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# The DNA unwinding element: a novel, *cis*-acting component that facilitates opening of the *Escherichia coli* replication origin

**David Kowalski and Martha J.Eddy**

Molecular and Cellular Biology Department, Roswell Park Memorial Institute, Buffalo, NY 14263, USA

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**We have discovered that DNA supercoiling, in the absence of replication proteins, induces localized unwinding in the *Escherichia coli* replication origin (*oriC*) at the same sequence opened by the dnaA initiator protein. The DNA helix at the tandemly repeated, 13mer sequence is thermodynamically unstable, as evidenced by hypersensitivity to single-strand-specific nuclease in a negatively supercoiled plasmid, and demonstrated by stable DNA unwinding seen after two-dimensional gel electrophoresis of topoisomers. A replication-defective *oriC* mutant lacking the leftmost 13mer shows no nuclease hypersensitivity in two remaining 13mers and no detectable DNA unwinding on two-dimensional gels. The replication defect in the *oriC* mutant can be corrected by inserting a dissimilar DNA sequence with reduced helical stability in place of the leftmost 13mer. Thus, the helical instability of the leftmost 13mer, not the specific 13mer sequence, is essential for origin function. The rightmost 13mer exhibits helical instability but differs from the leftmost 13mer in its strict sequence conservation among related bacterial origins. The repeated 13mer region appears to serve two overlapping functions: protein recognition and helical instability. We propose that the *cis*-acting sequence whose helical instability is required for origin function be called the DNA unwinding element (DUE).**

*Key words:* helical instability/localized unwinding/replication initiation/supercoiled DNA

## Introduction

DNA replication of the *Escherichia coli* chromosome originates at a unique site (Bird *et al.*, 1972; Hohlfeld and Vielmetter, 1973) called *oriC*. Cloned *oriC* (Yasuda and Hirota, 1977; Messer *et al.*, 1978; Miki *et al.*, 1978; von Meyenburg *et al.*, 1978) functions on plasmids with the same cell cycle regulation as the chromosomal origin (Leonard and Helmstetter, 1986). A 245 bp DNA sequence, the minimal *oriC*, contains all of the *cis*-acting information for autonomous replication (Oka *et al.*, 1980).

Mutational analyses of the DNA sequences required for origin function have led to the proposal that *oriC* consists of two essential sequence elements: (i) protein recognition elements, and (ii) spacer elements that maintain proper distance between protein recognition elements (Asada *et al.*, 1982; Oka *et al.*, 1984). This proposal is consistent with phylogenetic comparisons of origin DNA sequences and available biochemical data: purified initiator protein, dnaA (Fuller and Kornberg, 1983), binds to four 9 bp sequences (Fuller *et al.*, 1984; Matsui *et al.*, 1985) which are evolutionarily conserved in the chromosomal replication origins of six bacterial species examined (Zyskind *et al.*, 1983). Among these species, the sequences separating a given pair of dnaA binding sites are conserved in length but vary in primary structure.

In addition to binding the initiator protein, origin DNA must unwind prior to the actual initiation of DNA synthesis. DNA unwinding is required for the replication machinery to access and copy the individual strands. The dnaB helicase, which is responsible for unwinding DNA ahead of the replication fork (Baker *et al.*, 1986, 1987; Dodson *et al.*, 1986; Echols, 1986) is itself incapable of initiating unwinding within a duplex molecule (LeBowitz and McMacken, 1986). Recently, it has been shown that binding of the dnaA protein to *oriC* in the presence of HU protein leads to the specific duplex opening of a tandemly repeated, 13mer DNA sequence (Bramhill and Kornberg, 1988). Formation of this 'open complex' requires a negatively supercoiled template (Baker and Kornberg, 1988). Similarly, the λ phage initiator protein induces a localized destabilization of the helix at the λ origin in a reaction requiring negatively supercoiled DNA (Schnos *et al.*, 1988). The DNA sequence at the site of specific duplex opening in *oriC* is known to be essential for origin function *in vivo* (Oka *et al.*, 1980). However, the molecular basis for this DNA sequence requirement has not been established. Furthermore, the precise mechanism by which DNA supercoiling contributes to specific duplex opening is unclear.

Using single-strand-specific nuclease hypersensitivity to assay for localized DNA unwinding (Kowalski, 1984; Kowalski *et al.*, 1988), we show here that specific duplex opening of *oriC* is induced by DNA supercoiling alone and is localized to the same 13mer repeats that are opened by dnaA protein binding. Using two-dimensional (2D) gel electrophoresis of topoisomers containing wild-type or mutant *oriC* inserts, we demonstrate that the supercoil-induced opening of *oriC* is the consequence of a low free energy cost for unwinding the 13mer repeats, i.e. reduced helical stability. Functional replacement of the leftmost (L) 13mer by a dissimilar sequence with reduced helical stability demonstrates that the helical instability of the L 13mer, not the specific 13mer sequence, is essential for origin function *in vivo*. The repeated 13mer sequence is a complex genetic element that appears to serve two, overlapping functions: protein recognition and helical instability. We propose that the *cis*-acting sequence whose helical instability is required for origin function be called the DNA unwinding element (DUE).

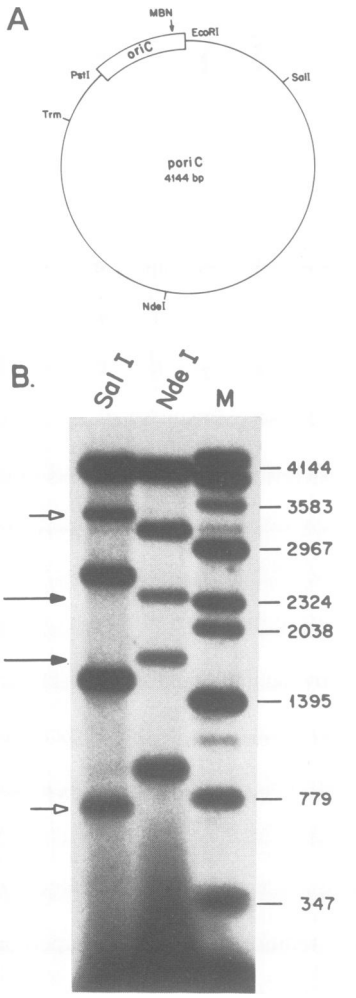


**Fig. 1.** Single-strand-specific nuclease hypersensitivity of *oriC* in supercoiled plasmid DNA. (**A**) Map of the *oriC* plasmid showing the locations of the mung bean nuclease-hypersensitive site (MBN) and pertinent restriction enzyme sites. The *oriC* box represents positions 493 to −41 in the chromosomal origin sequence (Meijer *et al.*, 1979; Sugimoto *et al.*, 1979). The thin line represents the cloning vector which is the *Eco*RI−*Pst*I fragment of pBR322. The Trm site corresponds to the previously characterized nuclease-hypersensitive site in the pBR322 vector (Sheflin and Kowalski, 1985). (**B**) Mapping the mung bean nuclease-hypersensitive sites in the *oriC* plasmid. Supercoiled p*oriC* was reacted with limiting mung bean nuclease to produce singly nicked circular DNA. The DNA was subsequently linearized and 5′-$^{32}$P-end-labeled at either the *Sal*I or *Nde*I site. The labeled DNA was irreversibly denatured with glyoxal, electrophoresed in a 1.4% agarose gel and autoradiographed. Two hypersensitive sites are revealed by two pairs of specific bands whose DNA fragment sizes add up to the plasmid unit length (4144 bp). The band pair indicated by the open arrows in the *Sal*I lane and the one indicated by the closed arrows in the *Nde*I lane, taken together, assign a nuclease-hypersensitive site to *oriC*. The other band pair maps to the Trm site in the vector. The unit length band (4144 bp) in the *Sal*I and *Nde*I lanes is derived from the intact strand of the singly nicked plasmid DNA. **Lane M** contains single-stranded DNA size markers, the number of bases in some of which are indicated on the right.

## Results

### *Specific duplex opening of E.coli oriC in supercoiled DNA*

We used mung bean nuclease (Kowalski *et al.*, 1976) to probe for the location of DNA unwinding in a negatively supercoiled plasmid containing *oriC*. The single-strand-specific nuclease can be used to probe supercoiled DNA structure at neutral pH (Kowalski and Sanford, 1982). The site specificity of the enzyme acting on supercoiled DNA is identical to that of P1 nuclease (Kowalski, 1984), which was used to identify the specific duplex opening induced by dnaA protein in *oriC* (Bramhill and Kornberg, 1988). The plasmid studied, p*oriC*, is a pBR322 derivative containing a 530 bp segment of the *E.coli* chromosome which includes the replication origin (Figure 1A). The supercoiled plasmid was quantitatively converted to nicked circular DNA by mung bean nuclease. The site specificity of the nuclease nicks was determined by agarose gel electrophoresis of DNA linearized and 5′-$^{32}$P-end-labeled at a unique restriction enzyme site and denatured in glyoxal. A mung bean nuclease site nicked at equal frequency in either DNA strand is expected to generate two bands of equal intensity by this procedure. As seen in Figure 1(B), two band pairs are revealed after *Sal*I or *Nde*I digestion. The pair of DNA fragments seen after cutting with *Sal*I (Figure 1B, open arrows) or with *Nde*I (closed arrows) assign a mung bean nuclease site to *oriC* (see Figure 1A). The other pair of DNA fragments in lanes 1 and 2 result from nuclease cleavage at a previously characterized site in the Amp$^r$ gene terminator region (Figure 1A, labeled 'Trm') in pBR322 (Sheflin and Kowalski, 1985).

Specific nicking of *oriC* is absolutely dependent on DNA supercoiling, as previously demonstrated for other single-strand-specific nuclease-hypersensitive sites detected under the same digestion conditions used here (Kowalski, 1984; Sheflin and Kowalski, 1985). Removal of supercoils, by prior relaxation or linearization of the DNA, results in no bands at the positions expected for specific cutting in *oriC* (not shown). Combined with the results in Figure 1(B), the data indicate that negative supercoiling in the absence of replication proteins can lead to specific duplex opening in *oriC*.

### *Duplex opening is localized in the tandemly repeated, 13mer sequence in the minimal oriC*

We determined the precise location and nucleotide sequence of the mung bean nuclease site in *oriC* by preparing singly end-labeled restriction fragments containing the nuclease nicks and analyzing them on DNA sequencing gels. The autoradiogram in Figure 2(A) shows a nucleotide-level analysis of the mung bean nuclease nicks alongside the DNA sequencing ladders of the same *oriC* restriction fragment. The region of the sequence cleaved is revealed in the +MBN lane (bracketed by the arrow in Figure 2A). A similar span of the sequence is cleaved in the same region in the opposite DNA strand (not shown). The locations of the nuclease nicks in the DNA sequence are shown in Figure 2(B) in relation to a partial map of *oriC*. The hypersensitive site occurs at the left end of the minimal sequence required for *oriC* function.

The nuclease-hypersensitive sequence (Figure 2B, positions 21−67) is localized to three tandem repeats of a 13mer sequence (positions 23−65). The 13mers conform to the consensus sequence GATCTNTTNTTTT and were identified by Bramhill and Kornberg (1988) as sites of dnaA/HU protein-induced opening in supercoiled DNA. Remarkably, DNA supercoiling itself induces the same sequence to open in the absence of dnaA/HU proteins.

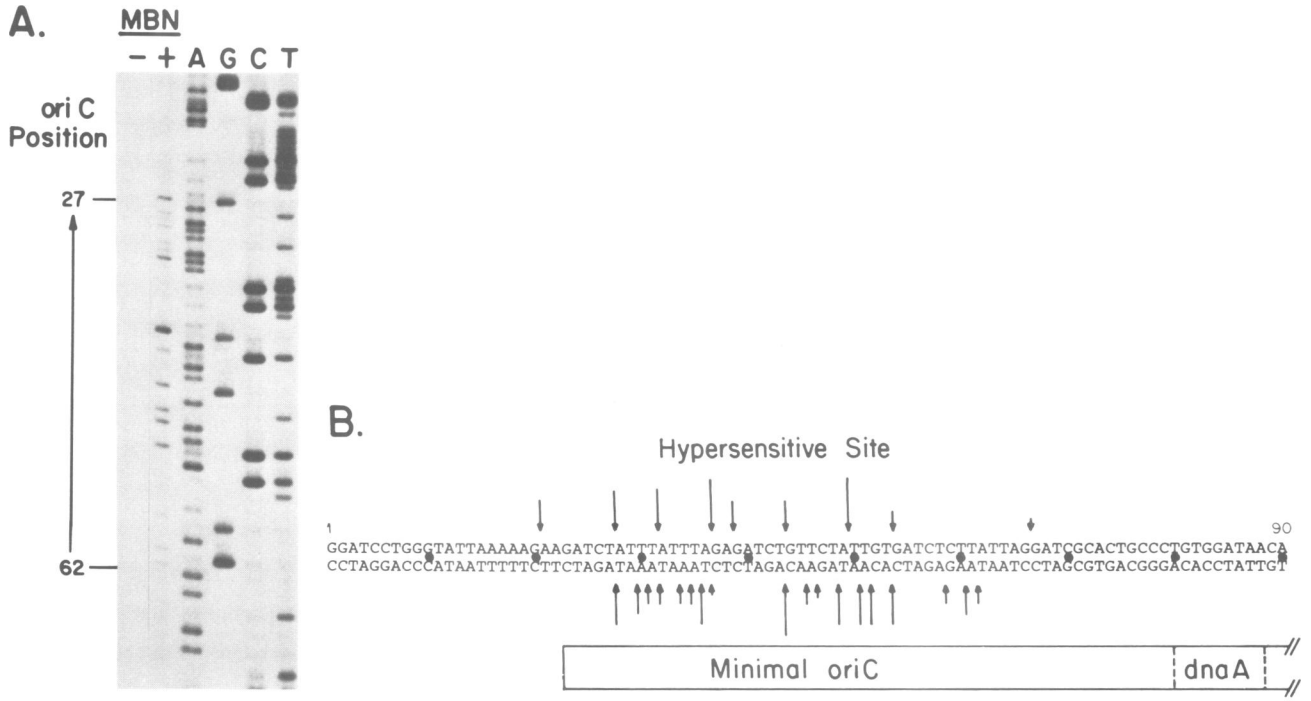


**Fig. 2.** Nucleotide level map of the single-strand-specific nuclease-hypersensitive site in *oriC*. (**A**) DNA sequence analysis of the mung bean nuclease-hypersenstivie site. **Lane MBN+** shows the locations and relative intensities of the mung bean nuclease nicks within the DNA sequence of the lower strand of *oriC* (see panel B). The nucleotide positions that encompass the *oriC* region nicked are shown at the left. Supercoiled p*oriC* was reacted with mung bean nuclease and a DNA segment containing the nuclease-hypersensitive region was excised with *Msp*I, 5′-end-labeled, isolated and then cleaved at the unique *Fok*I site. The isolated singly end-labeled fragment containing the hypersensitive site was denatured, electrophoresed in a 10% polyacrylamide−8 M urea gel and autoradiographed (lane MBN+). Included in the autoradiogram are the products of the Maxam and Gilbert (1980) sequencing reactions [**lanes A** (A > G), **G**, **C** and **T** (T+C)] performed on the same DNA without mung bean nuclease treatment. Also shown is a control reaction (**lane MBN−**) containing the same singly end-labeled restriction fragment derived from supercoiled p*oriC* that was not treated with mung bean nuclease. (**B**) The single-strand-specific nuclease-hypersensitive site maps in a DNA sequence at the left end of the minimal essential *oriC*. The left end of the minimal *oriC* is represented by the box below the DNA sequence and the location of the leftmost dnaA protein binding site is shown for reference. The locations of the mung bean nuclease nicks derived from DNA sequence analysis of the lower strand (panel A) and the upper strand (not shown) are indicated by the arrows. The length of the arrows reflect differences in nicking intensity on a non-linear scale. The numbers at the ends of the sequence refer to nucleotide position within *oriC* (Sugimoto *et al.*, 1979; Meijer *et al.*, 1979).

### *A replication-defective derivative lacking one 13mer shows no nuclease hypersensitivity in oriC*

The locations of the mung bean nuclease (MBN) hypersensitive site and the dnaA protein footprinting sites (Fuller *et al.*, 1984; Matsui *et al.*, 1985) on a map of the *oriC* insert are shown in Figure 3(A). The left side of the nuclease-hypersensitive site maps with two *Bgl*II sites (B in Figure 3A). Deletion of the 16 bp sequence separating the *Bgl*II sites (Figure 3A, DEL16) is known to inactivate replication from *oriC in vivo* (Oka *et al.*, 1980). When the supercoiled plasmid DEL16 is probed with mung bean nuclease, no bands are seen in the gel at the positions expected for *oriC* cleavage (Figure 3B, arrows). Instead, cleavage occurs exclusively at the Trm site of pBR322 (band pair flanking the arrows).

The 16 bp sequence removed from *oriC* in pDEL16 contains one of the three copies of the 13mer consensus sequence, specifically the L 13mer. Thus, deletion of the L 13mer from *oriC* leads to loss of nuclease hypersensitivity *in vitro* and loss of origin function *in vivo*.

### *Replication origin function is reactivated in the 13mer deletion derivative after inserting a dissimilar DNA sequence that restores nuclease hypersensitivity*

Based on their deletion analysis, Bramhill and Kornberg (1988) suggested that the specific 13mer sequence is required for *oriC* function. However, our finding that nuclease hypersensitivity of *oriC* and DEL16 in supercoiled plasmids correlates with origin function suggests a higher-order DNA structural role for the 13mer repeats (or two, overlapping roles; see Discussion). To assess whether it is the specific primary structure or a higher-order structural property of the sequence which is important for replication *in vivo*, we replaced a 13mer sequence with dissimilar sequences and analyzed both nuclease hypersensitivity and origin function. We inserted two different DNA sequences in both possible orientations in the *Bgl*II site of pDEL16 as shown in Figure 3(A). The AT105 sequence contains the nuclease-hypersensitive site Trm (Figure 1A) of pBR322 (Sheflin and Kowalski, 1985). The GC91 sequence, derived from the Tet$^r$ gene of pBR322, is not nuclease hypersentisive in supercoiled pBR322. As shown in Table I, the DNA sequences that replace the deleted 13mer repeat are all poor matches to the consensus sequence.

The insertion derivatives were probed with mung bean nuclease and the location of the hypersensitive sites in the plasmid were determined. Insertion of the GC91 sequence in pDEL16 results in no detectable change in the nuclease hypersensitivity at the position of *oriC* (Figure 3B, arrows). In contrast, insertion of the AT105 sequence in either orientation restores nuclease hypersensitivity in the vicinity of *oriC* (Figure 3B).

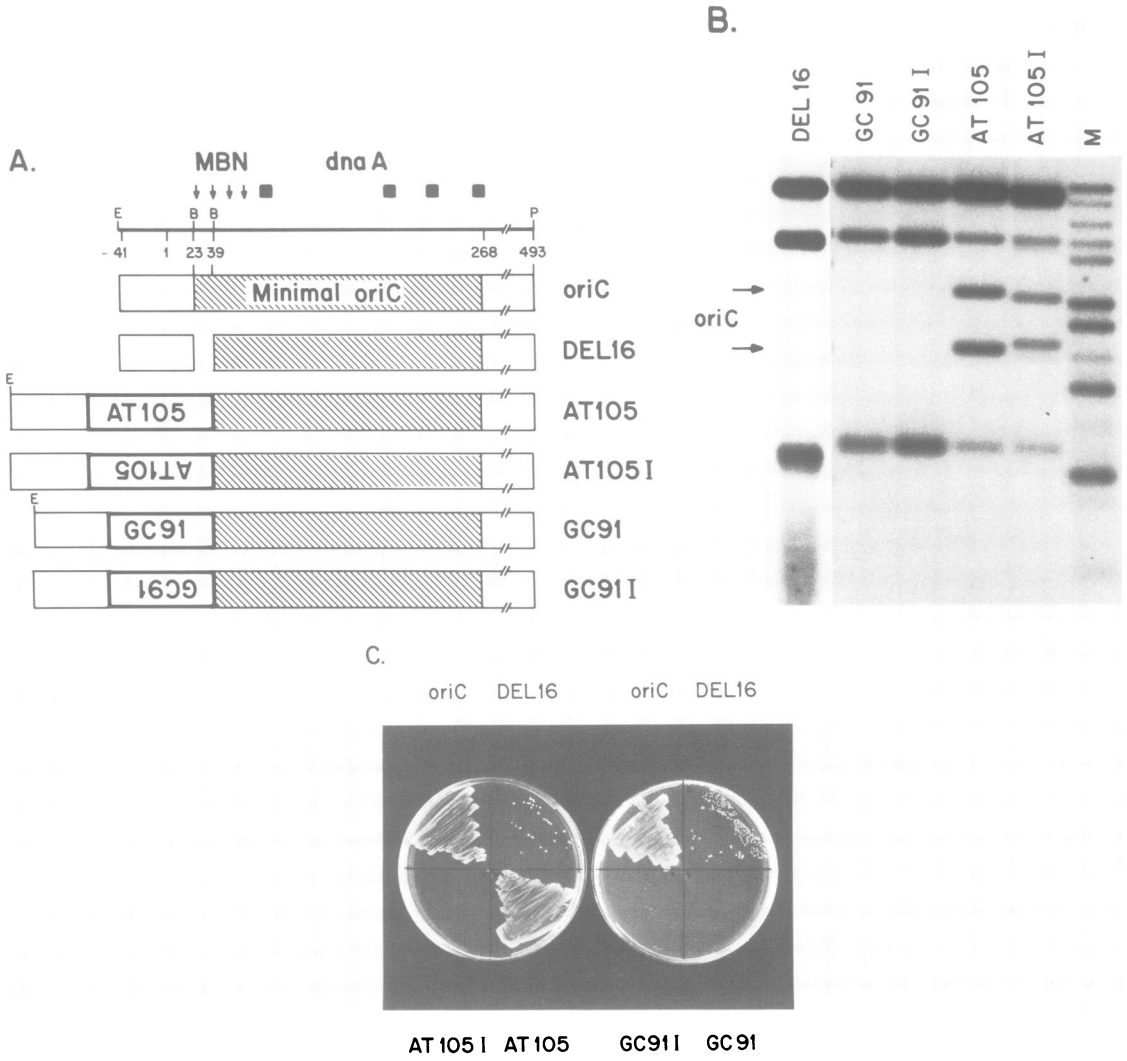


**Fig. 3.** Nuclease hypersensitivity and origin function of mutant *oriC* plasmids. (**A**) The top line is a map of the *oriC* insert showing the locations of relevant restriction sites (E, *Eco*RI; B, *Bgl*II; P, *Pst*I) and their nucleotide positions (Sugimoto *et al.*, 1979; Meijer *et al.*, 1979). Above the *oriC* map, the mung bean nuclease (MBN)-hypersensitive region (↓↓↓↓) and the dnaA protein-binding sites (■) are indicated. Below the *oriC* map are schematic diagrams of the inserts in the wild-type and mutant *oriC* plasmids studied. The cross-hatched region denotes the minimal region essential for origin function *in vivo* (Oka *et al.*, 1980). See Results and Materials and methods for a full description of the mutant derivatives. (**B**) Mapping the mung bean nuclease-hypersensitive sites in mutant *oriC* plasmids. Supercoiled plasmids containing the mutant *oriC* inserts diagrammed in panel A were reacted with limiting mung bean nuclease to produce singly nicked circular DNA. The DNA was subsequently linearized and 5′-$^{32}$P-end-labeled at the *Nde*I site. The labeled DNA was irreversibly denatured with glyoxal, electrophoresed in a 1.4% agarose gel and autoradiographed. The arrows indicate the location of the band pair that assigns a nuclease-hypersensitive site to the *oriC* region of the AT105 and AT105I derivatives (see Figure 1B, lane *Nde*I for comparison to the wild-type *oriC*). The other band pair which is common to all the plasmids maps to the Trm site in the vector. **Lane M** contains the single-stranded DNA size markers described in Figure 1(B). (**C**) Origin function of wild-type and mutant *oriC* plasmids in polA$^-$ *E.coli* cells. The polA$^-$ *E.coli* strain p3478 (DeLucia and Cairns, 1969) was transformed with the plasmids listed in panel A carrying the tetracycline-resistance gene. A portion of the transformed cell suspension was added to Luria broth containing tetracycline and incubated at 37°C for 18 h. The only turbid cultures which resulted were from cells transformed with the *oriC* and the AT105 plasmids. The figure shows the result of streaking a sample of each of the transformed cell cultures on tetracycline plates and then incubating the plates for 24 h at 37°C. The few colonies that arise after transformation with pDEL16 are spurious since, in contrast to colonies containing p*oriC* and pAT105, they do not grow when inoculated in Luria broth containing tetracycline and incubated at 37°C for 18 h.

The *oriC* plasmid as well as the deletion and insertion derivatives were assayed for *oriC*-dependent initiation of replication *in vivo* (Sugimoto *et al.*, 1979; Oka *et al.*, 1980). Plasmids containing a functional copy of the chromosomal origin replicate in the polA$^-$ *E.coli* strain p3478 (DeLucia and Cairns, 1969) and confer vector-encoded antibiotic resistance. In contrast, plasmids without a functional *oriC* do not replicate in this strain and do not confer antibiotic resistance. The failure to replicate the antibiotic resistance marker under selection stops cell proliferation. Figure 3(C) confirms that the wild-type *oriC* plasmid replicates in this strain and that deletion of a single 13mer (DEL16) inactivates replication. Insertion of the GC91, GC91I or AT105I sequences into DEL16 fails to restore replication function. In contrast, insertion of the AT105 sequences, in the opposite orientation of the AT105I sequence (Figure 3A), restores *oriC*-dependent replication (Figure 3C).

The transformation efficiencies for the *oriC* and AT105 plasmids are similar to each other ($5 \times 10^3$ and $3 \times 10^3$ respectively) but are $>1000$ times greater than those for DEL16 and the other plasmids tested. The reduced transformation efficiencies of the DEL16, GC91, GC91I and AT105I plasmids relative to the *oriC* and AT105 plasmids do not result from a defect in expression of antibiotic

**Table I.** DNA sequences at the L 13mer position in wild-type and mutant *oriC* derivatives

| *oriC* derivative | DNA sequence[a] | Consensus match[b] |
|---|---|---|
| *oriC* | G A T C T a T T t a T T T | 10 |
| DEL16 | t g g g T a T T a a a a a | 3 |
| AT105 | a t T t T t a a t T T a a | 4 |
| AT105I | a g g C a c c T a T c T c | 4 |
| GC91 | t g c g T c c g g c g T a | 2 |
| GC91I | c A T C a c c g a T g g g | 4 |
| Wild-type 13mer consensus[c] | G A T C T n T T n T T T T | |

[a]An upper-case letter indicates a match to the wild-type 13mer consensus sequence at one of the 11 specific positions.
[b]Number of matches to the 11 specific positions of the consensus.
[c]From Bramhill and Kornberg (1988).

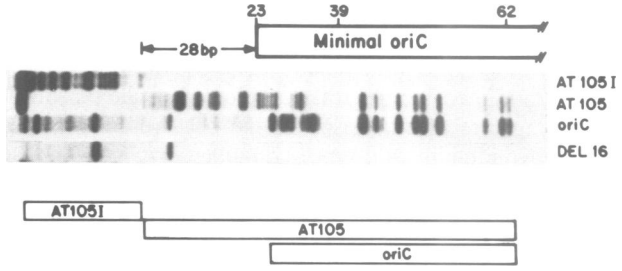


**Fig. 4.** Precise location of nuclease-hypersensitive sites in *oriC* mutants. The supercoiled plasmids listed in the figure were probed with mung bean nuclease and a singly $^{32}$P-end-labeled DNA fragment containing the hypersensitive region was isolated from each of the plasmids as described in Figure 2(A) for p*oriC* except that *Eco*RI was used in place of *Fok*I. The labeled DNA fragment from each plasmid was denatured, electrophoresed in a 15% 'wedge' polyacrylamide −8 M urea gel and autoradiographed. The direction of electrophoresis, as displayed in the autoradiogram shown, was from left to right. Above the autoradiogram is a map of the left end of the minimal *oriC* showing pertinent nucleotide positions in the lower strand sequence examined. The L, M and R 13mers in *oriC* begin at map positions 23, 39 and 54 respectively. The M and R 13mers are present in all the *oriC* derivatives listed. DEL16 lacks map positions 23−38 which include the L 13mer. The AT105 and AT105I inserts were joined to the residual *oriC* sequence in DEL16 at map position 39. Below the autoradiogram are boxes which delimit the sequence cleaved by the nuclease. Not included in these boxes are artefactual bands that arise from DNA renaturation and secondary structure formation (Sheflin and Kowalski, 1985). Such artefactual bands are observed in DNA not treated with nuclease (not shown) and are clearly seen in DEL16, which is not susceptible to nuclease cleavage in *oriC* (Figure 3B).

resistance since all of these plasmids transformed the isogenic polA$^+$ *E.coli* strain W3110 *thy*$^-$ at similar high efficiencies under tetracycline selection ($10^4$ transformants/$\mu$g DNA). The origin mutation in DEL16 is the same one known to result in a defect in *oriC*-dependent replication *in vivo* (Oka *et al.*, 1980) and *in vitro* (Bramhill and Kornberg, 1988). The results support the hypothesis that the AT105 insert rescues *oriC*-dependent replication and not some unrelated defect in DEL16.

In the AT105 insert, the DNA sequence that replaces the L 13mer repeat in *oriC* is a poor match to the consensus sequence (Table I). We conclude that a specific DNA sequence in the position of the L 13mer repeat is not essential for origin function *in vivo*. Instead, the ability to reactivate origin function with a dissimilar sequence that restores nuclease hypersensitivity indicates a higher-order DNA structural role for the L 13mer.

### *Replication competence of the insertion mutants is associated with nuclease hypersensitivity within, but not just outside, the repeated 13mer region*

Unlike the AT105 insert, the inverted insert, AT105I, cannot function in place of a 13mer (Figure 3C). Both inserts are nuclease hypersensitive in *oriC* (Figure 3B). However, the location of the *oriC* hypersensitive site differs between the two plasmids as indicated by the slightly different mobilities of the band pairs which map to *oriC* (Figure 3B). This indicates that the precise position of the nuclease hypersensitive site is likely to be an important correlate of origin function.

We determined the precise positions of the origin-hypersensitive sites in pAT105 and pAT105I by single-nucleotide-resolution mapping of the nuclease nicks. The results are shown in Figure 4 in relation to a map of the left end of minimal *oriC*. For clarity of presentation, the regions containing bands resulting from nuclease cleavage are delimited by a box for each plasmid (below the autoradiogram; see Figure 4 legend). Cleavages in the replication-competent pAT105 map primarily within the minimal *oriC* and, additionally, just outside the left boundary (position 23). In contrast, the replication-deficient pAT105I is not cleaved within the minimal *oriC*. Instead, the cleavages in pAT105I map outside minimal *oriC*, starting at 28 bp and leftward from the minimal *oriC* boundary. These findings demonstrate that a nuclease-hypersensitive site within, but not nearby and outside, the minimal *oriC* sequence of the insertion mutant is associated with origin function.

The nuclease cleavage pattern of the replication-competent AT105 mutant shows a remarkable resemblance to that of the wild-type *oriC* (Figure 4). The nuclease cleavages to the right of position 39 are identical between the two plasmids. The cleavages from positions 39 to 62 (Figure 4) map to the middle (M) 13mer repeat (positions 39−51) and right (R) 13mer repeat (positions 54−66). Thus, just as for the wild-type *oriC*, replication competence of the mutant origin in pAT105 is associated with detection of a nuclease-hypersensitive site that includes the 13mer repeat region in the minimal *oriC*.

The same two 13mer repeats present in pAT105 are present in pDEL16 but are not cleaved (Figure 4). Apparently, the sequence of the AT105 insert that replaces the L 13mer and is fused with *oriC* position 39 in pDEL16 co-operates with the M and R 13mers to form the nuclease-hypersensitive site. The restoration of nuclease hypersensitivity in the M and R 13mers in the replication-competent insertion mutant is consistent with a higher-order structural role for the M and R 13mers, in addition to the L 13mer (see above), in origin function.

### *Helical instability of the 13mer repeat region accounts for nuclease hypersensitivity and is linked to origin function*

The susceptibility of *oriC* to single-strand-specific nucleases in negatively supercoiled DNA presumably reflects localized DNA unwinding. Nuclease susceptibility alone, however, cannot distinguish whether the localized DNA unwinding is thermodynamically stable (predominates over normal helical DNA at equilibrium) or is a kinetic intermediate (a transient departure from stable helical DNA). Deciding between these mutually exclusive alternatives has important

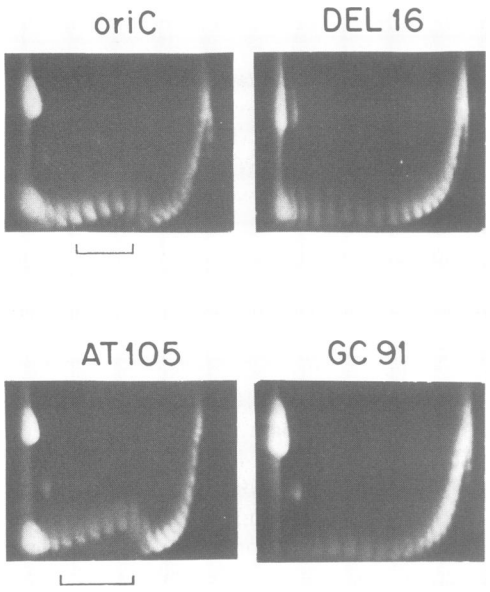


**Fig. 5.** Two-dimensional gel electrophoresis of plasmid topoisomers containing *oriC* and mutant derivatives. A spectrum of topoisomers, prepared by mixing artificially supercoiled plasmids which differed in linking number (Keller, 1975), were electrophoresed in a 1.0% agarose gel. The first dimension (top to bottom) was performed at 37°C in nuclease digestion buffer. The second dimension (left to right) was performed at 23°C in a buffered solution containing 60 μg/ml chloroquine. Stable DNA unwinding in the *oriC* and AT105 plasmid topoisomers is indicated by the retarded mobility of the bracketed topoisomers, relative to those immediately to the right of the bracket, in the first-dimension electrophoresis. The intense spot at the upper left of each panel is nicked-circular DNA. The line from the nicked-circular DNA spot to the bottom of each panel results from spontaneous nicking of topoisomers during first-dimension electrophresis at 37°C. The isolated spot just to the right of the line is linear DNA.

consequences for understanding the basis and biological implications of the nuclease hypersensitivity of *oriC*. To distinguish whether *oriC* DNA is, or is not, stably unwound in our nuclease assay condition, we performed 2D gel electrophoresis of plasmid topoisomers (Gellert *et al.*, 1983; Wang *et al.*, 1983) containing the *oriC* or mutant derivatives. To ensure that stable DNA unwinding in *oriC* would not be confused with that in the Trm hypersensitive site (Figure 1A; Kowalski *et al.*, 1988), we deleted the Trm site from the vector.

Plasmid topoisomers prepared *in vitro* were electrophoresed in an agarose gel under nuclease digestion conditions in the first dimension (Kowalski *et al.*, 1988) and in the presence of chloroquine in the second dimension, perpendicular to the first (Gellert *et al.*, 1983; Wang *et al.*, 1983). In the resolving region of a 2D gel, the more supercoiled topoisomers migrate faster during electrophoresis. In the first dimension, a topoisomer containing a thermodynamically stable unwound region does not migrate as fast as a topoisomer with the same linking number that is not stably unwound. For each helical turn stably unwound, one superhelical turn is lost and the mobility is correspondingly reduced. In the second dimension, the chloroquine reverses any stably unwound conformation, permitting resolution of topoisomers based on linking number differences alone.

Figure 5 shows the 2D gel analyses of four different origin-containing plasmids. The topoisomer linking number difference relative to the relaxed topoisomer increases starting from the fluorescent stain at the upper right of each panel and proceeding through the curved pattern of topoisomer spots. The *oriC* panel shows a group of topoisomers (bracketed region) whose mobility is retarded in the first dimension (top to bottom) relative to that of topoisomers of lower linking number difference immediately to the right of the bracket. The mobility retardation of topoisomers with sufficient linking number difference demonstrates that the *oriC* plasmid exhibits stable DNA unwinding in the first-dimension electrophoresis conditions. Second-dimension electrophoresis at a lower chloroquine concentration (not shown) reveals that the transition to stable unwinding occurs at superhelical density −0.049, well below that of plasmids extracted from *E.coli* by our procedure (−0.067).

We determined the effects of *oriC* mutations on stable DNA unwinding by performing 2D gel electrophoresis of plasmid topoisomers containing mutant *oriC* inserts. The same first-dimension gel was used for all of the plasmids, permitting direct comparison of the occurrence of stable DNA unwinding in wild-type and mutant *oriC* inserts. The DEL16 and GC91 mutants do not show relative mobility retardations among the most negatively supercoiled topoisomers resolved in the gel (Figure 5; topoisomers along the bottom of both panels). Thus, in contrast to the wild type *oriC* plasmid, stable DNA unwinding is not detectable at comparable levels of supercoiling in the replication-defective DEL16 and GC91 mutants. The fact that these mutations in the 13mer repeat region suppress the stable DNA unwinding detected in the wild-type *oriC* plasmid localizes the unwinding to the 13mer region of *oriC*.

The replication-competent AT105 plasmid, like the *oriC* plasmid, shows a group of topoisomers (bracketed region) retarded in mobility relative to that of topoisomers of lower linking number difference immediately to the right of the bracket (Figure 5). The results demonstrate that insertion of the AT105 sequence into DEL16 restores the stable DNA unwinding detected in the wild-type *oriC*.

The use of nuclease digestion conditions for the first-dimension electrophoresis of the 2D gels permits us to directly relate nuclease hypersensitivity to stable DNA unwinding. The data in Figure 5 show that stable DNA unwinding occurs in *oriC* and in the AT105 insertion mutant under the specific conditions used for nuclease digestion. The occurrence of stable DNA unwinding in the four plasmids examined (Figure 5) correlates perfectly with the detection of nuclease hypersensitivity (Figures 3B and 1B). Finally, both stable DNA unwinding and nuclease hypersensitivity are independently localized to the *oriC* 13mer region. The detection of stable DNA unwinding in *oriC* under the specific nuclease digestion conditions excludes transient unwinding ('breathing') as the basis for the nuclease hypersensitivity, as previously demonstrated for the pBR322-hypersensitive site (Kowalski *et al.*, 1988).

We conclude that thermodynamically stable DNA unwinding is the basis for the nuclease hypersensitivity of the 13mer repeat region. Detection of stable DNA unwinding indicates that the 13mer repeat region has a reduced helical stability, reflecting a low free energy cost for unwinding relative to other regions in *oriC* and in the plasmid in general. The helical instability of the 13mer repeat region in wild-

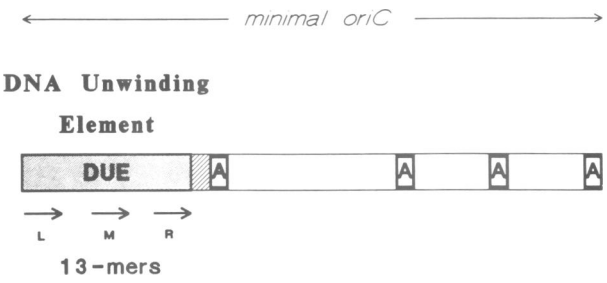


**Fig. 6.** Organization of genetic elements in the minimal *oriC*. The dnaA protein recognition elements are shown as boxed A's and the known spacer elements are shown as open boxes (Asada *et al.*, 1982; Oka *et al.*, 1984). The DNA unwinding element (DUE) identified here co-localizes with the three 13mer repeats (Bramhill and Kornberg, 1988). The cross-hatched box indicates a spacing element identified by phylogenetic comparisons of bacterial origin sequences (see text).

type and mutant *oriC* derivatives (Figure 5) is linked to origin function (Figure 3C).

## Discussion

### *The DNA unwinding element: a cis-acting component that facilitates localized origin unwinding*

Our investigation into the DNA structural properties of *oriC* present on a negatively supercoiled plasmid reveals that the repeated 13mer region has a low free energy cost for DNA unwinding, i.e. reduced helical stability. This is demonstrated by detection of thermodynamically stable origin unwinding in sufficiently supercoiled topoisomers after 2D gel electrophoresis (Figure 5, *oriC*). The helical instability of all three 13mers accounts for the single-strand-specific nuclease hypersensitivity of *oriC* in a negatively supercoiled plasmid (Figures 4 and 1B).

The replication-defective phenotype resulting from deletion of the L 13mer (DEL 16) is associated with an increase in the free energy cost for origin unwinding, i.e. increased helical stability. Both stable DNA unwinding (Figure 5) and nuclease hypersensitivity in the residual origin (Figure 3B) are undetectable. These findings suggest that the helical instability of the wild-type 13mer region is important for origin function.

The replication defect in DEL16 can be corrected by replacing the missing L 13mer with a dissimilar sequence (Table I, AT105) that restores nuclease hypersensitivity (Figure 3B) and stable DNA unwinding (Figure 5) in the origin. This finding strongly supports the importance of helical instability in *oriC* for origin function. The segment of the AT105 sequence that functionally replaces the L 13mer is part of a larger sequence that exhibits helical instability in supercoiled pBR322 DNA (Kowalski *et al.*, 1988). We conclude that the helical instability of the L 13mer, not the specific 13mer DNA sequence, is essential for origin function.

Given the repeated nature of the 13mer sequence and the nuclease hypersensitivity of all three 13mers in *oriC*, it is plausible that the helical instability of the hypersensitive region containing all three 13mers, not just the L 13mer, is important for origin function. Consistent with this interpretation, reactivation of origin function after insertion of the AT105 sequence in place of the L 13mer is accompanied by the precise restoration of the M and R 13mer cleavage pattern seen in the wild-type *oriC* (Figure 4; compare *oriC* and AT105 band patterns to the right of map position 39). Conversely, origin function is not detectable when the AT105 sequence is inserted in the inverted orientation (AT105I) and no nuclease cleavages are seen in the M and R 13mers.

We propose that the helical instability of the repeated 13mer region contributes to localized origin unwinding during initiation of DNA replication in living cells. The unwinding is initiated by dnaA protein recognition (Bramhill and Kornberg, 1988; see below). A genetic contribution of helical instability to *oriC* function is novel since previously described *cis*-acting elements function in either protein recognition or spacing (Asada *et al.*, 1982; Oka *et al.*, 1984). We call the *cis*-acting sequence whose helical instability is required for origin function the DNA unwinding element (DUE).

While the *oriC* DUE occurs in an AT-rich sequence, it is important to note that AT content alone cannot be used to identify the essential origin sequence with reduced helical stability. An earlier analysis of the DNA sequence of *E.coli oriC* specified several extended AT-rich regions (Meijer *et al.*, 1979), only one of which corresponds to the DUE identified here. Also, the sequence of DEL16 which substitutes for the deleted 13mer is AT-rich (Table I) but unwinds relatively poorly (Figure 5). Thus, the energetic requirement for unwinding the DUE in supercoiled DNA is governed by base sequence, not simply base composition. However, the DNA sequence requirements for at least the L 13mer portion of the DUE are not strict, as is evident from the capacity of the AT105 sequence (Table I) to work in place of the L 13mer.

### *The oriC DUE mediates supercoil-induced unwinding initiated by dnaA protein*

Remarkably, the DUE, localized here in negatively supercoiled DNA in the absence of replication proteins, corresponds precisely to the tandemly repeated, 13mer sequence that is induced to form an open duplex by the dnaA protein *in vitro* (Bramhill and Kornberg, 1988; Figure 6). To account for the site specificity of the duplex opening and the apparent requirement for the 13mer sequence, Bramhill and Kornberg (1988) proposed that the dnaA protein recognizes the 13mer sequences, in addition to the 9mer sequences on which it footprints (Fuller *et al.*, 1984; Matsui *et al.*, 1985). Our finding that a dissimilar DNA sequence (Table I, AT105) can functionally substitute for the L 13mer (Figure 3C, AT105) provides evidence that specific protein recognition of the L 13mer is not necessary for origin function.

Our discovery of the DUE suggests a plausible mechanism to account for specific duplex opening in *oriC* without requiring specific protein recognition of all three 13mers: limited DNA unwinding initiated by dnaA recognition of a single 13mer (or part of one) could nucleate supercoil-induced unwinding to the remaining, non-contacted 13mer region by virtue of the intrinsic helical instability of the region. This mechanism is consistent with the findings of the Kornberg lab that (i) the R 13mer, but not the L and M 13mers, is required for dnaA/HU protein-induced opening of *oriC* (Bramhill and Kornberg, 1988), and (ii) formation of the open complex requires negative supercoiling of the DNA (Baker and Kornberg, 1988). The proposed mechanism is also consistent with our findings that (i) the

helical instability of the L 13mer, not the specific DNA sequence, is essential for origin function, and (ii) the L, M and R 13mers exhibit helical instability in negatively supercoiled DNA.

Can similar sequences, such as the L and R 13mers, play distinct mechanistic roles? The R 13mer is clearly distinct from the L and M 13mers in its evolutionary conservation. Inspection of the DNA sequence data of Zyskind *et al.* (1983) reveals that the L and M 13mers have tolerated certain base substitutions and small deletions/insertions during evolution of six dnaA-dependent bacterial origins compared. Thus, strict sequence conservation of the L and M 13mers is not necessary for their biological function. In contrast, the R 13mer is identical in all six origins, apart from a single point mutation in the distantly related marine bacterium *Vibrio harveyi*. The identity extends immediately rightward from the R 13mer into a 5mer sequence, identical to the first five residues in the 13mer. The strict sequence conservation suggests a role for the R 13mer as well as the 5mer in specific protein recognition. We note that the spacing between the R 13mer + 5mer and the leftmost dnaA protein binding site is absolutely conserved (Figure 6, cross-hatched box), although the sequence varies in all six origins examined (Zyskind *et al.*, 1983). The conserved spacing suggests an interaction between proteins that recognize the R 13mer + 5mer and the dnaA boxes, compatible with recognition by the same or different subunits of the multimeric dnaA protein complex (Bramhill and Kornberg, 1988). Specific dnaA protein recognition of the R 13mer might account for the finding that dnaA protein interacts in some way with a repeated 13mer oligonucleotide (Yung and Kornberg, 1989).

Our experimental findings and our inferences derived from phylogenetic comparisons, combined with the findings of the Kornberg lab, indicate that the 13mer region is a complex genetic element that plays at least two essential and overlapping roles: (i) protein recognition and (ii) helical instability. Specific protein recognition, essential to initiate unwinding in the R 13mer region, may not be essential to propagate unwinding through the M 13mer, and is dispensable to complete origin unwinding in the L 13mer region, as long as the sequence that replaces the L 13mer has reduced helical stability. Helical instability of all three 13mers that comprise the DUE facilitates the dnaA-dependent unwinding of the origin.

Our discovery of a direct link between DNA supercoiling and *oriC* unwinding suggests the supercoiling could modulate replication initiation via the DUE. This hypothesis is consistent with the observation that genetic inactivation of topoisomerase I can suppress a *dnaA* temperature-sensitive defect in initiation of chromosome replication (Louarn *et al.*, 1984): the resulting increase in negative supercoiling might assist the defective initiator protein in unwinding the DUE. This hypothesis could also help account for the *in vivo* mechanism by which transcription activates replication initiation (Lark, 1972; Baker and Kornberg, 1988 and references therein) since (i) the strongest promoters in the *E.coli* genome, as well as most promoters near *oriC*, direct transcription away from *oriC* (Brewer, 1988), and (ii) RNA polymerase movement away from *oriC* would lead to negative supercoiling in the replication origin domain (Liu and Wang, 1987; Wu *et al.*, 1988; Pruss and Drlica, 1989).

### *The DUE is the entry site for replication machinery into the DNA helix*

The DNA sequence requirement for nuclease hypersensitivity of the DUE is the same as that for the open duplex detected in the stable, pre-priming complex (Bramhill and Kornberg, 1988), which includes dnaA and the dnaB helicase (van der Ende *et al.*, 1985; Funnell *et al.*, 1987). In both cases, the replication-defective *oriC* mutant lacking the L 13mer shows no stable duplex opening in the origin. Bramhill and Kornberg (1988) proposed that the opening of all three 13mers in the prepriming complex provides an entry site for the dnaB helicase, which itself is incapable of initiating unwinding within duplex DNA (LeBowitz and McMacken, 1986). Our discovery of the functional importance of DNA helical instability in the origin region containing the three 13mers provides independent support for this proposal.

We envision that the requirement for a DUE may be general for a variety of replication origins since unwinding the DNA helix is essential for the replication machinery to access the individual DNA strands. Other origins (e.g. SV40, phage λ) at which initiator protein binding leads to localized DNA unwinding (Borowiec and Hurwitz, 1988; Schnos *et al.*, 1988) may also require a DUE. Our nuclease hypersensitivity assay using naked, supercoiled DNA correctly identifies the origin sequence induced to unwind by the *E.coli* initiator protein. This discovery lends support to our earlier hypothesis that in yeast replication origins, for which the initiator protein has yet to be isolated, the nuclease-hypersensitive site in supercoiled DNA localizes the critical DNA sequence required for the initial unwinding event (Umek and Kowalski, 1987, 1988).

## Materials and methods

### *Enzymes*

Mung bean nuclease was isolated and purified to homogeneity as described by Kowalski *et al.* (1976). Enzymes from commercial suppliers were as follows: restriction enzymes, T4 DNA ligase and *E.coli* DNA polymerase I, Klenow fragment (New England Biolabs), T4 polynucleotide kinase (United States Biochemicals), bacterial alkaline phosphatase (Worthington Biochemicals), calf thymus DNA topoisomerase I (Bethesda Research Labs).

### *DNA purification and topology*

Plasmids were grown in *E.coli* HB101 cells in Luria–Bertani medium and amplified using 150 μg/ml chloramphenicol. DNA was obtained from cells lysed by boiling in the presence of lysozyme (Holmes and Quigley, 1981). DNA was purified by two rounds of equilibrium centrifugation in cesium chloride density gradients containing ethidium bromide (Radloff *et al.*, 1967). The superhelical densities (Wang *et al.*, 1983) of different plasmids prepared by this procedure were −0.067 as determined by 2D gel electrophoresis (see below) using the single-strand-specific nuclease reaction conditions at 37°C to define the relaxed state. The location of mung bean nuclease-hypersensitive sites was consistent among various DNA preparations of the same plasmid.

### *Construction of wild-type and mutant oriC plasmids*

The wild-type *oriC* plasmid includes the *Pst*I–*Hae*III fragment of *oriC* (Sugimoto *et al.*, 1979; Meijer *et al.*, 1979) inserted between the *Pst*I site and the filled-in *Eco*RI site of pBR322. The DEL16 plasmid is a 16 bp deletion in p*oriC* resulting from cleavage of p*oriC* at the two *Bgl*II sites and recircularizing by ligation at low DNA concentration. The plasmids were a gift from Ben Munson, Roswell Park Memorial Institute.

The plasmid DEL16 was used as the vector to construct the insertion mutants. The DEL16 plasmid was first cleaved at the *Bgl*II site (A↓GATCT) and dephosphorylated using alkaline phosphatase. The inserts, AT105 (positions 3225–3329 of pBR322; Sutcliff, 1978) and GC91 (pBR322 positions 376–466), were isolated after PAGE (Sheflin and Kowalski, 1984)

of a *Sau*3AI (↓GATC) digest of pBR322. The isolated inserts were fused to the vector using T4 ligase (Maniatis *et al.*, 1982). The ligated DNA was used to transform *E.coli* JW355 using the $CaCl_2-RbCL_2$ procedure (Kushner, 1978). Monomeric plasmids with the appropriate size inserts were isolated from selected tetracycline-resistant transformants. The insert orientations were determined by restriction enzyme mapping. Selected plasmids were subsequently propagated in *E.coli* HB101.

Origin inserts (*oriC*, DEL16, AT105 and GC91) were also cloned in a high-copy pBR322 derivative in which the Trm-hypersensitive site (Figure 1A) was deleted. Deletion of the Trm site was previously described (Kowalski *et al.*, 1988; pDEL-AT1). The high-copy plasmid was prepared by deleting the small *Bsp*MII−*Tth*111-I fragment of pBR322 (positions 1664−2219) which includes the repressor of primer (*rop*) gene (Cesareni *et al.*, 1982). The large *Bsp*MII−*Tth*111-I fragment, isolated after agarose gel electrophoresis, was blunt-ended using Klenow DNA polymerase I and circularized using T4 DNA ligase. The resulting plasmid is pBT3A (3655 bp). The small *Pst*I−*Eco*RI fragment from individual plasmids containing *oriC* and mutant derivatives was isolated by PAGE, combined with a *Pst*I + *Eco*RI digest of pBT3A (alkaline phosphatase treated), and ligated. The resulting plasmids were transformed into *E.coli* HB101 and grown on tetracycline plates. Replica plating using ampicillin selection revealed the tetracycline-resistant colonies containing plasmids with DNA inserts at the *Pst*I site and *Eco*RI site of pBT3A. Selected colonies were grown in culture and the isolated plasmids screened for DNA inserts of the appropriate size. The *oriC* phenotypes of origin inserts in the pBT3A background are the same as those in the pBR322 vector.

### *Plasmid-level mapping of single-strand-specific nuclease-hypersensitive sites*

To identify the hypersensitive sites in a plasmid, we limited the mung bean nuclease activity such that supercoiled DNA was converted to only nick-circular DNA (and not linear DNA). These conditions permit identification of the first nick introduced into the supercoiled DNA molecule. The first nick relaxes the DNA, rendering it resistant to further nuclease attack (Kowalski and Sanford, 1982). Nuclease reactions were carried out in 10 mM Tris−HCl (pH 7.0), 1 mM $Na_2EDTA$ at 37°C. Supercoiled plasmid DNA (1.6 μg) in 18 μl solution was pre-incubated at 37°C for 15 min. Mung bean nuclease (0.05 units contained in 2 μl; enzyme units as defined in Kowalski *et al.* 1976) was added to the DNA solution and the reaction mixture was kept at 37°C for 10−30 min. The nuclease reaction was quenched as previously described (Kowalski, 1984) and the enzyme was removed by phenol extraction. The nicked-circular DNA produced was cleaved and $^{32}P$-end-labeled at a unique restriction enzyme site (Sheflin and Kowalski, 1984). The labeled DNA was irreversibly denatured in glyoxal, electrophoresed in an agarose gel (22 cm, 30 V, 18 h) and autoradiographed as described elsewhere (Umek and Kowalski, 1988). Radiolabeled restriction fragments of known length were also prepared and denatured to serve as size markers and used for graphical estimation of the number of base pairs in DNA fragments resulting from mung bean nuclease nicks.

### *Nucleotide-level mapping of single-strand-specific nuclease-hypersensitive sites*

Supercoiled plasmids were probed with mung bean nuclease as described above. After restriction enzyme digestion, DNA fragments were $^{32}P$-end-labeled at dephosphorylated 5′ ends, separated by PAGE and isolated as previously described (Sheflin and Kowalski, 1984). Singly end-labeled restriction fragments were generated by cleaving end-labeled DNA with a second restriction enzyme and were isolated. DNA fragments containing mung bean nuclease nicks were denatured and electrophoresed alongside the products of Maxam and Gilbert (1980) sequencing reactions performed on the same fragment without nuclease nicks. The nucleotide positions of the nicks were read from the sequencing gels as previously described (Sheflin and Kowalski, 1984).

### *Analysis of the oriC phenotype*

*Escherichia coli* strain p3478 [polA1 thyA deoC *lambda*$^-$ IN(*rrnD-rrnE*)1] (DeLucia and Cairns, 1969) was used to test for *oriC* function. *Escherichia coli* strain W3110 *thy*$^-$ (*polA*$^+$, otherwise isogenic with p3478), which permits replication from the pBR322 vector origin, was used as a control. Cells was transformed with the wild-type and mutant *OriC* plasmids using the $CaCl_2-RbCL_2$ procedure (Kushner, 1978). Plasmid-containing cells were selected using tetracycline (10 μg/ml). Plasmids were analyzed for transformation efficiency (number of transformants/μg DNA) as well as the ability to confer cell growth under selection both in culture and on agar plates.

### *Two-dimensional gel electrophoresis of DNA topoisomers containing oriC and mutant inserts*

DNA topoisomers were prepared by treating plasmids with calf thymus topoisomerase I in the presence of various concentrations of ethidium bromide (Keller, 1975). Electrophoresis was performed in 1.0% horizontal agarose gels submerged in recirculated (100 ml/min) solution. Topoisomers were separated in the first dimension (4.8 V/cm, 6.5 h) under the same solution conditions as the single-strand-specific nuclease reaction at 37°C. The gel box was kept in a temperature-regulated water bath to maintain 37°C during electrophoresis. A 1 cm wide strip containing the topoisomers from the first-dimension gel was fused to the top of the second-dimension gel, perpendicular to the direction of electrophoresis. The hybrid gel was soaked in a Tris−phosphate buffer containing chloroquine phosphate and electrophoresed at 2.4 V/cm for 16 h in the same solution at 23°C.

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