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Rifampicin-resistant initiation of DNA synthesis on the isolated strands of ColE plasmid DNA

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ABSTRACT

The opposite strands of the ColE1 and ColE3 plasmids were isolated as circular single-stranded DNA molecules. These molecules were compared with M13 and ϕX174 viral DNA with respect to their capacity to function as templates for in vitro DNA synthesis by a replication enzyme fraction from Escherichia coli. It was found for both ColE plasmids that the conversion of H as well as L strands to duplex DNA molecules closely resembles ϕX174 complementary strand synthesis and occurs by a rifampicin-resistant priming mechanism involving the dnaB, dnaC, and dnaG gene products. Restriction analysis of partially double-stranded intermediates indicates that preferred start sites for DNA synthesis are present on both strands of the ColE1 HaeII-C fragment. Inspection of the nucleotide sequence of this region reveals structural similarities with the origin of ϕX174 complementary strand synthesis. We propose that the rifampicin-resistant initiation site (rri) in the ColE1 L strand is required for the priming of discontinuous lagging strand synthesis during vegetative replication and that the rri site in the H strand is involved in the initiation of L strand synthesis during conjugative transfer.

INTRODUCTION

The circular single-stranded DNA molecules of the phages M13, G4, and ϕX174 have proven extremely useful for unravelling the priming mechanisms involved in the initiation of DNA synthesis in Escherichia coli (1,2). Both M13 and G4 complementary strand synthesis starts at unique origins. M13 DNA synthesis is primed by the rifampicin-sensitive RNA polymerase, whereas G4 DNA synthesis is initiated by the DNA primase encoded by the dnaG gene. Rifampicin-resistant priming of ϕX174 DNA synthesis also involves the dnaG primase but depends in contrast to the G4 system on six additional prepriming proteins (dnaB, dnaC, n, n', n", i). These proteins are assembled on ϕX DNA coated with single-stranded DNA binding protein (SSB) as a nucleoprotein complex termed primosome

(3). It has further been shown that ϕX DNA contains a unique recognition site for the prepriming protein n' which is at or near the primosome assembly site. From this site the primosome moves in the 5' → 3' direction of the template thereby enabling primase to synthesize RNA primers at multiple preferred regions of the template DNA (4). The unique primosome assembly site may be considered as the origin of ϕX complementary strand synthesis (3). Thus in contrast to the M13 and G4 systems the origin of ϕX complementary strand synthesis is not necessarily identical with a priming site for DNA synthesis.

It is generally assumed that lagging strand synthesis of chromosomal and plasmid DNA replication occurs by the same mechanism as ϕX174 complementary strand synthesis (5). The ColE1 plasmid, the strands of which can be easily separated by poly(U,G)-CsCl gradient centrifugation into heavy (H) and light (L) strands, provides a suitable model replicon to test this assumption. ColE1 replication proceeds unidirectional from an origin (ori) located in the HaeII-E fragment with the L strand as leading strand and the H strand as lagging strand (6,7). In contrast to the rifampicin-sensitive initiation of L strand synthesis priming of the H strand was found to resemble the ϕX system by requiring the dnaB, dnaC, and dnaG gene products (8,9). Consistent with these findings, Nomura and Ray (10) have shown that the L strand of the ColE1 HaeII-E fragment, which had been cloned into M13 DNA, contains a region (designated rri-1) which is capable of promoting the rifampicin-resistant initiation of complementary strand synthesis. Furthermore Zipursky and Marians (11) have identified two n' recognition sites near the replication origin of the related plasmid pBR322. One site was found in the L strand of the HaeII-E fragment and appears to be identical with rri-1. The other n' effector site is located in the H strand of the pBR322 HaeII-B fragment, which corresponds to the origin-proximal half of the ColE1 HaeII-C fragment.

In this paper we describe the isolation of the opposite strands of the ColE1 and ColE3 plasmids as circular single-stranded DNA molecules. These circles are then compared with M13 and ϕX174 viral DNA with respect to their capacity to function as templates for in vitro DNA synthesis. Evidence is presented indi-

cating that both strands contain rri sites for rifampicin-resistant initiation of DNA synthesis analogous to the priming of øX174 complementary strand synthesis. The possible structure and function of these rri sites will be discussed.

EXPERIMENTAL PROCEDURES

Preparation of circular single-stranded plasmid DNA. E.coli strains YS10(ColE1) and W3110(ColE3) were grown in L-broth to OD_{600} = 1.0. Chloramphenicol (120 µg/ml) was then added and aeration continued for 2.5 h (ColE3) or 5 h (ColE1). Cells were harvested and supercoiled plasmid DNA was isolated by the method of Humphreys et al. (12). Plasmid DNA (4 mg) was incubated with Neurospora crassa endonuclease (2 units per mg DNA) in 4 ml 25 mM Hepes pH 8.0 — 0.2 M NaCl — 10 mM $MgCl_2$ for 20 min at 37°C. Under these conditions approximately 95% of the supercoiled DNA was converted to the open circular form. The reaction was stopped by the addition of 0.3 ml 0.5 M EDTA. After addition of 1.1 ml 1 N NaOH aliquots of 0.45 ml (containing 0.33 mg DNA) were layered on alkaline sucrose gradients (5-20% sucrose in 36 ml 0.3 M NaOH). The gradients were run in a Beckman SW27 rotor at 25,000 rpm for 40 h at 4°C. Fractions were collected from the bottom of the tubes and the optical density determined. The faster sedimenting peak of circular single strands was pooled, neutralized with 1 M KH_2PO_4, and dialysed against 25 mM Hepes pH 8.0 — 10 mM NaCl — 1 mM EDTA (buffer A). The yield was between 0.5 - 1.0 mg DNA.

Strand separation by poly(U,G)-CsCl equilibrium centrifugation. Circular single strands (0.5 mg) were diluted into 100 ml 0.13 N NaOH and incubated for 5 min at 37°C. 2 mg poly(U,G) were added and the incubation was continued for 1 min at 37°C followed by neutralization with 1 M KH_2PO_4 and rapid cooling in an ice bath. 3 ml 5% Sarkosyl was then added and the sample filled up to 150 ml with 5 mM EDTA. After addition of CsCl (1.34 g/ml) centrifugation was performed in a Beckman VTi50 rotor (36 ml per tube) at 40,000 rpm for 40 h at 15°C. The optical density profile of the gradient showed two peaks of H and L strand DNA respectively. The peaks were pooled separately and contaminating H strand DNA was removed from the pooled L strand fraction by a second poly(U,G)-CsCl gra-

dient centrifugation. The separated DNA strands were concentrated by ethanol precipitation and taken up in 0.8 ml buffer A. 1/25 volume of 5 N NaOH was added and each sample was layered on two 5 - 20% sucrose gradients in 10.6 ml 50 mM Na-phosphate pH 12.5 — 1 M NaCl — 5 mM EDTA — 0.1% Sarkosyl. Centrifugation was performed in a Beckman SW41 rotor at 35,000 rpm for 18.5 h at 5°C. The peaks of circular single-strands were pooled, neutralized with 1 M KH_2PO_4, dialysed against 50 mM NH_4HCO_3 — 0.1 mM EDTA, lyophilized, and taken up in 250 µl buffer A. The yield was 40 - 60 µg DNA of each strand.

Assay of DNA synthesis. Standard reaction mixtures (50 µl) contained 25 mM Hepes pH 8.0, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM spermidine, 12.5 µM NAD, rifampicin (25 µg/ml), 2 mM ATP, 120 µM each of CTP, GTP, and UTP. 12.5 µM each of dATP, dCTP, dGTP, and ^{3}H-dTTP (1000 cpm per pmol), 0.3 µg circular single-stranded DNA, plus replication enzyme fraction (approximately 4 mg protein per ml assay mixture). Reaction mixtures were incubated for 30 min at 30°C and assayed for incorporation of ^{3}H-dTMP into acid-insoluble material as described previously (13).

Enzymes and chemicals. Replication enzyme fraction was prepared by ammonium sulfate fractionation (0 - 40% saturation) of E.coli extracts (14). dnaC protein was purified as described previously (8). Purification of dnaG primase and preparation of antisera will be described elsewhere (E.Lanka, unpublished). Plasmid primase was isolated as described by Wilkins et al. (15). E.coli RNA polymerase was prepared by the procedure of Sternbach et al. (16). DNA polymerase I was purchased from Worthington. Restriction endonucleases EcoRI and HaeII were from BRL. N.crassa endonuclease, T4 DNA ligase, poly(U,G), and ddTTP were obtained from Boehringer.

RESULTS

Isolation of circular single-stranded plasmid DNA

The isolation of single-stranded circles from supercoiled plasmid DNA requires the introduction of a single strand interruption into one of the opposite DNA strands. We observed that supercoiled form I DNA of ColE1 and ColE3 can be converted almost

completely to singly nicked open circular form II molecules by incubation with the single strand specific endonuclease from Neurospora crassa. Upon prolonged incubation, the form II DNA is converted to doubly hit linear form III molecules. Similar results have been obtained previously for øX174 and f1 duplex DNA (17,18).

Circular single strands isolated after denaturation of form II DNA were further fractionated into H and L strands by poly(U,G)-CsCl equilibrium centrifugation. The use of a vertical rotor was found to be mandatory for preparative strand separation, since we were unable to achieve a satisfactory resolution of the poly (U,G) complexed DNA strands by isopycnic centrifugation in fixed angle rotors or by zone sedimentation (18). The single-stranded ColE1 circles obtained by this procedure were free of contaminating opposite strands as demonstrated by gel electrophoresis (Fig.1). Single-stranded circles of comparable purity were also isolated from supercoiled ColE3 DNA. In contrast to f1 replicative form DNA (18), no strand bias was noticed in the cleavage of ColE DNA by N.crassa endonuclease and circular H and L strands were obtained with nearly identical yields from both plasmids.

DNA synthesis on isolated strands of ColE plasmid DNA

Circular H and L strands from ColE1 and ColE3 plasmids were compared with single-stranded phage DNA with respect to their capacity to function as templates for DNA synthesis in vitro. An ammonium sulfate fraction from E.coli extracts that has been previously shown to contain DNA polymerase III, SSB, and the components of the primosome (19) was used as source of replication enzymes. It was found that both strands of ColE1 DNA can support a significant amount of incorporation when incubated with this enzyme fraction (Table 1). Similar results were obtained with ColE3 single strands. The ColE specific DNA synthesis resembles øX174 complementary strand synthesis and clearly differs from the M13 system in not being dependent on rifampicin-sensitive primer RNA synthesis. Although the relative efficiencies of the DNA templates varied somewhat with different replication enzyme preparations, the extent of incorporation supported by ColE H and L strand DNA was comparable to that obtained with øX174 DNA (Tables 1 - 3).

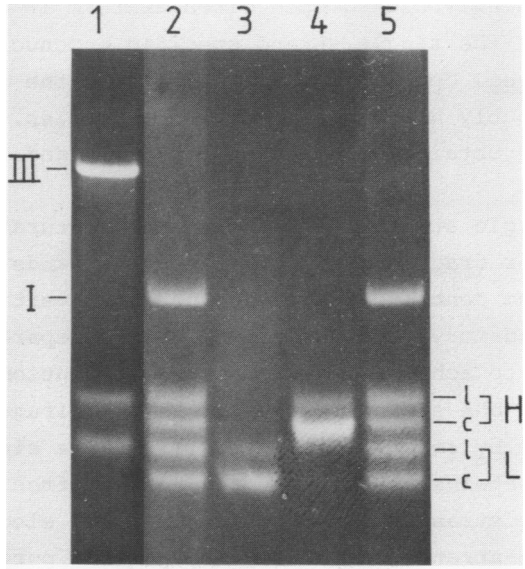

Fig. 1. Agarose gel electrophoresis of single-stranded ColE1 DNA. Samples of ColE1 DNA were analysed by gel electrophoresis in the presence of poly(U,G) according to the method of Goldbach et al. (27). 1 µg DNA was mixed with 8 µg partially fragmented poly(U,G), heated for 5 min at 100°C, and cooled in ice. The samples were then mixed with 1/4 volume 20% ficoll — 0.1% bromophenol blue and electrophoresed in vertical 1% agarose gels for 14 h at 20 mA in a running buffer containing 40 mM Tris-acetate pH 7.9 — 5 mM Na-acetate — 1 mM EDTA. The following DNA samples were used: ColE1 DNA linearized with EcoRI (lane 1), ColE1 DNA nicked with N.crassa endonuclease (lane 2 and 5), L strand circles (lane 3), and H strand circles (lane 4). The arrows on the left give the positions of supercoiled form I and linearized form III duplex DNA. The positions of linear (l) and circular (c) H and L strand DNA are indicated on the right.

We next tested the inhibitory effects of antisera directed against dnaB and dnaG protein respectively on ColE specific DNA synthesis. It can be seen from the data presented in Table 2 that ColE DNA synthesis and øX DNA synthesis were inhibited to a similar extent by both antisera, whereas M13 complementary strand synthesis was clearly unaffected. This dependency on the dnaB and the dnaG gene products was confirmed by complementation studies with enzyme preparations from conditional lethal E.coli mutants (data not shown). Furthermore ColE DNA synthesis was found to be markedly reduced when the incubation was carried out

Table 1. Effect of rifampicin on DNA synthesis.

Template ssDNA	^{3}H-dTMP incorporated (pmol)	
	Addition	
	RNA polymerase	Rifampicin
ColE1 H	48.7	49.7
ColE1 L	38.5	37.1
ColE3 H	26.3	22.1
ColE3 L	25.1	22.1
øX174	111.8	87.7
M13	212.8	3.9

Standard reaction mixtures (50 µl) supplemented with RNA polymerase (0.5 units) contained no rifampicin. Incubations were carried out with 0.3 µg single-stranded (ss) template DNA and replication enzyme fraction from E.coli H560 polA1 (240 µg protein) for 30 min at 30°C.

with a protein fraction prepared from a thermosensitive dnaC mutant (Table 3). The residual incorporation was stimulated severalfold by addition of purified dnaC protein. As expected, supplementing dnaC protein had a strong stimulatory effect on the øX system but not on M13 complementary strand synthesis. On the other hand, DNA synthesis could be primed efficiently by the

Table 2. Effect of dnaB and dnaG antisera on DNA synthesis.

Template ssDNA	^{3}H-dTMP incorporated (pmol)			
	Addition			
	dnaB		dnaG	
	Control serum	Anti-serum	Control serum	Anti-serum
ColE1 H	105.4	4.4	158.3	13.0
ColE1 L	76.9	12.0	132.7	20.2
ColE3 H	62.6	5.7	83.9	8.3
ColE3 L	41.3	5.4	52.2	9.5
øX174	94.5	2.7	98.1	6.2
M13	320.1	342.3	270.9	264.7

Enzyme fraction from E.coli H560 polA1 (240 µg protein in 10 µl) was preincubated with either 0.6 µl control serum or antiserum for 10 min at 0°C. Template DNA and reaction ingredients were then added and standard reaction mixtures (50 µl) incubated for 30 min at 30°C. In the case of M13 DNA rifampicin was omitted.

Table 3. Complementation of a dnaC receptor fraction.

	^{3}H-dTMP incorporated (pmol)		
	Addition		
	None	dnaC protein	Plasmid primase
ColE1 H	4.8	20.2	89.7
ColE1 L	3.5	13.7	50.6
ColE3 H	5.5	22.4	98.1
ColE3 L	3.7	14.5	50.0
øX174	2.1	34.1	153.6
M13	2.7	3.9	321.6

Standard reaction mixtures (50 µl) containing enzyme fraction from E.coli PC22 polA1 dnaC2 (160 µg protein) were incubated for 30 min at 30°C with or without addition of E.coli dnaC protein (2 µg) or IncIα-plasmid primase (5 ng) in the presence of rifampicin.

IncIα-plasmid primase (15,20) with all circular single-stranded DNA templates tested.

Characterization of the reaction products

The ColE1 DNA synthesized in vitro was subsequently characterized by velocity sedimentation in neutral and alkaline sucrose gradients. As shown in Fig. 2a for H strand DNA, the majority of the labeled DNA molecules synthesized by replication enzyme fraction from a polA1 mutant deficient in DNA polymerase I sedimented in a neutral sucrose gradient as open circular DNA. Alkaline gradient centrifugation of these molecules indicated that most of the radioactivity was present in linear strands of unit length (not shown). After addition of DNA polymerase I and DNA ligase to the incubation mixture a large portion of the reaction product was converted to closed circular DNA (Fig. 2b). Similar sedimentation patterns were observed with L strand DNA (not shown). To decide whether the strand interruption in the open circular molecules formed in the absence of DNA polymerase I was located in a unique position, we employed the labeling procedure of Nordheim et al. (21). No evidence was obtained for a unique location of the discontinuity in either H or L strand DNA (data not shown).

The newly synthesized ColE1 DNA was further analysed by isopycnic banding of the labeled DNA strands in poly(U,G)-CsCl gra-

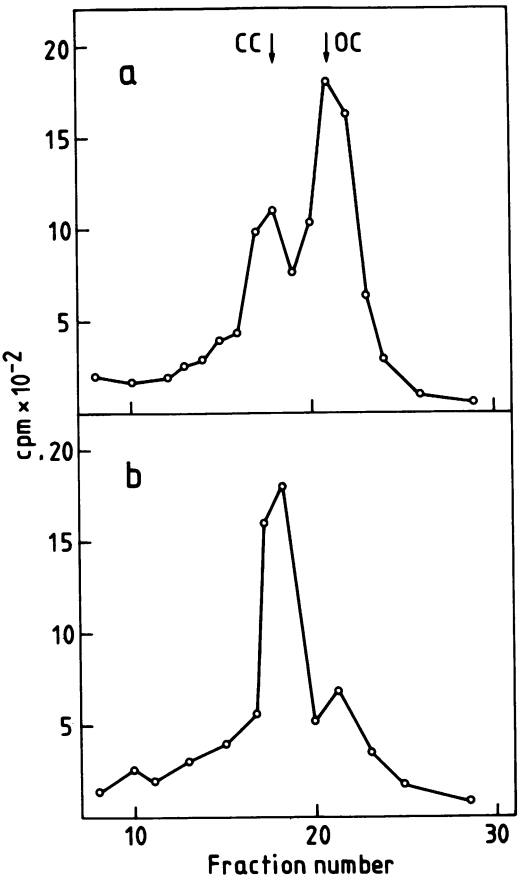

Fig. 2. Neutral sucrose gradient centrifugation of reaction products. Standard reaction mixtures (500 µl) containing ColE1 H strand circles (3 µg) and replication enzyme fraction from E.coli H560 polA1 (2.4 mg protein) were incubated for 30 min at 30°C without (a) or with (b) addition of E.coli DNA polymerase I (10 units) and T4 DNA ligase (2 units). Incorporation was stopped with 25 µl 0.5 M EDTA and the reaction mixtures were passed through a Sepharose 6B column (1.3 x 3.5 cm) in buffer A. Fractions containing ^{3}H-labeled DNA were pooled and centrifuged through 4-ml 5 - 20% sucrose gradients in 50 mM Tris-HCl pH 7.6 — 1 M NaCl — 5 mM EDTA in a Beckman SW60 rotor (50,000 rpm, 4 h, 4°C). Fractions were collected from the bottom of the tubes. Aliquots were spotted on filter paper disks and assayed for radioactivity. Sedimentation is from right to left. The arrows indicate the positions of closed circular (cc) form I and open circular (oc) form II DNA.

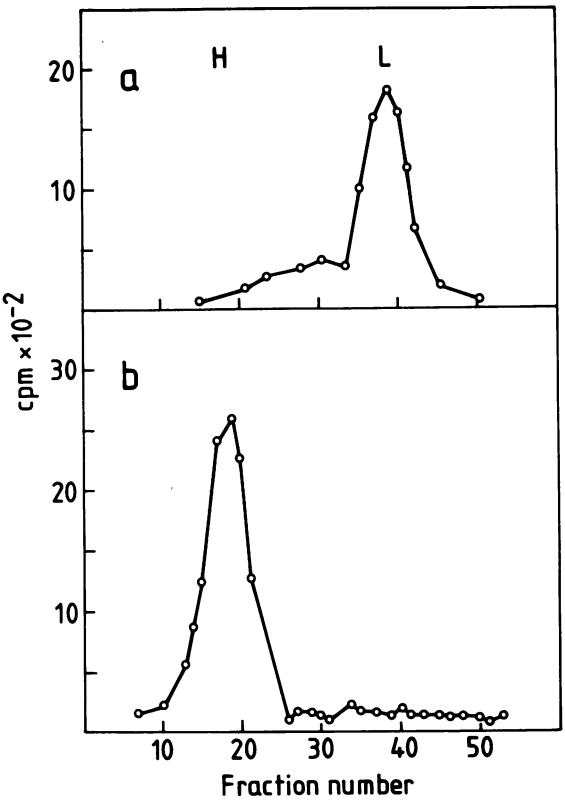

Fig. 3. Strand separation of reaction products. Standard reaction mixtures (500 µl) containing either 3 µg H strand circles (a) or L strand circles (b) of ColE1 were incubated and the reaction products fractionated by neutral sucrose gradient centrifugation as described in Fig. 2. Fractions containing closed circular DNA were pooled and dialysed against 50 mM Tris-HCl pH 7.6 — 50 mM KCl. 1-ml reaction mixtures were incubated with EcoRI (100 units) in the presence of 10 mM $MgCl_2$ for 15 min at 37°C. The incubation was stopped by addition of 50 µl 0.5 M EDTA. The samples were mixed with 850 µl 5 mM EDTA, 50 µl 5% Sarkosyl, and 50 µl poly(U,G) (2 mg/ml), heated for 5 min at 100°C, and chilled in ice. The sample volume was adjusted with 5 mM EDTA to 5 ml and 6.7 g CsCl were added. Centrifugation was performed in a Beckman Ti50 rotor at 40,000 rpm for 36 h at 15°C. Fractions were spotted on filter paper disks and assayed for radioactivity. Density increases from right to left. H and L indicate the positions of ^{32}P-labeled ColE1 single strands used as reference DNA.

dients. As shown in Fig. 3, the incorporated radioactivity was found exclusively in the respective complementary strand. This provides further evidence for the purity of the DNA strands employed and clearly demonstrates that both strands are functional as templates for the rifampicin-resistant initiation of DNA synthesis.

Restriction analysis of replicative intermediates

In order to obtain information as to the location of the starting points for DNA synthesis on the opposite strands of ColE1 DNA, the conversion of single-stranded DNA to duplex DNA was partially blocked by addition of the chain-terminator ddTTP. The replicative intermediates were then cleaved by double-digestion with the restriction endonucleases HaeII and EcoRI and the distribution of label between the various restriction fragments was determined by autoradiography as described in Fig. 4. For the interpretation of these results, it must be kept in mind that only fully synthesized restriction fragments are detectable as discreet bands. Nevertheless, the radioactivity profiles obtained for H and L strand directed DNA synthesis are distinctly different. With H strand circles, labeling of the HaeII-D fragment becomes most prominent upon increasing the ddTTP concentration (Fig. 4c), whereas in the case of L strands, the E fragment is preferentially labeled (Fig. 4f). Comparatively little label is found in the A1 and B fragments, even at low ddTTP concentrations.

These findings indicate, taking into account the opposite polarity of the strands, that preferred start sites for DNA synthesis are located on both ColE1 strands within the HaeII-C fragment (see Fig. 5). It can further be seen from the ColE1 cleavage map that the labeling patterns are clearly incompatible with unique priming events. Thus, DNA synthesis appears to be initiated on each strand non-randomly at multiple starting points.

DISCUSSION

By converting double-stranded plasmid DNA into circular single-strands the synthesis of plasmid DNA can be reduced to the simplicity of the single-stranded phage systems. It was found thereby that both strands of the ColE1 as well as the unrelated

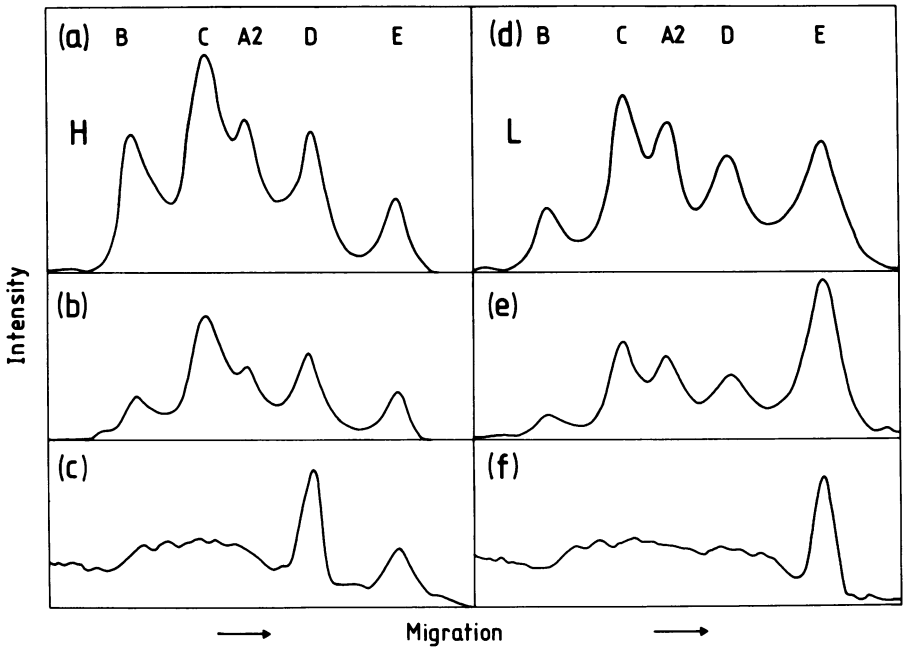

Fig. 4. Gel electrophoresis of cleavage products of replicative intermediates. 0.6 µg H strands (a - c) and L strands (d - f) of ColE1 were used as templates for DNA synthesis. Standard reaction mixtures (100 µl) contained ^{32}P-dCTP (40 µCi, 2500 Ci/mmol), unlabeled dTTP (12.5 µM), and ddTTP at the concentrations 5 µM (a,d), 25 µM (b,e), and 50 µM (c,f). After a 30-min incubation at 30°C the incorporation was stopped with EDTA and the reaction mixtures were passed through a Sepharose 6B column. Fractions containing labeled DNA were pooled, dialysed against 50 mM NH_4HCO_3 — 0.1 mM EDTA, lyophilized, and taken up in 10 mM Tris-HCl pH 7.5 — 10 mM NaCl — 10 mM $MgCl_2$ — 6 mM mercaptoethanol. Samples (30 µl) were mixed with 1 µg unlabeled ColE1 DNA and incubated with HaeII (8 units) for 2 h at 37°C. EcoRI (6 units) was then added, the NaCl concentration was raised to 50 mM, and the incubation continued for 4 h. Cleavage products were electrophoresed in a 1.4% agarose gel for 14 h at 20 mA. The gel was stained with ethidium-bromide and autoradiographed. The figure shows densitometer tracings of the autoradiogram. The letters indicate the positions of unlabeled HaeII / EcoRI restriction fragments. Fragment A1 migrates between C and A2. The small F fragment was not detectable.

ColE3 plasmid can function as templates for DNA synthesis by a replication enzyme fraction from E.coli. Considering the extensive homology between ColE2 and ColE3 DNA (22), one may expect that this holds true for all three ColE plasmids.

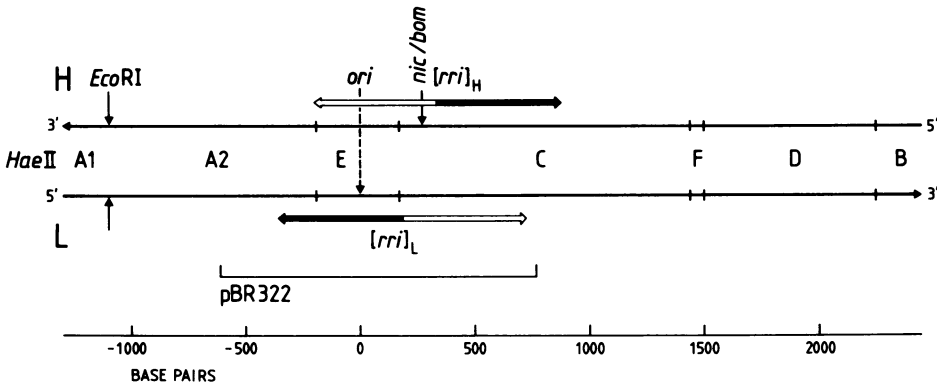

Fig. 5. Cleavage map of the ColE1 plasmid. The map is oriented with respect to the EcoRI site assigning the 5' terminus of the L strand to the left end. Only the left half of the plasmid is shown and the A1 and B fragments are therefore not drawn to scale. The bracket indicates the region of the pBR322 plasmid which is homologous with ColE1. ori: origin of vegetative replication, nic: location of the relaxation complex corresponding to the bom site required in cis for conjugal mobilisation (26), rri: probable sites for primosome assembly as deduced from the DNA sequence (see Fig. 6). Open arrows indicate the direction of primosome migration on each strand and full arrows show the direction of primosome-mediated DNA synthesis.

Like G4 and øX174 complementary strand synthesis priming of DNA synthesis on ColE single-strands does not involve RNA polymerase but is carried out by the DNA primase encoded by the dnaG gene. Furthermore rifampicin-resistant initiation of DNA synthesis on both H and L strands was shown to depend on the dnaB and dnaC gene products. In this respect ColE-directed DNA synthesis clearly differs from G4 complementary strand synthesis and strongly resembles the øX system. Although it remains to be shown whether the proteins n, n', n", and i are also involved, the presence of n' effector sites on both pBR322 strands (within the region homologous to ColE1 DNA) suggests that priming is carried out by the primosome complex described by Arai et al. (4). This of course does not exclude the possibility that a more detailed biochemical analysis will reveal replicon specific differences in primosome assembly.

The finding of preferred start sites for DNA synthesis within the ColE1 HaeII-C fragment prompted us to examine the published

sequences of that region for similarities with the origin of ϕX complementary strand synthesis (Fig. 6). As pointed out previously by Bastia (23,24) the nucleotide sequence downstream from the replication origin contains two prominent inverted repeats which have the potential of forming stem-loop structures in the single-stranded ColE1 DNA molecules. Moreover, the L-strand sequence in one of these inverted repeats located at the HaeII-E / HaeII-C junction (positions 161-171) bears a striking resemblance to a sequence found within the hairpin structure of the intercistronic region of ϕX DNA (positions 2314 - 2324). Furthermore, inspection of the DNA sequences at the 5'-termini of these hairpin regions reveals similar but not identical sequences of 12 nucleotides length. The homologous sequences are located in the H strand of the HaeII-C fragment at positions 382-371 and in the L strand of the HaeII-E fragment at positions 143-154 (see Fig. 6). Interestingly, in the L strand this sequence appears to be repeated further upstream towards the replication origin at positions 102 - 113. The nucleotide sequences are largely conserved in the pBR322

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                    5'                                                                    3'
ϕX174  (2297-2351)  TGTGAGGTTATAACGCCGAAGCGGTAAAAATTTTAATTTTTGCCGCTGAGGGGTT

ColE1 H (382-328)   TGTGAGCGGATGCCGGGAGCTGACAAGCCCGTCAGGGCGCGTCAGCAGGTTTTAG
pBR322 H (393-339)  TGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGG

ColE1 L (102-113)   TGTGAGTCAGTA
pBR322 L (113-124)  AGTGAGCTGATA

ColE1 L (143-197)   AGCGAGTCAGTGAGCGAGGAAGCGGAAAAGCGCCTGGACGTGCATTTTCTCCTTA
pBR322 L (154-208)  AGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTA
                                                        ↑
                                                      HaeII
Consensus           T
sequence :          A GYGAG...RTR
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Fig. 6. Nucleotide sequences of potential rri sites. Corresponding sequences of ϕX174 (28), ColE1 (23,24,29), and pBR322 (30) are shown. The ColE1 and pBR322 nucleotides are numbered from the origin of replication as defined by Tomizawa et al. (31). Bases are aligned with respect to a boxed concensus sequence presumably involved in recognition by the prepriming protein n' (R, purine bases; Y, pyridine bases). Underlined sequences represent regions of hyphenated dyad symmetry for which a base-paired secondary structure has been proposed (23,24,32).

plasmid and their positions are in agreement with the locations of n' effector sites determined by Hae-II restriction mapping (11). It should be noted however that the hairpin structure in the L strand spans the HaeII-E / HaeII-C junction and is consequently destroyed upon HaeII cleavage. Formation of a hairpin may therefore not be required for recognition by the n' protein.

On the basis of these data we propose that the boxed sequences shown in Fig. 6 represent n' recognition signals whereas the regions of hyphenated dyad symmetry located near the 3'-end of these sites are involved in subsequent primosome assembly. Both structures combined would thus constitute a rifampicin-resistant initiation site (rri). This conclusion is not necessarily in conflict with the results of Nomura and Ray (10), which indicate the occurrence of a priming site (rri-1) within the HaeII-E fragment of the ColE1 L strand. Conceivably, cloning of this fragment by insertion into the intergenic space of M13 could reconstitute a functional rri site either by regenerating the cleaved stem-loop structure or by joining the n' recognition signal of ColE1 to a hairpin present in the M13 origin region (25).

The location of an rri site in the L strand at the HaeII-E / HaeII-C junction just 170 nucleotides downstream from the replication origin (ori) is clearly consistent with the postulated function of the primosome in lagging strand synthesis of the Col E1 plasmid. Upon initiation of leading L strand synthesis at ori an early replicative intermediate with a D-loop structure is formed (6) which exposes the rri site on its single-stranded branch making it available for primosome assembly. Once assembled the primosome will migrate in the 5' → 3' direction on the parental L strand concomitant with replication fork movement thereby generating multiple primers for discontinuous synthesis of the lagging H strand. Furthermore, processive migration of the primosome may also facilitate leading strand synthesis by participating in the unwinding of the duplex at the replication fork (9).

The function of the rri site in the H strand of the HaeII-C fragment is less obvious. Zipursky and Marians (11) suggested that it might be involved in the switch from a continuous to a discontinuous mode of leading strand synthesis. However, such a

function is not easily reconciled with the established directionality of primosome movement (3) since any primosome attached to the H strand would migrate opposite to the direction of fork movement. Moreover, leading strand synthesis occurs in the absence of functional DNA primase and therefore does not require multiple priming events (9). On the other hand, the close vicinity of the rri sequence to the site of the relaxation nick (nic/bom) points to a possible role in transfer replication. A model has been proposed for ColE1 mobilisation according to which the H strand is nicked at the nic/bom site and displaced into the recipient (6,26). It seems therefore plausible that this rri site is required for the initiation of L strand synthesis on the transferred H strand in the process of mobilisation.

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