Functionality of the dnaA Protein Binding Site in DNA Replication Is Orientation-dependent*

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We analyzed the functionality of different dnaA protein binding sites by assaying in vitro dnaA-dependent replication of pBR322 derivatives. Single dnaA sites from oriC and from the mioC and dnaA gene promoters were active when combined with the primer generating element of pBR322 in a proper distance. Pre-replisome assembly did not require sequences in addition to the 9-base pair consensus dnaA binding site. Inversion of the structurally asymmetric dnaA site relative to its orientation in wild type pBR322 resulted in a marked reduction in replication efficiency, as observed with five different dnaA sites studied. The direction of DNA replication was not affected.

During initiation of Escherichia coli chromosomal DNA replication, a multiprotein complex, the pre-replisome, is formed at the origin, dnaA protein initially recognizes a 9-bp consensus sequence (TTAGGGCAACA) in the origin (Fuller et al., 1984). A dnaB-dnaC protein complex interacts with dnaA protein bound to oriC (Kornberg et al., 1986). In the presence of DNA gyrase, dnaB helicase action results in bidirectional DNA unwinding from oriC (Baker et al., 1987). Start sites for in vitro bidirectional DNA replication were mapped close to dnaA binding sites in oriC (Seufert and Messer, 1987b). Most likely, dnaG protein synthesizes initial primers at the site of pre-replisome assembly.

Another line of evidence for dnaA-dependent pre-replisome assembly comes from in vitro replication studies with plasmid pBR322. During initiation, proteins dnaB, dnaC, and dnaG are directed to the pBR322 origin in a process called primsome assembly (Arai and Kornberg, 1981), involving proteins n, n', n", i, and a single-stranded n' recognition site (Mindern and Mariants, 1985). In the presence of dnaA protein and its binding site, the primosomal proteins and their binding sites are no longer essential for replication (Seufert and Messer, 1987a). Primsome assembly and dnaA-dependent pre-replisome assembly are obviously functionally equivalent. This type of initiation may be relevant also for other dnaA protein-dependent replicons, like R1, pSC101, F, and P1 (Ortega et al., 1986; Masai and Arai, 1987; Frey et al., 1979; Felton and Wright, 1979; Hansen and Yarnolinsky, 1986; Murakami et al., 1987; Wickner and Chattoral, 1987).

In this study we analyzed whether different dnaA binding sites from oriC and from the mioC and dnaA gene promoter regions can support dnaA-dependent in vitro replication. All dnaA sites were functional in replication when combined in a proper distance with the primer generating element of pBR322. The pre-replisome complex, which is assembled at the structurally asymmetric dnaA protein binding site, is functionally asymmetric, i.e. its direction of movement is predetermined. We, therefore, analyzed whether the orientation of the different dnaA binding sites affects the efficiency or the mode of replication.

MATERIALS AND METHODS

Construction of Plasmids—Restriction enzyme digestes, agarose or polyacrylamide gel electrophoresis, phosphorylation of linkers, ligation, and purification of DNA with CsCl gradients were all performed using standard protocols (Maniatis et al., 1982) and following instructions of the manufacturers. Restriction fragments were isolated from acrylamide gels after transfer to DE81 paper (Whatman) as described by Dretzen et al. (1981). For generating a unique cloning site in the origin region, plasmid pBR322 (Fig. 1) was partially cut with Thal, linear unit length material was isolated from an agarose gel and ligated with Smal linkers (CCCGGG, Amersham Corp.). Plasmid pBR38S13 is identical to pBR38 except for a Smal linker inserted into the Thal site at position 2077 (Sutcliffe, 1978; Peden, 1983). The dnaA binding site of pBR322 was isolated on a 73-bp Alul-HaeIII fragment (positions 2418-2490; Sutcliffe, 1978; Peden, 1983), two dnaA sites from oriC on a 29-bp DpnI or a 39-bp Alul-Acl fragment (positions 99-94 and positions 24-28, respectively, Buhr and Messer, 1983), the dnaA binding sites from the mioC gene and dnaA gene promoters on a 59-bp Thal-ResI fragment (positions 790-838, Buhr and Messer, 1983) or a 68-bp DpnI fragment (positions 637-704, Hansen et al., 1982). A XmnI-PstI fragment (positions 2031-3599; Sutcliffe, 1978; Peden, 1983) of the recombinant plasmids was inserted into M13mp8 cut with Smal-PstI (Mensing and Vieira, 1982) and sequenced (Sanger et al., 1977). The sequences corresponded to the published data. Plasmid pBR38 was found to contain a 12-bp Alul-PvuII deletion in addition to the Alul-HaeIII deletion described (Seufert and Messer, 1987a; positions 2057-2068, positions 2117-2120; Sutcliffe, 1978; Peden, 1983).

DNA Replication in Vitro—dnaA-dependent replication of pBR322 derivatives was performed as described (Seufert and Messer, 1987a) using the in vitro system developed for oriC replication (Fuller et al., 1981).

Analysis of Replicative Intermediates—A standard in vitro replication assay was performed in the presence of 10 μM dTTP. DNA was purified from the assay mixture by phenolization, followed by RNase A treatment (20 μg/ml; 20 min at 30 °C), again phenolization, and passage through Sephadex LH20 (Pharmacia). The DNA was cut with PstI, phenolized, and precipitated with ethanol. DNA was prepared for electron microscopy by cytochrome c spreading in 50% formamide and carbonate buffer on a water hypophase (Burbardt and Lurr, 1984). The spreading film was picked up with Parlodion-coated copper grids and shadowed with platinum/iridium (80:20) at an angle of ~6°. Micrographs were recorded with a Philips EM400 on Agfa Scientia RA710P 35-mm film at × 4000 primary magnification and measured × 16 enlarged using an electronic digitizer (LM4, Bruhl, Nürnberg, Germany). Evaluation of the data and graphic representation was done with the help of a computer program. Cleaved replicative intermediates were selected as linear molecules showing a symmetrical loop ("eye") and two unreplicated branches or composed of two Y-shaped structures connected by the unreplicated region (~<~).

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The abbreviation used is: bp, base pair.
RESULTS

Functionality of the dnaA Site Is Position-dependent—During previous studies (Seufert and Messer, 1987a) concerning the role of dnaA protein in replication, we constructed pBR322 derivatives with deleted n' recognition sites. These derivatives had an absolute requirement for dnaA protein and its binding site for efficient in vitro replication. Initial steps in pBR322 replication, transcription by RNA polymerase, and primer extension by DNA polymerase I after its processing by RNase H (Itoh and Tomizawa, 1978), were still required. As indicated from these enzymatic studies, the dnaA binding site and the primer generating element of pBR322 are interdependent. We now checked on the DNA level whether the distance between these two elements is critical for functionality in replication.

Plasmid pBR3A8, from which the dnaA binding site is missing, is replication-defective in vitro, even in the presence of dnaA protein, in contrast to pBR3A6 retaining the dnaA site (Seufert and Messer, 1987a; Fig. 1). A single Smal site was created by linker insertion in plasmid pBR3A8 at either one of three positions (positions 2077, 1635, and 1390; pBR322 positions refer to Sutcliffe, 1978; Peden, 1983). An AluI-HaeII restriction fragment (positions 2418–2490) from wild type pBR322 containing the dnaA binding site was inserted into these Smal sites. In the resulting constructs pW072, pW030, and pW04 (Fig. 1A), the dnaA binding site is moved 16, 457, or 702 bp downstream from its original location without changing its orientation. dnaA-dependent replication was fully restored in pW072 as compared to pBR3A6, but it was virtually absent in pW030 and pW04 (Fig. 1B). These data show that neither the primer generating element nor a single dnaA binding site on its own is able to function in replication in vitro. But a dnaA-dependent replicon is created when both elements are put together in a proper distance.

Different dnaA Sites Are Functional in Replication—There are four dnaA binding sites within the minimal sequence of the E. coli chromosomal origin, oriC (Fuller et al., 1984). We analyzed whether oriC fragments with a single dnaA binding site give rise to a functional origin when combined with the primer generating element of pBR322. A 26-bp DpnI fragment (pW026; positions 69–94; oriC positions refer to Buhk and Messer, 1983; see also Meijer et al., 1979; Sugimoto et al., 1979) or a 39-bp AluI-AccI fragment (pW043; oriC positions 247–286) was inserted into the unique Smal site (pBR322 position 2077) of pBR3A8S13. The efficiency of in vitro replication in the presence of dnaA protein was checked with the resulting recombinant plasmids. dnaA-dependent replication proceeded efficiently in both cases. pW043 (oriC dnaA box 4) reached the level of plasmids pBR3A6 and pW072 containing the natural pBR322 dnaA binding site, pW026 (oriC dnaA box 1) gave 69% of this activity (Fig. 2). These data demonstrate the functionality in replication of a single dnaA binding site from oriC.

In addition to its role in replication, dnaA protein is a repressor of transcription of its own gene and the mioC gene close to oriC (Atlung et al., 1985; Braun et al., 1985; Kücherer et al., 1986; Lother et al., 1985; Rokeach and Zyskind, 1986; Stuitje et al., 1986; Løbner-Olesen et al., 1987; Schauzu et al., 1987). We investigated whether fragments from these dnaA-regulated promoters containing a consensus dnaA binding site are functional in replication. A 68-bp DpnI fragment (pW068; positions 637–704 in Hansen et al., 1982) from the dnaA gene promoter or a 59-bp Thal-RecI fragment (pW059; oriC positions 780–838) from the mioC gene promoter was inserted into the unique Smal site of pBR3A8S13. Again, the replication-defective vector plasmid became a potent dnaA-dependent replicon upon insertion of these dnaA binding sites (Fig. 2). We conclude that dnaA protein does not require sequences in addition to the 9-bp consensus sequence in order to exert its function in replication. As expected, fragments which do not contain a consensus sequence for dnaA binding, e.g. a 49-bp AluI fragment from a trnA operon (positions 227–275 in Hsu et al., 1984), were inactive in supporting dnaA-dependent replication in vitro.

Functionality of the dnaA Site Is Orientation-dependent—During analysis of the replication efficiency we realized that insertion of a single dnaA binding site gave rise to two functionally different populations of recombinant plasmids; type I constructs replicating at wild type efficiency in the presence of dnaA protein and type II constructs which exhibit...
Different dnaA binding sites are functional and their functionality is orientation-dependent. The in vitro DNA replication efficiency upon addition of dnaA protein was measured with plasmids carrying dnaA sites from different sources: from the E. coli chromosomal origin (oriC-1, oriC-4) and from the dnaA gene and mioC gene promoter regions (dnaA prom., mioC prom.). The orientation of different dnaA binding sites was identical to that in wild type pBR322 or reversed (R).

Analysis of Replication Products and Intermediates—In addition to quantitative measurements we analyzed the structure of replication products synthesized with templates containing different dnaA binding sites in different orientations. Complete products, i.e. full length synthesis of both strands, were obtained with all clones, as has been shown previously for dnaA-dependent replication of pBR322 (Seufert and Messer, 1987a).

The start site and direction of DNA replication of different constructs was determined by analyzing replicative intermediates in the electron microscope. Plasmids were isolated from a standard in vitro replication assay run in the presence of deoxyxynucleotides to enrich for intermediates and cut at the unique PstI site (pBR322 position 3609). The linearized plasmids were spread, shadowed, and the contour lengths of intermediates were measured in electron micrographs. The data obtained for plasmids pWO72 and pWO72R, containing the dnaA binding site of pBR322 in original or reversed orientation, are shown in Fig. 3. The left-hand branch point was located at a distance of 26% of total molecule length (4025 bp) from the PstI site and did not move with increasing extent of replication, whereas the position of the right-hand branch point depended linearly on the extent of replication. With respect to start site and direction of DNA replication, dnaA-dependent in vitro replication of these constructs was identical to ColEl replication in vivo (Lovett et al., 1974; Tomizawa et al., 1977). Inversion of the dnaA binding site did not alter the start site or direction of DNA replication (Fig. 3), but reduced the replication efficiency (Figs. 1 and 2). This is true for all different dnaA sites studied here. From these data it is obvious that the functionality of a dnaA binding site depends on its orientation relative to the primer generating element.

Insertion of multiple dnaA sites into pBRΔS13 gave rise to bidirectional replication only when a functional oriC fragment (oriC positions −44–288) was used (pMO2; Fig. 4A). Both branch points moved during replication, and the start site was shifted to the oriC position (28–34% relative length). A 16-bp BglII deletion (oriC positions 22–38), which destroys oriC function without affecting any of the dnaA binding sites, resulted in unidirectional counterclockwise replication (pMOB2; Fig. 4B), as observed with single dnaA binding sites. In this construct, only the right branch point moved with increasing extent of replication, whereas the left branch point stayed constant at the pBR322 origin position (25% relative length). Likewise, insertion of an AluI-Accl fragment (oriC positions 167–288) with three dnaA binding sites as well as of dimers of a dnaA site did not alter unidirectionality of
Fig. 3. Replicative intermediates of plasmids plasmid pW072 and pW072R. A, the maps of the plasmids are shown linearized at the PstI (P) site. Restriction sites for enzymes BamHI (B) and EcoRI (E) are shown, as well as the ampicillin (Ap) and tetracycline (Tc) resistance determinants, the primer transcript, and the dnaA protein binding site. The triangle points 5'→3' in the sequence 5'-TTATCCACA-3', which is in original orientation in pW072, in reversed orientation in pW072R. B, replicative intermediates, ordered by increasing extent of replication, are represented schematically (one line: unreplicated region; two lines: replicated region). C, positions of the left-hand (stars) or right-hand (circles) branch points are plotted versus the extend of replication.

replication (data not shown). Obviously, the primer generating element determines the direction of replication in these constructs where it is an essential part of the replicon.

DISCUSSION

The pre-replisome complex assembled at the pBR322 origin during initiation is functionally asymmetric, i.e. its movement is unidirectional counterclockwise, as well as unwinding of parental DNA by dnaB protein. Primer synthesis by dnaG protein is restricted to the lagging strand template. It is an attractive hypothesis to assume that the structural asymmetry of the dnaA binding site mediates the functional asymmetry of the prereplisome complex assembled there.

Different dnaA binding sites, including those from the mioC and dnaA gene promoter regions, where dnaA protein acts as a transcriptional repressor, were inserted instead of the cognate pBR322 dnaA box. These different restriction fragments, which did not contain any homology except the consensus sequence for dnaA protein binding, were fully functional in DNA replication. Obviously, sequences sufficient for dnaA binding are sufficient as well for pre-replisome formation, and no additional sequences are required.

We analyzed quantitative and qualitative effects arising from an inversion of the dnaA binding site in dnaA-dependent pBR322 derivatives. The start site and direction of DNA replication, which corresponded to in vivo data obtained with ColEl plasmid (Lovett et al., 1974; Tomizawa et al., 1977) were not altered upon inversion of the dnaA binding site as revealed by electron microscopic analysis of replicative intermediates (Fig. 3). Likewise, full length synthesis of both strands was observed. But inversion of the dnaA binding site resulted in a distinct reduction of the replication efficiency, as observed with five different dnaA binding sites studied here (Figs. 1 and 2). The observation that the reverse orientation is functional, albeit with a reduced efficiency, may be due to the binding of dnaA protein multimers at dnaA binding sites (Fuller et al., 1984). We conclude that the orientation of the dnaA binding site relative to the direction of DNA replication is critical for its efficient functioning.

Our results are corroborated by a comparison of the orien-
tation of dnaA binding sites relative to the replication direction in various naturally occurring dnaA-dependent replicons. Replication of plasmids pSC101, mini-F(oriV), P1, and R1 are unidirectional and dnaA protein-dependent and, with the exception of R1, the dnaA binding site is oriented as in the case of plasmid pBR322 (Hansen and Yarmolinsky, 1986; Ortega et al., 1986; Masai and Ara; 1987; Wickner and Chatteraj, 1987).

FIG. 4. Replicative intermediates of plasmid pMO2 (A) and pMOB2 (B). Plasmid pMO2 carries the functional oriC region (oriC position -44-288), in plasmid pMOB2 a 16-bp BglII fragment was deleted from pMO2 (oriC positions 22-38), which inactivates oriC. Positions of rightward (circles) or leftward (stars) moving branch points are plotted versus increasing extension of replication.

REFERENCES


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