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Detection of DNA looping due to simultaneous interaction of a DNA-binding protein with two spatially separated binding sites on DNA

(DNA knotting/action at a distance/catenation enhancement/enhancer of replication/plasmid R6K)

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ABSTRACT **We describe different and relatively rapid biochemical techniques to detect protein-mediated DNA looping. These techniques, based on enhancement of DNA knotting and that of ligase-catalyzed cyclization, were used to show that the replication initiator protein of plasmid R6K can bring together two intramolecular γ origin of replication sequences located as far apart as 2 kilobases. The site–site interaction causes looping out of the intervening DNA sequence as visualized by electron microscopy. Because the autoregulatory sequence of the initiator cistron also binds initiator protein, we investigated whether the γ origin-bound protein can participate in autoregulation by interaction of the two sites through a protein bridge. We discovered that the two sites do not interact *in vitro* at their natural locations when on opposite faces of the double helix. Moving the two sites to the same face of the double helix by introducing a half turn into the intervening sequence allows protein-mediated site–site interaction to occur.**

Interaction of a DNA-binding protein simultaneously with two noncontiguous binding sites on the DNA would cause bridging of the two DNA binding sites by the protein dimer or tetramer and looping out of the intervening DNA sequence flanked by the two binding sites. The process of DNA looping is of importance in molecular biology because of its presumed role in the interaction between eukaryotic enhancer elements and promoters (1, 2) and the demonstrated role of DNA loops in controlling prokaryotic transcription (3, 4) and DNA replication (5).

Previously, DNA looping was detected by DNase protection techniques (6) and by electron microscopy (7). Although effective, these procedures are too time-consuming to be amenable as a useful way to detect proteins that promote DNA looping. Development of a rapid assay procedure to detect DNA-looping proteins would help unravel the regulatory mechanism of eukaryotic and prokaryotic genes.

In this paper, we introduce two different and rapid biochemical techniques, with which we demonstrate DNA looping caused by interaction of the replication initiator protein of plasmid R6K with two copies of the γ origin of replication or a single copy of γ origin and a copy of the autoregulatory site of the initiator protein cistron (8, 9) when the two sites are separated by as much as 2000 base pairs (bp) of DNA.

We also studied the interaction between the γ origin and the autoregulatory site (operator) of the initiator cistron in their natural locations and discovered that the sites do not interact with each other because they are positioned on different faces of the DNA. However, introduction of a *Bam*HI site of 6 bp (one-half turn of the helix) brought the two sites onto the same DNA face and allowed interaction of the two sites in the presence of the initiator protein. The natural relative location of the operator with respect to the γ origin thus explains why the operator sequence apparently does not interfere with the interaction between the γ-origin enhancer and β-origin sequences even though the operator is located between the γ-origin and β-origin sequences and it has a much stronger affinity for the initiator protein than the β-origin sequence (5, 10, 11).

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MATERIALS AND METHODS

Plasmids and Bacterial Host Strains. 0γ refers to pBR322 DNA that does not have any binding sites for initiator protein. 1γ and 2γ substrates refer to pBR322 recombinants containing a single copy of the 277-bp γ-origin sequence inserted at the *Pvu* II site or two copies of the same sequence inserted, one at the *Eco*RI site and the other at the *Pvu* II site, respectively. Additional substrates were constructed by putting *Eco*RI linkers onto a 0.8-kb DNA fragment containing the natural R6K sequence from γ origin of replication to the autoregulatory site (Fig. 1). In one fragment, named F, the *Eco*RI linkers were introduced ≈125 bp upstream of seven repeats of γ origin at one end and 365 bp downstream from the eighth repeat and two half repeats that constitute the autoregulatory site at the other end. We used site-directed mutagenesis (13) to introduce a *Bam*HI site of 6 bp (one-half turn of helix) immediately downstream from the seventh repeat in fragment F, thus generating the fragment F^R. The fragment F had ≈30 bp of vector DNA on either end of the R6K sequence and near *Eco*RI linkers. We also constructed a recombinant clone that had a single copy of γ-origin sequence at the *Pvu* II site and a 0.45-kb DNA fragment of R6K containing the autoregulatory sequences between the *Eco*RI–*Bam*HI sites of pBR322 vector DNA (called substrate γ-8R:1.6kb, because of the 1.6-kb distance separating the two sites).

Purification of the Initiator Protein. The procedure is published (14).

Catenation Enhancement. Supercoiled DNA containing a single γ origin site (pSM103) was treated with 40–50 units of T4 topoisomerase II with or without various amounts of initiator protein in 100 μl of T2 buffer (40 mM Tris/60 mM KCl/10 mM $MgCl_2$/0.5 mM EDTA/0.5 mM dithiothreitol/0.5 mM ATP, pH 7.4; ref. 15) for 30 min at 37°C. The reaction was stopped by adding EDTA to 25 mM. The reaction products containing catenanes and topoisomers were nicked

Abbreviations: 0γ, pBR322 DNA that lacks binding sites for initiator protein; 1γ and 2γ, pBR322 recombinants containing one or two copies, respectively, of the γ-origin binding site for initiator protein; F and F^R, specifically designed linear DNA fragments (see *Plasmids and Bacterial Host Strains*); γ-8R:1.6kb, recombinant clone with one γ-origin sequence and R6K autoregulatory sequence spaced 1.6 kb apart.

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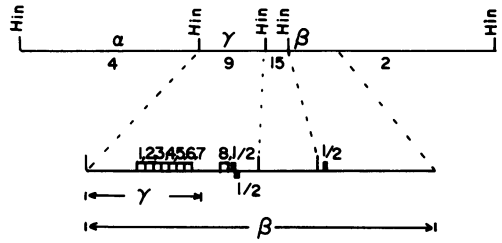

FIG. 1. Physical map of the replication control region of R6K. The *Hin*dIII (Hin) fragments 4, 9, 15, and 2 contain all cis- and trans-acting elements thus far known to control the replicon. α and β are frequently used origins, whereas γ is either an infrequently activated (12) or a silent origin (13). □, Seven 22-bp repeats that bind the initiator protein; the eighth repeat (□) and the two (one inverted) half (½) repeats (▮) at the autoregulatory site are shown. There is another half (½) repeat (■) at or near the β origin. Although the γ origin and β origin are physically separate, minimal β replicon activity requires the cis-located presence of fragment 9–15 at its 5′ region (5) (indicated at bottom of diagram).

with DNase I on the average of once per DNA molecule in the presence of ethidium bromide at 300 μg/ml. The DNA was phenol-extracted three times, precipitated, and dissolved in TEA (40 mM Tris/5 mM sodium acetate/1 mM EDTA, pH 7.6) buffer and electrophoresed in 1% agarose gels in the same buffer at 2 V/cm for 36 hr.

DNA Knotting. Knotted DNA was identified by its survival after nicking with DNase and by its mobility (one-half unit faster than the corresponding topoisomer) in a 1% agarose gel (16).

Cyclization Enhancement. Intramolecular reaction (DNA cyclization) was promoted over intermolecular ones by keeping the concentration of linear DNAs at <1 μg/ml. All DNA substrates (0γ, 1γ, 2γ, 1γ-8R:1.6kb), with the exception of the 0.8-kb linear DNAs carrying the origin of replication near one end and the autoregulatory site near the other (F and F^R), were linearized by digestion with *Ava* I. The other DNAs (F and F^R) were cut out from the recombinant clones with *Eco*RI and purified by gel electrophoresis and DEAE-cellulose column chromatography. A typical cyclization reaction mixture of 100 μl volume was 50 mM Tris, pH 7.4/10 mM $MgCl_2$/30 mM NaCl/gelatin at 100 μg/ml/20 mM dithiothreitol/1 mM ATP/0.1 μg of linear DNA and enough initiator protein to saturate the γ origin sites. After incubating the reaction mixture for 15 min at 20°C, the temperature was reduced to 18°C, and 1 unit of T4 ligase was added. Then after further incubation at 18°C for various time intervals, $NaDodSO_4$ was added to 0.5%, and the DNA was phenol-extracted, precipitated, dissolved in a convenient volume of TEA buffer, and analyzed by agarose gel electrophoresis. The gels were photographed; DNA bands were measured by densitometry (15).

Electron Microscopy. Naked DNA and DNA–protein complexes in a 30% (vol/vol) glycerol solution were spread on freshly cleaved mica, dried in a vacuum, and rotary-shadowed with platinum (5).

RESULTS

Detection of Intermolecular Site–Site Interaction by Catenation Enhancement. We previously reported that the initiator protein enhances the catenation of closed circular DNA catalyzed by T4 topoisomerase II provided that the closed circular DNA has a specific binding site for the initiator protein (15). We extended the investigation further by verifying an important prediction of the model shown in Fig. 2 *Left*—namely, that the principal product of the catenation reaction is a catenated dimer, as described below.

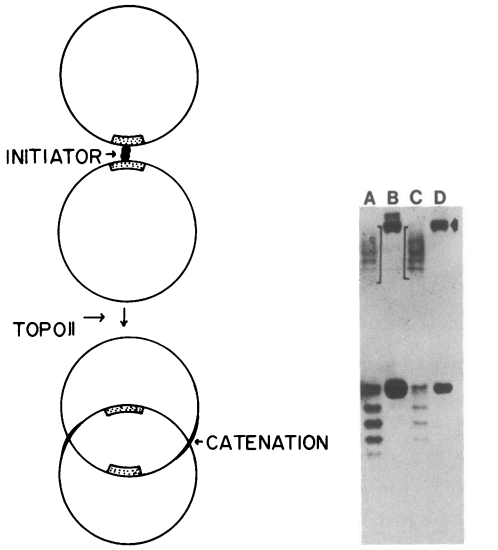

FIG. 2. Detection of intermolecular, protein-mediated site–site interaction of pSM103 DNA by catenation enhancement. (*Left*) Schematic showing the principle behind the assay (see ref. 15 for details). (*Right*) One percent agarose gel showing topoisomerase II-generated reaction products in saturating amounts (lane A) and two times the saturating amounts of initiator (lane C). The bracket shows catenanes; lanes B and D show the same reactions as lanes A and C but after DNase nicking, revealing mainly catenated dimers (arrow) as identified by the mobility (see ref. 17). Previous work (15) has shown that few catenanes are formed in DNA circles without a binding site with or without the initiator or in pSM103 DNA circles without the initiator protein. Bands at gel bottom are topoisomers of monomer circles that, upon nicking, get converted to nicked monomer circles.

A recombinant DNA (pSM103) consisting of a single copy of the γ-origin region, cloned into the *Eco*RI site of the vector pHW1 (minipBR322) was incubated in the binding buffer with topoisomerase II with enough initiator protein to fill the seven binding sites in γ origin (saturating amount; Fig. 2, lane A) and also twice the saturating amount of the protein (Fig. 2, lane C). The reaction products, upon analysis by 1% agarose gel electrophoresis, showed some topoisomers and catenanes (Fig. 2, lanes A and C). Very low levels of catenanes were produced under these conditions when the circles lacked a specific binding site for the initiator protein (data not shown; see ref. 15). Part of the reaction mixture was singly nicked with DNase I before electrophoresis and showed, principally, catenated dimers, identified by their electrophoretic mobility with respect to known markers (Fig. 2, lanes B and D) as previously determined by Sundin and Varshavsky (17). The apparent complexity of the catenanes shown here (Fig. 2, lanes A and C) and reported before (15), therefore, was from various degrees of superhelicity of component monomers of the dicatenanes and not from formation of catenanes larger than dimers. Thus, results strongly support the model of Fig. 2 *Left*.

Detection of DNA Looping by Enhancement of Knot Formation. The objective of this procedure was to determine whether the initiator protein could bring together two intramolecular γ-origin sequences located 2000 bp apart in closed circular DNA (Fig. 3*A*).

The rationale of the procedure is based on the observation that a DNA chain upon random circularization will form knots with a frequency directly proportional to the number of statistical segments (Kuhn's segments) present in that DNA (16). The longer the DNA chain length, the higher the probability of knot formation. An ≈5000-bp DNA circle, upon breakage and rejoining with catalytic amounts of topoisomerase II, in the presence of ATP, should produce

few knots (16). Detectable knot formation under these conditions would require equal weights of DNA and topoisomerase II and no ATP or the presence of DNA condensing agents such as PEG (16). However, if the initiator protein bound to both γ-origin sites in the DNA circle and brought together the two binding sites by protein–protein interaction and thereby reduced the effective statistical segment length, then catalytic amounts of topoisomerase II with ATP should generate DNA knots (Fig. 3*A*). The theory predicts that knots would be generated under these conditions only when the DNA circle has two binding sites and the initiator protein brings these two sites together.

DNA knots were visualized by their resistance to nicking by DNase I and their mobility in the agarose gel with respect to those of a topoisomerase II-generated ladder of the same DNA (16). The results clearly show that only the circles with two sites (2γ or 2S) generated knots in the presence of the initiator protein (Fig. 3*B*). No knots were produced from circles with no or one site either with or without initiator protein. Thus, the results are consistent with the predicted scheme (Fig. 3*A*).

DNA Looping in Linear DNA Detected by Cyclization Enhancement. The objective was to test whether initiator protein could bring together two γ-origin sites located near two ends of linear DNA and reveal this interaction by enhancing circle formation catalyzed by DNA ligase, as the two ends of linear DNA would be pulled closer by this interaction. The rationale underlying the detection procedure for DNA looping in linear DNA is shown (Fig. 4*A*). All three types of substrates—namely, 0γ (0S), 1γ (1S), and 2γ (2S) were linearized by cutting at the *Ava* I site. The linear 2γ (2S) DNA had one γ-origin sequence 1400 bp from one end and the other 600 bp away from the opposite end. The binding sites were not placed too close to the two ends of the linear DNA

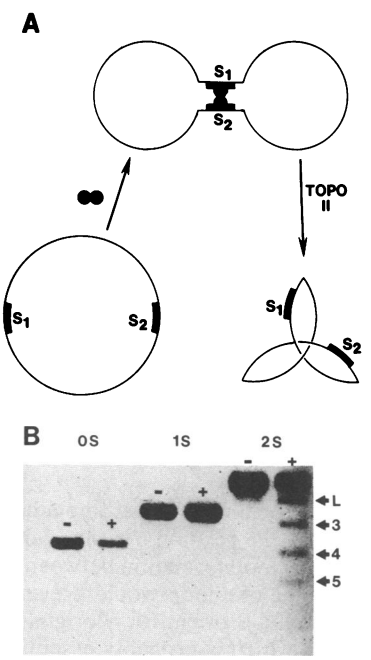

FIG. 3. Enhancement of DNA knotting as an assay of protein-mediated DNA looping. (*A*) Schematic of the underlying rationale. S_1 and S_2, two identical 277-bp γ-origin sequences located 2000 bp apart in a 2γ recombinant DNA substrate (2S); (●) protein dimer (see text for details). (*B*) Photograph of a 1% agarose gel showing lack of knots in 0γ (0S) and 1γ (1S) DNA with (+) or without (−) initiator protein. Note knots formed in 2γ (2S) substrate only with initiator protein; L, linear; 3, trefoils; 4, tetrafoils; and 5, pentafoils. Knots were identified by their survival after DNase I nicking and their mobility with respect to a topoisomer ladder (16).

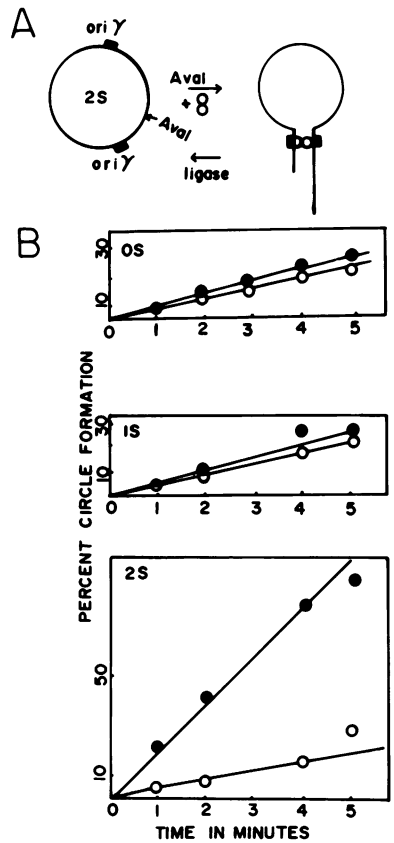

FIG. 4. DNA looping in linear DNA (2S) as revealed by cyclization enhancement. (*A*) Schematic showing the underlying rationale of the technique. (*B*) Kinetics of T4 DNA ligase-induced DNA cyclization of 0S, 1S, and 2S DNA substrates under identical conditions with or without the initiator. In 0S and 2S cyclization is shown with (●) and without (○) the initiator. In 1S, symbols are reversed. Note that only in the 2S substrate does initiator enhance DNA cyclization by a factor of ≈5. S, γ origin.

because of possible interference with ligation from initiator protein-mediated untwisting of the DNA (15). Equal amounts of the three substrates were treated with equal amounts of ligase under identical conditions with or without saturating amounts of initiator protein. The initiator protein had very slight or no effect on the rate of circularization or 0γ (0S) DNA; with 1γ (1S) DNA, rate of cyclization was reduced with protein because protein induced the formation of some amounts of linear oligomers. In contrast, the circularization rate of 2γ (2S) DNA was enhanced almost 5-fold with the initiator protein (Fig. 4*B*). Therefore, results are consistent with the depicted scheme to detect DNA looping.

Confirmation of DNA Looping by Electron Microscopy. We attempted to confirm the DNA looping caused by initiator-protein interaction with the two γ-origin sequences in 2γ (2S) DNA substrates by electron microscopy. 2γ, 1γ, and 0γ DNA substrates were linearized with *Ava* I and spread on freshly cleaved mica surfaces with or without complex formation with saturating amounts of initiator protein. Loops of expected dimension and tail lengths were seen in 30% of the 2γ DNAs complexed with protein (Fig. 5; ref. 5). Without initiator protein, loops were absent. Approximately 300 molecules of 2γ DNA each with or without protein were examined; similar numbers of 1γ and 0γ DNA with or without initiator protein also were examined. No loops of the expected dimension were detected in the 1γ (1S) and 0γ (0S) DNA. Therefore, electron microscopy confirmed the looping of 2γ DNA induced by the initiator protein, as detected by the enhancement of knot formation and cyclization catalyzed by topoisomerase II and DNA ligase, respectively.

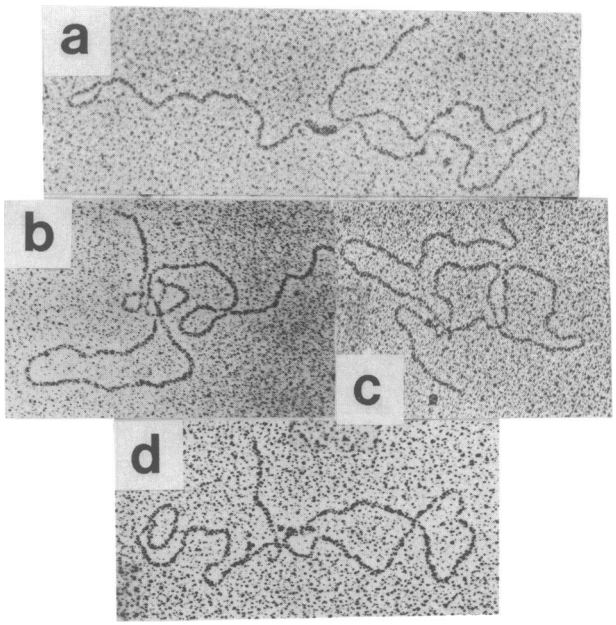

FIG. 5. Representative electron micrographs of linear 2γ (2S) DNA-forming loops formed by initiator-mediated γ-origin–γ-origin interaction. (*a–d*) 2γ (2S) DNA loops. No loops were seen in 1γ (1S) or 0γ (0S) DNA or in 2γ DNA without initiator. Three hundred molecules each of 0γ, 1γ, and 2γ substrate with or without the initiator were examined. As shown in Fig. 4*A*, the DNAs were linearized at the *Ava* I site. Dimensions of the loops and the tails, within experimental errors, were markedly close to that expected from the known sequence. (×117,450.)

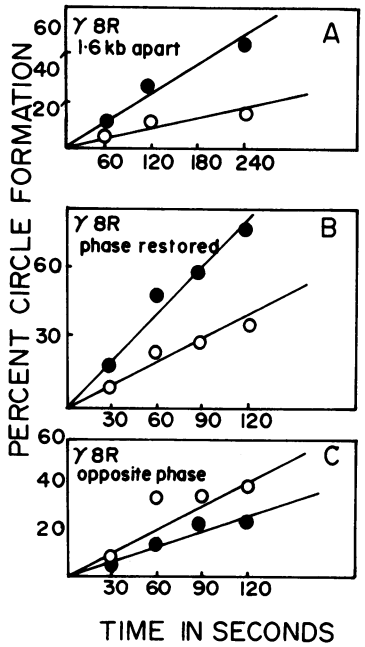

FIG. 6. (*C*) Enhancement of DNA cyclization experiments showing that the γ origin and the autoregulatory sites form no loops (even though both individually bind to the initiator) when in their natural locations—i.e., when the seventh and eighth repeats are 115 bp apart (γ8R, opposite phase). (*A*) Separating the two sites by 1600 bp promotes protein-mediated DNA looping (γ8R1.6kb apart). (*B*) Introduction of 6 bp into the DNA sequence that normally separates the two sites promotes DNA looping (γ8R, phase restored). Circle formation with (●) and without (○) the initiator protein.

Interaction Between the γ Origin and the Autoregulatory (Operator) Sequence of the Initiator Cistron. The operator of the initiator protein lies between γ origin and β origin, ≈100 bp from the seventh repeat of γ origin (Fig. 1; ref. 8). The initiator protein binds to the operator sequence to autoregulate its own synthesis at the transcriptional step (8, 9). The protein has no detectable affinity for β origin alone. However, with γ origin in cis location, protein binding to the γ-origin sequence causes cooperative protein binding to β origin by DNA looping (5). Because the operator sequence has much stronger affinity for the initiator protein than has β origin, the operator sequence would be expected to compete with β origin for interaction with γ origin by DNA looping when initiator is available and, thus, inhibit γ origin–β origin interaction. Does the autoregulatory sequence interact with γ origin?

To answer this question, we studied possible DNA looping by circularization assay between γ origin and the operator sequence (8, 9) at their natural locations on the R6K DNA. An 0.8-kb-long DNA fragment (F), described earlier, containing the R6K sequence from γ origin to the 8th repeat as well as two half repeats constituting the operator sequence and with *Eco*RI ends was the substrate for cyclization assay with and without enough inititator protein to saturate γ origin. The kinetics of circle formation as determined by agarose gel electrophoresis revealed that the initiator not only failed to enhance circle formation but actually reduced cyclization by a factor of 0.66 over that of naked DNA (Fig. 6*C*). This reduction was due to formation of oligomers promoted by protein-mediated intermolecular ligation. Because both the operator sites and γ origin independently bind to the initiator (10, 11), the lack of loop formation, as suggested by kinetics of circularization, could be due to lack of enough spacing between the two sites and/or the location of the two sites on opposite faces of the DNA double helix. Indeed, checking the sequence from the adenine residue of the GAG sequence (contact points of protein) of the seventh repeat at γ origin to that of the eighth repeat at the autoregulatory site revealed that the two sites are separated by 10 full turns and a partial turn, or half turn, of the double helix (8). But, protein-contact points of all other seven repeats at γ origin are exactly on the same DNA face because they are separated by two full turns with respect to each other (Fig. 7*i*).

We tried to bring the two sites (γ origin and the operator site) to the same DNA face by introducing 6 bp in the form of a *Bam*HI site immediately after the seventh repeat of the γ origin (F^R DNA) and then examined the ability of the two sites to form DNA loop by the cyclization test. The two sites could interact with each other (Fig. 6*B*) as indicated by a 2- to 3-fold increase in the rate of cyclization with initiator over that of the same DNA lacking initiator.

Separation of the two sites by ≈1800 bp also allowed site–site interaction and DNA looping as revealed by the cyclization test (Fig. 6*A*). Here, the cyclization rate was increased by a factor of 3 by adding initiator protein to the linear DNA substrate.

Thus, the relative location of the autoregulatory site on the opposite DNA face in relation to the γ-origin sequence ensures lack of DNA loop formation between these two sites (Fig. 7). This absence of a loop would prevent any interference between γ origin–β origin (or γ origin–α origin) DNA loop formation (5), which is probably essential for initiating replication form β origin or α origin.

DISCUSSION

We described different and rapid biochemical techniques to detect intermolecular DNA site–site interaction by enhancing catenation and intramolecular site–site interactions by increased knotting in covalently closed circular DNA and by increased DNA cyclization of linear DNA. In comparison with more conventional techniques of electron microscopy

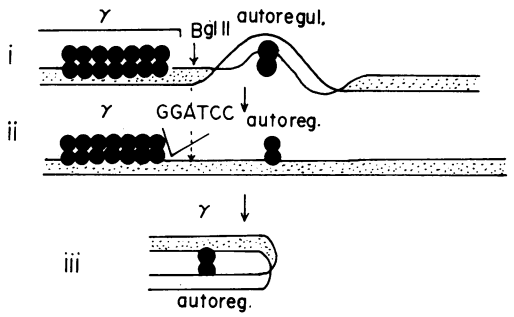

FIG. 7. (*i*) Schematic showing the location of γ origin and the autoregulatory sites on opposite faces of the DNA helix. (*ii*) The two sites are moved to the same face by introducing a *Bam*HI site (half turn of the helix), (*iii*) thus allowing the two sites to interact with the initiator (●), forming a loop.

and DNase protection assay, the procedures described here are simple and rapid and can be used, in principle, as assay procedures for detecting loop-promoting proteins. The results show that the initiator protein of R6K can bridge two noncontiguous origin sequences separated by 2000 bp. The initiator can also bridge, *in vitro*, pairs of γ-origin sequences located on different DNA molecules. It is not clear now whether a dimeric or a tetrameric form of the initiator interacts with these two sites.

A biologically interesting question is whether the initiator bound to γ origin can also bind to the autoregulatory sequence—thereby enhancing or stabilizing the transcriptional repression of the initiator-protein synthesis (8, 9). The results show that initiator bound to γ origin cannot bring that origin and the autoregulatory site together by DNA looping because the two sites are located on different DNA faces. Relocating the two sites to the same DNA face (Fig. 7 *ii* and *iii*) or increasing separation between the two sites by 2000 bp allowed site–site interaction between these two sequences. We also constructed F^{RR} by insertion of 4 bp at the *Bgl* II site of F^{R}, which relocated the operator to a double-helix face opposite from that of γ origin. As expected, the pattern of initiator-mediated cyclization rate of F^{RR} was the same (data not shown) as that of the DNA substrate F, although F^{RR} is longer than F by 10 bp. This result confirms the critical role played by phasing in protein-mediated site–site interaction.

A second biological implication of this result is that the operator's location (DNA face opposite from that of γ origin) is such that the autoregulatory site should not compete with β origin for interaction with γ origin, even though the autoregulatory site has much stronger affinity for initiator protein than the β-origin sequence (5, 8, 10, 11). That γ origin and β origin can interact by DNA looping has been shown *in vitro* (5).

Unlike the R6K system, recent work suggests that the autoregulatory site of P1 interacts with the repeats of the incompatibility locus by DNA looping (18) as revealed by electron microscopy. Therefore in P1, unlike R6K, action at a distance may also modulate autoregulation of initiator-protein synthesis.

Although both the DNA cyclization and knotting enhancement are highly sensitive techniques for detecting site–site interactions, the knotting process is limited by the DNase nicking step to resolve knotted DNAs from topoisomers. Excessive DNase digestion can lead to double-strand breaks and loss of knotted molecules. We suggest that this potential difficulty could be minimized by using a single-cut restriction enzyme (e.g., *Eco*RI or *Bam*HI), for which a single site is present in the DNA substrate, and by controlling the enzyme digestion by adding excess ethidium bromide (e.g., 200 μg/ml). The development of biochemical assays that detect DNA looping furthers the study of regulatory proteins and DNA sequences that interact from a distance.

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