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Review

The epigenetic regulation of autonomous replicons

Claudia Hagedorn, Hans J. Lipps and Sina Rupprecht*

Institute of Cell Biology, ZBAF University Witten/ Herdecke, 58453 Witten, Germany

*Corresponding author
e-mail: sina.ronzon@nuigalway.ie; sina.ronzon@uni-wh.de; sina.ronzon@gmx.de

Abstract

The discovery of autonomous replicating sequences (ARSs) in *Saccharomyces cerevisiae* in 1979 was considered a milestone in unraveling the regulation of replication in eukaryotic cells. However, shortly afterwards it became obvious that in *Saccharomyces pombe* and all other higher organisms ARSs were not sufficient to initiate independent replication. Understanding the mechanisms of replication is a major challenge in modern cell biology and is also a prerequisite to developing application-oriented autonomous replicons for gene therapeutic treatments. This review will focus on the development of non-viral episomal vectors, their use in gene therapeutic applications and our current knowledge about their epigenetic regulation.

Keywords: epigenetic regulation; plasmid vector pEPI; replication; replicon origin.

Introduction

Over the last few years many clinical gene therapy trials with beneficial results for patients have taken place. However, during these trials severe problems became obvious with regard to safety aspects of the technologies used.

In most gene therapy trials being virus-based vectors, such as adenoviral and retroviral vectors, have been mainly used for the delivery of genetic material to the cells (1). Owing to their high transduction efficiency these vectors have been found to have broad applications in gene therapeutic treatments; even so, the appliance of these viral vectors is not without safety risks (2). Despite their steady improvement they can still lead to insertional mutagenesis when integrating into the host genome or can induce immunological reactions in the recipient organism. Therefore, the use of viral vectors in gene therapy approaches is recommended only after a careful risk-benefit analysis.

Non-viral, autonomous replicating vectors are considered to be a safe alternative but owing to their low efficiency they have not yet been used routinely in gene therapy trials (3). Therefore, it is of immense importance to understand the underlying regulatory mechanisms involved in the establishment, replication and stability of autonomous replicons. Recently, the relevance of epigenetic parameters, such as chromatin structure, interplay between transcription and nuclear localization, has been analyzed in various organisms demonstrating that epigenetic factors play an important role in regulating DNA replication in higher organisms. In budding yeast, origin licensing is determined by the binding of the origin recognition complex (ORC) to a specific DNA sequence, which has been identified as autonomously replicating sequence (ARS). In contrast, the primary sequence of DNA alone is not sufficient for origin licensing in higher eukaryotic cells. Metazoan origins exhibit an extended structure and replication can be initiated at several different locations within (4). Moreover, in these cells cell cycle-dependent alterations of histone modifications and nucleosomal remodeling have been shown to regulate pre-replication factor assembly. Furthermore, recent studies provide evidence that cellular differentiation of embryonic stem cells is accompanied by controlled changes in replication timing, transcription and nuclear localization (5).

Therefore, understanding the epigenetic regulation of replication is essential for the rational design of episomally replicating vectors. This should not only lead to vector improvement but might also provide new insights into the regulatory mechanisms of replication. In this review, we will first summarize the main characteristics of eukaryotic origins of replication followed by discussing recent experiments that address progress towards understanding the epigenetic regulation of replication. We will then focus on advances in the construction of autonomous replicons used as model systems in basic cell and molecular biology, as well as in gene therapeutic applications.

Eukaryotic origins of DNA replication

The origins of DNA replication are defined as genomic sites at which DNA replication is initiated during the S-phase. Almost 50 years ago Jacob and Brenner (6) suggested the replicon model: replication initiation occurs when a sequence-specific DNA binding protein, the initiator, binds to a defined DNA sequence, the replicator. This binding is followed by the recruitment of other factors required for unwinding DNA and initiating DNA replication. In bacteria, the protein DnaA binds to the bacterial origin of replication, which is defined by a specific DNA sequence, leading to local unwinding of the DNA. DnaA, together with DnaC, loads the replicative helicase DnaB onto the melted DNA

allowing replication to start (7). It has been assumed that the replicon model not only explains initiation in bacterial or some viral systems but also explains it in higher organisms.

The first eukaryotic origins of replication which were isolated from the yeast *Saccharomyces cerevisiae* resulted in autonomously replicating vectors. Thus, DNA sequences having this property were called ARSs (8). These sequences are approximately only 140 bp in size and consist of various modules, the A element or ARS consensus sequence (ACS) and the B elements (in the case of ARS1, B1 to B3). The most important element is the 11 bp ACS found in all ARSs. Mutation in any nucleotide of this 11 bp leads to a loss of function although the ACS is not sufficient for origin formation. The combination of A and B elements modulates origin strength and efficiency (Figure 1). It has been estimated that approximately 450 ARSs are present in the yeast genome (10).

The ACS and the B1 element are the binding sites for the six-subunit protein complex, the ORC, required for replication in yeast. In *S. cerevisiae* the various ORC subunits interact with the DNA. ORC recruits other proteins such as Cdc6m, Cdt1 and six MCM proteins, MCM2–MCM7, to form the pre-replication complex (pre-RC) during the early G1-phase of the cell cycle. Transition into the S-phase is achieved by the activation of specific protein kinases and the conversion of the pre-RC to the initiation complexes. The cell cycle specific regulation of the ORC ensures that DNA replication occurs only in the S-phase and that a single origin of replication is activated only once during the cell cycle (11, 12). All the factors required are highly conserved in eukaryotic organisms indicating that the mechanisms of origin licensing and activation are conserved from yeast to human.

There are many more ARSs present in the yeast genome than are actually activated *in vivo*. Recent analyses suggest that approximately one-third of the genomic ARSs are late firing or inefficient (13). For instance, in simple eukaryotes selection of origins seems to depend on epigenetic factors as described below. For example, ARS sequences are located in all intergenic spacers of the ribosomal DNA (rDNA) locus but only those ARSs which are located immediately downstream of transcribed rDNA genes become activated (14). In the same locus, active rDNA ARSs are clustered and interspersed with large silenced domains in which no initiation of DNA replication is observed. Silencing is predominantly achieved by the Sir2p histone deacetylase, implying that chromatin structure plays a crucial role in origin selection and activation (15).

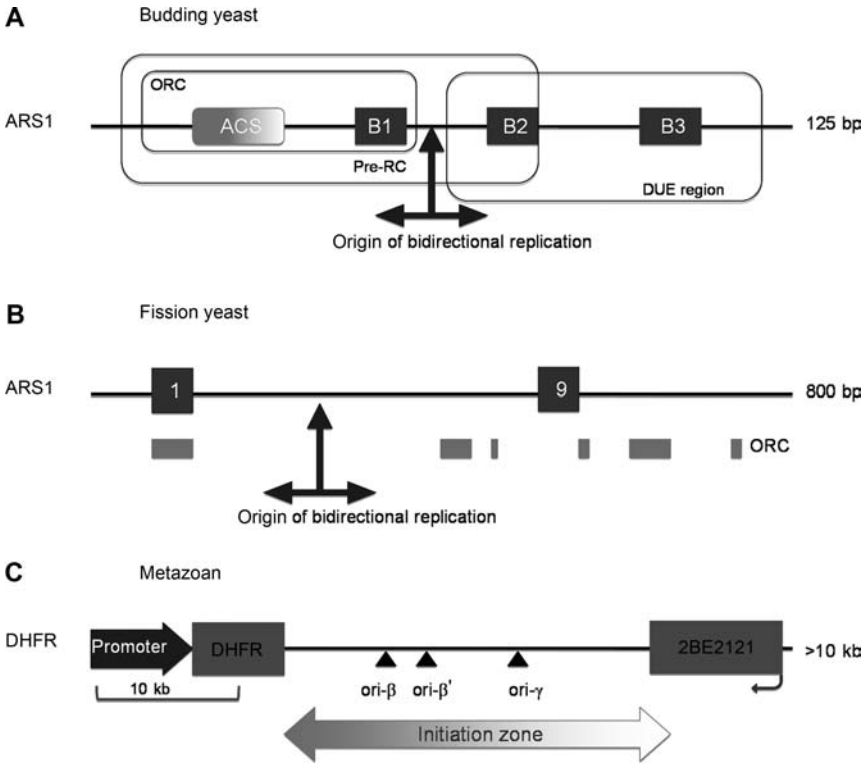

Figure 1 Origins of replication according to ''DNA replication and human disease'' (9).
(A) Replicator of budding yeast showing the essential functional elements. ACS and B elements are marked as black boxes, DNA regions capable of binding purified ORC or protected by ORC and pre-RC are indicated by unfilled rectangles. DNA unwinding element is indicated by a similar rectangle. Arrows indicate the origins of the bidirectional replication. (B) Replicator of fission yeast showing the essential elements. Important regions are marked as numbered black boxes, whereas regions capable of binding purified ORC are indicated by smaller rectangles below the DNA strand. Arrows indicate the origins of bidirectional replication. (C) Replicator of metazoans. The light, gray, two-sided arrow indicates the initiation zone containing multiple origins of replication shown by the black triangles. The unfilled rectangle depicts the DHFR coding region. The promoter is marked as a dark arrow pointing in the direction of transcription.

Whereas *S. cerevisiae* represents the prototype for sequence-specific binding of the eukaryotic initiator protein complex, this is not the case for higher eukaryotes (Figure 1). Initially, ARS elements in *S. pombe* were also identified by their ability to promote autonomous replication of plasmids (16). The ARS elements of *S. pombe* are much larger (0.5–1 kb) than those of *S. cerevisiae* (100–200 bp) and no conserved ACS essential for ARS function could be defined in *S. pombe*. In this yeast ARS elements are characterized by extended (AT)-hook motifs. Deletion of small elements of these motifs leads to a loss of function but can sometimes be replaced by poly(dA.dT) repeats (17). The number of ARS elements is approximately the same in *S. pombe* as in *S. cerevisiae* (18) but only approximately 160 of them are used in every cell cycle (18). Whereas in *S. cerevisiae* various ORC subunits have contact with the DNA, only ORC4 mediates DNA binding in *S. pombe*. Here, ORC4 shows a unique feature having a terminal extension of an AT-hook binding motif (19). ORC can bind to various elements within a single ARS and this multiple binding seems to be important for MCM recruitment and origin activation (20) (Figure 1).

The observation that there are already more replication origins in the yeast genome than are actually used is in agreement with the ''Jesuit model'' proposed by DePamphilis (21), which describes selection of an active origin as being crucial for correct replication. This is especially true for replication origins of higher metazoan cells. It has been estimated that replication initiates from approximately 30 000 sites in the mammalian genome (22) but various lines of evidence suggest that many more potential origins of replication are present in the genome and that, in contrast to yeast, an origin of replication is not defined by its primary sequence. In metazoans for example, the ORC has lost its specific DNA binding property but shows a preference for negatively and unwound DNA sequences (23). To identify human origins, several approaches were developed, as discussed below.

The average distance of initiation sites in differentiated cells is between 50 and 100 kb. These distances are considerably shorter in gene-rich regions and can be up to 500 kb in gene poor regions (24). Analyses of mapped origins of replication also revealed more common structural features such as AT-rich regions, CpG islands, bent DNA, the presence of scaffold/matrix attached regions (S/MARs), and the occurrence of initiation at various sites, than consensus sequences in mammalian origins (8, 20). This implies that ORC binding is not sequence-specific and that licensing and activation of mammalian origins of replication are strongly influenced by epigenetic factors.

The epigenetic regulation of replication

It is now increasingly obvious that epigenetic factors have a strong impact on origin selection and origin activation. Recent studies revealed that the selection and actual firing of an origin depends on ''environmental'' effects, such as transcriptional activity, chromatin organization and nuclear localization (25, 26), although the detailed mechanisms remain elusive. In the following section we will discuss recent studies in yeast and mammalian cells examining the epigenetic characteristics of replication (22, 25, 27–29). We will focus on the contribution of chromatin structure to origin selection and activation, and the impact of nuclear localization and transcription on replication.

The contribution of chromatin structure to origin selection and activation

Histone modifications, such as acetylation, are relevant for the initiation of replication already found in yeast genomes, as was recently demonstrated by Weber et al. (29). While analyzing replication initiation events and their influence on origin firing, they identified the origin-binding protein SumI. As part of the SumI/RfmI/HstI complex it is connected to an efficient replication initiation and represses meiotic gene expression during vegetative growth by histone deacetylation. A specific deacetylation of lysine 5 on histone 4 (H4K5) by histone deacetylase (HDAC) HstI was observed (29). By knocking out SumI or HstI and mutating lysines to glutamines in the H4 tail, which mimics a continuously acetylated state, a decrease of origin activity was observed (29). Whereas HstI deficient yeast strains have been shown to exhibit a decreased origin activity, knocking out the HDAC Sir2 results in a stronger origin activity (30). This effect could be explained by the fact that both HDACs target different histone modifications (29). Whereas HstI deacetylates H4K5, Sir2 mainly acts on H4K16, which is known to be enriched in early but is absent in late replicating chromatin (31). This observation demonstrates that histone acetylation is involved in the regulation of replication, but very much depends on the chromosomal context. Accordingly, Knott et al. were able to find a similar correlation between regulation of replication and epigenetic mechanisms. By knocking out the HDAC Rpd3L, it was possible to change the timing of a certain amount (32) of late-firing origins to an earlier time point (33) (Figure 2).

Recently, Wu and Nurse have studied the ORC and replication factor binding on replication initiation sites and compared the results with analyses of origin timing and efficiency in the yeast *S. pombe* (34). They unraveled a correlation between origin efficiency and early replication timing determined by AT-rich sequences and the availability of replication factors during M/G1-phase, when assembly of the pre-RC complex takes place (34). By connecting the level of pre-RC assembly to origin efficiency and replication timing, a higher ordered mechanism for replication is described, which can already be observed in yeast. Connections between changes in replication timing and chromatin structure have also been observed in higher organisms (5, 35). Analyzing embryonic stem cells revealed that during cellular differentiation replication domains undergo temporal reorganization processes, accompanied by a spatial reorganization in the nucleus (5). Evidence was provided for alterations of transcriptional activity of the relevant domains. These alterations were only present when a change of replication timing took place during a certain time period of the S-phase

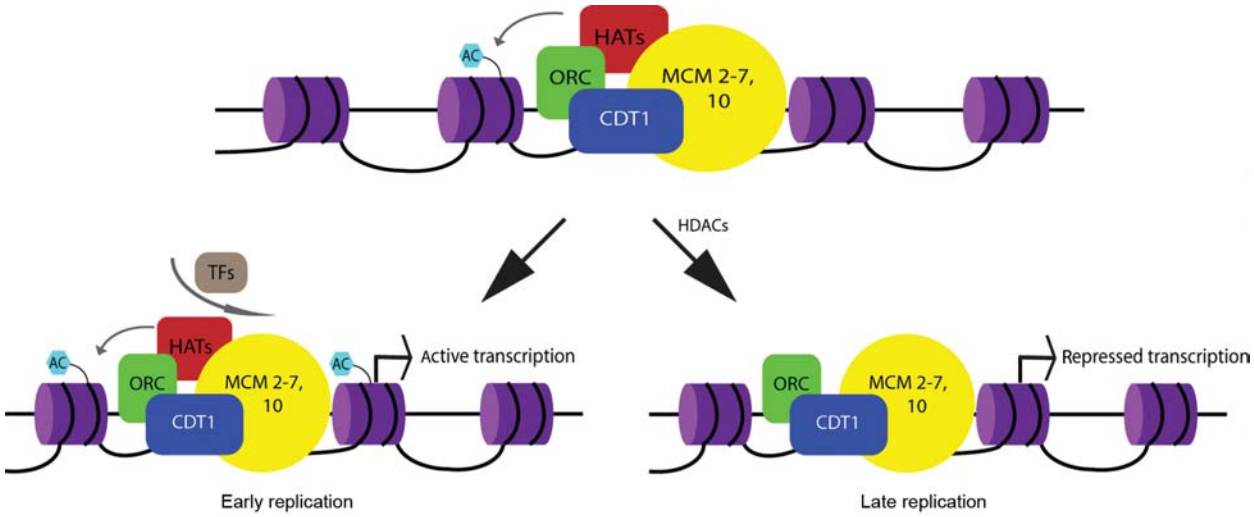

Figure 2 Molecular mechanisms of epigenetic replication.
The ORC complex binds to an origin of replication in proximity to a promoter region (gray rectangle). By an interplay between histone acetyltransferases (HATs), which acetylate surrounding nucleosomes, and transcription factors (TFs), the pre-RC activation is facilitated. Thus, early origin firing and an active transcription are promoted. Contrariwise leads the activity of HDACs to deacetylation and late replication.

or were accompanied by changes in their subnuclear positions (5). Overall, it is tempting to assume that replication timing facilitates the dissemination of chromatin states during DNA synthesis. But unfortunately this hypothesis seems to be premature, as no correlations between repressive chromatin histone modifications, such as H3K27me3, H3K9me3 and H4K20me3, and late S-phase replication were observed (5). In contrast, in embryonic fibroblasts the polycomb protein BMI1 was discovered to interact with CDC6, an essential regulator of DNA replication and thought to be recruited to the INK4a/ARF locus by CDC6. During this recruitment, this locus is usually transcriptionally silent and replicates during late S-phase. The authors suggest that during senescence BMI1 is replaced from the locus, whereupon the locus becomes transcriptionally activated and replicates during the early S-phase (35). These results provide evidence for the integration of epigenetic modifiers in regulating replication in eukaryotic cells (35). The relevance of chromatin structure for replication timing and origin activation has also been observed in the viral EBV replicon (25). These studies provide evidence that epigenetic mechanisms are involved in both origin firing activity (22) and replication timing (33). Moreover, the extent of the influence of chromatin structure on replication timing seems to be highly cell-type specific. Additionally, connecting these processes with replicon functioning in eukaryotic cells demonstrates the importance of understanding the control of epigenetic regulation of replication in higher organisms (36). Nonetheless, no general connection between these processes can be proposed, because contradictory results exist, as discussed below.

Nuclear organization and replication

It is well accepted that actively transcribed regions are replicated during the early S-phase, whereas DNA sequences with repressed transcription are replicated late in the S-phase. Accordingly, an association of replication timing with nuclear organization can be observed (37). Chromosomes that are localized in the center of the nucleus are replicated early during the S-phase, whereas late replicated heterochromatin rich chromosomes are preferentially found at the nuclear periphery (38, 39). This correlation of nuclear localization and replication timing, and the clustering of replication organization in so-called replication factories, can already be observed in yeast (26). In 1989, Nakamura et al. described distinct spots in replicating mammalian nuclei using BrdU-pulse labeling experiments (40). These foci were shown to co-localize with immunolabeled replication-associated proteins, such as DNA polymerase α and PCNA (41). Nowadays, live cell-imaging allows observation of specific assembly and disassembly of these replication foci during the S-phase (26) and their dynamics throughout the cell cycle. As the S-phase proceeds, the genome is replicated according to an organized temporal and spatial pattern. Therefore, replication foci show an assembly dynamic that depends on the chromatin state of the replicated DNA (42, 43).

In 1996, Friedman and colleagues discovered specific *cis*-acting sequences which influence both localization to the nuclear periphery and late replication timing (44). But while Raghuraman et al. were able to change the replication timing of a late-firing subtelomeric origin to the early S-phase by excising it from its chromosomal locus prior to the G1-phase (45), contrary observations had also been made. Zappulla et al. could not change replication timing by tethering an origin to the nuclear periphery (46). It appears that neither nuclear localization nor replication timing is sufficient to completely alter the fate of an origin. Only by changing the chromatin state itself, e.g., by inhibiting histone deacetylation, an influ-

ence on the spatial and temporal replication can be observed (47, 48). To date, it is unknown exactly how the spatial and temporal organization of replication influence each other. It is possible that nuclear localization is not only a downstream effect but also possesses the possibility of mediating a certain amount of influence by a feedback loop (Figure 3). That means that the localization of the regulated genomic region can be influential on the regulation of DNA replication in terms of which nuclear compartment is being occupied. The subnuclear localization itself does not seem to determine the timing of DNA replication (26), but if the regulated genomic region is located within a transcription factory it can be assumed that the active transcription correlates with early replication.

Transcription and replication

In 1999, DePamphilis observed that replication origins and transcriptional units are often found in a spatial vicinity to each other (50). Ongoing research revealed the importance of origin positioning relative to active genes. Although the existence of a transcription unit is important for origin activity (50), an origin can become inactivated as soon as transcription is running through an initiation site (51). Thus, an interesting link between replication and transcription might exist that is more complicated than previously thought. Recent studies in *S. cerevisiae* revealed that the chromatin environment of an active promoter attracts and facilitates origin activity (52). It has been observed that binding of RNA polymerases II and III, but not active transcription, positively regulates the initiation of the yeast origin ARS1, which resides in close proximity to these binding sites (52). In *Drosophila melanogaster*, transcription factors bind to the amplification control element (ACE), suggesting a role for these factors in the initiation of replication (53). Aladjem demonstrated that transcription factor binding sites appear close to origins, but failed to provide evidence for an existing connection between activation of origins and transcription factor binding (54). A correlation between origin and promoter density could be demonstrated by mapping genomic mouse origins of replication (55). Again this reflects a coordinated organization and mechanistic connection of replication and transcription (55). Furthermore, the observation that the distribution of replication initiation sites exactly resembles the spreading of transcriptional start sites supports the suggestion that transcriptional start sites influence origin selection and ORC assembly, probably by attributing specific epigenetic marks such as histone acetylation (25). Consequently, it was recently shown that histone modifications at early firing origins of mammalian cells strongly resemble the histone modification pattern at transcriptional start sites (56).

As recently reviewed elsewhere (25), the hypothesis arose that a permissive chromatin environment is supportive for origin selection and ORC assembly. By recruiting histone modifiers and transcription factors, an epigenetic environment is created that unravels the chromatin and facilitates binding of components of the replication machinery. But only potential transcription sites will provide this type of support. In contrast, active transcription would, similar to heterochromatin, create a non-permissive situation (51). As will be described in detail later, a correlation clearly pointing towards an interconnection of transcriptional activity and replication timing, reflected by the chromatin structure, was demonstrated by Zhang et al. (57). However, all studies

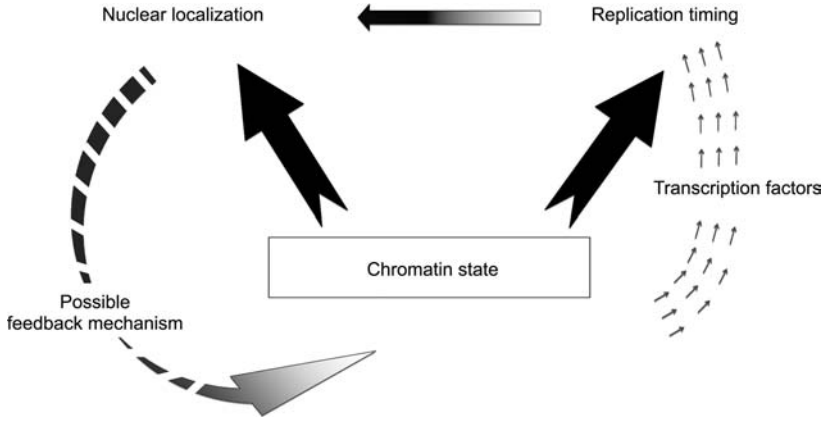

Figure 3 Schematic interplay of epigenetic mechanisms.
Changes of the chromatin state, e.g., by incorporating histone acetylation, have been shown to influence the spatial and temporal organization of DNA replication (47, 48). The detailed mechanism of how replication, localization, and timing depend on one another is still unknown, but experiments provide evidence that alterations of the timing of replication lead to altered spatial patterns of replication within a nucleus. Although evidence is missing, a reasonable feedback loop of nuclear localization-dependent DNA replication is thought to act on the chromatin structure. As transcription units often have been found in proximity to origins of replication, the act of transcription and the availability of transcription factors might also be functionally important for the regulation of DNA replication. In yeast it was discovered that an overrepresentation of binding sites of the transcription factors Smp1 and Swi6 correlates with replication origins that are regulated by the HDAC Rpd3L (49). Thus, a possible influence of these and other transcription factors is conceivable either by acting on the gene expression of the HDAC itself or by changing the transcriptional activity of the replicated and transcribed region. This schema demonstrates not a given interconnection, but rather a conceivable network of mechanisms that needs to be analyzed further.

underscore the significance of understanding the impact of epigenetic factors on episomally replicating vectors. Only by unraveling this will it be possible to design controllable vectors for use in gene therapy.

Epigenetic regulation in episomally replicating vectors

Viral vectors

Viruses are highly evolved natural vectors for the transfer of foreign DNA into cells. In the case of viral gene therapy vectors, the virus particles must be able to infect cells and to transport the recombinant viral DNA into the nucleus. But for safety reasons they must be unable to produce and release new infectious particles. For that purpose replication-defective viruses are generated. These virions (virus-like particles) exhibit all viral properties for transduction but lack the ability to replicate and produce progeny. Generally, coding sequences work in *trans* and are expressed by a helper plasmid. The viral *cis*-acting sequences could be linked to the therapeutic gene and can be introduced into the same cell, resulting in the production of replication-defective particles which are able to specifically transduce the new genetic information into target cells (58). Although a number of viruses have been developed, interest has centered on four types: retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses and herpes simplex virus type. These viruses differ specifically in their gene transfer efficiency, their target cells and whether they integrate or not into the host genome. Adeno-associated viruses (AAVs) are *per se* non-integrating but are able to integrate with low frequency into chromosome 19 (59, 60). The AAV is currently not known to cause diseases but causes a very mild immune response (61). In contrast, adenoviral DNA does not integrate into the host genome. Adenoviruses possess a linear dsDNA genome and are able to replicate in the nucleus of mammalian cells using the replication machinery of the host. Furthermore, retroviruses have the ability to integrate into the host genome in a stable manner in any arbitrary position in the host genome. Following these integration events, harmful mutations or even cancer can occur, as observed in clinical trials for X-linked severe combined immunodeficiency (62).

In addition to the replication-deficient viruses described above, *viral plasmid replicon based vectors* have been generated. These vectors lack the ability to transduce target cells and thus need to be delivered as naked DNA. Once delivered into the nucleus they exhibit highly efficient strategies for DNA replication. In particular, the replication strategies of simian virus 40 (SV40), bovine papillomavirus (BVP) and Epstein-Barr virus (EBV) have been investigated with great enthusiasm: the SV40 origin of replication is a 64-bp DNA sequence that contains a binding site for the large T-antigen. This protein is virally encoded and is the only factor required for replication (63). It functions as a replicative helicase allowing replication of SV40 independently from genomic replication and is uncoupled from the ''once per cell cycle'' licensing (64, 65). Hence, SV40 replicon-based vectors exhibit high copy numbers (several thousands) per transfected cell (66). This ensures a highly efficient segregation and episomal persistence during cell divisions. As in SV40, the replication of BVP also occurs uncoupled from the ''once per cell cycle'' licensing of MCM2–MCM7 mediated replication initiation (67) and is mediated by the virally encoded proteins E1 and E2 (68). The mitotic stability of BVP plasmid replicon is caused by the ability of the E2 protein to bind to metaphase chromosomes (69). However, in contrast to SV40, BVP plasmid replicon-based vectors exhibit an intermediate copy number (50–150) per transfected cell (70).

The EBV utilizes different origins of replication in latent and lytic cycle, respectively. In the lytic cycle, the viral origin binding protein binds to the lytic origin (*ori-Lyt*) and recruits the core replication machinery initiating a rolling-circle amplification of the viral genome (71). During the latent cycle, the EBV genome is maintained as a large episome. Synthesis and maintenance are thereby mediated by a *cis*-acting sequence (oriP) and the Epstein-Barr viral nuclear antigen 1 (EBNA-1) (72). Replication from the oriP is coupled to host ORC and MCM2–MCM7 complexes and occurs ''once per cell cycle'', utilizing the host replication machinery (73). Structurally, the 1.7-kb oriP is a bipartite sequence, consisting of the dyad symmetry element (DS) and the family of repeats element (FR) (Figure 4). The DS element consists of four overlapping, palindromic EBNA-1 binding sites of intermediate affinity. First, EBNA-1 binds to the DS and recruits the cellular ORC to initiate replication. The FR consists of 20 copies of a 30-bp repeat, each representing a high-affinity site for EBNA-1. The EBNA-1/FR interaction serves as a replication enhancer (74, 75). Furthermore, EBNA-1 molecules interact with each other by a DNA-looping mechanism thereby linking the various binding sites, and this might stabilize EBNA-1/DS binding (76, 77). To construct ''ori-fishing'' vectors, the DS element in the oriP region of EBV plasmid replicons was deleted. These DS element depleted vectors are correctly segregated during cell division based on the property of EBNA-1 to bind both the FR element and metaphase chromosomes (78–80). But deletion of the DS element abrogates the ability of the vector to self-replicate, and thus chromosomal DNA is required to support the episomal replication. Based on this observation, so-called origin-trapping assays were designed to identify new human origins of DNA replication. In early attempts to adapt the ARS assay to define mammalian origins of replication, genomic sequences were inserted into plasmids. Krysan and colleagues screened random mammalian DNA fragments using a replication-defective (DS-depleted) EBV (81) and found selected DNA sequences that mediated autonomous replication over a period of several months under selection. But only fragments of 6 kb and larger displayed replication activity in this assay. In both short-term and long-term assays, random human DNA fragments displayed increased replication efficiency with increased fragment length. Despite their length, other activity conferring features, could not be identified (82). In another approach, a library of

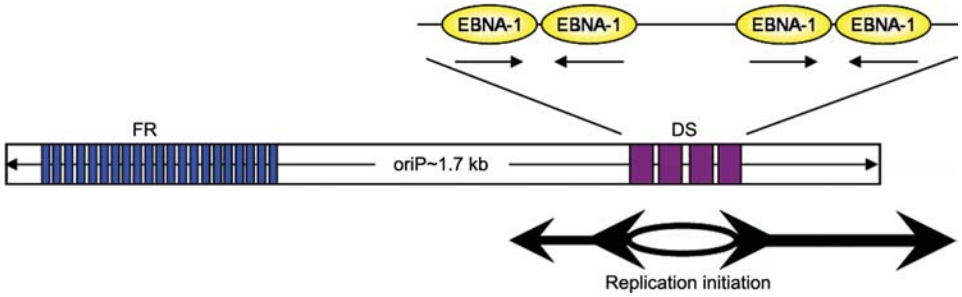

Figure 4 Replication of the EBV genome is mediated by oriP and EBNA-1.
The oriP of EBV consists of the dyad element (DS, purple) and the family of repeats element (FR, blue). The FR harbors 20 copies of the EBNA binding site and functions as a replication enhancer. The DS element harbors four palindromic EBNA-1 binding sites and represents the replication initiation and start site of EBV (black arrows).

potentially ORC-binding DNA fragments, enriched by chromatin immunoprecipitation (ChIP) was generated (23). Recently, a two-step origin-trapping assay was developed combing both approaches described above (78–80). In this assay, ORC-binding DNA is firstly enriched by ChIP and, in a second step, selected by its ability to rescue transient replication of a replication-defective EBV plasmid (83). Gerhardt et al. demonstrated ORC assembly at the chromosomal sequences in these EBV-based vectors (83). However, no common sequence motifs could be identified suggesting that the potential to initiate replication depends on size and can occur on multiple sites (84). This is in agreement with results obtained in embryonic systems, such as the *Xenopus* system, in which almost any sequence can be used as the origin of replication (4). Only later during development, is replication initiated at more specific sites. Sequence independent initiation of DNA replication has also been demonstrated in a non-viral episomal plasmid (85).

As the EBV origin of replication uses the cellular licensing machinery to regulate replication it is also a valuable model to study epigenetic changes in chromatin structure during the cell cycle. Zhou and colleagues found that the DS region is flanked by nucleosomes. These nucleosomes undergo cell cycle-dependent chromatin changes, including chromatin remodeling and histone H3 deacetylation, leading to origin ORC assembly, but also to a delayed origin activity (36). They suggest that deacetylated histones contribute to an origin licensing by providing the possibility of ORC loading, but the same mechanism stops origin firing until the late S-phase (36). More recently, using a combination of fluorescence *in situ* hybridization (FISH) and confocal microscopy it was shown that EBV genomes reside preferentially in perichromatic regions of the nucleus of the host (86). The perichromatin is characterized by open chromatin and is highly accessible for the assembly of replication and transcription machineries, as well as for chromatin modifying proteins (87). Moreover, the authors specified the preferential localization of EBV to H3K4me3 and H3K9ac domains. These histone modifications are linked to active chromatin. Accordingly, an association with the heterochromatic H3K9me3 was not observed (86). Using various EBV mutants it was further shown that the FR portion of oriP is essential for its nuclear localization, whereas the DS seems to have a minor impact on the epigenetic environment (86). These findings are similar to a non-viral extrachromosomal replication model (88), as discussed below (Figure 5).

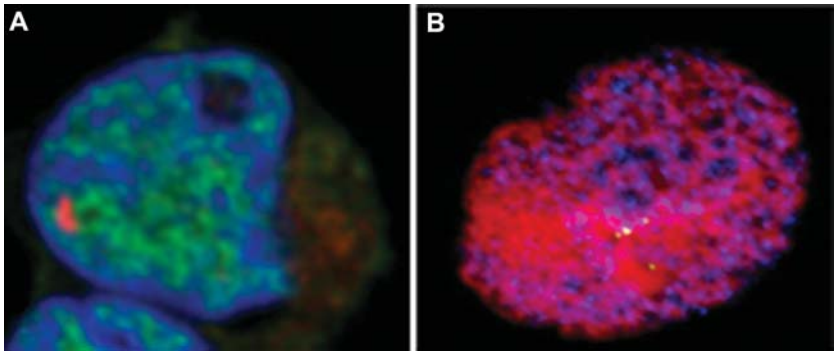

Figure 5 EBV genomes and pEPI are associated with active histone modifications.
(A) EBV is shown to associate with histone 3 lysine 9 acetylation (H3K9ac). The colocalization of EBV genomes with H3K9ac was determined with a combination of immunofluorescence techniques using Raji cells. H3K9ac is shown in green, the EBV genome of Raji cells in red, and the DNA counterstain in blue [with permission from A. Schepers (86)]. (B) pEPI molecules (green/white) colocalize with H3K9ac and H3k14ac (blue) in CHO cells. CHO genome is stained red [with permission from J. Postberg (88)].

Non-viral vectors

Artificial chromosomes Since the 1990s, human artificial chromosomes (HACs) have been under development following the success of yeast and bacterial artificial chromosomes (YACs and BACs), respectively (89, 90). With the first construction of an HAC in 1997 (91) a breakthrough in gene therapy was expected. HACs offered an alternative approach addressing the complex requirements of an idealized vector (92). In general, the key advantages of HACs include their mitotic stability and maintenance as non-integrating vectors, their large capacity allowing the insertion of therapeutic genes in their natural context including distal regulatory elements, and long-term gene expression (92).

There are two approaches to generate mammalian artificial chromosomes: fragmenting a natural chromosome or generating *de novo* chromosomes. The ''top down'' approach – telomere-associated chromosome fragmentation or telomere-directed truncation – was developed in 1991 (93–95). Natural chromosomes are reduced to a minimum length by telomere fragmentation (94). These mini-chromosomes are usually 0.5–1 Mb, linear, contain telomeres at their termini, and centromeric regions with a minimum of 100 kb of the centromeric α-satellite DNA (92, 96). Within the alternative ''bottom up'' approach artificial chromosomes are generated by introducing cloned centromeric and telomeric DNA into human cultured cells. HACs are formed *de novo* and range in size from 1 to 10 Mb. Using this approach, Harrington et al. generated the first *de novo* HAC in HT1080 cells (91). Despite the presence of telomere sequences these HACs generate cell lines that contain circular artificial chromosomes (92, 96).

As it has been shown that replication origins occur on average every ~100 kb throughout the genome, sufficiently large DNA fragments will be competent for origin function (97, 98). Nevertheless, some specific mammalian sequences, e.g., the well-characterized β-globin locus, proved to show origin activity when moved to an ectopic chromosomal localization (99), and indeed artificial chromosomes containing a β-globin mini-cassette have been successfully cloned (100). However, HACs segregate correctly during each cell division in human cells, but somehow display a slight increase of segregation instability when compared to human chromosomes (101). In contrast, in murine cells they show an increased and variable rate of loss (102, 103). Recently, Moralli et al. found that HAC segregation in murine cells correlates with their position in the murine nucleus. HACs that preferentially associate with the chromocenter, a densely staining mass of heterochromatin in the chromosomes with six arm-like extensions of euchromatin, displayed a lower loss rate. Whereas the HACs that localized more frequently outside of the chromocenter were associated with variable amounts of H3K9me3, and these variable amounts in turn correlate with the loss rates of HACs (28). Furthermore, Nakano et al. generated a HAC containing a tetracycline operator. They reported missegregation and loss of the HAC when the chromatin state was altered to a more open configuration using the transcriptional activator (rTA). The same was observed when the chromatin was altered to a more closed configuration using the transcription silencer tTS. Binding of tTS caused, among other effects, the loss of H3K4me2, a marker for transcriptionally competent or neutral chromatin, and was accompanied by an accumulation of H3K9me3, a marker of repressive chromatin (104). Thus, Nakano and colleagues were the first to manipulate the epigenetic status and provided evidence that formation of heterochromatin is incompatible with an appropriate kinetochore function (104).

A non-viral, episomally replicating vector – pEPI Based on the observation that the binding of an origin of replication to the nuclear matrix precedes the onset of the S-phase (105), an episomally replicating plasmid was designed in our laboratory (32). The simian virus large T-antigen was replaced by a nuclear S/MAR (32). The resulting vector pEPI was shown to replicate episomally at copy numbers of ~5–10 in Chinese hamster ovary (CHO) cells and is mitot-

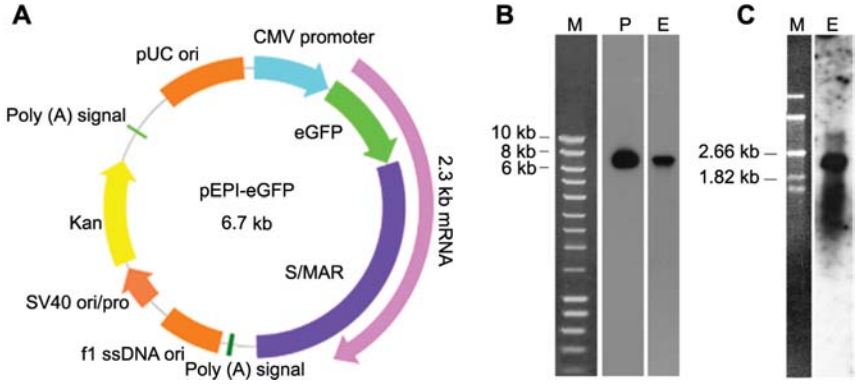

Figure 6 Autonomously replicating vector pEPI.
(A) Vector map of pEPI. Transcription of the transgene (eGFP) running into the S/MAR sequence is required for episomal maintenance (pink arrow). Plasmid and eGFP-S/MAR transcript were detected in CHO cells after transfection with pEPI-eGFP using Southern blot (B) and Northern blot (C), respectively. M, ladder; P, plasmid control; E, extract.

ically stable over hundreds of generations in the absence of selection (32) (Figure 6).

The vector replicates like chromosomal DNA once per cell cycle in the early S-phase (106). Schaarschmidt et al. found that Orc1, Orc2 and MCM3, which are components of the pre-RC, bind to multiple sites of the plasmid in G1-phase cells. They present further evidence that these pre-RCs bound to various regions of pEPI are functional, because they partially disassemble in the S-phase as is known for cellular pre-RCs. The authors conclude that chromatin-binding sites for ORC and other components of the pre-RC do not much depend if at all, on the underlying DNA sequence. Rather, sites of replication initiation can be determined by epigenetic mechanisms (85). We could show that established vector molecules are marked by active histone modifications, whereas integrated molecules display an enrichment of repressive histone modifications (88). Histone modifications that are associated with transcriptionally competent chromatin (H3K4me3, H3K4me1) (107) and are enriched in genes replicating during the S-phase (H3K4me3, H3K36me3) (5) were also found to be significantly enriched on the S/MAR element in asynchronous cell culture and in cells during the S-phase. During mitosis, the S/MAR element loses most of its H3K4me3 and H3K4me1 modifications and all of the histone modifications are decreased (108). This is probably due to a different chromatin structure of the S/MAR element required for co-segregation of pEPI during mitosis. FISH analyses as well as *in vivo* crosslinking studies demonstrated that pEPI interacts with compounds of the nuclear matrix (109) and a specific interaction with scaffold attachment factor-A (SAF-A), one of the major matrix proteins, was shown (110). It has been assumed that this interaction is responsible for the mitotic stability of pEPI and that the vector uses the replication machinery of the host. The functional elements that could be involved in autonomous replication are the SV40 origin of replication and the S/MAR. But both elements had been inserted in numerous vectors without resulting in episomal replication of the respective constructs. Stehle et al. (111) attended to that aspect and surprisingly found that an active transcription running into the S/MAR is required for episomal replication of pEPI (111). They generated various deletion constructs and demonstrated that pEPI derivates in which transcription of the transgene (eGFP) running into the S/MAR was abrogated, e.g., by poly(A) termination signals, failed to replicate episomally. As a consequence, vector molecules became lost from the transfected cell or occasionally integrated into the host genome (111).

But despite these efforts, the stable establishment of pEPI, as of other extra-chromosomal replicons (112), is a rare event and occurs in only 1–5% of transfected cells (111). Analyses of vector localization at various time points post-transfection revealed an initially large number of vector molecules reaching the nucleus but become lost during subsequent cell divisions.

A sequence-independent connection between the time point of transfection and transcriptional competence of transfected vectors was demonstrated by Zhang et al. (57). Exogenous genes were up to 10-fold more efficiently transcribed when transfected into early S-phase cells, whereas cells transfected in the late S-phase displayed low transcription rates. These transcriptional states are retained even 48 h post-transfection, respectively (57). This effect of transcriptional competence is associated with distinct histone modifications. Whereas exogenous DNA transfected in early S-phase cells was associated with acetylated histones, those transfected in late S-phase cells were packaged in chromatin containing deacetylated histones. That deacetylation of histones is partly responsible for repression of late S-phase-transfected DNA was confirmed by pretreating cells prior to transfection with trichostatin A, a HDAC inhibitor. This pretreatment led to an increased expression of late S-phase-transfected DNA (57). However, in established pEPI clones vector molecules are predominantly found at the border between condensed chromatin and the perichromatin domain (88, 108), whereas integrated vector molecules are located in highly condensed chromatin (108). Further findings indicate that pEPI can associate with any active locus, but once associated it seems to be amazingly non-dynamic (108).

Nonetheless, pEPI has already been successfully used to generate genetically modified pigs. Expression of the transgene could be demonstrated in nine out of 12 modified fetuses. In these positive animals, expression was shown in all tissues with up to 79% of positive cells (113). In this experiment, pEPI was delivered to female pigs using sperm-mediated gene transfer. A method that uses the nature of sperm cells to bind and internalize DNA, which is then transferred into eggs (114). However, establishment efficiency of approximately 79% was surprisingly high and is probably due to dynamic epigenetic processes during germ cell proliferation. Chromatin undergoes epigenetic remodeling during mammalian spermatogenesis or oogenesis, mediated by a decrease of histone modifications and DNA methylation and resulting in imprinting or X-chromosome inactivation (115). In mouse germ cells a so-called erasure process takes place. DNA is genome-wide demethylated by embryonic day 12–13; afterwards, epigenetic marks are established *de novo* in differentiating germ cells according to their sex (116–118). Concerning the data of Manzini et al. (113), this indicates that DNA was delivered at a very unique point of the epigenetic modification processes, implying an important role of epigenetic modifications for episomal vector establishment and maintenance (119).

Scaffold/matrix attachment regions – S/MARs As previously mentioned, based on the observation that binding of an origin of replication to the nuclear matrix precedes the onset of the S-phase (105), we constructed an episomally replicating vector that contains an S/MAR sequence (32). Owing to the protocols that led to their detection, S/MARs are implicated in a wide variety of biological activities compatible with an affinity to the nuclear matrix. These include origin of replication function (110, 120), augmentation of transcription (121), insulator function (122) and long-term maintenance of high transcription levels by counteracting DNA methylation (123, 124). In the above described non-

viral vector pEPI, the large T-antigen of SV40 was replaced by a S/MAR sequence from the human β-interferon gene (32). Several AATATATTTA elements of this S/MAR function as DNA unwinding elements and enable a stress-induced DNA duplex destabilization of dsDNA up to 200 bp, whereas introduced mutations prevent complete unwinding to ssDNA (125). This increased propensity to unwind the DNA strands can promote the formation of secondary structures, which are recognizable by DNAses, topoisomerases, and enzymes of the histone acetylation and DNA methylation apparatus. In past years, several S/MAR binding proteins have been identified, of which one of the first included topoisomerase II (126), histone H1 (127) and SAF-A (128–130). However, it was also reported that transcription factor p300 strongly interacts with SAF-A and that both proteins bind to the S/MAR. This binding seems to be restricted to the transcriptional inactive state and is accompanied by acetylated histone H3. It seems likely that the SAF-A/p300 binding to the S/MAR of non-transcribed genes set up these genes for activation (131). Similar results were obtained when the cell cycle dependent histone dynamics of pEPI, as described above, were investigated (108). Histone modifications associated with transcriptionally competent chromatin were found to be enriched on the S/MAR element during the early S-phase, an observation that supports the relevance of an ongoing transcription into the S/MAR for vector maintenance (108, 111). Consistent with these results, Dang et al. reported that the S/MAR has the ability to inhibit *de novo* DNA methylation (123). DNA methylation in turn is able to recruit histone deacetylases, which is a first step in repressive chromatin formation (132). Altogether, these results indicate that the meaning of S/MARs for epigenetic regulation is most probably more important than assumed so far.

Conclusion

Despite increasing experimental evidence that epigenetics play an important role in regulating DNA replication, the details of interconnection are not clear and thus our understanding of epigenetic regulation of autonomous replicons still remains fragmentary. Understanding these details is one of the major intellectual challenges in modern cell biology but is also a necessary prerequisite for the rational design of autonomously replicating vectors to be used in gene therapy.

Nevertheless, some progress towards the construction of non-viral and episomally replicating vectors has been made within the past years. These constructs will have to be improved with regard to the establishment and expression efficiency before they can be used in gene therapeutic applications.

The observation that a transcription unit linked to an S/MAR sequence is sufficient for episomal replication and retention demonstrates that no specific *cis*-acting sequence but rather an interplay between transcription and a functional chromosomal element is required for these processes (111). Furthermore, the relevance of chromatin structure and histone modifications for vector functioning was shown (88, 108, 111). The epigenetic regulation of vector functioning was not only demonstrated in S/MAR based vectors (36). Nucleosomes that flank the oriP of EBV-based vectors were found to undergo epigenetic alteration in the course of origin licensing. Hence, the state of histone acetylation is thought to be important for ORC assembly and timing of replication (36).

Our current view is that epigenetic mechanisms play a crucial role in the regulation of DNA replication in the genome and therefore also in the regulation of autonomous replicons. But analyzing the extent to which these mechanisms are involved in the functioning of these vector systems remains the challenge of the future.

Outlook

By considering the relevance of epigenetic mechanisms, such as replication timing and its connection to transcriptional activity, it is our conviction that it will be possible to further improve plasmid vectors for their use in gene therapeutic applications. During the past decade, the success of gene therapy trials has suffered from ignoring the importance of connected disciplines. Either because of the lack of technologies or the just recently developed interest for epigenetic influences, successful progress in developing efficient plasmid vectors has been missing. But we are convinced that the recent progress in the analysis of the epigenetic regulation of replication will have an immense influence on the development of gene therapeutic competent plasmid vectors.

Highlights

- Discovery of ARS:
  - In *S. cerevisiae* specific *cis*-acting elements (ARSs) act as origins of replication, but ARS assays were not successful in higher eukaryotes
- Jesuit model proposed by DePamphilis:
  - Many origins exist, but only a few are selected during DNA replication. Their correct selection is crucial for functional DNA replication
- Assembly of telomeres, centromeres and genomic DNA leads to construction of the first human artificial chromosome in 1997
- Description of the ORC cycle
- Selection of human sequences that replicate autonomously using EBV-based vectors
- Construction of one of the first non-viral, episomally replicating vector, pEPI
- A transcription unit upstream of an S/MAR element is sufficient for episomal replication and retention
- Epigenetic regulation of autonomous replicons:
  - The relevance of chromatin structure and nuclear localization for DNA replication demonstrated for EBV-based and non-viral vectors

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