# An *Escherichia coli* replication protein that recognizes a unique sequence within a hairpin region in φX174 DNA

(DNA-dependent ATPase/sequence specificity/protein n'/origin of φX DNA complementary strand replication)

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ABSTRACT    Protein n', a prepriming DNA replication enzyme of *Escherichia coli*, is a φX174 DNA-dependent ATPase. Restriction endonuclease fragmentation and exonuclease VII digestion of φX174 DNA have led to the identification of a 55-nucleotide fragment that carries the protein n' recognition sequence. Molecular hybridization and sequence analysis have located this sequence within the untranslated region between genes F and G, a map location analogous to that of the unique complementary strand origin of phage G4 DNA. Within the 55-nucleotide fragment is a sequence of 44 nucleotides that forms a stable hairpin structure. This duplex may be the signal for protein n' to initiate the prepriming events that lead to the start of φX174 complementary DNA strand replication.

Conversion of the single-stranded chromosomes of phages M13, G4, and φX174 (φX) to their duplex replicative forms are model systems for studies of the mechanisms of initiation of DNA synthesis in *Escherichia coli*. The more complex φX system appears to be particularly pertinent to the discontinuous phase of *E. coli* chromosomal replication (1, 2).

The M13, G4, and φX templates differ primarily in their enzymatic requirements for the initiation of primer synthesis. Coated with single-stranded-DNA binding protein (SSB), M13 DNA can be primed directly by RNA polymerase (3) and G4 DNA, by primase (4, 5). However, SSB-coated φX DNA, although primed by the same *E. coli* primase, must first be activated in a prepriming stage. In this prepriming reaction, the *E. coli* proteins n', n and n", i, dnaB, and dnaC form an activated complex with φX DNA (2, 6–8).

The distinctions in primer synthesis on these three phage templates are probably due to structural differences in "promoter"-like sites recognized by the distinctive priming systems. Although the origin of complementary DNA strand replication is unique and well characterized for M13 (9) and G4 (10–14), it does not appear to be at a unique site for φX, as judged by *in vivo* and *in vitro* studies (7, 9, 11, 15).

We will describe elsewhere the purification of protein n' to near homogeneity, its φX DNA-dependent ATPase activity, and its capacity to destabilize an SSB–φX DNA complex. In this paper we report the recognition by protein n' of a specific sequence in φX DNA located at an intergenic region and suggest that it may be the signal that leads to the initiation of φX complementary DNA strand replication.

## MATERIALS AND METHODS

Nucleic Acids, Enzymes, Resins, and Nucleotides. φX, φX replicative form (RF)I, G4, and M13 DNAs were prepared as described (16). *E. coli* SSB was 4 × 10⁴ units/mg (17); *Hae* III and *Hin*fI restriction endonucleases were from Bethesda Research Laboratories, Rockville, MD, and *Mbo* II was from New England BioLabs; exonuclease VII preparations were gifts from S. P. Goff (Massachusetts Institute of Technology) and from J. W. Chase (Albert Einstein Medical School); T4-polynucleotide kinase was from P-L Biochemicals. Hydroxyapatite was obtained from Bio-Rad, agarose was from Bethesda Research

Table 1. Localization of the n' recognition site to a particular nuclease restriction fragment

| DNA effector | ATP hydrolyzed, pmol |
|---|---|
| None | <1 |
| φX174 | 132 |
| G4 | 3 |
| *Hae* III fragments of φX | |
| Z1 | 114 |
| Z2 | 8 |
| Z3 | 13 |
| Z4 | 9 |
| Z5-11* | 8 |

$^{32}$P-labeled single-stranded φX DNA (200 μg) was incubated with 450 units of *Hae* III nuclease in 50 mM Tris·HCl (pH 7.5), 5 mM MgCl$_2$, and 0.5 mM dithiothreitol at 37°C for 20 hr. Reactions were stopped by additions of EDTA, Sarkosyl, glycerol, and bromphenol blue to final concentrations of 50 mM, 2%, 10% and 0.01%, respectively. Digestion products were electrophoresed in a 2.5% agarose gel in Tris acetate and stained with ethidium bromide (1 μg/ml). DNA bands were sliced and extracted. Purified DNA fragments at 0.8 nM covered by SSB (one SSB per nine nucleotides) were assayed as effectors for n'-ATPase activity by using the standard assay conditions.
* Analyzed separately or collectively, the values for these *Hae* III fragments were 8 or less.

Laboratories, and polyethyleneimine-cellulose was from Brinkmann. 1-[(m-Nitrobenzyloxy)methyl] pyridinium chloride was a gift from G. M. Wahl of this department.

Assays for Protein n'. (i) Reconstitution of a single-stranded circular DNA (SS) → RF φX DNA replication reaction. The reconstituted enzymatic reaction was as described (17).

(ii) DNA-dependent ATPase. The assay measures the production of labeled ADP from [³H]ATP or [α-³²P]ATP and could also be applied to the production of ³²Pᵢ from [γ-³²P]ATP. Standard assays were carried out in 25-μl reaction mixtures containing 10 mM KCl, 1 mM MgCl₂, 1 mM labeled ATP or dATP, 120 pmol of φX DNA (as nucleotide), 50 mM Tris·HCl (pH 7.5), 6% (wt/vol) sucrose, and bovine serum albumin at 0.2 mg/ml. Samples to be assayed were diluted in 50 mM imidazole·HCl, pH 6.8/25% glycerol/100 mM ammonium sulfate/bovine serum albumin at 0.2 mg per ml/1 mM EDTA. When SSB was used, 1 μg was added, unless otherwise noted. Reactions were carried out at 30°C unless otherwise noted. Aliquots of 2 μl were applied to polyethyleneimine-cellulose strips (0.6 × 6 cm) together with unlabeled ATP, ADP, and AMP markers. The strips were developed with 1 M formic acid/0.5 M LiCl at room temperature, dried, and examined with UV light to locate and cut out the ATP and ADP spots. Radioactivity was determined in scintillation fluid without

Abbreviations: φX, φX174; SS, single-stranded circular DNA; RF, double-stranded DNA of circular replicative form; SSB, single-stranded-DNA binding protein; DBM, diazobenzyloxymethyl.
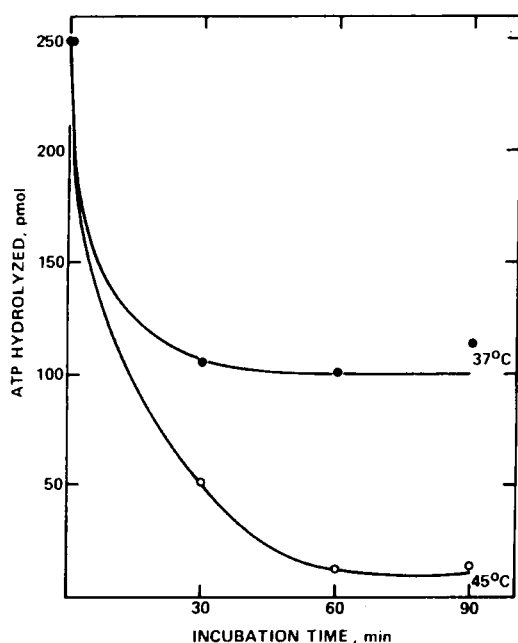
FIG. 1. Exonuclease VII resistance of DNA effector activity. The *Hae* III Z1 DNA fragment (300 pmol in nucleotides, Table 1) was incubated with 0.3 unit of *E. coli* exonuclease VII in 13 $\mu$l of 67 mM potassium phosphate, pH 7.9/8.3 mM EDTA/10 mM 2-mercaptoethanol at 37°C or 45°C. At indicated times, reactions were prepared for ATPase assays by the following additions: $MgCl_2$ to 21 mM, KCl to 10 mM, sucrose to 6%, bovine serum albumin to 0.1 mg/ml, SSB to 10 $\mu$g/ml, and [$^3$H]ATP (20,000 cpm/pmol) to 1 mM. ATP hydrolysis at 30°C was measured 15, 30, and 45 min after the addition of protein n' (fraction VIII) (unpublished results). Values represent rates of ATP hydrolysis corrected for background ATPase activity of the exonuclease VII preparation.

elution. One unit of n' ATPase activity represents the cleavage of 1 pmol of ATP or dATP per min. The ATPase assay conditions, designed to resemble those of the replication assay, were not optimal with respect to pH (85–90% of maximal activity) or $\phi$X DNA concentration (about 65% of maximal activity).



FIG. 2. Separation of exonuclease VII-resistant sequences in *Hae* III DNA fragment Z1 in a polyacrylamide gel. $^{32}$P-labeled *Hae* III Z1 fragment (2.9 $\mu$g) was incubated at 37°C for 60 min with 5 units of *E. coli* exonuclease VII as in Fig. 1. Reaction products were precipitated overnight at −20°C in 0.3 M sodium acetate (pH 5.5) with 2 vol of isopropanol. The precipitate was collected by centrifugation in the SW56 rotor at 50,000 rpm and 0°C for 2 hr, dissolved in 80% deionized formamide, heated for 3 min at 100°C and cooled in an ice-water bath, adjusted to 0.01% in bromphenol blue and xylene cyanol FF, and electrophoresed on a preparative 12% polyacrylamide/7 M urea gel (20) at a constant voltage of 200 V for 8 hr at room temperature. DNA bands were detected by autoradiography of the wet gel. Numbers in parentheses indicate fragment size in residues.
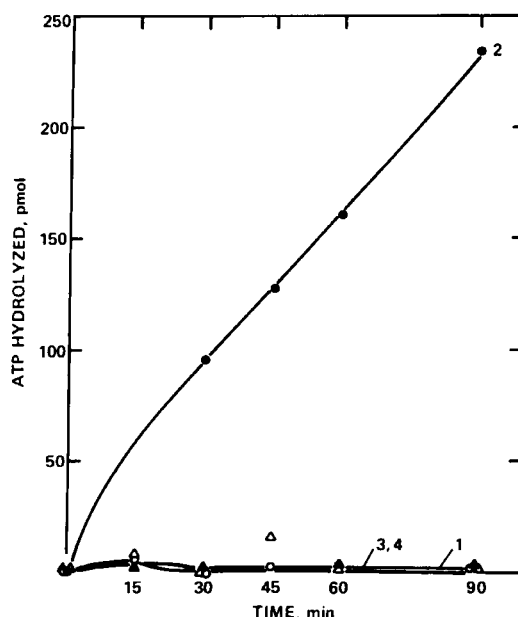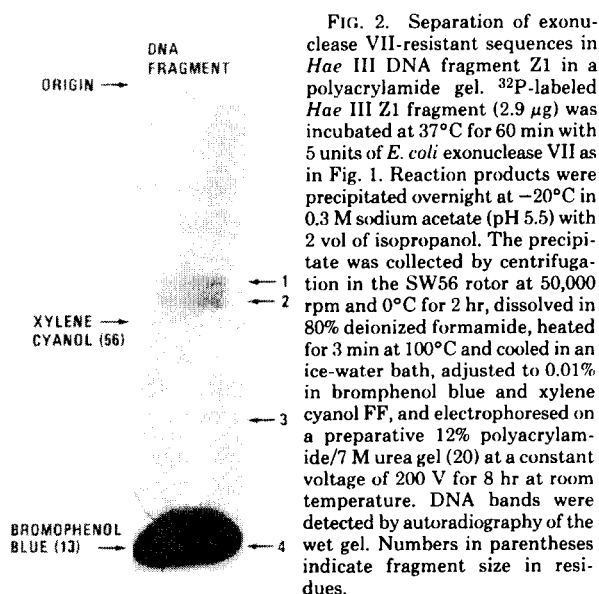
FIG. 3. DNA effector activity for n'-ATPase in exonuclease VII-resistant DNA sequences of *Hae* III Z1 fragment. The *Hae* III Z1 fragment (Table 1) was digested with exonuclease VII; the products were separated by electrophoresis in 12% polyacrylamide/7 M urea gels as in Fig. 2. DNA fragments 1, 2, 3, and 4 were electroeluted from the gel, concentrated by the hydroxyapatite procedure, and assayed for effector activity in the n'-ATPase reaction. Assays covered a range of DNA concentrations (0.2–1.6 nM) in the presence of SSB (one SSB per nine DNA nucleotides). Values were obtained at 1.6 nM DNA.

**Exonuclease VII Assay.** *E. coli* exonuclease VII was assayed as described by Chase and Richardson (18, 19). Reaction mixtures of 25 $\mu$l were used at 37°C, unless otherwise noted.

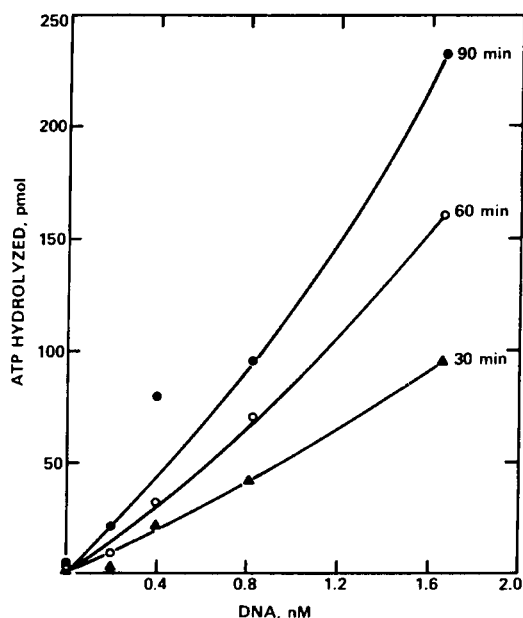**Gel Electrophoresis.** Electrophoresis of DNA was carried



FIG. 4. Influence of concentration of fragment 2 on n'-ATPase reaction. Exonuclease-resistant fragment 2 (Fig. 3) was assayed for its activity as a DNA effector in the n'-ATPase reaction after 30, 60, and 90 min of incubation under standard assay conditions.

out in 0.7–3.5% agarose gels at constant voltage (1 V/cm) at room temperature in either 90 mM Tris borate, pH 8.3/2.5 mM EDTA or in 40 mM Tris acetate, pH 7.8/5 mM sodium acetate/1 mM EDTA. Electrophoresis of DNA fragments in 12% or 20% polyacrylamide/7 M urea gels was as described (20).

Other Procedures. Labeling of DNA 5′ ends with ${}^{32}$P by T4 polynucleotide kinase and determination of DNA sequence were as described by Maxam and Gilbert (21). Preparation of diazobenzyloxymethyl (DBM) paper, transfer of DNA to DBM paper, and hybridization procedures were as described by Alwine et al. (22) and by Wahl et al. (23). To isolate DNA fragments from agarose gels, the gels were digested with 1.5 vol of 6 M NaClO₄ at 65°C for 10–20 min (or at 37°C for 2 hr). The DNA was adsorbed on hydroxyapatite columns in 10 mM sodium phosphate buffer (pH 7) and maintained at 65°C. Loaded columns were washed with several bed volumes of this buffer and eluted with a gradient of 0.3–0.5 M sodium phosphate buffer (pH 7). Alternatively, the same procedure was used batchwise at room temperature (G. Weinstock, personal communication); DNA was extracted from gels by electroelution.

## RESULTS

A Unique Region in φX Chromosome is Recognition Site for Protein n′. ATP or dATP hydrolysis catalyzed by the E. coli prepriming enzyme n′ was DNA-dependent, used φX DNA preferentially as an effector, but was inactive with other DNAs in the presence of SSB (Table 1). Among the separated single-

stranded Hae III fragments of φX DNA assayed as effectors for n′-ATPase activity, only one (Z1, which includes about one-fourth of the φX chromosome as seen in Fig. 6) could serve and was as effective on a molar basis as intact φX DNA.

Recognition Site for Protein n′ in φX SS Has Secondary Structure. Activity of an SSB-coated φX DNA fragment as an effector in the n′-ATPase reaction, when other coated single-stranded DNAs are completely inactive (unpublished observations), suggests a recognition site with persistent duplex structure. For this reason, the isolated Hae III Z1 fragment was digested by exonuclease VII, which degrades single-stranded DNA from both the 3′ and 5′ ends but does not digest double-stranded DNA (19). The digest retained effector activity (>45%) even after prolonged incubation at 37°C, but at 45°C the activity of Z1 was destroyed (>95%) (Fig. 1). Resistance of the activity of the Z1 fragment at 37°C and susceptibility at 45°C suggested that the effector activity of this single-stranded DNA fragment is in a duplex denatured at 45°C.

Isolation of Duplex DNA Region that Carries Protein n′-Recognition Site. The limit-digestion products of exonuclease VII hydrolysis of fragment Z1 separated by electrophoresis were mainly oligomers 8–12 nucleotides long (Fig. 2). However, 7.5% of the digested DNA was found in longer chains: fragment 1, 65–70 residues (2.5%); fragment 2, 55–60 residues (3.6%); and fragment 3, 18–22 residues (1.4%). Only fragment 2, representing about 1% of the original φX chromosome, was active as an effector for n′-ATPase (Fig. 3); the longest fragment (fragment 1) was inactive. ATP hydrolysis rate with fragment 2 (Fig. 3) was 52% of that with intact φX DNA. ATPase activity
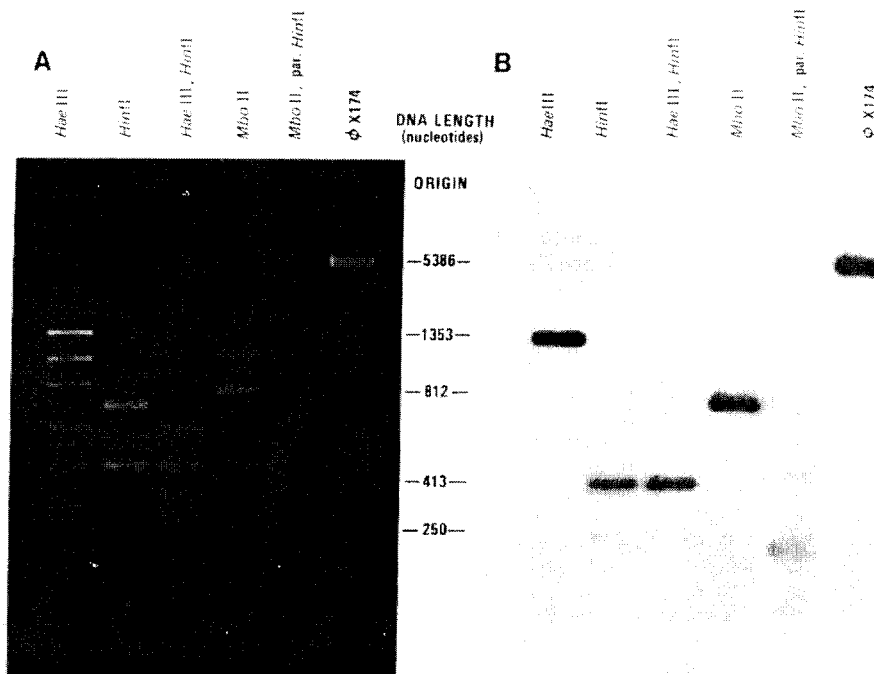


FIG. 5.    Sequence homology of exonuclease VII-resistant fragment 2 as judged by hybridization to various regions of φX DNA. φX RFI DNA (0.8 μg) was incubated at 37°C for 1 hr with restriction endonucleases as follows: Hae III (3 units) in 50 mM Tris·HCl, pH 7.5/5 mM MgCl₂/0.5 mM dithiothreitol; HinfI (3 units) or Hae III and HinfI simultaneously (each 3 units) in 6 mM Tris·HCl, pH 7.5/6 mM MgCl₂/6 mM 2-mercaptoethanol/100 mM NaCl; or Mbo II (3 units) or HinfI and Mbo II simultaneously (each 3 units) in 10 mM Tris·HCl, pH 7.9/6 mM KCl/10 mM MgCl₂/1 mM dithiothreitol. Reactions were stopped by addition of EDTA, Sarkosyl, glycerol, and bromphenol blue to final concentrations of 50 mM, 2%, 10% and 0.01%, respectively. (A) DNA fragments were electrophoresed in 2% agarose gels and stained with ethidium bromide (1 μg/ml). DNA was denatured in the gel and transferred to DBM paper (22). (B) 5′-${}^{32}$P-labeled exonuclease VII-resistant DNA fragment 2 (Fig. 2) was hybridized (23) in the presence of 10% dextran sulfate-500 at 42°C for 40 hr to the DNA fragments bound to DBM paper; the paper was washed (23) and subjected to autoradiography at −80°C by using an intensifying screen (Cronex, Du Pont). In the far right lane, ${}^{32}$P-labeled φX DNA was used as a size and transfer marker.
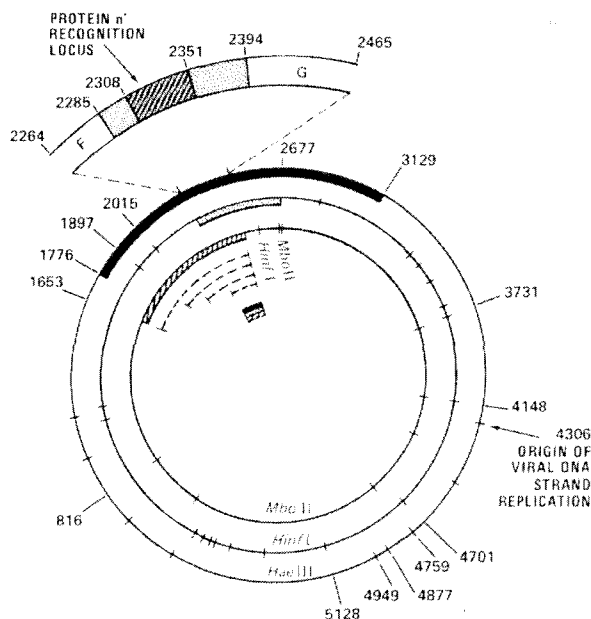
FIG. 6. Location of protein n′ recognition locus on φX chromosome. Heavy lines represent restriction fragments of φX RFI DNA, to which exonuclease VII-resistant DNA fragment 2 was hybridized (Fig. 5B). Broken lines indicate products of a partial digestion of an *Mbo* II fragment (1653–2465) by *Hin*fI, to which the radioactive DNA probe was hybridized (Fig. 5B). The magnified region (2264–2465) closest to the center of the diagram is the hybridized sequence common to all three restriction digests. The n′ recognition site is located within this 201-nucleotide region. It contains an untranslated sequence of 110 nucleotides (2285–2394) between structural genes F and G; within this intergenic region is the hairpin sequence of 44 nucleotides (see Fig. 7) in the exonuclease-resistant DNA fragment 2 (2308–2351).



FIG. 7. Possible secondary structure in recognition locus of protein n′. The proposed structure of base-paired regions separated by two internal loops and a hairpin loop is based on a calculation of free-energy contributions of base-paired regions and loops of the sequence at position 2308–2351 (Fig. 6) within the 55-nucleotide fragment possessing the protein n′ recognition site.

is a function of the concentration of the DNA effector, but the dependence is more nonlinear at low concentrations (Fig. 4).

**Location of Isolated Protein n′ Recognition Sequence in φX Chromosome.** Molecular hybridization and sequence analysis were used to map the location of the exonuclease VII-resistant fragment 2 (above). Products of digestion of φX RFI DNA by the restriction endonucleases *Hae* III, *Hin*fI, and *Mbo* II were separated on 2% agarose gels (Fig. 5A), denatured, and transferred to DBM paper (22). Fragment 2, labeled with $^{32}$P at its 5′ end by polynucleotide kinase, was hybridized to the denatured, paper-bound φX DNA fragments (Fig. 5B) (23).

With the *Hae* III digest separated into nine bands (Fig. 5A), the fragment 2 probe hybridized, as expected, only to fragment Z1 (24), 1353 residues at position 1777–3129 (Fig. 6).

Of the ten bands produced by *Hin*fI digestion (Fig. 5A), the probe hybridized mainly to fragment F5b (24), 413 nucleotides long (Fig. 5B) at position 2265–2677 (Fig. 6). F5b was part of a triplet band with fragments F5a and F5c (24), 417 and 427 nucleotides long, respectively; but these two fragments (at positions 3732–4148 and 4702–5128) are well outside the *Hae* III Z1 sequence. Fragment F5a is also excluded by the results of the double digestion by *Hae* III and *Hin*fI. Although the F5a sequence contains three *Hae* III sites (at positions 4759, 4877, and 4949) (Fig. 6), hybridization of the probe to a fragment about 413 residues long was not affected. Slight hybridization to a *Hin*fI (or *Hin*fI plus *Hae* III) digestion product about 250 nucleotides long (Fig. 5B) will be considered below.

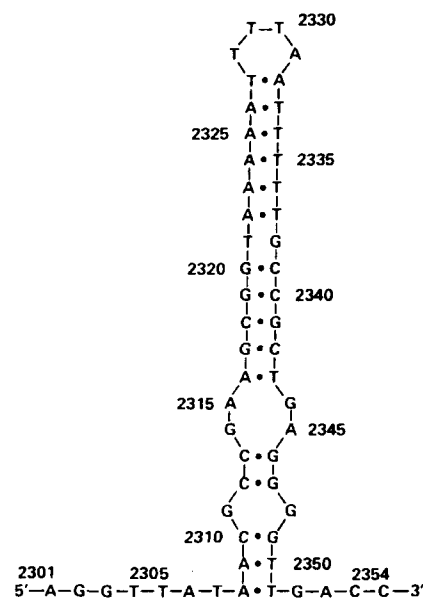With the *Mbo* II digest, which yielded six bands (Fig. 5A), the fragment 2 probe hybridized to fragment M4 (24), 812

nucleotides long (Fig. 5B) at position 1654–2465 (Fig. 6). M4 migrated in the agarose gel as part of a doublet band with M3, 837 nucleotides long (at position 817–1653) (Fig. 6). Not only is M3 outside the *Hae* III Z1 sequence, but it is also eliminated by the results of a double digestion (completely with *Mbo* II and partially with *Hin*fI). Fragment 2 hybridized to another set of fragments generated by the incomplete digestion of the *Mbo* II fragment M4 by *Hin*fI (Figs. 5B and 6). Hybridization was observed with the 812-nucleotide fragment when no cuts were introduced in this region by *Hin*fI, and with fragments 201, 450, and 568 nucleotides long, produced by *Hin*fI cuts within M4 at positions 2264, 2015, and 1897.

In summary, the hybridization experiments limit the location of the exonuclease VII-resistant fragment 2 to a 201-residue region at position 2265–2465. It remained to be determined whether the sequence of fragment 2 contains the potential for secondary structure.

**A Hairpin Structure in Recognition Site.** Exonuclease VII-resistant fragment 2 upon electrophoresis in 20% polyacrylamide/7 M urea contained a major component 55 nucleotides long and a minor one, one or two nucleotides shorter. To map this fragment precisely, the DNA sequence was determined. Analysis of a 52-nucleotide region in this fragment (data not shown) and comparison of the sequence with the Sanger map (24) locates it at position 2304–2355. Within this fragment is a 44-nucleotide-long sequence at position 2308–2351 with a strong potential for secondary structure (Fig. 7). Calculations of the free energy contributions of the base-paired regions and loops, based on studies with polyribonucleotides (25), yield a value of −14 kcal/mol at 25°C and 1 M NaCl. This value is consistent with a duplex form for this sequence (Fig. 7) under the experimental conditions in which it resists destabilization by SSB and digestion by exonuclease VII (Fig. 2).

The *Hin*fI (or *Hin*fI plus *Hae* III) fragment about 250 nucleotides long that showed some homology to fragment 2 (see

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above) is the 249 nucleotide-long HinfI fragment F7 (24) at position 2016–2264, as judged by its size and its location within a sequence that overlaps both the Hae III Z1 and the Mbo II M4 DNA fragments that were shown to be the only Hae III and Mbo II φX DNA fragments with homology to the DNA probe (Fig. 5B). The F7 DNA fragment contains (in its viral strand) a sequence of eight nucleotides, G-C-T-G-A-G-G-G (at position 2039–2046), identical to one on the exonuclease VII-resistant DNA fragment (position 2341–2348); this may explain its hybridization to the DNA probe.

## DISCUSSION

A prepriming system reconstituted from six E. coli proteins is needed to prepare the single-stranded, binding-protein-covered φX DNA circle for primer synthesis by primase to initiate DNA replication (26, 27). This system is specific for φX DNA and does not operate on the SSs of phages G4 and M13 (26, 27). Unlike phages G4 and M13, which possess unique origins for complementary strand starts, both in vivo and in vitro studies indicate multiple starts on φX DNA (7, 9, 11, 14). However, the strict specificity of the prepriming system for φX DNA suggests that a particular locus is recognized by one or more of the proteins of the prepriming system. Protein n' is a candidate for this role.

Protein n', now available in homogeneous form (unpublished observations), is an ATPase dependent on the presence of DNA. Unlike most other such ATPases, protein n' is strikingly specific in its DNA dependence. Among a large number of binding-protein-coated single strands and duplexes, only φX DNA is active (unpublished observations). Because recognition of coated G4 DNA by primase and M13 DNA by RNA polymerase depends in each instance on a unique hairpinlike structure, it seemed that protein n' might also depend on such a helical structure that resists destabilization by SSB. Exonuclease VII, an enzyme that acts on single-stranded DNA from both 3' and 5' termini (18, 19), leaves a resistant 55-nucleotide stretch that retains the full effector capacity of the φX circle to support protein n' ATPase activity (Figs. 1–4).

Within the exonuclease VII-resistant, 55-nucleotide fragment there is a 44-nucleotide sequence with a potential for forming a relatively stable hairpin structure (Fig. 7). Because its size distinguishes this sequence neither from other duplex regions in φX that also resist exonuclease VII action (Fig. 2) nor from the duplex origins in G4 and M13 DNAs, the basis for recognition by protein n' must depend on other features, such as a particular sequence within the hairpin, mismatched regions, the terminal loop, or neighboring regions of the hairpin. Available methods for chemical synthesis of polydeoxynucleotides (28) offer an approach for settling this question.

The 55-nucleotide recognition sequence for protein n' is contained within a 107-nucleotide, untranslated region of the φX chromosome between genes F and G, a location analogous to that of the complementary strand origin of phage G4 (12). This is remarkable in view of the low degree of overall sequence homology between these phages and the lack of resemblance between the hairpins recognized by protein n' in φX and by primase in G4. It seems likely that the series of reactions that culminates in the priming of φX complementary strand synthesis is initiated by protein n' at a specific locus, and that subsequent events, such as movement of dnaB protein around the φX circle, may obscure this unique start.

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