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Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells

Research Article

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Abbreviations: Luria-Bertani media, (LB); origins of replication, (ORIs); population doublings, (PDs); prototype episome, (pEPI); scaffold/matrix attachment region, (S/MAR); stress-induced duplex destabilization, (SIDD)

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Summary

Nonviral replicating circular episomes are a rather new addition to the field of mammalian expression vectors. After their establishment, which conventionally requires an initial phase under selection pressure, these entities utilize the replication apparatus of the host cell to replicate in accord with the cell cycle. The requirements of a selection agent, the gradual inactivation by cellular defense mechanisms, and a limited cloning capacity (up to 5 kb could be realized for the prototype) have remained the critical parameters. Here we introduce a site-specific recombination-based strategy that permits the excision of prokaryotic vector parts after the parental construct has been amplified as a plasmid. The remaining 4 kb ´minicircle´ consists of only one active transcription unit and a scaffold/matrix attachment region (S/MAR). In contrast to the parent plasmid vector it can be established in the absence of selection, it is not subject to epigenetic silencing and it replicates stably without a sign of integration. In further contrast to available minicircles that are maintained only in non-dividing tissues our minicircle represents the first example that is suited for the modification of dividing cells and tissues due to its association with the nuclear matrix and its authentic segregation.

I. Introduction

Gene therapy is dedicated to the treatment or prevention of disease through gene transfer. To this end, several methods are explored based on viral vectors or "naked" DNA. Viruses have the natural inclination to invade human cells and deposit their genome in the nucleus. They would be the preferred vectors for applications in gene therapy in the absence of distinct drawbacks: viruses may trigger the immune system and some of them interfere with the expression of essential genes by integration. Although the past decade has brought vector technology a long way from the early days of using wild-type viruses, even today the associated problems could not be fully resolved and this is one reason that alternatives gain increasing attention. As a potential solution episomes have emerged. However, until recently the only replicating episomes were of viral origin and needed viral (and thereby oncogenic) factors for their propagation (Bode et al, 2001).

A more general problem goes back to the fact that eukaryotes have evolved elaborate defense systems to protect the integrity of their genomes and to fight the expression of ectopic transcription units. In mammals, the insertion of retroviral DNA, the incorporation of repeat arrays and the co-introduction of prokaryotic vector parts are the major triggers of transcriptional silencing processes. In case of retroviruses it has been suggested that the cell recognizes structural features of integration intermediates. Additional defense strategies go back to the fact that dinucleotide frequencies in mammals differ from

those of other organisms, especially regarding the abundance of CpG dinucleotides. In general, the DNA of higher eukaryotes is impoverished in these motifs relative to bacteria, for which the abundance is in accord with statistical expectations. Most silencing processes are accompanied by the methylation of CpGs, which may be preceded by histone H3 methylation at Lys-9 (Fuks et al, 2003). A methylation center in turn can trigger chromatin condensation spreading to a downstream promoter to provide it with a heterochromatin-like structure – at least in the cases where such a process is not blocked by an intervening insulator element (Goetze et al, 2005).

Typically, a high level of transgene expression is detected shortly after DNA has been delivered to target cells, but this expression is silenced, within a few weeks, even though vector DNA may remain in an extrachromosomal state. The short duration and the shutdown of transgene expression are important limitations that have to be overcome for many potential clinical gene therapy applications. We and others have applied chromosome-based and epigenetic principles for the optimal utilization of the transcription and replication apparatus of mammalian cells (review: Bode et al, 2003; Jackson et al, 2006). According to this concept, transgenes are introduced in the form of an autonomous domain, which, in its extreme, is a circular, nonviral episome with a single domain boundary (S/MAR).

One of the fundamental properties ascribed to S/MARs is their strand-separation potential (Bode et al, 1992, 2006), which is the likely reason for the fact that these elements are regularly found in association with origins of replication (ORIs). This ORI-support capacity has been exploited to develop pEPI, one of the first examples of a plasmid-based episomal vector that replicates extrachromosomally (Piechaczek et al, 1999). Available evidence indicates that this vector class recruits, via the huIFN-β 5´ S/MAR, components of the cellular replication apparatus to support an authentic segregation (Baiker et al, 2000). Following these pilot studies we have started to refine the system by reducing its size to the absolutely required minimum. We demonstrated that for pEPI most sequences apart from the (correctly oriented) *egfp* gene and the S/MAR element are not required for episomal maintenance and expression (Nehlsen, 2004) and that a largely functional S/MAR can be assembled from 150 bp modules (Jenke et al, 2004, Bode et al, 2006). The latter plasmid performed replication comparable to pEPI but it did not express the *egfp*-gene to any measurable extent – possibly due to the fact that transcription of the 150 bp repeats leads to mRNA instability.

Here we resume these efforts by the generation of "minicircles" *via* a deletion of prokaryotic sequences after the vector has been amplified, as a plasmid, in a bacterial producer strain. The deletion comprises the resistance marker, which, in case of the original pEPI-vector, is essential for establishing the plasmid in the recipient cell (**Figure 1** and Papapetrou et al, 2006). Although convenient and efficient, such a selection routine would not be compatible with most gene therapeutic regimens. We will demonstrate that the deletion strategy supports the establishment and maintenance of functional, replicating episomes in the absence of selection pressure even in rapidly dividing cells. It also overcomes the rapid epigenetic inactivation, which presents a major impediment to the application of the parent plasmid-type "pEPI"-vector. These and related properties of the minicircle will be demonstrated exemplarily for three prototype cell lines (CHO – transformed chinese hamster ovary cells; HEK293 - human transformed primary embryonal kidney cells; NIH3T3 – immortal but non-transformed clone from mouse embryonic fibroblasts) where it can be established in the absence of any selection pressure.

II. Materials and methods

A. Plasmids and strains

Escherichia coli strain MM294Flp (MM294 (CGSC #6315 (294-FLP: F^-, λ^-, supE44, endA1, thi-1, hsdR17, lacZ:cI857-FLP)) (Buchholz et al, 1996) was kindly provided by Francis Stewart (University of Dresden): Flp recombinase gene under the control of λP_R-promoter was inserted into the bacterial lacZ gene using the gene replacement technique as described by Buchholz et al (Buchholz et al, 1996).

B. Minicircle production and purification

A single colony of *Escherichia coli* MM294Flp was transformed with the maxicircle (**Figure 3A**) and grown overnight in a shaking incubator at 30°C in Luria-Bertani media (LB) containing 25 mg/ml Kanamycin. Cells were pelleted at 4000 rpm before resuspension in 4:1 (v/v) LB. After washing, cells were re-pelleted at 4000 rpm and resuspended in 2:1 (v/v) fresh LB. Flp expression was initiated by incubation at 40°C for 20 min. Incubation of bacteria was continued for 2.5 h at 35°C in a shaking incubator (180 rpm). This period was succeeded by a second initiation step at 40°C for 20 min and incubation was continued for an additional 1.5 h at 35°C.

The superhelical status of the vector turned out to be a relevant parameter for episomal establishment and therefore various procedures, CsCl gradient centrifugation, Qiagen mediprep system and Capillary Gel Electrophoresis, were initially explored for its preparation. It was found that all three procedures could be applied with similar success in the context of our protocol. For present work the pool of DNA products was digested by *Hin*dIII, which linearizes the maxicircle and the miniplasmid but not the minicircle. Undigested supercoiled minicircle could then be separated from the linearized maxicircle and the bacterial miniplasmid by agarose gel electrophoresis (**Figure 3B´**). The respective band was excised from the gel and the DNA was extracted using the Qiagen Gel Purification Kit. A further purification step, the application of ATP dependent nuclease, could be applied to free the gel-extracted minicircle from nicked or linear contaminants (**Figure 3B´´**). To this end 42 ml of the extract were provided with 5 ml 10×Plasmid Safe™ reaction buffer, 2 ml of 25 mM ATP and 1 ml Plasmid-Safe™ ATP-dependent DNAse (all materials from Epicentre / Biozym Scientific GmbH). After shaking (37°C) supercoiled DNA was recovered by the QIAquick PCR purification kit according to the manufacturer´s instructions.

C. Cell culture

-*NIH 3T3* cells (ACC59; population doubling time 20 h) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 20 mM glutamine, 60 μg of penicillin/ml, and 100 μg of streptomycin/ml.

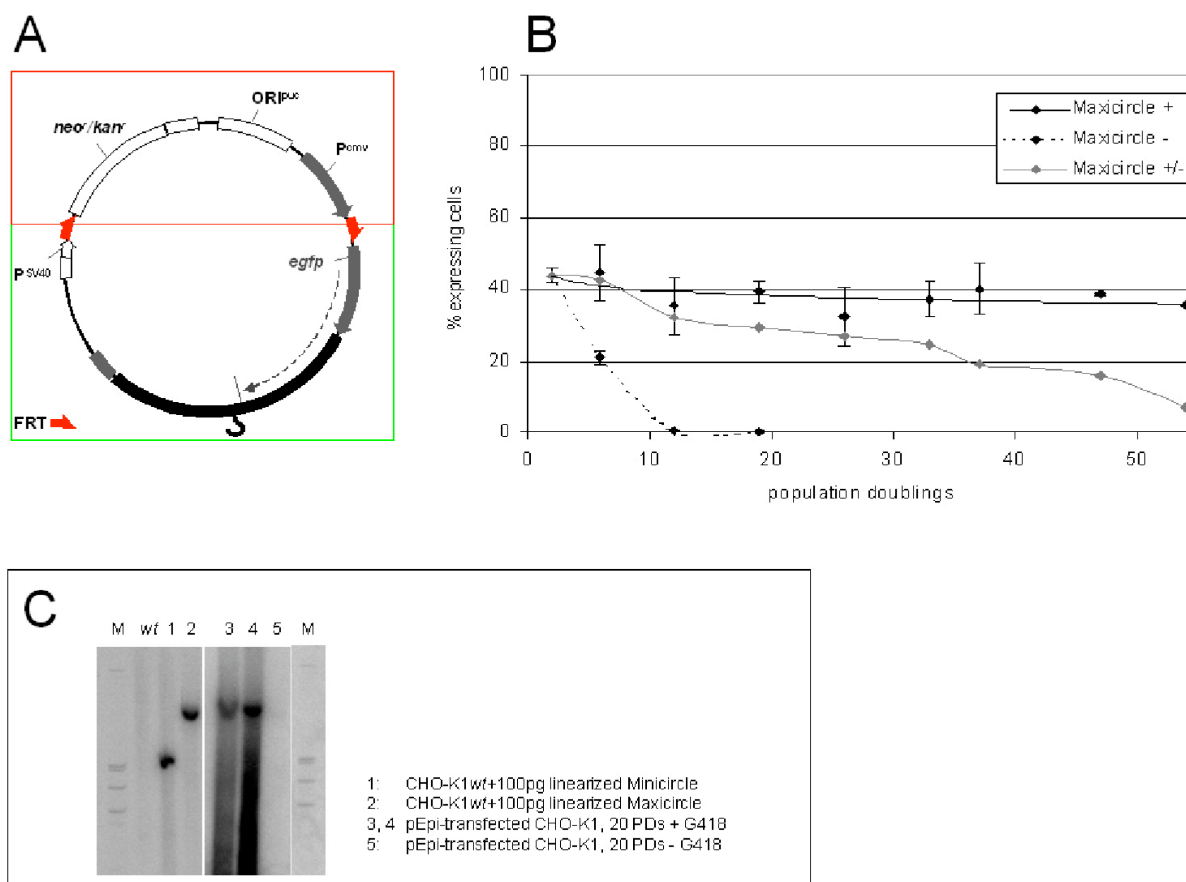

Figure 1. Performance of a 6.4 kb plasmid as replicating episome. A. Constitution of the parental vector. The function of the well-characterized pEPI-vector (here called "maxicircle") depends on a S/MAR (here the 2 kb element upstream to the human interferon-b gene, symbolized by the hook symbol) and a transcription unit (here: *egfp*). Transcription has to traverse part of the S/MAR as indicated. The role (if any) of the second transcription unit (*neo*^{r}) and of the plasmid (pUC) origin (upper box) in episomal maintenance is one subject of this study. The basic vector has been provided with two identical 48 bp FRT wildtype-sites (half arrows) permitting the Flp-induced deletion of the intervening sequence, i.e. the conversion of the maxicircle into a minicircle and a miniplasmid (**Figure 3**). Abbrevations: P^{SV40}, SV40 promoter/enhancer driving the neomycin/kanamycin encoding gene as a selection marker for mammalian cells or E. coli, resp.; ORI^{puc}, plasmid origin of treplication; P^{cmv}, CMV promoter driving the *egfp* coding unit; FRT, full (48 bp) Flp-recombinase target sites. **B. Maxicircles are lost in the absence of selection**. After lipofection according to the GenePorter^{TM} protocol the persistence of maxicircles strictly depends on selection in G418 (500µg/ml) (compare traces "Maxicircle +" and "Maxicircle – "). Minor expression levels of the *neo*^{r}/*kan*^{r} unit are known to suffice for G418 resistance. Among the resistant cells 40% also express measurable levels of *egfp* as indicated. If an initial selection period is discontinued after 12 population doublings ("Maxicircle +/-") a decrease of expression levels and of episomal persistence is noted at a rate that is largely reduced relative to the "Maxicircle -" case. **C. Dependence of pEPI-type maxicircles on selection.** Lanes 2 contains a loading control, i.e. 100 pg of linearized episome DNA in the presence of 4 µg genomic DNA from non-transfected CHO K1 cells. Lane 1 is a corresponding control for the minicircle. Lanes 3 and 4 (taken from cells 20 PDs after lipofection) show that pEPI can be maintained if CHO-K1 cells are kept under selection pressure (+G418). Lane 5 demonstrates the total loss of the maxicircle after 20 PDs in the absence of the drug (-G418). Left and right panels show size markers, lane *"wt"* shows non-transfected ("empty") cells.

-*CHO-K1* cells (ACC110; population doubling time 24 h) were cultured in a 1:1 mixture of Nut. Mix F12 (HAM) medium with GlutaMAX (Gibco) and Dulbecco's modified Eagle's medium which were both supplemented with 10% fetal calf serum, 20 mM glutamine, 60 µg of penicillin/ml, and 100 µg of streptomycin/ml.

-*HEK293* cells (ACC305; average population doubling time 24 h) were cultured in Minimal Essential Medium containing Earle's salts supplanted by 20 mM glutamine, 60 µg of penicillin/ml, 100 µg of streptomycin/ml and 10% fetal calf serum.

D. Transfection

Since electroporation was found to seriously interfere with the superhelical status and thereby to promote integration into the genome, we optimized a lipofection protocol as this method generated the highest proportion of cells for which expression was exclusively due to the episome and not accompanied by inadvertent integration events. For mini- and maxicircle-transfer to 5×10^4 cells on a 6-well culture plate 1 µg of DNA was diluted to 50 ml by 'DNA diluent' and left for 5 min at room temperature. In a different vial 10 µl of GenePORTER^{TM2} -reagent (Genlantis) were mixed with 40 µl of serum free medium. The DNA-solution was added, without vortexing, to the GenePORTER^{TM2} -solution. After a 10 minute incubation at

room temperature the mixture was carefully pipetted onto the cells with 1 ml of serum free medium. After 4 hours an additional ml (containing 20 % FCS) was added to reach a final concentration of 10 % FCS. Medium exchange was performed the following day and selection with G418 (CHO-K1: 500 µg/ml; NIH3T3: 700 µg/ml) was applied where applicable.

E. FACS analysis

The intrinsic fluorescence of the GFP-protein is used for the analysis of expression levels in living cells. To this end non-confluent cells were trypsinized and collected in EPICS (PBS, 10 % heat inactivated FCS) buffer. Cells were collected by centrifugation (5 min, 1000 rpm) in a Heraeus-Christ minifuge before they were diluted to 1×10^7 ml in EPICS-buffer. Propidium iodide (2 mM) was used to stain and exclude dead cells. eGFP fluorescence was excited by irradiation at 488 nm. Sorted cells were kept for four days in the presence of Gentamycin (5 µl/ml of a stock containing 10 mg/ml).

F. Localization and episomal status of transgenes

FISH-analysis: Cells were grown to 60-80 % confluence and split one day before the preparation of metaphase spreads. Colcemide was added to a final concentration of 40 ng/ml medium and the culture was subjected to a 4 hour incubation at 37°C. After trypsinization the cell pellets were incubated in 2M NaCl/KCl (1:1) for 1 minute, centrifuged at 1200 rpm for 5 minutes, fixed three times in MeOH/Acetic acid (3:1) and incubated overnight at 4°C. An additional three fixation steps with MeOH/Acetic acid were performed by applying the solution on pre-cooled slides. Hybridization of the slides was done with a labelled nick-translated pEpi-plasmid DNA probe using SpectrumRed (Invitrogen) and counter stained with 10 µl of DAPI (0.187 µg/ml in Vectashield mounting medium).

G. Southern blot analysis

High molecular weight DNA was harvested from 1×10^6 cells and digested with the respective restriction enzyme. The genomic as well as extrachromosomal DNA was then separated on 0.8 % agarose gels, blotted and hybridized with a ^{32}P-labeled SV40-DNA probe.

F. Epigenetic reactivation experiments

Cells were seeded at a densitiy of 1×10^5 and incubated in medium containing either Butyrate (5 mM), TSA (165 nM) or 5-AzaC (24µM). Reactivation of eGfp-expression is determined by FACS-analysis after 48 hours.

III. Results

A. General properties

The prototype episome (pEPI) does not depend on any viral factor and it divides in synchrony with cellular replication (Schaarschmidt et al, 2004). Its function depends on a S/MAR element by which the replication apparatus of the host cell can be recruited and utilized (see **Figure 1A**). Authentic segregation into daughter cells is supported by the S/MAR´s capacity to act as a maintenance element (Bode et al, 2001). Beyond these properties S/MARs are proven tools to restrict epigenetic silencing via DNA-methylation/histone deacetylation (Dang et al, 2000).

Previous experiments have shown that in this case the S/MAR function depends on its (at least partial) transcription, which may support its conversion to the single-stranded state. For pEPI an artificial termination site has been localized within the 2 kb S/MAR sequence after 800 bp (**Figure 1A** and Nehlsen 2004). In addition, the direction of transcription was shown to matter: if the *egfp* transcription unit was inverted by the use of Cre recombinase in combination with two inversely oriented lox P sites, only the original orientation was maintained as an episome whereas the inverse one was lost without indications for an integration.

Meanwhile pEPI-type vectors have emerged as potential tools for applications in gene therapy and their performance has recently been evaluated for dividing cells of the haematopoietic system (Papapetrou et al, 2006; review: Papapetrou et al, 2005). These studies show that, in the absence of initial selection, at most 1% of replicating cord blood cells retain the vector after 28 days suggesting that it is poorly maintained in progeny cells. In fact, a selection step has to be applied for establishing this episome, which may be compatible with the modification of cells *ex vivo* but not *in vivo*. Our data in **Figure 1B** support these principles by using rapidly dividing CHO cells, for which most data of pEPI-type vectors have been derived. In our model experiment the vector is lost during 12 population doublings (PDs; see **Figure 1B** and lane 5 in **Figure 1C**). On the other hand, an initial selection in G418 is sufficient to enrich a subpopulation of cells that continues to propagate even in the absence of the drug, although, under these conditions, a >50% loss of expressing cells is observed over 50 PDs ("Maxicircle +/- "). A stable subpopulation of cells can finally be obtained if transfected cells are selected in the permanent presence of 500 µg/ml of G418 (**Figure 1B**, trace "Maxicircle +").

Over some years our experiments have indicated an inverse relation between episome size and –stability, especially regarding the superhelical state during freezing-thawing cycles, which frequently caused problems for the maxicircle (S. Broll, unpublished; see Discussion). To improve this situation we started different approaches such as the design and construction of a minimal S/MAR element that could be obtained by oligomerizing a S/MAR-module, a so called ´unpairing element´ (UE; see Jenke et al, 2004), or the deletion of sequences that are exclusively needed for plasmid amplification in bacteria. For present work we decided to leave the S/MAR element constant since artificial S/MARs with repetitive sequences caused unexpected complications regarding *egfp* expression (Nehlsen, unpublished). As a consequence, we will compare below a pEPI-like ´maxicircle´ with a ´minicircle´ that is the result of excising all sequences that are only required for producing the plasmid precursor.

B. Scaffold/Matrix attachment regions (S/MARs)

All S/MARs, whether they are located within a chromatin domain or at its borders, share a common criterion: they consist of a more or less regular succession of DNA-unpairing elements at which the double strand separates under negative superhelical tension (Bode et al, 2006). These UEs together constitute the architecture that

is required for the accommodation of prototype nuclear matrix proteins (Bode et al, 2003). This feature is illustrated by the SIDD (stress-induced-duplex destabilization) profiles in **Figures 2A and 2B,** which are routinely recorded for the negative superhelicity that is typically present in a plasmid (Bode et al, 2006). The coding region (*egfp*) has no propensity to separate strands, in contrast to the transcriptional termination site, which is highly destabilized. Previous contributions have demonstrated that these are common features found for any gene and have revealed the functional background of such an architecture (Bode et al, 2006). In this respect it is intriguing to note that the minicircle is destabilized over its entire length with the sole exception of the *egfp*-tract.

C. Minicircles generated in bacteria

An E. coli strain (MM294Flp) with the Flp-recombinase gene under the control of a heat-inducible promoter (Buchholz et al, 1996) was kindly provided by Francis Stewart (University of Dresden). We applied this system for the amplification of the 6.4 kb pEPI-derivative in **Figure 3A**, which had been provided with equi-directed FRT-sites (half-arrows) and for which the *egfp* coding unit was promoter-free. This setup guarantees that the only specimens expressing eGFP will be those that underwent excision and it overcomes any ambiguity that could be ascribed to remainders of the educt.

The Flp-mediated recombination between the FRT sites was triggered by a shift to 40°, which served to eliminate the intervening sequences and to pose the *egfp* unit under the control of the SV40 promoter. As a result, a fluorescent 4.1 kb episome (´minicircle´) and a 2.3 kb

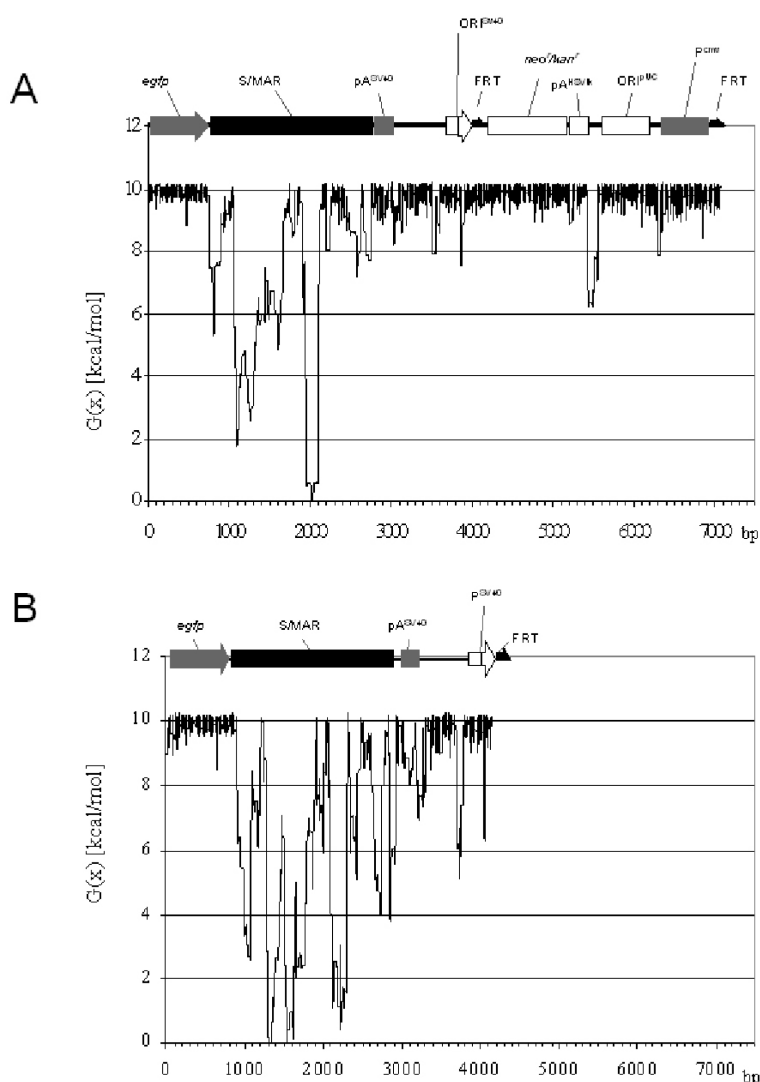

Figure 2. Molecular components necessary for episomal replication: Structural analyses. The molecular constitution and stress-induced duplex destabilization (SIDD-) profiles are shown for the 6.4 kb parental plasmid from **Figure 3A** (**A**) and for the minicircle (**B**). A value G(x)=0 kcal/mol would mean strand separation at the respective site under a standard superhelix density of σ = -0.05 (Bode et al, 2006). Note that the minicircle is destabilized throughout with the exception of the *egfp* coding region.

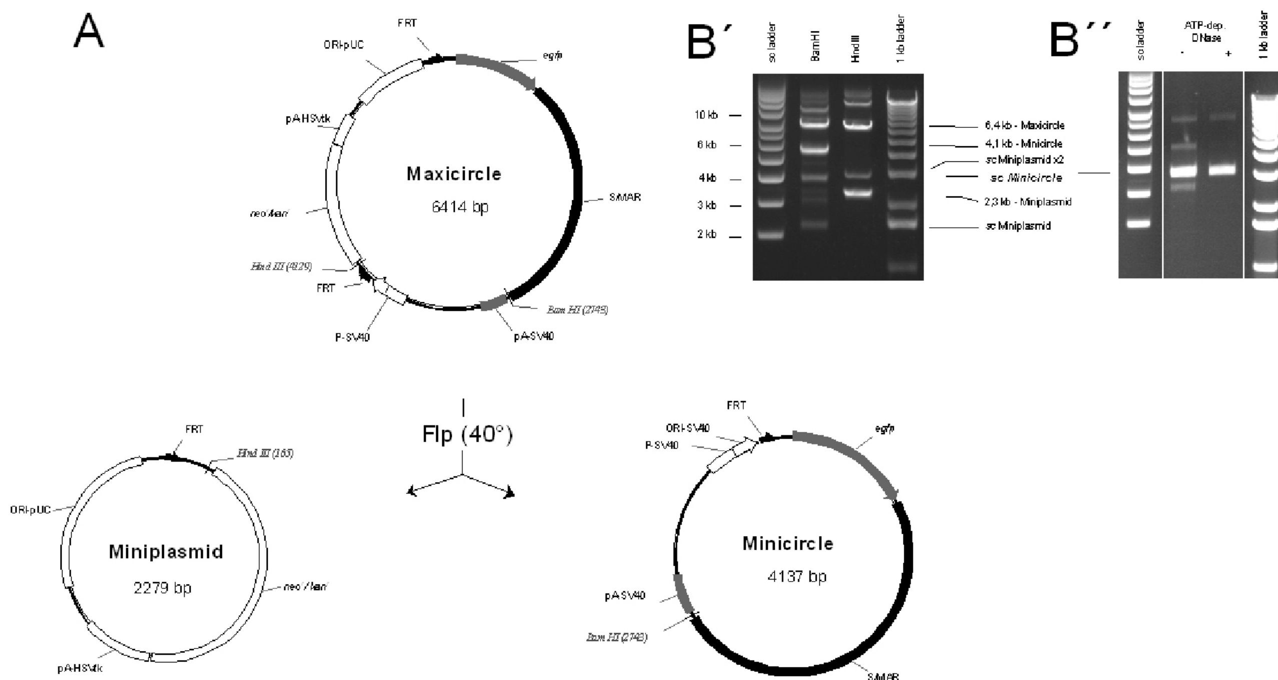

Figure 3. Generation of the minicircle by Flp-mediated recombination A. The principle. Flp recombinase is induced in *E. coli* strain MM294-Flp by the temperature shift cycle (30° → 40° → 35°) described in "Materials and Methods". The "minicircle" is generated from the eukaryotic sequence parts (lower box in **Figure 1A**) and a "miniplasmid" from the plasmid parts. This process places the *egfp* reporter gene under the control of the SV40 promoter. Abbreviations other than those **in Figure 1A**: PA-SV40 and PA-HSV-tk: polyadenylation signals derived from SV40 or the HSV-tk gene, resp.. **B. Analyses (B´)** The reaction mixture is treated with *Hin*dIII (lane 3) whereby the educt and the miniplasmid are linearized and converted to substrates of ATP-dependent DNase; the supercoiled minicircle remains unaffected. For analytical purposes lane 2 shows a digest by *Bam*HI which linearizes all species except the miniplasmid. **(B´´) Supercoiled minicircles after extraction from an electrophoretic gel; the effect of ATP-dependent DNase**. The lane marked "-" shows (from top to bottom) traces of a *sc* minicircle-dimer, the *lin* minicircle, the *sc* minicircle and traces of *lin* miniplasmid.

´miniplasmid´ were generated (**Figure 3A**). While the minicircle contains the S/MAR and the *egfp*-tract, the miniplasmid carries the prokaryotic sequences together with the *neo*^{r}/*kan*^{r} selection gene. This situation is analyzed in **Figure 3B´** after digestion with *Hin*dIII (single cut in the parental construct and the miniplasmid leaving a supercoiled minicircle) and with *BamH*I (single cut in the parental plasmid and in the minicircle).

Figure 3B´´ demonstrates that superhelical, circular DNA can efficiently be purified by ATP-dependent DNase (Wilcox et al, 1976). Under standard reaction conditions this enzyme rapidly degrades duplex linear DNA. It also utilizes energy from ATP hydrolysis to move along the DNA and to unwind regions of the molecule, releasing large partially or totally single-stranded fragments on which it acts as an endonuclease; duplex circular DNA is not a substrate (Wilcox e t al, 1976)). In our example we demonstrate the ultimate purification of a minicircle that has been separated by gel electrophoresis, followed by extraction using the QUIAquick procedure. Alternatively, crude plasmid DNA was treated with *Hin*dIII as for **Figure 3B´** (trace 3) and all DNAs except the minicircle were directly removed by ATP-dependent DNAse. All following experiments are based on the first variant in order to suppress any kind of integration that might follow the transfer of linear DNA remainders.

Figure 4A compares situations in which either the maxicircle (**Figure 1A**) or preparations of the minicircle were transferred and analyzed at various time points. Starting with a 40-45% contribution of fluorescent cells (lipofection transfer efficiency) five population doublings (PDs) were allowed for the establishment of episomes before fluorescent cells were recovered by FACSorting. Detailed analyses started after 12 PDs, at which time a functional minicircle was left in 70% of the cells while fluorescent maxicircles persisted in less than 10% of the cell population. During the subsequent 40 PDs the minicircles showed a stable propagation whereas the fate of the maxicircles strictly depended on the treatment of cells: **Figure 4A** demonstrates a close to complete loss in the absence of selection while continued selection in G418 media led to the enrichment of a subpopulation in which both the *egfp* and the *neo*^{r} cassette were expressed.

Essentially similar results were obtained for human embryonic kindney (HEK293; **Figure 5A**) and murine NIH3T3 cells (**Figure 5B**). For HEK293 cells the minicircle is seen to yield a broad though stable population between 32 and 60 PDs whereas the maxicircle-transfected cells show a continuous drift to lower expression levels.

This drift can be reversed, to a large extent, by the addition of (R)-Trichostatin A (TSA), an established inhibitor of histone deacetylases that permits histone (re-)acetylation (Schlake et al, 1994). The same treatment for the minicircle population leaves the FACS-profile unchanged indicating that only the maxicircle is subject to epigenetic inactivation. NIH3T3 cells, an immortal but non-transformed, contact inhibited cell line, reveals the most clearcut differences between the systems: while the minicircle is stably expressed between 32 and 55 PDs, the maxicircle has undergone an almost complete shutoff already at 32 PDs.

The copy number of pEPI-type vectors is low (Baiker et al, 2000) but stably maintained during cell divisions (Schaarschmidt et al, 2004). In **Figure 6** we show FISH analyses comparing the properties of maxi- and minicircles. For the minicircles we consistently find sharp fluorescent spots in association with the metaphase chromosomes. The same is true for the majority of maxicircle-containing cells but there are notable exceptions, where intense doublets on both chromosome arms indicate occasional integration events of the plasmid-type vectors during continued cultivation. An example is given in the upper right section of **Figure 6**.

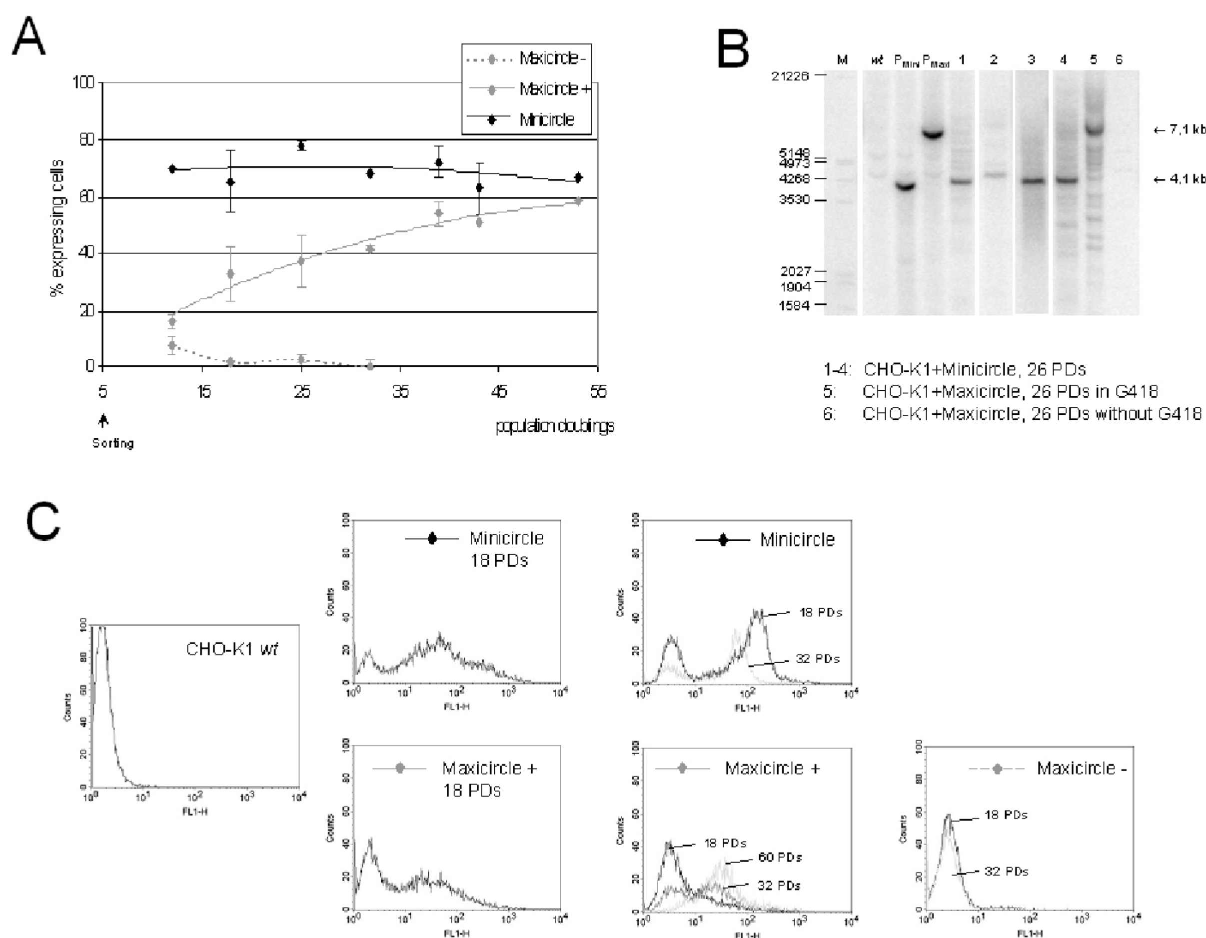

Figure 4. Long-term expression of replicating episomes in CHO-K1 cells after a single FACS-enrichment of eGfp-expressing cells. A. After a 5-days period of ´episome establishment´ fluorescent cells are recovered by FACSorting. Measurements start at day 12 when 65% of fluorescent cells are left for the minicicle, and 3% for the maxicircle (here: the pEPI-vector shown in **Figure 1A**). If the latter population is kept under selection pressure (G418) the fluorescent subpopulation becomes dominant and reaches 60% after 53 population doublings. In case of the minicircle selection is neither possible nor required as the population is perfectly stable at the 65% level over the entire time interval. **B.** Southern blot-analysis for episomally replicating mini- and maxicircles in CHO-K1-cells. Lanes 1-4: Minicircle from four separate transfection experiments of the minicircle after 26 PDs and linearization with BamHI. Lanes 5, 6 corresponding analyses for the maxicircle kept for 26 PDs in the presence or absence of G418, respectively. Size marks indicate the 7.1 kb pEPI-vector and the 4.1 kb minicircle derived from the pEPI-derivative as shown in **Figure 3A**; the corresponding lanes "P_{Mini}" and "P_{Maxi}" are loading controls i.e. 100 pg of linearized episome DNA in the presence of 4 µg genomic DNA from non-transfected CHO K1 cells, lane "wt" shows just the genomic DNA. **C**. Episomes (mini- or maxicircles as indicated) in CHO-K1 cells were analyzed in two parallel transfection experiments after 18 or 32 population doublings (18 PD or 32 PD, resp.) in the absence of selection pressure ("Minicircle" / "Maxicircle –") according to section A. Both experiments demonstrate a faster inactivation of the maxicircle. If selection pressure is applied from the time of sorting (5 PDs) on (situation "Maxicircle +"), an expressing population emerges that approaches the level of the minicircle population (see the continuous shift from trace 18 PD to 32 PD and 60 PD).

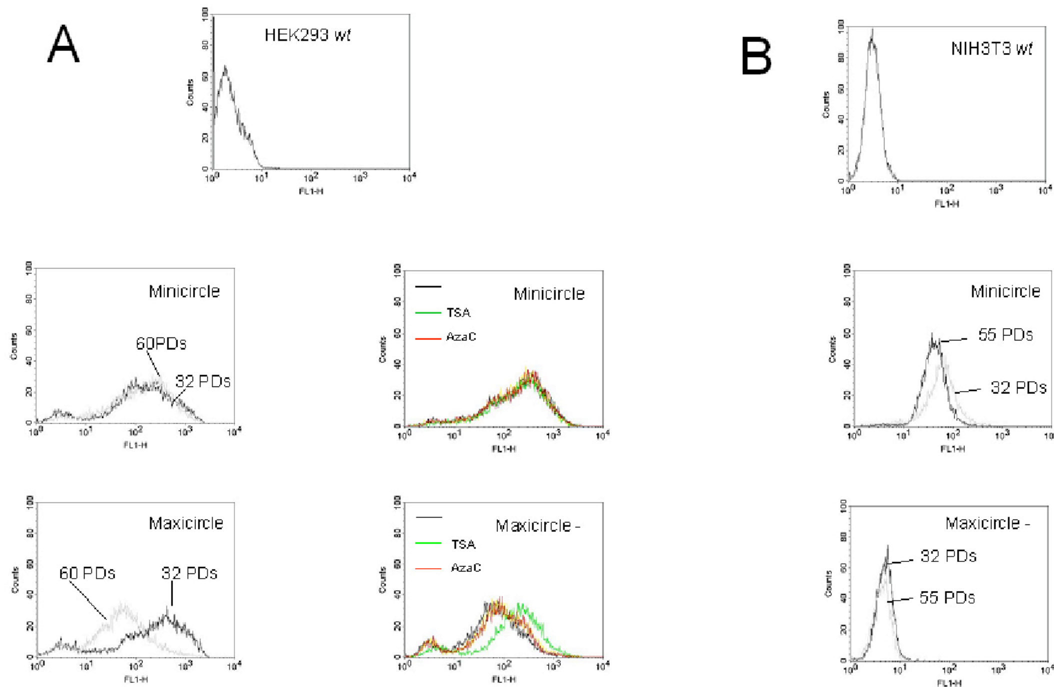

Figure 5. Persistence and expression of plasmid vectors and minicircles in two other cell lines. A. Analyses corresponding to **Figure 4** but for HEK293 cells. For the left-hand transfection experiments minicircles and maxicircles were analyzed after 32 and 60 PDs. After 60 PDs cells were subjected to treatments with either 165 nM (R)-Trichostatin A ("TSA") or 24 mM 5-Aza-cytidine ("AzaC") as indicated and re-analyzed after an additional 48 h in the presence of these drugs. **B.** NIH3T3 cells: FACS-analyses for mini- and maxicircles after 32 and 55 PDs. For the minicircle the profiles remain nearly unchanged in this interval. For the maxicircle a complete shutoff is noted already after 32 PDs.

IV. Discussion

While there is significant progress in the modification by episomal DNA of slowly-dividing tissues like liver, muscle and brain, maintenance problems have so far limited the use of nonviral episomes for dividing cells, for instance of the hematopoietic system (Papapetrou et al, 2005, 2006). For liver, the most advanced vehicles appear to be "minicircles", small circular vectors that are exclusively composed from eukaryotic sequences. In contrast to linear DNA, minicircles do not concatemerize and are less prone to integration. It is also known that, owing to their superhelical status, they are better transcriptional templates than linear DNA (Weintraub et al, 1986).

Based on this rationale M. A. Kay and coworkers could demonstrate that transgene expression levels in non-replicating minicircles are not only 45-560 fold higher but also more persistent compared to conventional plasmids (Chen et al, 2003; Riu et al, 2005). The authors applied a critical test to prove the episomal state of these vectors, i.e. a 2/3 hepatectomy upon which almost every hepatocyte undergoes one or two cell doublings until the liver mass is reconstituted. It was shown that during cell cycling the minicircles were lost in accord with their non-integrated (episomal) status (Chen et al, 2001). The results clearly demonstrate that this class of vectors is not functionally attached to chromosomal DNA, which would otherwise provide the required centromere function (Bode et al, 2001) and they anticipate the category of problems that have to be overcome if episomal vectors are to be used for the modification of proliferating cells. To be effective it is required that the new genetic material not only replicates but that it is also actively retained through cell division and passed on to daughter cells. These considerations have set the stage for the present study.

In past work we have already defined the essential components of an episome that replicates once per cell cycle (Schaarschmidt et al, 2004), i.e. an active transcription unit and a S/MAR while the SV40 origin function was found to be dispensable (Nehlsen 2004). In a computer-assisted way analogous to **Figure 2** S/MAR elements were designed such that they can accommodate components of the nuclear scaffold / nuclear matrix, among these scaffold-attachment factor A (SAF-A / hnRNP-U; Jenke et al, 2001, 2004). These interactions mediate the association of the vector with the chromosome arms enabling an effective segregation into the daughter cells (maintenance function, see Bode et al 2001). Other established S/MAR functions are the capacity to reduce epigenetic silencing and to promote histone hyperacetylation (Klehr et al, 1992). Interestingly, the performance of S/MARs can be boosted by the application of histone deacetylase inhibitors such as (R)-Trichostatin A (TSA), butyrate (Schlake et al, 1994) or by certain derivatives (e.g. phenylbutyrate) that have found use for therapeutical applications (Gore et al, 1997). These activities depend – at least in part – on S/MAR

conformational changes that are brought about by a nearby active transcription unit (see **Figure 1A**).

Our present series of experiments expands the knowledge about the essential vector components. It has been shown before that a S/MAR that is at least partially traversed by the transcription machinery is essential while either the deletion (Baiker et al, 2000) or the inversion (Nehlsen, 2004) of the transcription unit lead to integration. Here we show, for the first time, that minicircles but not maxicircles give raise to a stable population of cells as long as they contain a single active gene (*egfp*) and the S/MAR (**Figure 4A**). Together these results prove that, while a second transcription unit (here: neo^r/kan^r) is compatible with the episomal status, it is not required as it can be deleted together with the prokaryotic vector parts (including the pUC origin of replication, ORIpUC). The resulting minicircles provide an increased cloning capacity, which according to preliminary observations may be as high as 7 – 8 kb and even higher in cases the subunits of a protein can be encoded by separate episomes (Nehlsen, 2004). They also have an improved long-term- (**Figure 4A**) and physical stability (transformed cells resist multiple freezing-thawing cycles; S. Broll, unpublished). Most important, however, they can be transferred into the dividing cells and established in the absence of any selection pressure, meeting a major requirement of gene therapeutic applications.

The criteria that are sometimes used to establish the episomal status are subject to considerable contention. Among these is **i -** a full-length PCR amplification, which would give the same result in case the transgenes had integrated as a head-to-tail multimeric concatemer – a typical concomitant of the classical Ca^{++}-phosphate transfection procedure. **ii -** A clear-cut Southern-blot signal is a more stringent criterion as additional bordering fragments would arise in case of integration. Inspecting **Figure 4B**, we can state that, considering the low copy number in our clone mixtures, background-signals are negligible for the minicircles (lanes 1-4 in **Figure 4B** refer to four independently prepared clone mixtures, lanes 5 and 6 exemplify the maxicircle). Where present, most of this background arises during the establishment phase as a probable consequence of some non-superhelical contaminants. **iii -** The common extraction procedure according to Hirt leads to the enrichment of non-integrated DNA - at least at early passages. The efficiency of this protocol decreases with time since continued rounds of replication can give raise to extrachromosomal chains (concatenates) even in case of the viral systems (Klehr and Bode, 1988). **iv -** A plasmid-rescue, i.e. a re-transfer of circular episomes from CHO cells to E. coli, has been suggested as yet another criterion. This procedure is not feasible in our case since the present concept demands that minicircles do not contain the necessary bacterial DNA components. Even more important, it may be ambiguous again since integrated concatemers may generate circular specimens due to intramolecular recombination (Wegner et al, 1989).

For these reasons we have put emphasis on the FISH-visualization of transgenes on metaphase spreads, which had proven its potential before (Baiker et al, 2000). In this approach we either get multiple sharp spots in association with the chromosomes when we have to deal with intact episomes; this association is lost if the preparation involves shear forces (Baiker et al, 2000). Alternatively, we find a single intense signal indicating the typical co-integration of multiple copies immediately subsequent to DNA transfer (Baiker et al, 2000). In our present series of experiments (**Figure 6**) we find the first situation. For the maxicircle there are some exceptions where an additional intense doublet of spots (one on each chromatid) indicates integration events that happen during continued cultivation and replication.

In accord with current concepts (Chen et al, 2003, Riu et al, 2005) all our results suggest that the stability of the replicating minicircle can be ascribed to the absence of prokaryotic vector parts. The observation (**Figure 5A**) that an epigenetic re-activation by TSA is effective for the maxicircle (pEPI) but not for the minicircle is in accord with this explanation. We have to mention, however, that another difference exists between the episomes that we compare in **Figure 4**: the *egfp*-unit is driven by the CMV promoter in the maxicircle (pEPI) but by the SV40 promoter in the minicircle. This difference permitted our deletion strategy and the detection of fluorescence arising from this process (**Figure 3A**). Even more important, however, maintenance of the SV40 unit was dictated by the fact that nuclear transfer of plasmid DNA is facilitated by the association of ubiquitous transcription factors with this sequence and the subsequent exposure of their NLS signals (Vacik et al, 1999). This study also demonstrated that the CMV promoter is inactive in this respect (Vacik et al, 1999). Therefore, if we had chosen to drive the *egfp* unit by the CMV promoter in both cases, facilitated nuclear pore passage would have been abolished for the minicircle but maintained for the maxicircle where P^{SV40} drives the selection gene. A completely different series of experiments would have to be developed to trace promoter-specific susceptibilities to epigenetic silencing. We do not anticipate this kind of promoter-specific effects, however, since both the CMV- (Grassi et al, 2003) and the SV40-sequences (Broday et al, 1999) are subject to methylation-dependent inactivation.

While one transcription unit is sufficient to mediate episomal maintenance, the example of pEPI-type vectors shows that a second transcription unit is at least compatible with such a status. Experiments with pEPI (**Figure 1A**) derivatives and two antibody chain genes in place of the *egfp*-unit, each controlled by a separate promoter, point into the same direction (Nehlsen, unpublished). A logical extension of our findings will therefore be the generation of a two-transcription unit minicircle devoid of plasmid sequences. In this case *egfp* will be the ´gene on duty´ that provides for the required conformational changes at the S/MAR. A second complete transcription unit, the ´gene of interest´, will be added at an upstream position. Cells containing this vector can be traced or isolated by FACS as in the present study, while the GOI is expressed in parallel. Again, this approach will require knowledge on the performance of promoter(-combination)s in the context of a replicating episome.

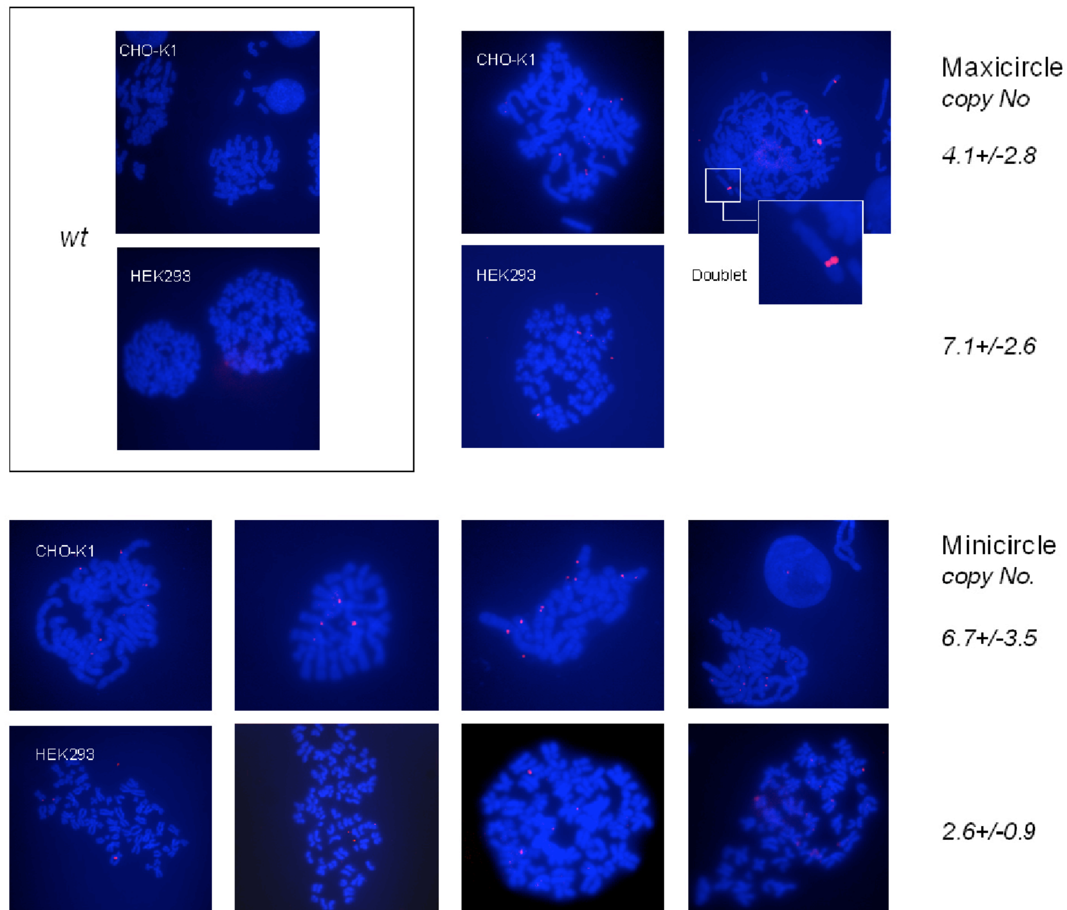

Figure 6. Copy numbers and status of maxi- and minicircles: FISH analyses. FISH-analysis were performed 55PDs after transfection (cf. the final situation in **Figure 4A**). The control (box with "empty" CHO-K1 and HEK293 cells) shows no signals. In contrast, the majority of transfected cells showed clear fluorescent signals. For the maxicircles there are single, chromosome-associated signals and, in about 40% of all cells, also intense doublets that cover corresponding positions on both chromosome arms and are therefore indicative of eventual integration. All minicircle preparations show signals throughout the metaphase spread and copy numbers that are comparable with the maxicircle situation. However, in the minicircle case there is no indication of integration events. Average copy numbers have been derived from 10-20 individual metaphase spreads and are given together with their standard deviation.

There are intriguing indications that multiple nuclear association sites may exist for the episomes, which vary in their properties. In the present study this has first become apparent during the **Figure 1B** experiments where we find a certain contribution of non-egfp expressing (but G418-resistant) cells. A similar phenomenon seems to hold for HEK293 cells (**Figure 5A**), where a narrow range of copy numbers (1-3 per cell) is associated with a wide range of expression levels (more than two orders of magnitude). On the basis of the FACS-profiles in **Figure 4C and 5** in comparison with the FISH analyses in **Figure 6**, it is therefore tempting to speculate that points of association are highly defined and maintained over many generations. Other classes of less appropriate sites may exist in the "transient expression phase" during which the non-functional sites are abandoned. In case of the minicircles this phase has terminated after 10 PDs or even before (**Figure 4A**). For maxicircles, on the other hand, active selection has to be applied in order provide a selective advantage for the rare subpopulation in which the maxicircle is propagated in an active state.

In summary, concepts have become available to improve plasmid-based, replicating episomes up to the stage where they support the predictable and long-term expression of transgenes also in dividing cells. These strategies will not only overcome detrimental effects of prokaryotic sequences but will also take into account the targeting capacity of S/MAR(-derivatives) or related elements by which subnuclear structures can be addressed for an optimized transcriptional capacity.

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