EP 1620559, priority date May 5, 2003. The corresponding US application (10/556069) is still pending. The subject matter refers to parent plasmids for the production of minicircles that also encode the required recombinase in the region outside of the recombination sequences [7], as initially disclosed by Cameron *et al.* (WO 96/26270, see above). Furthermore, a method for purification of the minicircle product is described in EP 1620559, wherein the minicircle is immobilized in the plasma membrane of the producing bacteria upon lysis and can be isolated in this manner.

Immediately thereafter, Schroff and Smith submitted their application with priority date June 10, 2003, which was published as WO 2004/111247. The application has been abandoned in the United States and granted in Europe (EP 1631672). Here, the authors present a method to obtain circular gene expression

cassettes by specific restriction digestion of plasmid DNA leading to two linear fragments, one containing the expression cassette and the other the nonintended sequences of the plasmid (e.g., backbone, resistance gene). The fragments are recircularized (but not supercoiled) by ligation. Subsequently, a second digestion step with a different restriction enzyme, cutting exclusively the nonintended molecule (the backbone sequences) at least once, results in a mixture of linear fragments deriving from the backbone molecule and an intact circular GOI molecule. Thereafter, the linear fragments are removed by digestion with an exonuclease, so that the circular GOI molecule is further purified, as disclosed already in 2001 by Bigger *et al.* (US application 11/249929).

US Patent 7,622,252, granted to Zechiedrich, priority date June 10, 2005, is directed to the production of minicircles in topoisomerase IV-deficient cells. This is said to result in a higher yield of supercoiled minicircles, although these are formed as catenates and have to be decatenated before use. A more recent application by Zechiedrich *et al.*, priority date October 16, 2009, is pending in the United States (12/905612) and in Europe (EP 10824202.5). It is directed to the use of "minivectors" (essentially minicircles) in gene therapy for the continuous expression of shRNA and miRNA in a target (see also Ref. 8).

Finally, two more recent applications are pending by Kay *et al.* One, by Chen and Kay, priority date July 3, 2008, is pending in Europe (EP 09773923.9) and has been granted in the United States (US Patent 8,236,548). A method is disclosed for the production of minicircles with a high purity. A precursor plasmid is cleaved in a host cell by site-specific recombination into a minicircle and a miniplasmid, the latter then being digested by an endonuclease encoded in the same host, similarly to the method of Bigger *et al.* (see above).

The other application, by Wu and Kay, is pending in the United States as 12/925483 with priority date October 23, 2009. It refers to the treatment of ischemic cardiovascular disease by transducing muscle cells of the patient with HIF-1-encoding minicircles.

It is important to note that according to general principles of patent law, a patent may depend on another patent. This means that if the teaching of a later patent is a further development of the teaching of an earlier patent that is still in force, the proprietor of the later patent can use its teaching only with the consent of the proprietor of the earlier patent. In the field of minicircle patents, numerous such dependences exist. For example, most minicircle patents are dependent on the patent of Cameron *et al.*, which covers all minicircles having a gene expression cassette. Another example is the use of restriction enzyme and/or exonuclease treatment of nonminicircle molecules during minicircle production, already disclosed in 2001 by Bigger *et al.* (US application 11/249929).

Figure 1.1 gives an overview of minicircle patent applications depending on their filing date.

The general idea of reducing the size of a circular DNA vector was shown to be successful (see certain examples within this book). The removal of CpG elements as initially described for plasmids and patented by Drocourt *et al.* as EP 1366176 was a significant improvement with respect to the state-of-the-art plasmids used before as shown for "zero-CpG plasmids" later [2]. However, this CpG-free backbone could be

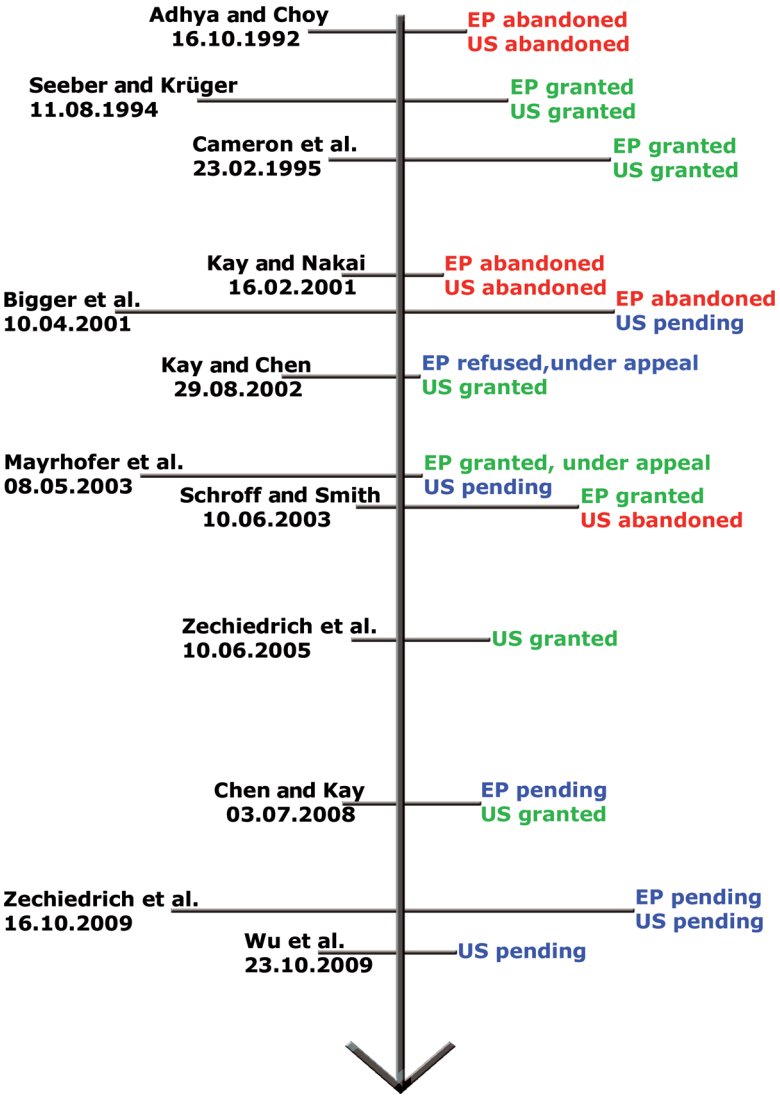

Figure 1.1 Overview of minicircle patents according to their filing date.

surrounded by the use of minicircle DNA, since the latter is – as long as the sequence of interest is CpG-free – in total almost free of any CpG.

Various applications for the use of minicircle DNA have been presented since their invention (see Tables 7.1 and 10.1). Since the size of minicircles cannot be reduced further, we expect the positive modulation of their functionality.

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