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Compartmentalization of prokaryotic DNA replication

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Abstract

It becomes now apparent that prokaryotic DNA replication takes place at specific intracellular locations. Early studies indicated that chromosomal DNA replication, as well as plasmid and viral DNA replication, occurs in close association with the bacterial membrane. Moreover, over the last several years, it has been shown that some replication proteins and specific DNA sequences are localized to particular subcellular regions in bacteria, supporting the existence of replication compartments. Although the mechanisms underlying compartmentalization of prokaryotic DNA replication are largely unknown, the docking of replication factors to large organizing structures may be important for the assembly of active replication complexes. In this article, we review the current state of this subject in two bacterial species, *Escherichia coli* and *Bacillus subtilis*, focusing our attention in both chromosomal and extrachromosomal DNA replication. A comparison with eukaryotic systems is also presented.

Keywords: DNA replication; Fluorescence microscopy; Membrane; Prokaryotes

Contents

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1. Introduction

In 1963, Jacob et al. [1] published their Replicon Theory, which postulated that the bacterial membrane plays a key role in prokaryotic DNA replication. These authors proposed that a replicon consists of a *cis*-acting sequence, the *replicator* (now termed origin of replication), and a *trans*-acting gene, coding for a protein that initiates replication at the *replicator*. In addition, this model assumed that the replicons are attached to specific sites on the bacterial membrane by its *replicator*. As pointed out by Nordström [2], although important aspects of the Replicon Theory still holds true, work on the involvement of membranes in DNA replication has been fairly rare.

Earlier, the putative role of the bacterial membrane in DNA replication was analyzed by cell fractionation techniques. Essentially, these investigations, which are covered in numerous reviews [3–7], revealed the presence of newly replicated DNA molecules and replication proteins in membrane fractions obtained by a variety of procedures. Moreover, DNA–membrane complexes isolated from several bacterial species were able to synthesize DNA in vitro without the addition of exogenous DNA or enzymes, suggesting that these components were already present and active in the complexes. Additional insights into the involvement of the bacterial membrane in DNA replication was obtained by genetic and biochemical studies, which led to the conclusion that chromosomal DNA replication, as well as plasmid and viral DNA replication, occurs associated to the bacterial membrane. However, despite these findings, a detailed molecular investigation concerning membrane-association of DNA replication has been performed in few systems.

Very little is known about the function of the bacterial membrane in DNA replication. The general idea is that the bacterial membrane provides a framework on which replication factors and DNA can bind and interact. This compartmentalization mechanism would enhance the efficiency of the replication process. Nevertheless, in *Escherichia coli*, it was shown that the bacterial membrane plays an important role in the control of chromosomal DNA replication. This regulation implies direct interactions between membrane components and replication factors. For example, accumulating evidence supports that acidic phospholipids modulate the activity of the replication initiation protein DnaA, both in vitro and in vivo [8]. Furthermore, unlike *Bacillus subtilis*, *E. coli* chromosomal DNA is methylated at adenine residues within the sequence GATC, which is frequent in the replication origin. Following initiation of DNA replication, hemimethylated origins are generated, and some reports support that their binding to the outer membrane prevents re-initiation of chromosomal DNA replication [9–15].

Over the last several years, the introduction of new in vivo fluorescence microscopy methods has made it possible to follow bacterial processes in space and time [16–18]. For example, the use of the green fluorescent protein (GFP) has made it possible to visualize the position of replication proteins in living cells. This experimental approach has revealed that particular replication proteins are localized at discrete intracellular positions [19,20]. In addition, particular DNA sequences have been visualized in fixed cells, using fluorescence *in situ* hybridization (FISH), and in living cells, by inserting tandem copies of the lactose operon operator (*lac*O) into the DNA molecule close to the region under study. In the latter case, expression of GFP fused to the lactose operon repressor (LacI) allows detection of the *lac*O array and, thereby, localization of the region under study (GFP-LacI tagging). The application of these cytological methods has shown that the replication origin site on chromosomal DNA has a precise subcellular location during the cell cycle [21–24]. Moreover, it has been shown that plasmid DNA molecules are specifically localized within the bacterial cell [23,25].

In this article, we review recent results that support the existence of DNA replication compartments in two bacterial species, *E. coli* and *B. subtilis*. We include data on in vivo replication of the broad-host-range plasmid RK2 and the *B. subtilis* phage ϕ29, where proteins likely involved in membrane-localization of replication complexes have been identified.

2. Chromosomal DNA replication in *E. coli*

Escherichia coli cells contain a single circular chromosome that is replicated bidirectionally from a unique 245 base pair sequence, termed *ori*C. Initiation of replication occurs only once per cell division cycle, and simultaneously at all origins present in a cell [26]. *E. coli* has

developed different mechanisms to control initiation of chromosomal DNA replication [for a review, see 27]. Below, we highlight the replication control strategies in which the bacterial membrane appears to have an important role. These findings, together with recent advances in the intracellular localization of the *ori*C region, support the notion that chromosomal DNA replication occurs at specific compartments.

2.1. Regulation of DnaA activity by acidic phospholipids

The role of DnaA protein in initiation of chromosomal DNA replication has been covered in a recent review [28]. DnaA protein cooperatively binds to five 9-mer DnaA boxes within the *ori*C sequence. This binding causes a local unwinding of an A–T rich region, which leads to the formation of the prepriming complex. The intracellular availability of DnaA protein determines the time of initiation and thereby the initiation mass [29]. DnaA binds the nucleotides ADP and ATP in vitro with high affinity. Although both ADP–DnaA and ATP–DnaA bind *ori*C, only ATP–DnaA is active for subsequent replication steps [30,31]. ATP promotes an allosteric modification and does not provide energy for the unwinding reaction since non-hydrolyzable ATP analogues are equally effective [30]. In vitro, the exchange of bound ADP for ATP is slow, with a half-life of ~30 min when ADP–DnaA is incubated with an excess of ATP [30]. Studies using replication cycle-synchronized cultures suggest that the number of ATP–DnaA molecules increases prior to initiation, and that ATP–DnaA is converted to the inactive ADP–DnaA form after initiation [32]. Moreover, a mutant DnaA protein defective in ATP hydrolysis promotes over-initiation of chromosomal DNA replication, supporting a crucial role for bound nucleotides in regulating DnaA activity [33].

Purified DnaA protein binds to phospholipid vesicles in vitro [34]. It was demonstrated that rejuvenation of the inactive ADP–DnaA form, by replacement with ATP, is catalyzed in vitro by acidic phospholipids (cardiolipin and phosphatidylglycerol) in a highly fluid bilayer [35]. During treatment with phospholipids, the presence of *ori*C DNA facilitates the binding of ATP to DnaA protein [36]. Moreover, phospholipids derived from membranes lacking an unsaturated fatty acid (e.g., oleic acid) are unable to promote the exchange of nucleotides [37]. Further studies of the interactions between DnaA protein and lipids showed that fluid membranes with acidic domains activate DnaA [38]. The membrane-mediated dissociation of the nucleotides bound to DnaA occurs with insertion of the DnaA protein into the hydrophobic region of the lipid bilayer [39]. Proteolytic fragments of DnaA protein, which retain tightly bound nucleotide, were examined for their ability to respond functionally to acidic phospholipids. This experimental approach defined a discrete region of DnaA critical for functional interaction with acidic membranes. The region of about 1–2 kDa included the residue Lys^{372} [40]. Subsequently, site-directed mutagen- esis experiments indicated that amino acids Arg^{328} and Lys^{372} of DnaA are involved in its functional interaction with acidic phospholipids [41,42]. These amino acids lie within two potential α-helices (amino acids 327–344 and 357–374).

Accumulating evidence supports that membrane phospholipids also modulate DnaA activity in vivo [8]. Immunofluorescence and immunoelectron microscopy techniques revealed that DnaA protein is located at the bacterial membrane [43]. Upon purification of DnaA, about half of the protein was found in an inactive, aggregated form, which contained phospholipids. Replication activity of this DnaA form was restored by treatment with either DnaK protein or phospholipase A_2 [44]. Furthermore, experiments performed by Fralick and Lark [45] indicated that inhibition of oleic acid synthesis specifically inhibits initiation of chromosomal DNA replication. Some twenty years later, it was reported that cells depleted of anionic phospholipids are arrested for growth, except when constitutive stable DNA replication, which is DnaA and *ori*C independent, is activated [46]. Thus, it appears that acidic phospholipids regulate DnaA-dependent initiation of replication in vivo. In fact, a single amino acid substitution in the membrane-binding domain of DnaA protein overcomes the growth arrest of cells deficient in acidic phospholipids [47]. Additional studies showed that normal replication initiation at *ori*C can take place in cells without detectable levels of phosphatidylglycerol and cardiolipin but with increased levels of phosphatidic acid [48]. Moreover, expression of certain *dna*A mutants appears to alter the physical properties of the bacterial membrane [49].

In a recent study, Makise et al. [50] examined the effects of various phospholipids on DnaA binding to *ori*C in vitro. These authors found that phospholipids required an acidic moiety and unsaturated fatty acids to inhibit DnaA binding to *ori*C. This negative effect caused by phospholipids is thought to prevent reinitiation of DNA replication. Furthermore, some mutant *dna*A genes, whose overexpression allows growth of *E. coli* cells unable to synthesize acidic phospholipids, have mutations in the *ori*C-binding domain of DnaA [47].

2.2. Binding of hemimethylated oriC to the outer membrane

Escherichia coli DNA is normally methylated at adenine residues within the sequence GATC by DNA adenine methylase, the product of the *dam* gene. The *ori*C sequence contains eleven GATC sites in 245 base pairs. Following initiation of DNA replication, the newly synthesized strands remain temporarily unmethylated. Campbell and Kleckner [51] found that methylation of

the hemimethylated *ori*C is significantly delayed relative to the time of methylation at GATC sites elsewhere in the chromosome, indicating that newly replicated origins are specifically sequestered from Dam methylase. It was reported that methylated *ori*C plasmids fail to undergo more than one round of replication when introduced into *dam*$^-$ cells [52]. In contrast, unmethylated *ori*C plasmids replicate under such conditions. Therefore, hemimethylation prevents initiation of DNA replication in vivo [52]. Moreover, the time between successive initiations on the same origin (the eclipse) is highly influenced by the intracellular level of Dam methylase, indicating that the eclipse corresponds to the period of origin hemimethylation [53]. Hemimethylated *ori*C sequestration is thought to be the first line of defence against over-initiation, providing a time window during which the initiation potential can be reduced by other means [27].

Since hemimethylated origins are active in vitro [54], other functions are required for origin inactivation in vivo. Some reports support that binding of hemimethylated *ori*C to the outer membrane is implicated in origin sequestration and thereby in control of chromosomal DNA replication. In 1988, Ogden et al. [9] showed that *E. coli* outer membrane preparations bind *ori*C DNA only when it is hemimethylated. Subsequently, it was demonstrated that binding of hemimethylated *ori*C to membranes inhibits the initiation of DNA synthesis in vitro [10]. Moreover, remethylation of the membrane-bound hemimethylated *ori*C results in reactivation [10]. DNase I footprinting analysis showed that the outer membrane interacts with *ori*C mainly at the left moiety of the minimal origin, where 10 out of 11 Dam methylation sites are located [11].

Identification of factors involved in hemimethylated *ori*C sequestration has been the focus of several studies. A genetic approach identified SeqA as a protein responsible for sequestering hemimethylated *ori*C DNA [55]. This study showed that methylation of the newly replicated origins is faster in *seq*A$^-$ than in *seq*A$^+$ cells. In addition, the synchrony of initiation at multiple copies of *ori*C is disrupted in *seq*A$^-$ cells [55]. More recently, it was found that an excess of SeqA protein prolongs sequestration of *ori*C [56]. Besides its role in origin sequestration, SeqA protein functions as a negative regulator of the primary initiation process [55,57] and its overproduction affects chromosome partitioning and cell division [53,56]. In vitro studies showed that SeqA protein binds hemimethylated *ori*C and non-*ori*C sequences, as well as fully methylated *ori*C. However, the affinity of SeqA for hemimethylated *ori*C is higher than for fully methylated *ori*C [12,58,59]. The Dam methylase is unable to displace SeqA protein already bound to hemimethylated *ori*C sequences in vitro [60]. It is known that purified SeqA protein inhibits replication initiated from *ori*C in vitro, both by interfering with the prepriming complex and by affecting DNA topology [61–63].

The specific binding of hemimethylated *ori*C to outer membrane fractions in vitro is drastically reduced when membranes are prepared from *seq*A$^-$ cells, suggesting that it is mediated by SeqA protein [12,13]. Membrane-association of SeqA depends on DNA replication cycle [15]. The maximal amount of SeqA in outer membrane fractions was detected at the initiation of DNA replication and then it gradually decreased. The same pattern of variation was found in the hemimethylated *ori*C binding activity of the membrane fractions [15]. This correlation supports the involvement of SeqA in membrane binding of hemimethylated *ori*C. However, the mechanism by which SeqA, a soluble protein, associates with the bacterial membrane is still unknown.

Shakibai et al. [14] proposed that a multiprotein complex would mediate binding of hemimethylated *ori*C to the bacterial membrane. These authors identified SeqB as a component of this membrane-associated system, which also includes SeqA protein. SeqB stimulates the *ori*C-binding activity of SeqA and is required for SeqA-*ori*C binding at low concentrations of SeqA. Furthermore, HobH was described as a component of the outer membrane involved in hemimethylated *ori*C binding based on studies performed with a LacZ-HobH fusion protein [11]. Later, it was reported that HobH is a non-specific acid phosphatase and it was termed NAP [64]. NAP protein was purified to near homogeneity and its hemimethylated DNA binding activity was examined [65]. Gel retardation experiments indicated that purified NAP is a DNA binding protein with an increased affinity for hemimethylated *ori*C DNA. However, footprinting experiments showed protection of hemimethylated *ori*C DNA by partially purified NAP, but not by purified preparations. This result was interpreted as indicative of the existence of other proteins required for *ori*C protection besides NAP [65].

2.3. *Intracellular localization of the* E. coli ori*C region*

Cytological methods, based on subcellular localization of specific chromosomal DNA segments, have revealed a dynamic organization of the bacterial chromosome. This dynamic organization seems to play a critical role in chromosome replication, chromosome partitioning and cell division (for a review, see [21, 22]).

By fluorescence in situ hybridization (FISH), Niki and Hiraga [66] analyzed the subcellular localization of the replication origin and terminus sites in rapidly growing *E. coli* cells (55 min doubling time). A scheme of their results is shown in Fig. 1(a). In newborn cells, a single *ori*C focus, which appears to be a pair of linked sister *ori*C segments, is preferentially localized near a nucleoid border. Later, one copy of the duplicated *ori*C region remains at the same position, whereas the other

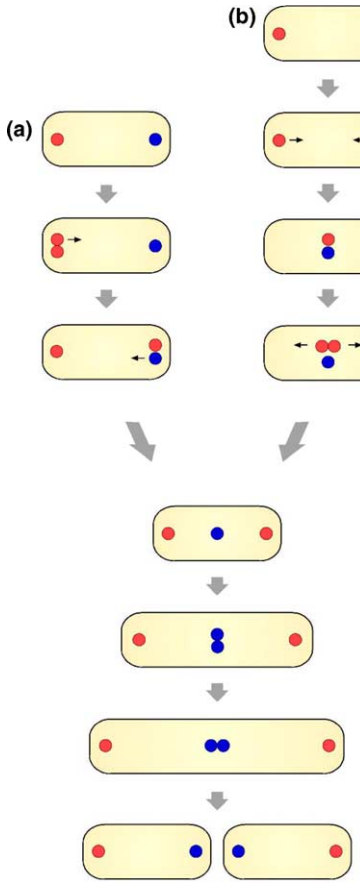

Fig. 1. Cartoon showing the intracellular localization of the replication origin and terminus sites during the *E. coli* cell cycle. The *ori*C region (red circle) and the terminus region (blue circle) are indicated. The horizontal arrows indicate migration of these regions. (a) Rapidly growing cells. Adapted from Niki and Hiraga [66]. (b) Slowly growing cells. Based on the study performed by Niki et al. [68]. See text for details.

migrates to the opposite nucleoid border, where the replication terminus is localized. The *ori*C copies are retained at both nucleoid borders, remaining at a constant distance from each cell pole. In an early stage of the cell division cycle, the terminus region migrates from the nucleoid border to midcell and is retained there until the terminus is duplicated [66]. The intracellular position of the *ori*C region was also analyzed by inserting tandem copies of the lactose operon operator (*lac*O) into the bacterial chromosome near *ori*C [67]. Expression of the GFP-LacI fusion protein allowed visualization of the *lac*O array and, thereby, *ori*C localization. This study indicated that the *ori*C region is closely associated with the cell pole throughout the cell cycle, even during origin duplication [67].

Further insight into the localization pattern of the *ori*C region was obtained by FISH analysis of slowly growing *E. coli* cells (80 min doubling time) [68]. In newborn cells, the *ori*C and terminus regions are differentially localized near opposite cell poles. However, before initiation of chromosome replication, both regions migrate towards the middle of the cell, suggesting an overall rearrangement of the chromosome (see Fig. 1(b)). Hence, under slow cell growth conditions, the *ori*C region is replicated at or near the midcell position. Then, during ongoing replication, the sister *ori*C regions migrate from midcell towards opposite cell poles. In contrast to *ori*C, the terminus region remains near midcell both during replication and between completion of chromosome replication and cell division [68].

The subcellular localization of *ori*C was also examined by FISH in *dna*A203 mutant cells, which are temperature sensitive for initiation of DNA replication [69]. In temperature-shift experiments, a single *ori*C focus was localized preferentially at midcell just before transfer to the permissive temperature (30 °C) and also for 10 min after the transfer, indicating that the *ori*C region is replicated at midcell.

By electron-microscopic autoradiography, the subcellular position of [^{3}H]thymidine incorporation during DNA replication was analyzed in *E. coli* cells growing with a 210 min doubling time [70]. According to this study, DNA replication starts in the cell center where it remains for a large part of the DNA replication period. Furthermore, FISH analysis of *E. coli* cells grown under steady state conditions with a doubling time of 79 min indicated that *ori*C is confined to the center of the prospective daughter cell when DNA replication starts [71]. Thus, these findings also support the occurrence of a centrally located DNA replication compartment.

Recently, the positions of DNA regions close to the chromosome replication origin and terminus have been visualized simultaneously in *E. coli* cells. Furthermore, the positions of these regions with respect to a replication factory-associated protein have been analyzed. The results obtained are consistent with a model in which a primary cellular positioning mechanism sequentially positions the newly replicated origins, the replication factory, the FtsZ ring and the replication terminus region at a place where cell division will occur as a consequence of contraction of the FtsZ ring [72].

It has been shown that the *ori*C region of *E. coli* localizes to the cell poles even when replication initiates elsewhere on the chromosome. Hence, positioning of the *ori*C region at the cell poles appears to be independent of the site of replication initiation [73]. This observation supports the idea that sequences outside the minimal *ori*C are used by host mechanisms to direct the proper placement of the origin region [73]. Recent studies showed that MreB protein, an actin homolog, forms helical filaments that extend along the long axis of the *E. coli* cells. These MreB filaments appear to participate in directional chromosome movement and segregation [74]. Moreover, MreB is present in all rod-shaped

bacteria and is required to maintain the non-spherical shape of the cells (for reviews, see [75–77]).

2.4. Intracellular localization of SeqA protein

As indicated above, SeqA protein negatively modulates initiation of chromosome replication [55,57]. It binds hemimethylated *ori*C and non-*ori*C sequences in vitro, as well as fully methylated *ori*C [12,58,59]. The subcellular localization of SeqA was examined in fast-growing cells (55 min doubling time) by indirect immunofluorescence microscopy [78]. SeqA protein was detected as discrete fluorescent foci throughout the cell cycle, but its localization pattern was different from that of the *ori*C region [66]. In newborn cells, a single SeqA focus is localized at midcell. The SeqA focus is duplicated and tethered at such a position until an FtsZ ring is formed. Then, the SeqA foci migrate in opposite directions towards the 1/4 and 3/4 positions of the cell, where they remain until the cell divides [78]. These results are consistent with the subcellular localization of a SeqA-GFP fusion protein in living cells [79]. Fluorescent foci of SeqA were also observed in an *ori*C-deleted mutant strain, indicating that the SeqA-associated DNA-protein complex is formed at a specific region(s) of the chromosome but not at *ori*C itself [78].

The subcellular localization of SeqA was also examined in synchronously replicating cultures of *dna*C2 and *dna*A203 cells [69]. This study showed that formation of discrete SeqA foci depends on ongoing chromosome replication, suggesting that SeqA foci are clusters of newly replicated hemimethylated DNA segments bound with SeqA molecules. Moreover, a single SeqA focus appears at midcell upon initiation of DNA replication. Then, the SeqA focus separates into two foci, which abruptly migrate in opposite directions during the course of the first round of replication. Bidirectional migration of SeqA foci occurs prior to separation of linked *ori*C copies [69]. Recently, a sequential binding of SeqA to hemimethylated nascent DNA segments at replication forks has been observed in synchronized cultures of *E. coli* (S. Hiraga, personal communication). Hiraga et al. [69] proposed two models of replication factories based on the bidirectional migration of the SeqA foci:

(a) Translocating replication factories. In this model (Fig. 2, Model 1), the SeqA clusters are always localized close to the replication apparatuses. Initially, a pair of replication apparatuses, which promote bidirectional replication, would be localized at the midcell position. After replication initiation, a number of SeqA molecules would bind to newly replicated hemimethylated DNA segments forming a SeqA–DNA cluster by protein–protein interactions. During DNA replication, the SeqA cluster at midcell separates into two clusters, which migrate in opposite directions to the 1/4 and 3/4 positions, together with each replication apparatus. Subsequently, the linked sister *ori*C copies separate and migrate asymmetrically in opposite directions towards both pole-proximal nucleoid borders. The finding that the β subunit (DNA sliding clump/DnaN) of DNA polymerase III holoenzyme is recruited near SeqA foci in synchronously replicating cells supports this model [80].

(b) Fixed replication factories. This model (Fig. 2, Model 2) was previously proposed by Lemon and Grossman [19] based on the intracellular localization of the replicative DNA polymerase in *B. subtilis* cells (see below). In this model, the pair of replication apparatuses remains at the midcell position until termination of DNA replication, whereas the SeqA clusters would migrate from midcell to the cell quarter positions during DNA replication. Hence, newly replicated hemimethylated double strands would be pulled from replication apparatuses located at midcell to the SeqA–DNA clusters located at the cell quarter positions.

Additional experiments would be needed to conclude which model applies to *E. coli* cells. Specifically, it will be critical to measure the number of origins and replication forks when thermosensitive strains containing mutations in the *dna*C gene are used for synchronization, since inhibition of initiation of chromosome replication at nonpermissive temperature, as well as reinitiation of replication at permissive temperature, are affected by a number of parameters [81].

In a recent study, Molina and Skarstad [82] analyzed the SeqA focus and replication fork distributions in four exponential cultures with three different replication patterns. These authors found that pairs of forks beginning from the same origin stay coupled for most of the cell cycle supporting the replication factory model. Furthermore, the nucleotide synthesis apparatus appears to colocalize with the replisomes, forming a "hyperstructure".

The subcellular localization of newly synthesized 5-bromo-2′-deoxyuridine (BrdU) pulse-labeled DNA has been recently analyzed in growing *E. coli* cells using immunofluorescence microscopy. This analysis indicates that the replication apparatuses involved in bidirectional replication of the chromosome are colocalized at midcell in the early replication stage (S. Hiraga, personal communication).

3. Extrachromosomal DNA replication in *E. coli*

The replicon model [1,2] was the first that postulated an essential role of the bacterial membrane for prokaryotic DNA replication. Since then, some reports support that plasmid and viral DNA replication, as well as chromosomal DNA replication, takes place associated to the bacterial membrane. This evidence mainly includes the isolation of DNA–membrane complexes, the

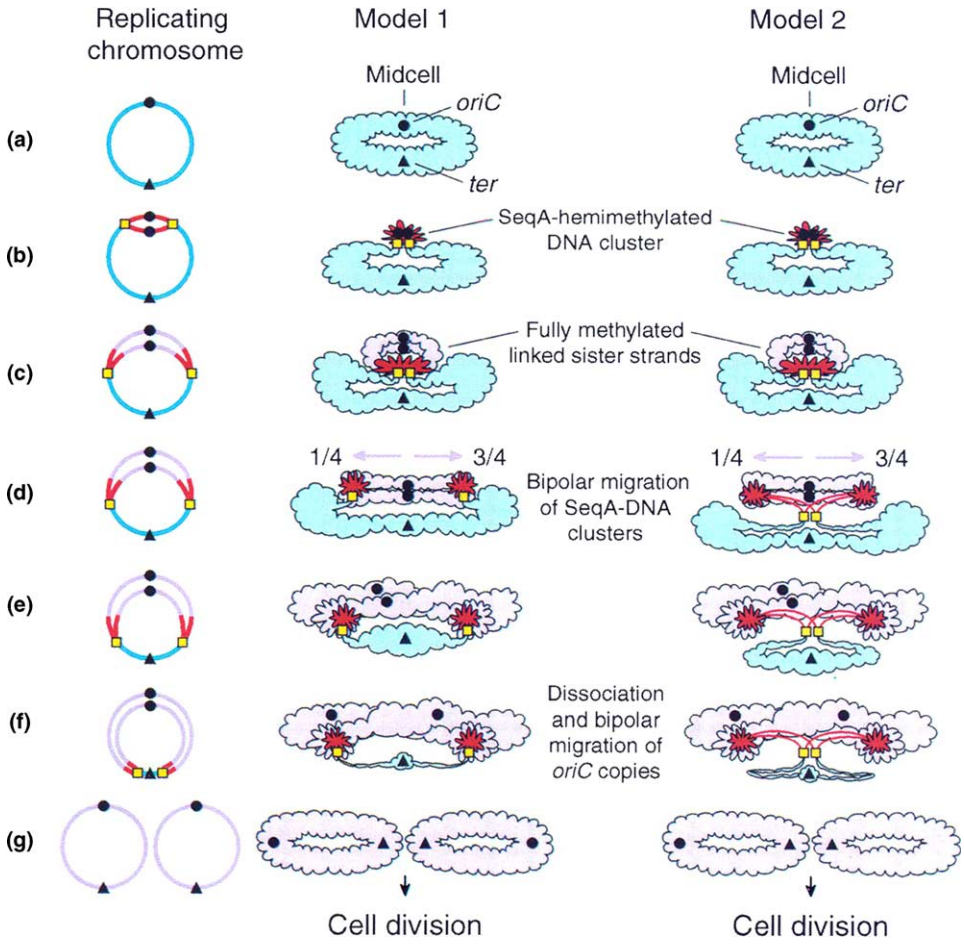

Fig. 2. Models for the positioning of the replication factories in *E. coli* cells. Reproduced with permission (Blackwell Publishing) from Hiraga et al. [69]. The bidirectional replication of the *E. coli* chromosome is shown at the left. Model 1: translocating replication factories. Model 2: fixed replication factories. Blue lines and areas represent unreplicated fully methylated chromosomal DNA. Red lines and areas represent nascent hemimethylated DNA strands. Purple lines and areas represent fully methylated sister DNA strands. Black circles represent *ori*C sites. Black triangles represent terminus sites. Yellow squares represent replication apparatuses. Purple horizontal arrows represent rapid bidirectional migration of SeqA clusters from midcell to the 1/4 and 3/4 positions of the cell. See text for more details.

identification of membrane-associated DNA replication proteins and, in few cases, the isolation of membrane complexes able to synthesize DNA in vitro without the addition of exogenous DNA and replication factors. Early studies on this subject have been previously reviewed [3,5,6]. We will deal here with studies on membrane-association of plasmid RK2 DNA replication, since it has been extensively investigated. In addition, we will review recent observations concerning the intracellular localization of plasmid DNA molecules.

3.1. Membrane-association of plasmid RK2 DNA replication

Replication of the broad-host-range plasmid RK2 initiates at a unique sequence, termed *ori*V, and requires the plasmid-encoded initiation protein TrfA. The *ori*V site and the *trf*A gene are the only plasmid-specific determinants required for initiation of RK2 DNA replication in a wide variety of bacterial hosts [83]. The initiation protein TrfA binds to *ori*V as a monomer [84]. In vitro replication experiments using a plasmid RK2 DNA–membrane complex indicated that TrfA was present and active in the complex [85]. Subsequently, cell fractionation studies performed in *E. coli* showed that TrfA protein was tightly bound to the inner membrane [86]. Moreover, it was shown that TrfA-dependent initiation of RK2 DNA replication was associated primarily with the inner membrane fraction [87]. Further cell fractionation studies identified an inner membrane subfraction that contains TrfA and is able to bind *ori*V DNA in vitro [88]. It was reported that this membrane subfraction, which represents less than 10% of the total membrane, also supports TrfA-dependent initiation of RK2 DNA synthesis [89]. Therefore, replication of plasmid RK2 is thought to take place at a specific site on the bacterial membrane [89]. In addition to *E. coli*, TrfA protein was found to fractionate with the

cell membranes of four different gram-negative hosts [90]. In all the cases, only the inner membrane fraction was capable of extensive TrfA-dependent RK2 DNA synthesis.

The TrfA protein has a 12-amino acid hydrophobic region [91]. By mutational analysis, it was found that changes of specific amino acids within such a region affect both membrane-association of TrfA and its ability to support replication of plasmid RK2 in vivo. Hence, the hydrophobic region may function as a membrane-targeting domain.

3.2. Intracellular localization of plasmid DNA

Recent advances in fluorescence microscopy have shown that plasmid DNA molecules are specifically localized within the bacterial cell. These localization studies suggest that plasmid DNA replication occurs at particular subcellular sites and establish that segregation of bacterial plasmids takes place in an active and directed fashion [21,22,25].

The low-copy-number plasmids F and P1 are present at one or two copies per chromosome equivalent in *E. coli* cells. Both plasmids are actively partitioned into daughter cells by the plasmid-encoded *sop*ABC and *par*ABS systems, respectively [21–23]. The intracellular localization of plasmids F and P1 during the cell cycle was examined by inserting tandem *lac* operator sequences into the plasmids [67]. Expression of the GFP-LacI fusion protein allowed detection by fluorescence microscopy of the tandem *lac* operator sequences and, thereby, localization of plasmid DNA molecules. In newborn cells, plasmids P1 and F are localized to the cell midpoint (see Fig. 3). Upon duplication at such a position, plasmid DNA molecules migrate bidirectionally to the 1/4 and 3/4 cell positions, which become the midpoints of the nascent daughter cells. This ensures that each daughter cell will receive at least one plasmid molecule. Using FISH techniques, Niki and Hiraga [92] reported a similar distribution pattern for a mini-F plasmid carrying the *sop*ABC system. Moreover, by synchronizing mini-F replication, it was shown that sister copies are separated shortly after replication at midcell [93].

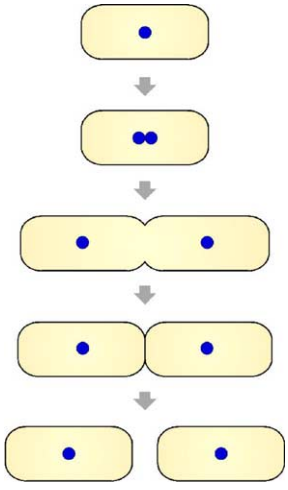

Fig. 3. Cartoon showing the intracellular localization of plasmids F and P1 (blue circles) during the cell cycle. Based on the results obtained by Gordon et al. [67] and Niki and Hiraga [92].

Positioning of plasmid molecules at specific cellular sites depends on the partitioning system. For example, mini-F plasmids lacking the *sop*ABC system are randomly localized in cytosolic spaces of the cell poles [92]. More recently, it was shown that plasmids constructed to replicate using the *E. coli ori*C site are localized to midcell and quarter cell positions only when they carry the *sop*ABC sytem of plasmid F [94]. Furthermore, a mini-F plasmid carrying the partitioning system (*par*A) of the *E. coli* plasmid R1 is targeted near the cell midpoint or cell poles, rather than near the cell quarters [95]. Thus, the *par*A system of plasmid R1 moves plasmid DNA from midcell, where plasmids are presumably replicated, to opposite cell poles [95]. ParM protein, a component of the *par*A system, forms dynamic F-actin-like filaments that are involved in plasmid partitioning [96,97]. A model for plasmid partitioning by the R1 *par*A system, which takes place independently of host cell factors, has been recently proposed [98]. In this model, ParM moves plasmid DNA molecules by an actin-like insertional polymerization mechanism.

The broad-host-range plasmid RK2 has around five to eight copies per chromosome equivalent in *E. coli* cells. The subcellular location of RK2 was examined using both GFP-LacI tagging and FISH techniques [99]. The number of fluorescent foci observed in each cell was considerably less than the copy number of RK2. Thus, many copies of RK2 seem to be replicated and partitioned in clusters targeted to specific subcellular locations. In newborn cells, RK2 is localized near midcell. After duplication, RK2 foci separate and migrate with rapid kinetics to the quarter cell positions. The pattern of RK2 localization is conserved in *Pseudomonas aeruginosa* and *Vibrio cholerae* [100]. Plasmid R1, which has an average copy number of four to five per cell, is also present as clusters in exponentially growing *E. coli* cells, as shown by FISH experiments [101]. Several lines of evidence suggest that high-copy-number plasmids lacking a partitioning system can also be targeted to specific sites within the cell [99,102,103].

Plasmids F, P1 and RK2 belong to different incompatibility groups. Ho et al. [100] compared the relative positions of these plasmids by dual labelling FISH experiments and found that, despite its similar distribution pattern, they are targeted separately to different positions in the vicinity of the cell midpoints or cell quarters. Moreover, these plasmids are not only spatially separated in the cell, but they segregate at different times relative to one another. Hence, compatible plasmids appear to be

effectively separated from one another during much of the cell cycle. This compartmentalization supports a model in which compatible plasmids interact with different subcellular structures [100]. The nature of these putative host cell receptors remains unknown.

4. Chromosomal DNA replication in *B. subtilis*

The circular chromosome of *B. subtilis* is replicated bidirectionally from a single origin (*ori*C) [104]. Like in *E. coli*, the *B. subtilis* DnaA protein binds to DnaA boxes found in the *ori*C site. This binding leads to a localized unwinding of *ori*C, which allows assembly of a large multiprotein machine, the replisome [105]. Several studies support that replication of the *B. subtilis* chromosome is associated to the bacterial membrane [for a review, see 7]. In this chapter, we will review recent experiments concerning the subcellular localization of the *ori*C region and some replication proteins during the *B. subtilis* cell cycle.

4.1. Intracellular localization of the B. subtilis ori*C region*

In *B. subtilis*, studies on chromosome segregation revealed that newly replicated *ori*C regions are in a defined orientation for most of the bacterial cell cycle [for recent reviews see 23,24]. The position of *ori*C, as well as that of the replication terminus site (*ter*C), was examined in vegetative cells by introducing an array of LacI-binding sites into the chromosome near *ori*C or *ter*C. A GFP-LacI fusion protein was then used to visualize the position of the operator array [106]. Early in the cell cycle, the *ori*C regions were predominantly observed towards the cell poles. By contrast, the *ter*C regions were usually located near the midpoint of the cell. Similar results were reported for *E. coli* based on both GFP-LacI tagging [67] and FISH techniques [66] (see Fig. 1). As pointed out by Webb et al. [106], cells with a bipolar arrangement of the newly replicated *ori*C regions could arise in one of two ways. One possibility is that before initiation of DNA replication, the *ori*C region is not attached near either pole. Only after replication starts do the origin regions migrate towards the cell poles. Alternatively, in a newborn cell, the *ori*C region could be positioned near a cell pole prior to replication. Then, after replication ensues, one of the two newly formed origin regions could move towards the opposite end of the cell [106]. The movement of the *ori*C and *ter*C regions of the *B. subtilis* chromosome was further visualized by time-lapse fluorescence microscopy [107]. According to this study, unduplicated origins, or origins that have undergone duplication but have not yet begun to separate from each other, move to a variable extent from a polar to a more central position in the cell. Then, the newly duplicated *ori*C regions separate and move rapidly towards the cell poles during a brief interval of the cell cycle. Termini also exhibit movement, but they do not move apart as far or as rapidly as do the origins. These findings supported the existence of a mitotic-like apparatus for rapid and directed origin movement [107].

Identification of factors involved in the precise subcellular localization of *ori*C has been the focus of several investigations. A candidate for such a function was the SpoOJ protein, which is related to the ParB family of plasmid-encoded partition proteins [108]. It was described that *spo*OJ null mutants produce a significant increase in the number of cells lacking DNA during vegetative growth [109]. Furthermore, SpoOJ is required for chromosome partitioning during sporulation [110]. SpoOJ is a DNA binding protein that recognizes a 16 bp sequence located within the *spo*OJ gene. This sequence, called *par*S, can function as a partitioning, or centromere-like, site [111]. Furthermore, ten potential SpoOJ binding sites were found in the origin-proximal 20% of the chromosome. Eight of these sites are bound to SpoOJ in vivo. Hence, SpoOJ binds to chromosomal sites close to *ori*C [111]. The subcellular position of SpoOJ was examined by immunofluorescence microscopy and visualizing fluorescence of a SpoOJ-GFP fusion protein [112,113]. These studies showed that SpoOJ forms discrete foci that tend to be localized towards the cell poles. In addition, the SpoOJ foci colocalize with the *ori*C regions throughout the cell cycle [114]. Duplication of SpoOJ foci occurs early in the DNA replication cycle and requires the initiation of DNA replication at *ori*C [115]. Soon after duplication, sister *ori*C/SpoOJ foci move rapidly apart to achieve a fixed separation of about 0.7 μm [115]. Nevertheless, using time-lapse microscopy, Webb et al. [107], visualized movement of the origin region towards the cell poles in the absence of SpoOJ.

Recently, it was shown that SpoOJ contributes to the positioning of sister origins at the cell quarters during vegetative growth. In *spo*OJ null cells, sister origin regions were often closer together, nearer to midcell [116]. However, it was found that the subcellular localization of SpoOJ depends on the chromosomal position of its binding sites, indicating that SpoOJ is not sufficient to recruit the origin regions to the cell quarters [116]. Therefore, additional factors must determine the specific positioning of the *ori*C region in vegetative cells. Recent evidence indicated that actin-like proteins MreB and MbI are required for bipolar positioning of replication origins in *B. subtilis* cells [117]. These proteins form dynamic helical filaments underneath the cell membrane and are also needed for proper cell shape [118–120].

During sporulation, newly replicated *ori*C regions migrate to extreme opposite ends of the cell where they appear to be anchored [121]. A large chromosomal region adjacent to, but not including the replicative origin, was

shown to be necessary for polar origin positioning during sporulation [122]. Furthermore, recent studies showed that the bacterial DNA binding protein RacA is required for anchoring the *ori*C regions at the cell poles [123]. RacA localization depends on the division site selection protein DivIVA [123], which also functions in chromosome segregation during sporulation [124]. The DivIVA/RacA and Soj/SpoOJ systems act in a concerted fashion to target the chromosomal origin to the cell pole [125]. A protein functionally equivalent to RacA has not yet been identified during exponential growth of *B. subtilis* cells.

4.2. Intracellular localization of B. subtilis replication proteins

The replicative DNA polymerase of *B. subtilis* has been visualized in living cells using a fusion protein consisting of the catalytic subunit (PolC) attached in-frame to GFP [19]. By this experimental approach, the DNA polymerase was localized at discrete intracellular positions, predominantly at or near midcell, rather than being randomly distributed along the nucleoid mass. Fast-growing cells with multiple chromosomes had additional PolC-GFP foci located at the cell one-quarter and three-quarter positions [19]. Moreover, the δ' and τ components of the DNA polymerase III holoenzyme were independently fused to GFP. Both fusion proteins were functional and had patterns of localization similar to those of PolC-GFP [19]. Lemon and Grossman [126] showed that the stationary, centrally positioned DNA polymerase represents the location of active replication forks. After a region of DNA is replicated, the two duplicated regions move away from the replisome towards opposite ends of the cell. These findings support the factory model for chromosome replication in which the replisome is anchored in place and the DNA template moves through it. The observation that DNA polymerase is a motor protein that resides in a stationary replisome led to the "extrusion-capture" model for chromosome segregation. This model proposes that DNA replication itself pushes newly replicated DNA towards opposite sides of the cell [19,127,128]. More recently, another polymerase (DnaE) replicating with the opposite polarity to PolC was also found at midcell [129].

The DnaB and DnaI primosomal proteins interact physically and functionally with the DnaC replicative helicase and mediate its functional delivery on *ori*C [130]. This delivery is a key step for initiation of chromosomal DNA replication. Imai et al. [20] examined the subcellular localization of DnaA, DnaB and DnaI by immunofluorescence microscopy. DnaB and DnaI, but not DnaA, were detected as foci during the cell-division cycle. Although the foci were not always colocalized with *ori*C, they seemed to be localized near the outer or inner edges of the nucleoids at initiation of replication. Furthermore, the DnaX subunit of DNA polymerase III was fused to GFP, and DnaX-GFP foci were detected near either edge of the nucleoids at the onset of chromosomal DNA replication [20]. Thus, these observations differ from those reported by Lemon and Grossman [19] and suggest that *B. subtilis* chromosomal DNA replication begins near either edge of the nucleoid.

Noirot-Gros et al. [131] used genome-wide yeast two-hybrid screens to identify the proteins that physically associate with known *B. subtilis* replication proteins. This experimental approach revealed interactions of replication proteins with membrane-associated protein complexes, supporting that bacterial DNA replication is linked with the membrane. For example, the DnaB initiator protein interacts with the chemotaxis proteins McpA and YvaQ. These authors propose that this interaction might determine the polar localization of DnaB reported by Imai et al. [20], since the methyl-accepting chemotaxis proteins form large clusters located at the cell poles in both *E. coli* [132] and *B. subtilis* [133]. In addition, earlier studies showed that DnaB is essential for the binding of *ori*C to the bacterial membrane [134,135].

5. Replication of the *B. subtilis* bacteriophage ϕ29

In prokaryotes, like in eukaryotes, replication of the viral genomes seems to occur at specific intracellular locations. In both cases, the use of large organizing structures to bring together replication factors may be a general mechanism to enhance the efficiency of the replication process. Some lines of evidence support that replication of phage DNA takes place in close association with the bacterial membrane [3,5]. For example, in phage T4, the gene 69 product (gp69) behaves as an integral membrane protein in the inner membrane of *E. coli*, and a model for anchoring of a replication initiation complex to the membrane by gp69 has been proposed [136,137]. In this chapter, we will review recent investigations concerning compartmentalization of phage ϕ29 DNA replication in *B. subtilis* cells. These studies identified p1 and p16.7 as membrane-localized, phage-encoded proteins likely involved in the membrane-association of ϕ29 DNA replication.

5.1. Genetic organization of the ϕ29 DNA: general features

The genome of ϕ29 is a linear double-stranded DNA molecule with a terminal protein (TP) covalently linked at each 5′-end. Its genetic and transcriptional organization has been covered in a recent review [138]. Therefore, only general features will be emphasized.

The early-expressed ϕ29 genes are organized in two operons, which are located at the right and left regions

of the genome. Both operons are transcribed leftward (see Fig. 4(a)). The right operon is transcribed from the strong C2 promoter, whose activity decreases rapidly 10 min after infection [139]. This promoter is repressed both in vivo and in vitro by the viral early protein p6 [140–142]. The right operon contains genes 17 and 16.7, in addition to four open reading frames of unknown function (16.9, 16.8, 16.6 and 16.5). There is an additional weak early promoter, termed C1, which is located within gene 16.7. Thus, this promoter may drive the expression of open reading frames 16.6 and 16.5. It was reported that protein p17 is required for efficient ϕ29 DNA synthesis when cells are infected at low but not at high multiplicities of infection [143]. In vitro, protein p17 interacts with the viral protein p6 [144]. Furthermore, recent studies showed that protein p17 participates in the injection of ϕ29 DNA, which occurs from the right end by a push-pull mechanism [145]. The role of protein p16.7 in ϕ29 DNA replication is described below.

The left early operon contains genes 6, 5, 4, 3, 2 and 1. It is transcribed from two strong promoters, termed A2c and A2b [reviewed by 138,146,147]. In addition, gene 1 would be transcribed from the weak promoter A1IV, which is located within gene 2 [148–150]. A Rho-independent transcriptional terminator, named TA1, is located within gene 4 [151]. With the exception of gene 4, which encodes a transcriptional regulator protein (p4) [146,147], the left early genes are involved in phage DNA replication. Specifically, genes 6 (protein p6, double-stranded DNA binding protein), 5 (p5, single-stranded DNA binding protein), 3 (TP) and 2 (DNA polymerase) are essential for in vivo ϕ29 DNA replication [reviewed by 138,152–154]. Protein p6 has also a role in transcriptional regulation. Gene 1 encodes protein p1, which enhances the rate of viral DNA replication in vivo (see below). An additional early promoter (A1) is located downstream gene 1. It is active throughout the infection cycle [139] and gives rise to a small RNA, which is required for packaging of ϕ29 DNA into proheads [155].

All late-expressed ϕ29 genes (7–16) are organized in a single operon, which is located at the central region of the genome (Fig. 4(a)). This late operon is transcribed rightward from the A3 promoter, whose expression is activated by the early viral proteins p4 and p6. Both proteins also repress the early A2c and A2b promoters [147,156–159]. Late genes encode components of the viral capsids, proteins involved in phage morphogenesis, and those required for cell lysis. A Rho-independent bidirectional transcription terminator (TD1) is located downstream gene 16 [151].

5.2. Protein-primed ϕ29 DNA replication

Initiation of DNA replication by a protein-priming mechanism occurs in linear double-stranded DNAs that have a TP covalently linked to each 5′-end (parental TP). In these DNAs, a free molecule of the TP (primer TP) provides the hydroxyl group needed by the DNA polymerase to start DNA synthesis [152,154,160]. The protein-priming replication mechanism has been

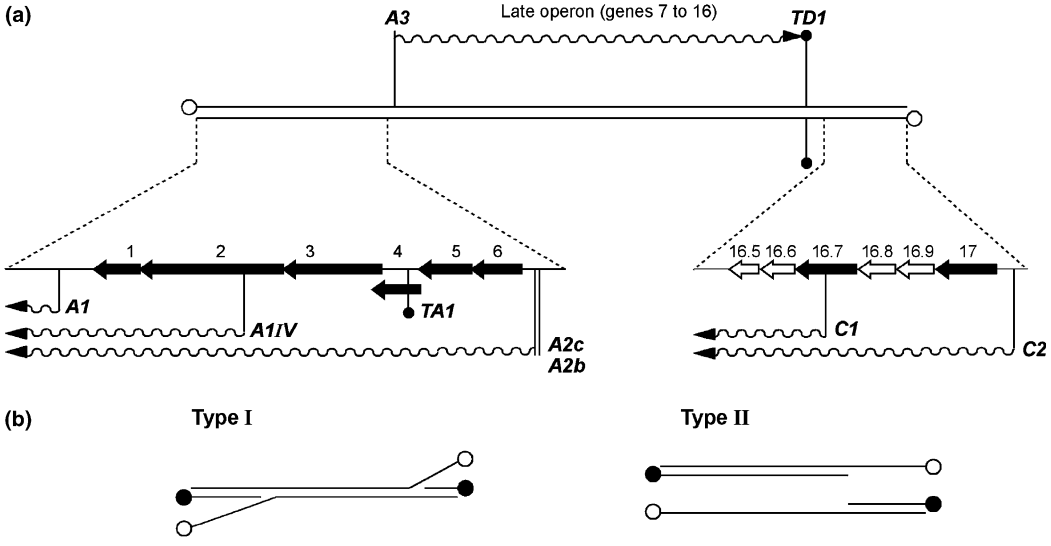

Fig. 4. (a) Genetic and transcriptional map of the ϕ29 genome. Only relevant features are shown. White circles represent the parental terminal proteins. The early promoters (*A1*, *A1IV*, *A2b*, *A2c*, *C1* and *C2*) and the late promoter (*A3*) are indicated. Wave lines represent transcripts from the different promoters. A significant number of transcripts starting from the early promoters *A2b* and *A2c* terminate at the transcriptional terminator *TA1*, contributing to the high intracellular levels of proteins p6 and p5. The transcriptional terminator *TD1* is bidirectional. The position of genes (black arrows) and open reading frames (white arrows) is indicated. (b) Types of ϕ29 replicative intermediates (see text for details). Black circles represent the terminal protein molecules used as primers.

extensively studied in phage ϕ29, which is being used as a model system. Electron microscopy studies revealed two main types of ϕ29 replicative intermediates in infected cells, named type I and type II [161,162] (Fig. 4(b)). Type I intermediates are unit-length linear double-stranded DNA molecules with one or more single-stranded branches of varying lengths. Type II intermediates are unit-length linear molecules in which a region of the DNA starting from one end is double-stranded, and the adjacent region containing the other end is single-stranded. Therefore, these studies showed that replication of ϕ29 DNA starts non-simultaneously at either DNA end, where the replication origins are located, and proceeds by strand displacement towards the other end.

Fig. 5 shows the current model for initiation of ϕ29 DNA replication. For simplicity, only one ϕ29 DNA end is represented. A detailed description of the proteins involved in the replication process can be found in numerous reviews [138,152–154,160]. The viral protein p6 activates the initiation of ϕ29 DNA replication in vitro by forming a multimeric nucleoprotein complex at the replication origins [163–166]. This activation requires specific recognition of the protein p6 nucleoprotein complex by the primer TP-ϕ29 DNA polymerase heterodimer [167]. After origin recognition, the DNA polymerase catalyzes the covalent linkage of dAMP to the hydroxyl group of Ser^{232} provided by the primer

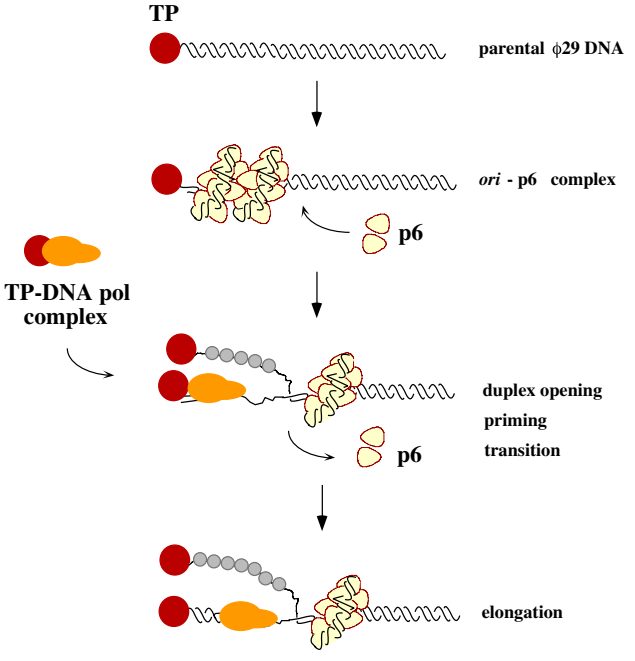

Fig. 5. Model for the initiation of in vitro ϕ29 DNA replication, adapted from Bravo and Salas [181]. The viral proteins involved in the initiation stages are indicated. TP: terminal protein, DNA pol: DNA polymerase. Grey circles represent the single-stranded DNA binding protein p5. For simplicity, only one ϕ29 DNA end (replication origin) is represented.

TP (TP-dAMP initiation complex) [168–170]. Phage ϕ-29 DNA has a 6-bp-long inverted terminal repeat (AAAGTA) [171,172]. Formation of the TP-dAMP initiation complex is directed by the second nucleotide at the 3'-end of the template. Then, the TP-dAMP complex slides back to recover the information of the first nucleotide (sliding-back mechanism) [173]. Before dissociating from the primer TP, the DNA polymerase synthesizes a short elongation product [174]. Subsequently, the same DNA polymerase catalyzes highly processive polymerization by a strand-displacement mechanism [175,176], and protein p5 binds to the displaced single-stranded DNA [177]. The extensive knowledge of the ϕ29 DNA replication mechanism has been fundamental to approach the in vivo localization of the replication process (see below).

5.3. Protein p1

In 1973, Ivarie and Pène [178] described the isolation of a rapidly sedimenting complex from ϕ29 infected cells using linear density gradients of Renografin. The complex, previously identified as membrane-bound bacterial DNA, contained parental ϕ29 DNA and was enriched for newly synthesized phage DNA. The parental ϕ29 DNA–membrane complexes were detected near the onset of viral DNA replication, and their formation required synthesis of early viral-encoded proteins. Moreover, a correlation between the formation of such complexes and synthesis of viral DNA was reported. For example, parental ϕ29 DNA encoding a thermosensitive DNA polymerase was not membrane-associated at the restrictive temperature, unless cells were co-infected with wild-type phage [178–180]. Based on these findings, it was postulated that in vivo ϕ29 DNA replication takes place associated to the bacterial membrane.

Some 20 years later, and as an approach to identify viral-encoded proteins involved in membrane-association of ϕ29 DNA, Bravo and Salas [181] examined whether ϕ29 replication proteins, encoded by the left early operon (Fig. 4(a)), were present in membrane fractions of infected cells. In this study, membrane fractions were obtained by a method that yields almost exclusively "right-side-out" membrane vesicles [182]. During viral DNA replication, large amounts of protein p1 and primer TP were recovered in membrane fractions, as well as a little amount of the ϕ29 DNA polymerase. In contrast, neither protein p6 (double-stranded DNA binding protein) nor protein p5 (single-stranded DNA binding protein) were membrane-associated. Interestingly, the number of ϕ29 DNA polymerase molecules relative to that of protein p1 and primer TP increased in membrane fractions when initiation of ϕ29 DNA replication was blocked. These results suggested that the ϕ29 replication machinery, constituted at least by primer TP and DNA polymerase, assembles on the cell membrane prior to

initiation of ϕ29 DNA replication. Furthermore, experiments performed with *B. subtilis* cells carrying a p1- or primer TP-encoding plasmid indicated that both proteins are membrane-associated even in the absence of other viral components [181]. Thus, protein p1 and primer TP were thought to mediate targeting of the ϕ29 replication machinery to the bacterial membrane.

In ϕ29 infected cells growing at 30 °C, the small viral protein p1 (85 residues) is present in about 10^4 molecules per cell at early stages of infection, and it increases up to 10^5 molecules per cell at late stages. The molar ratio of protein p1 to primer TP (about three) and to DNA polymerase (about 60) is kept constant throughout the course of infection [181]. To understand the role of protein p1, the kinetics of phage DNA synthesis was determined in the presence or absence of protein p1, and under different bacterial growth conditions [181,183]. To this end, the ϕ29 *sus*1(629) mutant phage, which carries a suppressor-sensitive nonsense mutation in gene 1 [184,185], was used. This study showed that protein p1 enhances the rate of ϕ29 DNA replication in vivo, playing a critical role when bacteria are growing at 37 °C, rather than at 30 °C.

As mentioned above, cell fractionation studies indicated that protein p1 is membrane-associated both during synthesis of ϕ29 DNA and after blocking ϕ29 DNA replication [181]. Membrane-association of p1 also occurs in the absence of other viral components, suggesting that protein p1 contacts the bacterial membrane directly [181,186]. The nature of this association was examined by phase partitioning with Triton X-114, a nonionic detergent widely used for the isolation of integral membrane proteins [187,188]. This biochemical analysis showed that protein p1 has an amphiphilic nature, suggesting that it associates with membranes as an integral protein [186]. The C-terminal sequence of p1 spanning residues Tyr^{68} to Ala^{84} is highly hydrophobic. This region could be involved in membrane-association of p1, since a truncated p1 protein that lacks the C-terminal 43 amino acids does not associate with membranes in vivo [181]. Further immunoelectron microscopy studies using affinity-purified p1 antibodies indicated that protein p1 is located at or close to the bacterial membrane during viral DNA replication [186].

Another interesting feature of protein p1 is its capacity to assemble into highly ordered structures in vitro. For example, protein p1 functions as a polymerization domain when it is fused to the maltose-binding protein, leading to the formation of long filamentous structures [183]. Moreover, a truncated p1 protein that lacks the N-terminal 33 amino acids (p1ΔN33) assembles into large polymers that show a parallel array of longitudinal protofilaments (Fig. 6). These structures are two-dimensional sheets whose length and width depends on the polymerization time [183]. The region of protein p1 located between residues Glu^{38} and Asn^{65} presumably forms an α-helical coiled-coil structure. A mutational analysis showed that this coiled-coil motif is involved in the formation of p1ΔN33 sheets [189]. Remarkably, a single conservative substitution targeting the hydrophobic core of the coiled-coil sequence is sufficient to change the pathway of molecular assembly from two-dimensional sheets to ~10-nm-wide filaments (Fig. 6) [189]. Although protein p1 has no sequence homology to cytoskeletal elements, the p1ΔN33 sheets, examined by negative-stain electron microscopy, resemble polymers formed under particular in vitro conditions by FtsZ protein, which forms a ring-like structure that mediates bacterial cell division [190,191]. However, unlike FtsZ, p1 polymerization is not regulated by GTP hydrolysis.

The ability of protein p1 to self-associate was also demonstrated in vivo [186]. By combining in vivo chemical cross-linking and cell fractionation techniques, it was shown that protein p1 assembles into large multimeric structures that are associated to the bacterial membrane. These structures exist both during viral

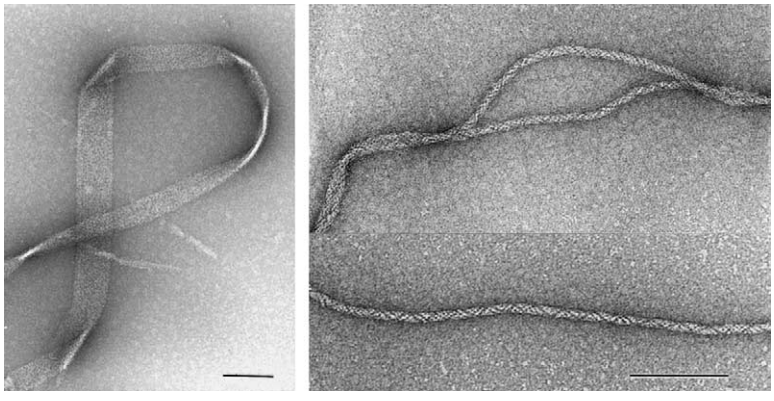

Fig. 6. Electron micrographs of negatively stained structures formed by p1 mutant proteins. Reproduced from Bravo et al. [189]. (Permission granted by The American Society for Biochemistry and Molecular Biology.) Left: protofilament sheet formed by protein p1ΔN33.L39A, in which residue Leu^{39} was changed to Ala. This structure is identical to the one formed by the wild-type p1ΔN33 protein [183]. Right: filamentous structures formed by p1ΔN33.L46V, in which residue Leu^{46} was changed to Val. The scale bars represent 100 nm.

DNA replication and when ϕ29 DNA synthesis is blocked due to the lack of viral replisome components. Membrane-associated p1 structures are also generated in cells harbouring a p1-encoding plasmid, indicating that their formation does not require other viral components. Moreover, these pre-assembled structures appear to support DNA replication of a p1-lacking mutant phage. It remains to be determined what kind of structures p1 would form in vivo.

By in vitro chemical crosslinking experiments, it was shown that a truncated p1 protein that lacks the C-terminal 43 amino acids interacts with the primer TP [150]. Such a protein neither associates with membranes in vivo [181] nor self-interacts in vitro [150]. In addition, truncated p1 proteins that retain the N-terminal 42 amino acids, when present in excess, interfere with the formation of the TP-dAMP initiation complex in a reaction that requires the efficient formation of a primer TP-ϕ29 DNA polymerase heterodimer. This in vitro interference can be explained as a competition between the truncated p1 protein and the ϕ29 DNA polymerase for binding to the primer TP. Thus, the N-terminal 42 amino acid residues of protein p1 are sufficient for binding to the primer TP in vitro [150].

A model for the role of protein p1 has been proposed in which a p1 multimeric structure assembled on the bacterial membrane provides a specific site for ϕ29 DNA replication [150,186]. According to this compartmentalization model (Fig. 7), the ϕ29 DNA replication machinery, constituted at least by the primer TP-ϕ29 DNA polymerase heterodimer, would be targeted to the membrane-associated p1 structure. This association could be achieved by protein–protein interactions between protein p1 and primer TP. In addition, direct contacts between primer TP and the bacterial membrane could take place. Once ϕ29 DNA replication starts by a protein-priming mechanism, the primer TP remains covalently bound to the newly synthesized DNA and, thereby, viral DNA becomes membrane-associated when it is replicated. The role proposed for the membrane-associated p1 structure resembles the function of the eukaryotic nuclear matrix in adenovirus DNA replication. Like phage ϕ29, the genome of adenovirus is a linear double-stranded DNA with a TP covalently attached to each 5′-end. Therefore, its replication proceeds via a protein-priming mechanism [192]. Cell fractionation studies indicated that adenovirus DNA is tightly bound to the nuclear matrix throughout the course of infection [193,194]. It has been shown that this attachment is mediated by the TP covalently bound to the DNA [195]. Furthermore, the precursor of the TP, which interacts with the viral DNA polymerase and primes DNA replication, binds to the nuclear matrix both in vivo and in vitro [196]. Hence, the nuclear matrix is thought to provide the structural framework on which the replication factors and DNA can bind and interact.

5.4. Protein p16.7

Gene 16.7 is located at the right early operon of the ϕ-29 genome (Fig. 4(a)). The intracellular amount of protein p16.7 (130 amino acids) throughout the infection cycle was determined by quantitative immunoblotting [197]. In cells growing at 37 °C, protein p16.7 was detected at 6 min post-infection, reaching its maximal intracellular level at 12 min. Then, the amount of p16.7 remained constant (about 65,000 molecules per cell). To understand the role of protein p16.7, the kinetics of phage DNA synthesis was determined in the presence or absence of p16.7. To this end, the *sus*16.7 mutant phage, which carries a suppressor-sensitive nonsense mutation in gene 16.7 [198], was used. In the absence of p16.7,

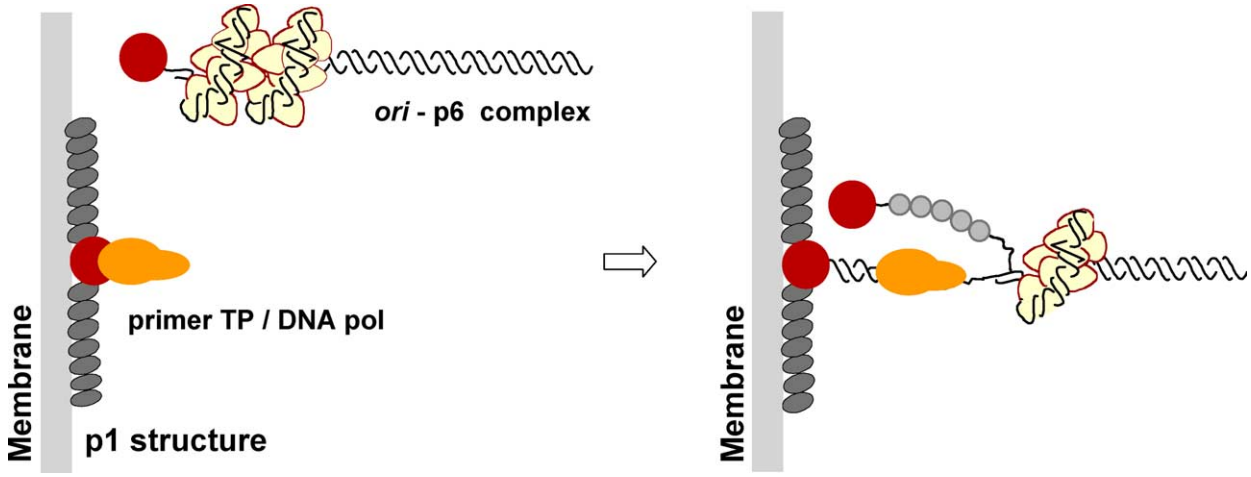

Fig. 7. Model for the role of protein p1 in ϕ29 DNA replication, adapted from Bravo et al. [150]. A membrane-associated multimeric p1 structure provides an anchoring site for the viral replisome, constituted at least by the primer TP-ϕ29 DNA polymerase heterodimer. The membrane-associated replisome would further interact with the replication origin. See legend to Fig. 5 and text for details.

the time post-infection necessary to detect amplified ϕ-29 DNA was delayed. This effect was moderately enhanced at low multiplicity of infection and at low growth temperatures. Thus, protein p16.7 appears to be required for efficient in vivo ϕ29 DNA replication under the different conditions tested [197]. Furthermore, the subcellular localization of ϕ29 DNA replication was examined in the presence or absence of p16.7 by immunofluorescence techniques [198]. According to this study, phage ϕ29 DNA replication occurs at sites different from the host cell replication factories. At the onset of phage ϕ29 DNA replication, and in the presence of p16.7, ϕ29 DNA nearly always localizes as a single focus towards one end of the host cell nucleoid. Later on, phage replication is redistributed to multiple sites around the periphery of the nucleoid. In the absence of protein p16.7, redistribution of phage DNA from the initial replication site to the sites surrounding the nucleoid is delayed. Therefore, protein p16.7 is thought to be involved in the spatial organization of ϕ29 DNA replication.

Computer-assisted analysis revealed that protein p16.7 has an N-terminal transmembrane-spanning domain (residues 4–22). This region is preceded by a negatively charged residue and followed by positively charged residues [197]. The subcellular localization of p16.7 throughout the infection cycle was examined by immunofluorescence techniques [198]. This analysis showed that protein p16.7 has a peripheral location around the cell, presumably at the bacterial membrane. A similar localization pattern was reported for a p16.7-GFP fusion protein [197]. In addition, cell fractionation studies showed that protein p16.7 is exclusively detected in membrane fractions of infected cells [197]. On the contrary, protein p16.7A, which lacks the predicted transmembrane region, is essentially recovered in the soluble fraction, indicating that the N-terminal region of p16.7 is required for its membrane localization [197].

Since protein p16.7 was insoluble, in vitro studies were carried out using the soluble protein p16.7A. Gel mobility shift assays revealed that p16.7A binds single-stranded DNA. Protein p16.7A also binds double-stranded DNA, although with lower affinity [199]. Moreover, in vitro ϕ29 DNA amplification assays showed that protein p16.7 functionally substitutes the ϕ29 single-stranded DNA binding protein p5. According to psoralen crosslinking experiments, protein p16.7A binds to the stretches of single-stranded DNA present in ϕ29 DNA replication intermediates [199]. However, unlike p5, protein p16.7A neither has helix-destabilizing activity nor stimulatory effect on in vitro ϕ29 DNA replication, indicating that they do not have overlapping functions [199]. Based on these findings, and taking into account the membrane localization of the native protein p16.7 [197,198], it was proposed that protein p16.7 is involved in the attachment of replicating ϕ29 DNA molecules to the membrane of infected cells [199].

The region of p16.7 spanning amino acids 19 to 60 has a high probability to form an α-helical coiled-coil structure [197]. Sedimentation assays through glycerol gradients showed that protein p16.7A forms dimers in solution [197]. In addition to dimers, protein p16.7A can form multimers, as shown by in vitro crosslinking studies [197,200]. Binding of protein p16.7A to single-stranded DNA enhances or stabilizes the formation of p16.7A multimers [200]. It was shown that the C-terminal 10 amino acids of p16.7A are required for multimerization but not for dimerization or single-stranded DNA binding [200]. Furthermore, electron microscopic analyses revealed that protein p16.7A is able to join small single-stranded DNA fragments generating

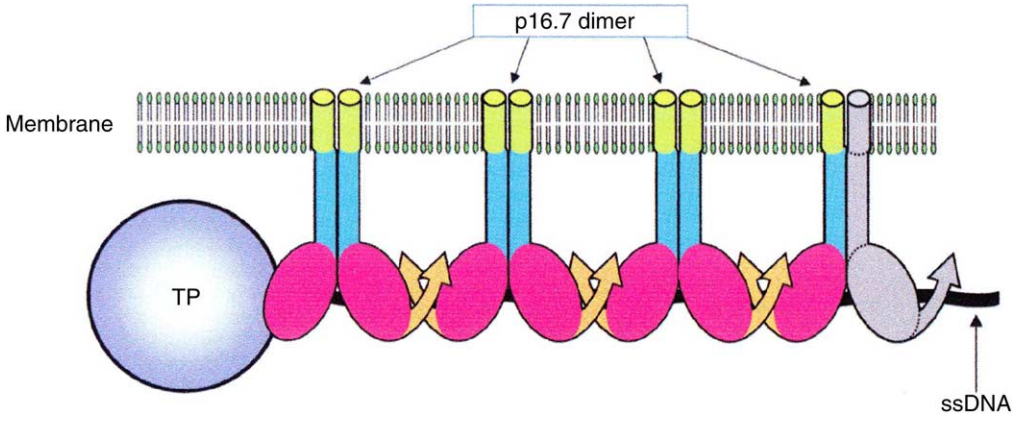

Fig. 8. Model for the role of protein p16.7 in ϕ29 DNA replication, reproduced with permission (Nature Publishing Group; http://www.nature.com/) from Serna-Rico et al. [200]. The membrane bilayer and protein p16.7 dimers are indicated. Only one end of a TP-containing displaced single-stranded DNA of a ϕ29 DNA replication intermediate is shown. A single p16.7 monomer, the one forming part of the right most dimer shown is indicated in grey. The N-terminal membrane anchor and the putative coiled-coil domain are shown as green and blue bars, respectively. The putative DNA-binding domains are shown as red ovals. Curved orange arrows illustrate the multimerization domains. For simplicity, the C-terminal end of the p16.7 monomer contacting the TP is omitted.

filaments of high molecular weight. Formation of these long nucleoprotein filaments depends on p16.7A multimerization [200].

In vitro chemical crosslinking experiments and sedimentation assays through glycerol gradients showed that protein p16.7A interacts with the TP [200]. Most likely, this activity of p16.7A facilitates its binding to the TP-containing single-stranded DNA segments of the ϕ-29 DNA replication intermediates. The C-terminal region of protein p16.7A is not required for its interaction with TP [200].

A model for the role of p16.7 in the organization of in vivo ϕ29 DNA replication was recently proposed [200]. In this model (Fig. 8), protein p16.7, localized to the membrane by its transmembrane-spanning domain and in a dimeric form through the formation of a coiled-coil, would recruit the ϕ29 DNA replication intermediates to the membrane by binding to both the parental TP and the displaced single-stranded DNA.

6. Comparison with eukaryotic systems

Studies with eukaryotic cells showed that chromosomal DNA replication occurs at numerous locations within the nucleus. Each site constitutes a replication factory containing many polymerizing machines working on different templates [201]. Replication factories fixed to a nucleoskeleton were visualized by electron microscopy techniques [202]. This finding supports a model for chromosomal DNA replication in which DNA polymerases are immobilized by attachment to larger structures, and DNA is pulled through (stationary replisome model) (reviewed by [203]). Attachment of replication complexes to specific cellular structures has also been reported for eukaryotic viral genomes. Replication of most DNA viruses occurs within the cell nucleus. In this case, nuclear substructures appear to be involved in the formation of DNA replication compartments [204–206]. For example, replication of adenovirus, a linear DNA genome with a terminal protein bound to the ends, takes place at distinct subnuclear sites on the nuclear matrix [207,208]. It was shown that the precursor of the terminal protein (pTP) binds to the nuclear matrix both in vivo and in vitro [196]. In addition, pTP interacts with the viral DNA polymerase and primes DNA replication [192]. Thus, the docking of adenovirus replication complexes to the nuclear matrix seems to be mediated by pTP.

The main steps in the lifecycle of positive-strand RNA viruses depend on cellular membranes. In these viruses, replication occurs in close association with intracellular membranes of diverse origin (e.g. endoplasmic reticulum, lysosome, chloroplast). In fact, almost all positive-strand RNA viruses induce proliferation or reorganization of membranous structures in the infected cells [209]. Membranes appear to function not just as a way of compartmentalizing virus RNA replication, but also appear to have a central role in the organization and function of the replication machinery [209]. Some progress has been made in understanding how the viral RNA replication apparatus is fixed to specific types of membranes. For example, the tobacco etch potyvirus 6-kDa protein is thought to be necessary for targeting viral RNA replication complexes to endoplasmic reticulum-derived membranes. This protein associates with membranes as an integral protein via a 19 amino acid hydrophobic domain [210]. In Semliki Forest virus, binding of the viral replicase to endosomal/lysosomal membranes is likely mediated by the RNA-capping protein Nsp1, which has affinity for negatively charged phospholipids [211,212]. In contrast to those viruses, host-encoded proteins appear to act as membrane anchors of the tobamoviruses replication complexes. Specifically, the seven-pass transmembrane protein TOM1, encoded by *Arabidopsis thaliana*, interacts with the helicase domain of tobamovirus-encoded replication proteins [213]. Moreover, TOM1 interacts with TOM2A, a four-pass transmembrane protein also encoded by the host cell [214]. Both proteins TOM1 and TOM2A are necessary for efficient intracellular multiplication of tobamoviruses and are mainly localized to the vacuolar membranes (i.e. the tonoplasts) [215]. TOM2A is thought to facilitate the formation of tobamoviral RNA replication complexes on TOM1-containing membranes [215].

In hepatitis C virus, fractionation experiments and immunoelectron microscopy studies revealed that nonstructural viral proteins are associated with membranes of the endoplasmic reticulum. Thus, either the endoplasmic reticulum or an endoplasmic reticulum-derived compartment provides a site for hepatitis C RNA replication [216,217]. It was shown that the C-terminal 21 amino acids of the RNA-dependent RNA polymerase, termed NS5B, are necessary and sufficient for its targeting to the cytosolic side of the endoplasmic reticulum membrane [218]. The C-terminal hydrophobic domain of NS5B traverses the phospholipid bilayer as a transmembrane segment [219]. Therefore, NS5B belongs to a relatively small class of membrane proteins termed "tail-anchored proteins" [220].

Infection of mammalian cells with poliovirus results in the proliferation of membranous vesicles in the cytoplasm. Poliovirus RNA replication complexes assemble on the surface of these vesicles, which derive from the endoplasmic reticulum [221,222]. Membranous vesicles isolated from poliovirus-infected cells contain all viral proteins required for RNA replication [223]. The poliovirus RNA-dependent RNA polymerase is not a membrane-associated protein. However, it binds specifically to another viral protein, termed 3AB, which associates with intracellular membranes [224–226]. By X-ray crystallography, the structure of the poliovirus polymerase

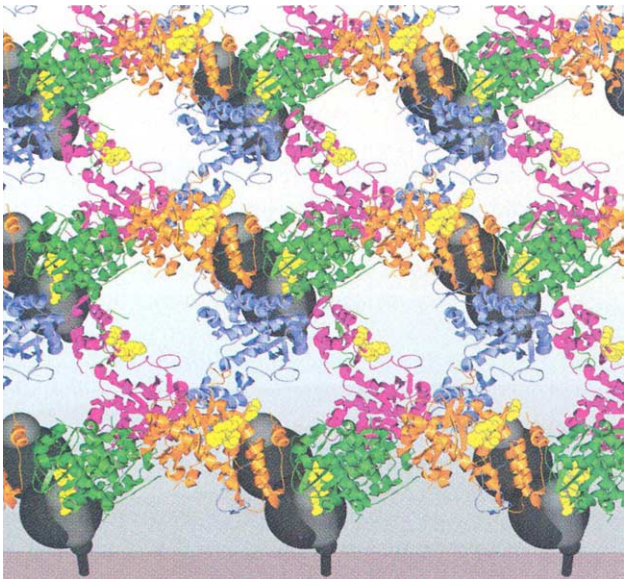

Fig. 9. Model for the formation of higher-order poliovirus polymerase structures in infected cells. Reprinted with permission from Lyle et al. [228]. Copyright (2002) American Association for the Advancement of Science (www.sciencemag.org). The four monomers of the poliovirus polymerase that compose the unit cell are shown in different colours (green, blue, orange and red). To model this structure on intracellular membranes, the angle of interaction of fibres interacting at interface II was altered to allow for the formation of a planar lattice. Protein 3AB is represented as a globular, integral membrane protein in black and grey.

was determined at 2.6 Å resolution [227]. Extensive regions of polymerase–polymerase interactions observed in the crystals suggested an unusual higher order structure likely important for polymerase function. Further electron microscopy studies revealed that poliovirus RNA-dependent RNA polymerase forms large two-dimensional sheets and tubes in solution [228]. Formation of these large assemblies seems to be critical for cooperative RNA binding and, therefore, for RNA elongation. Moreover, membranous vesicles isolated from poliovirus-infected cells contain structures consistent with the presence of two-dimensional polymerase arrays on their surfaces [228]. A model for the formation of a higher-order polymerase structure on intracellular membranes has been proposed [228]. In this model (Fig. 9), the polymerase molecules are attached to the membrane through their interaction with 3AB molecules. Lyle et al. [228] have proposed that host cytoplasmic membranes may function as physical foundations for two-dimensional polymerase arrays, conferring the advantages of surface catalysis to viral RNA replication.

7. Concluding remarks

In *E. coli* and *B. subtilis*, recent cytological studies have shown that several replication proteins, and even particular regions of the chromosome, are localized to specific subcellular sites during the cell division cycle. These findings support the notion that in prokaryotes, like in eukaryotes, DNA replication machineries assemble and function at discrete sites. Furthermore, compatible plasmids appear to be targeted to different positions near to the cell midpoint, where plasmid DNA replication is thought to take place. Further work should clarify the molecular nature of these putative replication sites. It is attractive to speculate that, like in the *B. subtilis* phage ϕ29, specific scaffold proteins associated to the bacterial membrane provide a site for attachment of replication complexes.

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References

[1] Jacob, F., Brenner, S. and Cuzin, F. (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28, 329–438.
[2] Nordström, K. (2003) The replicon theory 40 years: an EMBO workshop held in Villefranche sur Mer, France, January 18–23, 2003. Plasmid 49, 269–280.
[3] Siegel, P.J. and Schaechter, M. (1973) The role of the host cell membrane in the replication and morphogenesis of bacteriophages. Annu. Rev. Microbiol. 27, 261–282.
[4] Liebowitz, P.J. and Schaechter, M. (1975) The attachment of the bacterial chromosome to the cell membrane. Int. Rev. Cytol. 41, 1–28.
[5] Firshein, W. (1989) Role of the DNA/membrane complex in prokaryotic DNA replication. Annu. Rev. Microbiol. 43, 89–120.
[6] Firshein, W. and Kim, P. (1997) Plasmid replication and partition in *Escherichia coli*: is the cell membrane the key? Mol. Microbiol. 23, 1–10.
[7] Sueoka, N. (1998) Cell membrane and chromosome replication in *Bacillus subtilis*. Prog. Nucl. Acid Res. Mol. Biol. 59, 35–53.
[8] Crooke, E. (2001) *Escherichia coli* DnaA protein–phospholipid interactions: in vitro and in vivo. Biochimie 83, 19–23.
[9] Ogden, G.B., Pratt, M.J. and Schaechter, M. (1988) The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell 54, 127–135.
[10] Landoulsi, A., Malki, A., Kern, R., Kohiyama, M. and Hughes, P. (1990) The *E. coli* cell surface specifically prevents the initiation

of DNA replication at *ori*C on hemimethylated DNA templates. Cell 63, 1053–1060.
[11] Herrick, J., Kern, R., Guha, S., Landoulsi, A., Fayet, O., Malki, A. and Kohiyama, M. (1994) Parental strand recognition of the DNA replication origin by the outer membrane in *Escherichia coli*. EMBO J. 13, 4695–4703.
[12] Slater, S., Wold, S., Lu, M., Boye, E., Skarstad, K. and Kleckner, N. (1995) *E. coli* SeqA protein binds *ori*C in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. Cell 82, 927–936.
[13] Bahloul, A., Meury, J., Kern, R., Garwood, J., Guha, S. and Kohiyama, M. (1996) Co-ordination between membrane *ori*C sequestration factors and a chromosome partitioning protein, TolC (MukA). Mol. Microbiol. 22, 275–282.
[14] Shakibai, N., Ishidate, K., Reshetnyak, E., Gunji, S., Kohiyama, M. and Rothfield, L. (1998) High-affinity binding of hemimethylated *ori*C by *Escherichia coli* membranes is mediated by a multiprotein system that includes SeqA and a newly identified factor, SeqB. Proc. Natl. Acad. Sci. USA 95, 11117–11121.
[15] d'Alencon, E., Taghbalout, A., Kern, R. and Kohiyama, M. (1999) Replication cycle dependent association of SeqA to the outer membrane fraction of *E. coli*. Biochimie 81, 841–846.
[16] Losick, R. and Shapiro, L. (1999) Changing views on the nature of the bacterial cell: from biochemistry to cytology. J. Bacteriol. 181, 4143–4145.
[17] Jensen, R.B. and Shapiro, L. (2000) Proteins on the move: dynamic protein localization in prokaryotes. Trends Cell Biol. 10, 483–488.
[18] Phillips, G.J. (2001) Green fluorescent protein: a bright idea for the study of bacterial protein localization. FEMS Microbiol. Lett. 204, 9–18.
[19] Lemon, K.P. and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science 282, 1516–1519.
[20] Imai, Y., Ogasawara, N., Ishigo-Oka, D., Kadoya, R., Daito, T. and Moriya, S. (2000) Subcellular localization of Dna-initiation proteins of *Bacillus subtilis* : evidence that chromosome replication begins at either edge of the nucleoids. Mol. Microbiol. 36, 1037–1048.
[21] Hiraga, S. (2000) Dynamic localization of bacterial and plasmid chromosomes. Annu. Rev. Genet. 34, 21–59.
[22] Gordon, G.S. and Wright, A. (2000) DNA segregation in bacteria. Annu. Rev. Microbiol. 54, 681–708.
[23] Draper, G.C. and Gober, J.W. (2002) Bacterial chromosome segregation. Annu. Rev. Microbiol. 56, 567–597.
[24] Pogliano, K., Pogliano, J. and Becker, E. (2003) Chromosome segregation in Eubacteria. Curr. Opin. Microbiol. 6, 586–593.
[25] Pogliano, J. (2002) Dynamic cellular location of bacterial plasmids. Curr. Opin. Microbiol. 5, 586–590.
[26] Skarstad, K., Boye, E. and Steen, H.B. (1986) Timing of initiation of chromosome replication in individual *Escherichia coli* cells. EMBO J. 5, 1711–1717.
[27] Boye, E., Løbner-Olesen, A. and Skarstad, K. (2000) Limiting DNA replication to once and only once. EMBO Rep 1, 479–483.
[28] Messer, W. (2002) The bacterial replication initiator DnaA. DnaA and *ori*C, the bacterial mode to initiate DNA replication. FEMS Microbiol. Rev. 26, 355–374.
[29] Løbner-Olesen, A., Skarstad, K., Hansen, F.G., von Meyenburg, K. and Boye, E. (1989) The DnaA protein determines the initiation mass of *Escherichia coli* K-12. Cell 57, 881–889.
[30] Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) ATP activates DnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell 50, 259–265.
[31] Yung, B.Y., Crooke, E. and Kornberg, A. (1990) Fate of the DnaA initiator protein in replication at the origin of the *Escherichia coli* chromosome in vitro. J. Biol. Chem. 265, 1282–1285.
[32] Kurokawa, K., Nishida, S., Emoto, A., Sekimizu, K. and Katayama, T. (1999) Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in *Escherichia coli*. EMBO J. 18, 6642–6652.
[33] Nishida, S., Fujimitsu, K., Sekimizu, K., Ohmura, T., Ueda, T. and Katayama, T. (2002) A nucleotide switch in the *Escherichia coli* DnaA protein initiates chromosomal replication: evidence from a mutant DnaA protein defective in regulatory ATP hydrolysis in vitro and in vivo. J. Biol. Chem. 277, 14986–14995.
[34] Sekimizu, K., Yung, B.Y. and Kornberg, A. (1988) The DnaA protein of *Escherichia coli*. Abundance, improved purification, and membrane binding. J. Biol. Chem. 263, 7136–7140.
[35] Sekimizu, K. and Kornberg, A. (1988) Cardiolipin activation of DnaA protein, the initiation protein of replication in *Escherichia coli*. J. Biol. Chem. 263, 7131–7135.
[36] Crooke, E., Castuma, C.E. and Kornberg, A. (1992) The chromosome origin of *Escherichia coli* stabilizes DnaA protein during rejuvenation by phospholipids. J. Biol. Chem. 267, 16779–16782.
[37] Yung, B.Y. and Kornberg, A. (1988) Membrane attachment activates DnaA protein, the initiation protein of chromosome replication in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 85, 7202–7205.
[38] Castuma, C.E., Crooke, E. and Kornberg, A. (1993) Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. J. Biol. Chem. 268, 24665–24668.
[39] Garner, J., Durrer, P., Kitchen, J., Brunner, J. and Crooke, E. (1998) Membrane-mediated release of nucleotide from an initiator of chromosomal replication, *Escherichia coli* DnaA, occurs with insertion of a distinct region of the protein into the lipid bilayer. J. Biol. Chem. 273, 5167–5173.
[40] Garner, J. and Crooke, E. (1996) Membrane regulation of the chromosomal replication activity of *E. coli* DnaA requires a discrete site on the protein. EMBO J. 15, 3477–3485.
[41] Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2000) Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. J. Biol. Chem. 275, 4513–4518.
[42] Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2001) Molecular mechanism for functional interaction between DnaA protein and acidic phospholipids: identification of important amino acids. J. Biol. Chem. 276, 7450–7456.
[43] Newman, G. and Crooke, E. (2000) DnaA, the initiator of *Escherichia coli* chromosomal replication, is located at the cell membrane. J. Bacteriol. 182, 2604–2610.
[44] Hwang, D.S., Crooke, E. and Kornberg, A. (1990) Aggregated DnaA protein is dissociated and activated for DNA replication by phospholipase or DnaK protein. J. Biol. Chem. 265, 19244–19248.
[45] Fralick, J.A. and Lark, K.G. (1973) Evidence for the involvement of unsaturated fatty acids in initiating chromosome replication in *Escherichia coli*. J. Mol. Biol. 80, 459–475.
[46] Xia, W. and Dowhan, W. (1995) In vivo evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92, 783–787.
[47] Zheng, W., Li, Z., Skarstad, K. and Crooke, E. (2001) Mutations in DnaA protein suppress the growth arrest of acidic phospholipid-deficient *Escherichia coli* cells. EMBO J. 20, 1164–1172.
[48] Kikuchi, S., Shibuya, I. and Matsumoto, K. (2000) Viability of an *Escherichia coli pgs*A null mutant lacking detectable phosphatidylglycerol and cardiolipin. J. Bacteriol. 182, 371–376.

[49] Wegrzyn, A., Wrobel, B. and Wegrzyn, G. (1999) Altered biological properties of cell membranes in *Escherichia coli dna*A and *seq*A mutants. Mol. Gen. Genet. 261, 762–769.
[50] Makise, M., Mima, S., Katsu, T., Tsuchiya, T. and Mizushima, T. (2002) Acidic phospholipids inhibit the DNA-binding activity of DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. Mol. Microbiol. 46, 245–256.
[51] Campbell, J.L. and Kleckner, N. (1990) *E. coli ori*C and the *dna*A gene promoter are sequestered from Dam methyltransferase following the passage of the chromosomal replication fork. Cell 62, 967–979.
[52] Russell, D.W. and Zinder, N.D. (1987) Hemimethylation prevents DNA replication in *E. coli*. Cell 50, 1071–1079.
[53] von Freiesleben, U., Krekling, M.A., Hansen, F.G. and Løbner-Olesen, A. (2000) The eclipse period of *Escherichia coli*. EMBO J. 19, 6240–6248.
[54] Boye, E. (1991) The hemimethylated replication origin of *Escherichia coli* can be initiated in vitro. J. Bacteriol. 173, 4537–4539.
[55] Lu, M., Campbell, J.L., Boye, E. and Kleckner, N. (1994) SeqA: a negative modulator of replication initiation in *E. coli*. Cell 77, 413–426.
[56] Bach, T., Krekling, M.A. and Skarstad, K. (2003) Excess SeqA prolongs sequestration of *ori*C and delays nucleoid segregation and cell division. EMBO J. 22, 315–323.
[57] Boye, E., Stokke, T., Kleckner, N. and Skarstad, K. (1996) Coordinating DNA replication initiation with cell growth: differential roles for DnaA and SeqA proteins. Proc. Natl. Acad. Sci. USA 93, 12206–12211.
[58] Brendler, T. and Austin, S. (1999) Binding of SeqA protein to DNA requires interaction between two or more complexes bound to separate hemimethylated GATC sequences. EMBO J. 18, 2304–2310.
[59] Skarstad, K., Lueder, G., Lurz, R., Speck, C. and Messer, W. (2000) The *Escherichia coli* SeqA protein binds specifically and co-operatively to two sites in hemimethylated and fully methylated *ori*C. Mol. Microbiol. 36, 1319–1326.
[60] Kang, S., Lee, H., Han, J.S. and Hwang, D.S. (1999) Interaction of SeqA and Dam methylase on the hemimethylated origin of *Escherichia coli* chromosomal DNA replication. J. Biol. Chem. 274, 11463–11468.
[61] Wold, S., Boye, E., Slater, S., Kleckner, N. and Skarstad, K. (1998) Effects of purified SeqA protein on *ori*C-dependent DNA replication in vitro. EMBO J. 17, 4158–4165.
[62] Torheim, N.K. and Skarstad, K. (1999) *Escherichia coli* SeqA protein affects DNA topology and inhibits open complex formation at *ori*C. EMBO J. 18, 4882–4888.
[63] Taghbalout, A., Landoulsi, A., Kern, R., Yamazoe, M., Hiraga, S., Holland, B., Kohiyama, M. and Malki, A. (2000) Competition between the replication initiator DnaA and the sequestration factor SeqA for binding to the hemimethylated chromosomal origin of *E. coli* in vitro. Genes Cells 5, 873–884.
[64] Thaller, M.C., Schippa, S., Bonci, A., Cresti, S. and Rossolini, G.M. (1997) Identification of the gene (*aph*A) encoding the class B acid phosphatase/phosphotransferase of *Escherichia coli* MG1655 and characterization of its product. FEMS Microbiol. Lett. 146, 191–198.
[65] Reshetnyak, E., d'Alencon, E., Kern, R., Taghbalout, A., Guillaud, P. and Kohiyama, M. (1999) Hemi-methylated *ori*C DNA binding activity found in non-specific acid phosphatase. Mol. Microbiol. 31, 167–175.
[66] Niki, H. and Hiraga, S. (1998) Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. Genes Dev. 12, 1036–1045.
[67] Gordon, G.S., Sitnikov, D., Webb, C.D., Teleman, A., Straight, A., Losick, R., Murray, A.W. and Wright, A. (1997) Chromosome and low copy plasmid segregation in *E. coli* : visual evidence for distinct mechanisms. Cell 90, 1113–1121.
[68] Niki, H., Yamaichi, Y. and Hiraga, S. (2000) Dynamic organization of chromosomal DNA in *Escherichia coli*. Genes Dev. 14, 212–223.
[69] Hiraga, S., Ichinose, C., Onogi, T., Niki, H. and Yamazoe, M. (2000) Bidirectional migration of SeqA-bound hemimethylated DNA clusters and pairing of *ori*C copies in *Escherichia coli*. Genes to Cells 5, 327–341.
[70] Koppes, L.J.H., Woldringh, C.L. and Nanninga, N. (1999) *Escherichia coli* contains a DNA replication compartment in the cell center. Biochimie 81, 803–810.
[71] Roos, M., vanGeel, A.B., Aarsman, M.E., Veuskens, J.T., Woldringh, C.L. and Nanninga, N. (1999) Cellular localization of *ori*C during the cell cycle of *Escherichia coli* as analyzed by fluorescent *in situ* hybridization. Biochimie 81, 797–802.
[72] Lau, I.F., Filipe, S.R., Søballe, B., Okstad, O., Barre, F. and Sherratt, D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. Mol. Microbiol. 49, 731–743.
[73] Gordon, G.S., Shivers, R.P. and Wright, A. (2002) Polar localization of the *Escherichia coli ori*C region is independent of the site of replication initiation. Mol. Microbiol. 44, 501–507.
[74] Kruse, T., Møller-Jensen, J., Løbner-Olesen, A. and Gerdes, K. (2003) Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli*. EMBO J. 22, 5283–5292.
[75] Margolin, W. (2003) Bacterial shape: growing off this mortal coil. Curr. Biol. 13, R705–R707.
[76] Young, K.D. (2003) Bacterial shape. Mol. Microbiol. 49, 571–580.
[77] Egelman, E.H. (2003) Actin's prokaryotic homologs. Curr. Opin. Struct. Biol. 13, 244–248.
[78] Hiraga, S., Ichinose, C., Niki, H. and Yamazoe, M. (1998) Cell cycle-dependent duplication and bidirectional migration of SeqA-associated DNA–protein complexes in *E. coli*. Mol. Cell 1, 381–387.
[79] Onogi, T., Niki, H., Yamazoe, M. and Hiraga, S. (1999) The assembly and migration of SeqA–GFP fusion in living cells of *Escherichia coli*. Mol. Microbiol. 31, 1775–1782.
[80] Onogi, T., Ohsumi, K., Katayama, T. and Hiraga, S. (2002) Replication-dependent recruitment of the β-subunit of DNA polymerase III from cytosolic spaces to replication forks in *Escherichia coli*. J. Bacteriol. 184, 867–870.
[81] Withers, H.L. and Bernander, R. (1998) Characterization of *dnaC2* and *dnaC28* mutants by flow cytometry. J. Bacteriol. 180, 1624–1631.
[82] Molina, F. and Skarstad, K. (2004) Replication fork and SeqA focus distributions in *Escherichia coli* suggest a replication hyperstructure dependent on nucleotide metabolism. Mol. Microbiol. 52, 1597–1612.
[83] Ayres, E.K., Thomson, V.J., Merino, G., Balderes, D. and Figurski, D.H. (1993) Precise deletions in large bacterial genomes by vector-mediated excision (VEX). The trfA gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. 230, 174–185.
[84] Toukdarian, A.E., Helinski, D.R. and Perri, S. (1996) The plasmid RK2 initiation protein binds to the origin of replication as a monomer. J. Biol. Chem. 271, 7072–7078.
[85] Kornacki, J.A. and Firshein, W. (1986) Replication of plasmid RK2 in vitro by a DNA–membrane complex: evidence for initiation of replication and its coupling to transcription and translation. J. Bacteriol. 167, 319–326.
[86] Kostyal, D.A., Farrell, M., McCabe, A., Mei, Z. and Firshein, W. (1989) Replication of an RK2 miniplasmid derivative in vitro by a DNA/membrane complex extracted from *Escherichia coli*: involvement of the DnaA but not DnaK host proteins and

association of these and plasmid-encoded proteins with the inner membrane. Plasmid 21, 226–237.
[87] Michaels, K., Mei, J. and Firshein, W. (1994) TrfA-dependent, inner-membrane-associated plasmid RK2 DNA synthesis in *Escherichia coli* maxicells. Plasmid 32, 19–31.
[88] Mei, J., Benashski, S. and Firshein, W. (1995) Interactions of the origin of replication (*ori*V) and initiation proteins (TrfA) of plasmid RK2 with submembrane domains of *Escherichia coli*. J. Bacteriol. 177, 6766–6772.
[89] Kim, P.D. and Firshein, W. (2000) Isolation of an inner membrane-derived subfraction that supports in vitro replication of a mini-RK2 plasmid in *Escherichia coli*. J. Bacteriol. 182, 1757–1760.
[90] Banack, T., Kim, P.D. and Firshein, W. (2000) TrfA-dependent inner membrane-associated plasmid RK2 DNA synthesis and association of TrfA with membranes of different gram-negative hosts. J. Bacteriol. 182, 4380–4383.
[91] Kim, P.D., Rosche, T.M. and Firshein, W. (2000) Identification of a potential membrane-targeting region of the replication initiator protein (TrfA) of broad-host-range plasmid RK2. Plasmid 43, 214–222.
[92] Niki, H. and Hiraga, S. (1997) Subcellular distribution of actively partitioning F plasmid during the cell division cycle in *E. coli*. Cell 90, 951–957.
[93] Onogi, T., Miki, T. and Hiraga, S. (2002) Behavior of sister copies of mini-F plasmid after synchronized plasmid replication in *Escherichia coli* cells. J. Bacteriol. 184, 3142–3145.
[94] Niki, H. and Hiraga, S. (1999) Subcellular localization of plasmids containing the *ori*C region of the *Escherichia coli* chromosome, with or without the *sop*ABC partitioning system. Mol. Microbiol. 34, 498–503.
[95] Jensen, R.B. and Gerdes, K. (1999) Mechanism of DNA segregation in prokaryotes: ParM partitioning protein of plasmid R1 co-localizes with its replicon during the cell cycle. EMBO J. 18, 4076–4084.
[96] Møller-Jensen, J., Jensen, R.B., Löwe, J. and Gerdes, K. (2002) Prokaryotic DNA segregation by an actin-like filament. EMBO J. 21, 3119–3127.
[97] van den Ent, F., Møller-Jensen, J., Amos, L.A., Gerdes, K. and Löwe, J. (2002) F-actin-like filaments formed by plasmid segregation protein ParM. EMBO J. 21, 6935–6943.
[98] Møller-Jensen, J., Borch, J., Dam, M., Jensen, R.B., Roepstorff, P. and Gerdes, K. (2003) Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. Mol. Cell 12, 1477–1487.
[99] Pogliano, J., Ho, T.Q., Zhong, Z. and Helinski, D.R. (2001) Multicopy plasmids are clustered and localized in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 98, 4486–4491.
[100] Ho, T.Q., Zhong, Z., Aung, S. and Pogliano, J. (2002) Compatible bacterial plasmids are targeted to independent cellular locations in *Escherichia coli*. EMBO J. 21, 1864–1872.
[101] Weitao, T., Dasgupta, S. and Nordström, K. (2000) Plasmid R1 is present as clusters in the cells of *Escherichia coli*. Plasmid 43, 200–204.
[102] Eliasson, A., Bernander, R., Dasgupta, S. and Nordström, K. (1992) Direct visualization of plasmid DNA in bacterial cells. Mol. Microbiol. 6, 165–170.
[103] Reich, Z., Wachtel, E.J. and Minsky, A. (1994) Liquid-crystalline mesophases of plasmid DNA in bacteria. Science 264, 1460–1463.
[104] Yoshikawa, H. and Wake, R.G. (1993) Initiation and termination of chromosome replication In: Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A.L., Hoch, J.A., Losick, R., Eds.), pp. 507–528. American Society for Microbiology, Washington, DC.
[105] Moriya, S., Imai, Y., Hassan, A.K. and Ogasawara, N. (1999) Regulation of initiation of *Bacillus subtilis* chromosome replication. Plasmid 41, 17–29.
[106] Webb, C.D., Teleman, A., Gordon, S., Straight, A., Belmont, A., Lin, D.C., Grossman, A.D., Wright, A. and Losick, R. (1997) Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. Cell 88, 667–674.
[107] Webb, C.D., Graumann, P.L., Kahana, J.A., Teleman, A.A., Silver, P.A. and Losick, R. (1998) Use of time-lapse microscopy to visualize rapid movement of the replication origin region of the chromosome during the cell cycle in *Bacillus subtilis*. Mol. Microbiol. 28, 883–892.
[108] Williams, D.R. and Thomas, C.M. (1992) Active partitioning of bacterial plasmids. J. Gen. Microbiol. 138, 1–16.
[109] Ireton, K., Gunther IV, N.W. and Grossman, A.D. (1994) Spo0J is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. 176, 5320–5329.
[110] Sharpe, M.E. and Errington, J. (1996) The *Bacillus subtilis soj spo*0J locus is required for a centromere-like function involved in prespore chromosome partitioning. Mol. Microbiol. 21, 501–509.
[111] Lin, D.C. and Grossman, A.D. (1998) Identification and characterization of a bacterial chromosome partitioning site. Cell 92, 675–685.
[112] Lin, D.C., Levin, P.A. and Grossman, A.D. (1997) Bipolar localization of a chromosome partition protein in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 94, 4721–4726.
[113] Glaser, P., Sharpe, M.E., Raether, B., Perego, M., Ohlsen, K. and Errington, J. (1997) Dynamic, mitotic-like behavior of a bacterial protein required for accurate chromosome partitioning. Genes Dev. 11, 1160–1168.
[114] Lewis, P.J. and Errington, J. (1997) Direct evidence for active segregation of *ori*C regions of the *Bacillus subtilis* chromosome and co-localization with the SpoOJ partitioning protein. Mol. Microbiol. 25, 945–954.
[115] Sharpe, M.E. and Errington, J. (1998) A fixed distance for separation of newly replicated copies of *ori*C in *Bacillus subtilis*: implications for coordination of chromosome segregation and cell division. Mol. Microbiol. 28, 981–990.
[116] Lee, P.S., Lin, D.C., Moriya, S. and Grossman, A.D. (2003) Effects of the chromosome partitioning protein Spo0J (ParB) on *ori*C positioning and replication initiation in *Bacillus subtilis*. J. Bacteriol. 185, 1326–1337.
[117] Soufo, H.J. and Graumann, P.L. (2003) Actin-like proteins MreB and Mbl from *Bacillus subtilis* are required for bipolar positioning of replication origins. Curr. Biol. 13, 1916–1920.
[118] Jones, L.J., Carballido-López, R. and Errington, J. (2001) Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. Cell 104, 913–922.
[119] Carballido-López, R. and Errington, J. (2003) The bacterial cytoskeleton: in vivo dynamics of the actin-like protein Mbl of *Bacillus subtilis*. Dev. Cell 4, 19–28.
[120] Daniel, R.A. and Errington, J. (2003) Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. Cell 113, 767–776.
[121] Shapiro, L., McAdams, H.H. and Losick, R. (2002) Generating and exploiting polarity in bacteria. Science 298, 1942–1946.
[122] Wu, L.J. and Errington, J. (2002) A large dispersed chromosomal region required for chromosome segregation in sporulating cells of *Bacillus subtilis*. EMBO J. 21, 4001–4011.
[123] Ben-Yehuda, S., Rudner, D.Z. and Losick, R. (2003) RacA, a bacterial protein that anchors chromosomes to the cell poles. Science 299, 532–536.
[124] Thomaides, H.B., Freeman, M., El Karoui, M. and Errington, J. (2001) Division site selection protein DivIVA of *Bacillus subtilis*

has a second distinct function in chromosome segregation during sporulation. Genes Dev. 15, 1662–1673.
[125] Wu, L.J. and Errington, J. (2003) RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. Mol. Microbiol. 49, 1463–1475.
[126] Lemon, K.P. and Grossman, A.D. (2000) Movement of replicating DNA through a stationary replisome. Mol Cell 6, 1321–1330.
[127] Lemon, K.P. and Grossman, A.D. (2001) The extrusion-capture model for chromosome partitioning in bacteria. Genes Dev. 15, 2031–2041.
[128] Sawitzke, J. and Austin, S. (2001) An analysis of the factory model for chromosome replication and segregation in bacteria. Mol. Microbiol. 40, 786–794.
[129] Dervyn, E., Suski, C., Daniel, R., Bruand, C., Chapuis, J., Errington, J., Janniere, L. and Ehrlich, S.D. (2001) Two essential DNA polymerases at the bacterial replication fork. Science 294, 1716–1719.
[130] Velten, M., McGovern, S., Marsin, S., Ehrlich, S.D., Noirot, P. and Polard, P. (2003) A two-protein strategy for the functional loading of a cellular replicative DNA helicase. Mol. Cell 11, 1009–1020.
[131] Noirot-Gros, M.F., Dervyn, E., Wu, L.J., Mervelet, P., Errington, J., Ehrlich, S.D. and Noirot, P. (2002) An expanded view of bacterial DNA replication. Proc. Natl. Acad. Sci. USA 99, 8342–8347.
[132] Maddock, J.R. and Shapiro, L. (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. Science 259, 1717–1723.
[133] Kirby, J.R., Niewold, T.B., Maloy, S. and Ordal, G.W. (2000) CheB is required for behavioural responses to negative stimuli during chemotaxis in *Bacillus subtilis*. Mol. Microbiol. 35, 44–57.
[134] Winston, S. and Sueoka, N. (1980) DNA–membrane association is necessary for initiation of chromosomal and plasmid replication in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 77, 2834–2838.
[135] Hoshino, T., McKenzie, T., Schmidt, S., Tanaka, T. and Sueoka, N. (1987) Nucleotide sequence of *Bacillus subtilis dna*B: a gene essential for DNA replication initiation and membrane attachment. Proc. Natl. Acad. Sci. USA 84, 653–657.
[136] Mosig, G. and Macdonald, P. (1986) A new membrane-associated DNA replication protein, the gene 69 product of bacteriophage T4, shares a patch of homology with the *Escherichia coli dna*A protein. J. Mol. Biol. 189, 243–248.
[137] Mosig, G., Macdonald, P., Powell, D., Trupin, M. and Gary, T. (1987) A membrane protein involved in initiation of DNA replication from the *ori*A region of phage T4. DNA replication and recombination. UCLA Symp. Mol. Cell. Biol. 47, 403–414.
[138] Meijer, W.J., Horcajadas, J.A. and Salas, M. (2001) ϕ29 family of phages. Microbiol. Mol. Biol. Rev. 65, 261–287.
[139] Monsalve, M., Mencía, M., Rojo, F. and Salas, M. (1995) Transcription regulation in *Bacillus subtilis* phage ϕ29: expression of the viral promoters throughout the infection cycle. Virology 207, 23–31.
[140] Whiteley, H.R., Ramey, W.D., Spiegelman, G.B. and Holder, R.D. (1986) Modulation of in vivo and in vitro transcription of bacteriophage ϕ29 early genes. Virology 155, 392–401.
[141] Barthelemy, I., Mellado, R.P. and Salas, M. (1989) In vitro transcription of bacteriophage ϕ29 DNA: inhibition of early promoters by the viral replication protein p6. J. Virol. 63, 460–462.
[142] Camacho, A. and Salas, M. (2001) Repression of bacteriophage ϕ29 early promoter C2 by viral protein p6 is due to impairment of closed complex. J. Biol. Chem. 276, 28927–28932.
[143] Crucitti, P., Lázaro, J.M., Benes, V. and Salas, M. (1998) Bacteriophage ϕ29 early protein p17 is conditionally required for the first rounds of viral DNA replication. Gene 223, 135–142.
[144] Crucitti, P., Abril, A.M. and Salas, M. (2003) Bacteriophage ϕ29 early protein p17. Self-association and hetero-association with the viral histone-like protein p6. J. Biol. Chem. 278, 4906–4911.
[145] González-Huici, Salas, M. and Hermoso, J.M. The push-pull mechanism of bacteriophage ϕ29 DNA injection. Mol. Microbiol. 52, 529–540.
[146] Salas, M. (1998) Control mechanisms of bacteriophage ϕ29 DNA expression. Int. Microbiol. 1, 307–310.
[147] Rojo, F., Mencía, M., Monsalve, M. and Salas, M. (1998) Transcription activation and repression by interaction of a regulator with the alpha subunit of RNA polymerase: the model of phage ϕ29 protein p4. Prog. Nucl. Acid Res. Mol. Biol. 60, 29–46.
[148] Sogo, J.M., Lozano, M. and Salas, M. (1984) In vitro transcription of the *Bacillus subtilis* phage ϕ29 DNA by *Bacillus subtilis* and *Escherichia coli* RNA polymerases. Nucleic Acids Res. 12, 1943–1960.
[149] Barthelemy, I., Salas, M. and Mellado, R.P. (1986) In vivo transcription of bacteriophage ϕ29 DNA: transcription initiation sites. J. Virol. 60, 874–879.
[150] Bravo, A., Illana, B. and Salas, M. (2000) Compartmentalization of phage ϕ29 DNA replication: interaction between the primer terminal protein and the membrane-associated protein p1. EMBO J. 19, 5575–5584.
[151] Barthelemy, I., Salas, M. and Mellado, R.P. (1987) In vivo transcription of bacteriophage ϕ29 DNA: transcription termination. J. Virol. 61, 1751–1755.
[152] Salas, M. (1991) Protein-priming of DNA replication. Annu. Rev. Biochem. 60, 39–71.
[153] Salas, M. and Rojo, F. (1993) Replication and transcription of bacteriophage ϕ29 DNA In: Bacillus subtilis and other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A.L., Hoch, J.A., Losick, R., Eds.), pp. 843–857. American Society for Microbiology, Washington, DC.
[154] Salas, M., Miller, J.T., Leis, J. and DePamphilis, M.L. (1996) Mechanisms for priming DNA synthesis In: DNA Replication in Eukaryotic Cells (DePamphilis, M.L., Ed.), pp. 131–176. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[155] Anderson, D. and Bodley, J.W. (1990) Role of RNA in bacteriophage ϕ29 DNA packaging. J. Struct. Biol. 104, 70–74.
[156] Elías-Arnanz, M. and Salas, M. (1999) Functional interactions between a phage histone-like protein and a transcriptional factor in regulation of ϕ29 early–late transcriptional switch. Genes Dev. 13, 2502–2513.
[157] Camacho, A. and Salas, M. (2001) Mechanism for the switch of ϕ29 DNA early to late transcription by regulatory protein p4 and histone-like protein p6. EMBO J. 20, 6060–6070.
[158] Calles, B., Salas, M. and Rojo, F. (2002) The ϕ29 transcriptional regulator contacts the nucleoid protein p6 to organize a repression complex. EMBO J. 21, 6185–6194.
[159] Camacho, A. and Salas, M. (2004) Molecular interplay between RNA polymerase and two transcriptional regulators in promoter switch. J. Mol. Biol. 336, 357–368.
[160] Salas, M. (1999) Mechanisms of initiation of linear DNA replication in prokaryotes (Setlow, J.K., Ed.), Genetic Engineering, Vol. 21, pp. 159–171. Kluwer Academic/Plenum Publishers, New York.
[161] Inciarte, M.R., Salas, M. and Sogo, J.M. (1980) Structure of replicating DNA molecules of *Bacillus subtilis* bacteriophage ϕ-29. J. Virol. 34, 187–199.
[162] Sogo, J.M., García, J.A., Peñalva, M.A. and Salas, M. (1982) Structure of protein-containing replicative intermediates of *Bacillus subtilis* phage ϕ29 DNA. Virology 116, 1–18.
[163] Blanco, L., Gutiérrez, J., Lázaro, J.M., Bernad, A. and Salas, M. (1986) Replication of phage ϕ29 DNA in vitro: role of the viral

protein p6 in initiation and elongation. Nucleic Acids Res. 14, 4923–4937.
[164] Serrano, M., Gutiérrez, J., Prieto, I., Hermoso, J.M. and Salas, M. (1989) Signals at the bacteriophage ϕ29 DNA replication origins required for protein p6 binding and activity. EMBO J. 8, 1879–1885.
[165] Serrano, M., Salas, M. and Hermoso, J.M. (1990) A novel nucleoprotein complex at a replication origin. Science 248, 1012–1016.
[166] Serrano, M., Gutiérrez, C., Salas, M. and Hermoso, J.M. (1993) Superhelical path of the DNA in the nucleoprotein complex that activates the initiation of phage ϕ29 DNA replication. J. Mol. Biol. 230, 248–259.
[167] Freire, R., Serrano, M., Salas, M. and Hermoso, J.M. (1996) Activation of replication origins in ϕ29-related phages requires the recognition of initiation proteins to specific nucleoprotein complexes. J. Biol. Chem. 271, 31000–31007.
[168] Blanco, L. and Salas, M. (1984) Characterization and purification of a phage ϕ29-encoded DNA polymerase required for the initiation of replication. Proc. Natl. Acad. Sci. USA 81, 5325–5329.
[169] Hermoso, J.M. and Salas, M. (1980) Protein p3 is linked to the DNA of phage ϕ29 through a phosphoester bond between serine and 5′-dAMP. Proc. Natl. Acad Sci. USA 77, 6425–6428.
[170] Hermoso, J.M., Méndez, E., Soriano, F. and Salas, M. (1985) Location of the serine residue involved in the linkage between the terminal protein and the DNA of phage ϕ29. Nucleic Acids Res. 13, 7715–7728.
[171] Escarmís, C. and Salas, M. (1981) Nucleotide sequence at the termini of the DNA of *Bacillus subtilis* phage ϕ29. Proc. Natl. Acad. Sci. USA 78, 1446–1450.
[172] Yoshikawa, H., Friedmann, T. and Ito, J. (1981) Nucleotide sequences at the termini of ϕ29 DNA. Proc. Natl. Acad. Sci. USA 78, 1336–1340.
[173] Méndez, J., Blanco, L., Esteban, J.A., Bernad, A. and Salas, M. (1992) Initiation of ϕ29 DNA replication occurs at the second 3' nucleotide of the linear template: a sliding-back mechanism for protein-primed DNA replication. Proc. Natl. Acad. Sci. USA 89, 9579–9583.
[174] Méndez, J., Blanco, L. and Salas, M. (1997) Protein-primed DNA replication: a transition between two modes of priming by a unique DNA polymerase. EMBO J. 16, 2519–2527.
[175] Blanco, L. and Salas, M. (1985) Replication of phage ϕ29 DNA with purified terminal protein and DNA polymerase: synthesis of full-length ϕ29 DNA. Proc. Natl. Acad. Sci. USA 82, 6404–6408.
[176] Blanco, L., Bernad, A., Lázaro, J.M., Martín, G., Garmendia, C. and Salas, M. (1989) Highly efficient DNA synthesis by the phage ϕ29 DNA polymerase. Symmetrical mode of DNA replication. J. Biol. Chem. 264, 8935–8940.
[177] Martín, G., Lázaro, J.M., Méndez, E. and Salas, M. (1989) Characterization of the phage ϕ29 protein p5 as a single-stranded DNA binding protein. Function in ϕ29 DNA-protein p3 replication. Nucleic Acids Res. 17, 3663–3672.
[178] Ivarie, R.D. and Pène, J.J. (1973) DNA replication in bacteriophage ϕ29: the requirement of a viral-specfic product for association of ϕ29 DNA with the cell membrane of *Bacillus amyloliquefaciens*. Virology 52, 351–362.
[179] McGuire, J.C., Pène, J.J. and Barrow-Carraway, J. (1974) Gene expression during the development of bacteriophage ϕ29. Analysis of viral-specific protein synthesis with suppressible mutants. J. Virol. 13, 690–698.
[180] Mellado, R.P., Moreno, F., Viñuela, E., Salas, M., Reilly, B.E. and Anderson, D.L. (1976) Genetic analysis of bacteriophage ϕ29 of *Bacillus subtilis*:integration and mapping of reference mutants of two collections. J. Virol. 19, 495–500.
[181] Bravo, A. and Salas, M. (1997) Initiation of bacteriophage ϕ29 DNA replication in vivo: assembly of a membrane-associated multiprotein complex. J. Mol. Biol. 269, 102–112.
[182] Konings, W.N., Bisschop, A., Veenhuis, M. and Vermeulen, C.A. (1973) New procedure for the isolation of membrane vesicles of *Bacillus subtilis* and an electron microscopy study of their ultrastructure. J. Bacteriol. 116, 1456–1465.
[183] Bravo, A. and Salas, M. (1998) Polymerization of bacteriophage ϕ29 replication protein p1 into protofilament sheets. EMBO J. 17, 6096–6105.
[184] Reilly, B.E., Zeece, V.M. and Anderson, D.L. (1973) Genetic study of suppressor-sensitive mutants of the *Bacillus subtilis* bacteriophage ϕ29. J. Virol. 11, 756–760.
[185] Prieto, I., Méndez, E. and Salas, M. (1989) Characterization, overproduction and purification of the product of gene 1 of *Bacillus subtilis* phage ϕ29. Gene 77, 195–204.
[186] Serrano-Heras, G., Salas, M. and Bravo, A. (2003) In vivo assembly of phage ϕ29 replication protein p1 into membrane-associated multimeric structures. J. Biol. Chem. 278, 40771–40777.
[187] Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256, 1604–1607.
[188] Brusca, J.S. and Radolf, J.D. (1994) Isolation of integral membrane proteins by phase partitioning with Triton X-114. Methods Enzymol. 228, 182–193.
[189] Bravo, A., Serrano-Heras, G. and Salas, M. (2001) A single amino acid substitution within a coiled-coil motif changes the assembly of a 53-amino acid protein from two-dimensional sheets to filamentous structures. J. Biol. Chem. 276, 21250–21256.
[190] Erickson, H.P., Taylor, D.W., Taylor, K.A. and Bramhill, D. (1996) Bacterial cell division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. Proc. Natl. Acad. Sci. USA 93, 519–523.
[191] Rothfield, L., Justice, S. and García-Lara, J. (1999) Bacterial cell division. Annu. Rev. Genet. 33, 423–448.
[192] de Jong, R.N. and van der Vliet, P.C. (1999) Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication. Gene 236, 1–12.
[193] Younghusband, H.B. and Maundrell, K. (1982) Adenovirus DNA is associated with the nuclear matrix of infected cells. J. Virol. 43, 705–713.
[194] Bodnar, J.W., Hanson, P.I., Polvino-Bodnar, M., Zempsky, W. and Ward, D.C. (1989) The terminal regions of adenovirus and minute virus of mice DNAs are preferentially associated with the nuclear matrix in infected cells. J. Virol. 63, 4344–4353.
[195] Schaack, J., Ho, W.Y., Freimuth, P. and Shenk, T. (1990) Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA. Genes Dev. 4, 1197–1208.
[196] Fredman, J.N. and Engler, J.A. (1993) Adenovirus precursor to terminal protein interacts with the nuclear matrix in vivo and in vitro. J. Virol. 67, 3384–3395.
[197] Meijer, W.J., Serna-Rico, A. and Salas M (2001) Characterization of the bacteriophage ϕ29-encoded protein p16.7: a membrane protein involved in phage DNA replication. Mol. Microbiol. 39, 731–746.
[198] Meijer, W.J., Lewis, P.J., Errington, J. and Salas, M. (2000) Dynamic relocalization of phage ϕ29 DNA during replication and the role of the viral protein p16.7. EMBO J. 19, 4182–4190.
[199] Serna-Rico, A., Salas, M. and Meijer, W.J. (2002) The *Bacillus subtilis* phage ϕ29 protein p16.7, involved in ϕ29 DNA replication, is a membrane-localized single-stranded DNA-binding protein. J. Biol. Chem. 277, 6733–6742.

[200] Serna-Rico, A., Muñoz-Espín, D., Villar, L., Salas, M. and Meijer, W.J. (2003) The integral membrane protein p16.7 organizes in vivo ϕ29 DNA replication through interaction with both the terminal protein and ssDNA. EMBO J. 22, 2297–2306.
[201] Newport, J. and Yan, H. (1996) Organization of DNA into foci during replication. Curr. Opin. Cell Biol. 8, 365–368.
[202] Hozak, P., Hassan, A.B., Jackson, D.A. and Cook, P.R. (1993) Visualization of replication factories attached to nucleoskeleton. Cell 73, 361–373.
[203] Cook, P.R. (1999) The organization of replication and transcription. Science 284, 1790–1795.
[204] Verheijen, R., van Venrooij, W. and Ramaekers, F. (1988) The nuclear matrix: structure and composition. J. Cell Sci. 90, 11–36.
[205] Bridge, E. and Pettersson, U. (1995) Nuclear organization of replication and gene expression in adenovirus-infected cells. Curr. Top. Microbiol. Immunol. 199, 99–117.
[206] Lamond, A.I. and Earnshaw, W.C. (1998) Structure and function in the nucleus. Science 280, 547–553.
[207] Pombo, A., Ferreira, J., Bridge, E. and Carmo-Fonseca, M. (1994) Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. EMBO J. 13, 5075–5085.
[208] Angeletti, P.C. and Engler, J.A. (1998) Adenovirus preterminal protein binds to the CAD enzyme at active sites of viral DNA replication on the nuclear matrix. J. Virol. 72, 2896–2904.
[209] Buck, K.W. (1996) Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv. Virus Res 47, 159–251.
[210] Schaad, M.C., Jensen, P.E. and Carrington, J.C. (1997) Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J. 16, 4049–4059.
[211] Ahola, T., Lampio, A., Auvinen, P. and Kaariainen, L. (1999) Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J. 18, 3164–3172.
[212] Lampio, A., Kilpelainen, I., Pesonen, S., Karhi, K., Auvinen, P., Somerharju, P. and Kaariainen, L. (2000) Membrane binding mechanism of an RNA virus-capping enzyme. J. Biol. Chem. 275, 37853–37859.
[213] Yamanaka, T., Ohta, T., Takahashi, M., Meshi, T., Schmidt, R., Dean, C., Naito, S. and Ishikawa, M. (2000) TOM1, an Arabidopsis gene required for efficient multiplication of a tobamovirus, encodes a putative transmembrane protein. Proc. Natl. Acad. Sci. USA 97, 10107–10112.
[214] Tsujimoto, Y., Numaga, T., Ohshima, K., Yano, M.A., Ohsawa, R., Goto, D.B., Naito, S. and Ishikawa, M. (2003) Arabidopsis tobamovirus multiplication (TOM) 2 locus encodes a transmembrane protein that interacts with TOM1. EMBO J. 22, 335–343.
[215] Hagiwara, Y., Komoda, K., Yamanaka, T., Tamai, A., Meshi, T., Funada, R., Tsuchiya, T., Naito, S. and Ishikawa, M. (2003) Subcellular localization of host and viral proteins associated with tobamovirus RNA replication. EMBO J. 22, 344–353.
[216] Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K. and Bartenschlager, R. (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. 75, 1252–1264.
[217] Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M.R., Pedrazzini, E., Bonatti, S. and Migliaccio, G. (2002) Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 293, 31–43.
[218] Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C.M., Blum, H.E. and Moradpour, D. (2001) Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 276, 44052–44063.
[219] Ivashkina, N., Wolk, B., Lohmann, V., Bartenschlager, R., Blum, H.E., Penin, F. and Moradpour, D. (2002) The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. J. Virol. 76, 13088–13093.
[220] Wattenberg, B. and Lithgow, T. (2001) Targeting of C-terminal (tail) proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic 2, 66–71.
[221] Suhy, D.A., Giddings Jr., T.H. and Kirkegaard, K. (2000) Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J. Virol. 74, 8953–8965.
[222] Rust, R.C., Landmann, L., Gosert, R., Tang, B.L., Hong, W., Hauri, H.P., Egger, D. and Bienz, K. (2001) Cellular COPII proteins are involved in production of the vesicles that form the poliovirus replication complex. J. Virol. 75, 9808–9818.
[223] Egger, D., Pasamontes, L., Bolten, R., Boyko, V. and Bienz, K. (1996) Reversible dissociation of the poliovirus replication complex: functions and interactions of its components in viral RNA synthesis. J. Virol. 70, 8675–8683.
[224] Lama, J., Paul, A.V., Harris, K.S. and Wimmer, E. (1994) Properties of purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase 3Dpol. J. Biol. Chem. 269, 66–70.
[225] Hope, D.A., Diamond, S.E. and Kirkegaard, K. (1997) Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. J. Virol. 71, 9490–9498.
[226] Lyle, J.M., Clewell, A., Richmond, K., Richards, O.C., Hope, D.A., Schultz, S.C. and Kirkegaard, K. (2002) Similar structural basis for membrane localization and protein priming by an RNA-dependent RNA polymerase. J. Biol. Chem. 277, 16324–16331.
[227] Hansen, J.L., Long, A.M. and Schultz, S.C. (1997) Structure of the RNA-dependent RNA polymerase of poliovirus. Structure 5, 1109–1122.
[228] Lyle, J.M., Bullitt, E., Bienz, K. and Kirkegaard, K. (2002) Visualization and functional analysis of RNA-dependent RNA polymerase lattices. Science 296, 2218–2222.