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Discriminatory function of ribonuclease H in the selective initiation of plasmid DNA replication

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ABSTRACT

The initiation stage of ColE1-type plasmid replication was reconstituted with purified protein fractions from Escherichia coli. The reconstituted system included DNA polymerase I, DNA ligase, RNA polymerase, DNA gyrase, and a discriminating activity copurifying with RNAase H (but free of RNAase III). Initiation of DNA synthesis in the absence of RNAase H did not occur at the normal replication origin and was non-selective with respect to the plasmid template. In the presence of RNAase H the system was selective for ColE1-type plasmids and could not accept the DNA of non-amplifiable plasmids. Electron microscopic analysis of the reaction product formed under discriminatory conditions indicated that origin usage and directionality of ColE1, RSF1030, and ClodF13 replication were consistent with the normal replication pattern of these plasmids. It is proposed that the initiation of ColE1-type replication depends on the formation of an extensive secondary structure in the origin primer RNA that prevents its degradation by RNAase H.

INTRODUCTION

On the basis of their replicative behavior the plasmids of enterobacteria can be divided into two broad categories: (i) Amplifiable plasmids, which continue to replicate in the absence of de novo protein synthesis and do not require any plasmid-specific replication protein, and (ii) non-amplifiable plasmids, the replication of which appears to be more or less tightly coupled to the synthesis of plasmid-encoded initiator protein(s). The most extensively studied plasmid of the first category is the ColE1 plasmid and therefore other amplifiable plasmids (pMB1, RSF1030, ClodF13) are commonly referred to as ColE1-type plasmids. Since the replication of ColE1-type plasmids can be efficiently carried out by cell-free extracts of Escherichia coli, these plasmids have been used as model systems for biochemical studies on plas-

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mid DNA synthesis (1,2,3).

Plasmid DNA synthesis appears to be regulated by the frequency of initiation and the enzymology of the initiation process is therefore of particular interest (4,5). For ColE1 it was found that replication is initiated by an RNA primer, which is synthesized by RNA polymerase and elongated by DNA polymerase I. Evidence has also been presented that processing of transcripts by RNAase III might be required for ColE1-type replication (6,7). On the other hand, Itoh and Tomizawa (8) reported that the initiation of ColE1 replication catalyzed by purified polymerases is markedly stimulated by RNAase H. It was subsequently shown that RNAase H is required for the processing of a pre-primer RNA at the origin of replication (9). Furthermore, an involvement of DNA gyrase in the initiation of ColE1 replication has been inferred from the striking sensitivity of this process to changes in the superhelicity of the template DNA (10).

The work reported in this paper was carried out in order to clarify the functions of the various enzymes implicated in the initiation of ColE1-type DNA replication. For that purpose we fractionated the crude replication system into defined protein fractions catalyzing partial reactions of the initiation process. We thereby obtained a "priming fraction" containing RNA polymerase and DNA gyrase, which supported efficient DNA synthesis when supplemented with DNA polymerase I. However, in contrast to the unfractionated system, DNA synthesis did not start at the normal (vegetative) origin and was not restricted to amplifiable plasmids. Selective DNA synthesis could be restored by addition of a "discriminating fraction" containing RNAase H (but free of RNAase III), which suppressed unspecific initiation events. It will be shown that under discriminating conditions origin usage and directionality of ColE1, RSF1030, and CloDF13 DNA synthesis are fully consistent with previous studies on vegetative plasmid replication. These results strongly suggest a discriminatory function of RNAase H in plasmid replication. An analogous role has been previously proposed for RNAase H in the replication of single-stranded phage DNA (11,12).

EXPERIMENTAL PROCEDURES

Nucleic acids. Plasmid DNA was isolated by established procedures (13,14) from the E.coli strains listed in Table 1. ^{3}H-poly(A) was from Amersham-Buchler, poly(dT) and poly(U) were purchased from P-L.

Enzymes and materials. DNA polymerase I and the restriction endonucleases EcoRI, HpaI, and SalI were obtained from BRL. T4-DNA ligase and creatine kinase were from Boehringer. Heparin-Sepharose CL-6B, DEAE-Sephacel, and Sephadex G-75 were from Pharmacia. Hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad. Sources of all other reagents have been described (18).

Plasmid DNA synthesis. Reaction mixtures (25 µl) contained 20 mM Hepes-KOH pH 8.0, 100 mM KCl, 10 mM Mg-acetate, 2 mM ATP, 0.4 mM each of CTP, GTP, and UTP, 0.025 mM each of dATP, dCTP, dGTP, and ^{3}H-dTTP (500 cpm/pmol), 0.05 mM NAD, 0.05 mM cAMP, 15 mM creatine phosphate, 0.1 mg/ml creatine kinase, 40 mg/ml plasmid DNA, 1 unit DNA polymerase I, 1 unit T4-DNA ligase, plus protein fractions as indicated. Incubations were performed at 30°C for 60 min and the incorporation of ^{3}H-dTMP into acid-insol-

Table 1. Bacterial strains and plasmids

Strain designation		Plasmid	Source (Reference)
C600	thr-1,leu-6,thi-1, supE44,lacY1,tonA21	pSC138	R.Eichenlaub (15)
C600		pRE300	R.Eichenlaub
C600		pSE014	S.Ely (16)
C600		pSC101	R.Diaz
YS10	thr,leu,minA,str, thi,end	ColE1	J.Tomizawa
W3110	thy	ColE3	T.Hashimoto-Gotoh
W3110	thy,ΔtrpES	pBR322	R.Eichenlaub
W1485	thy	RSF1030	J.Crosa
1100	endI	pKN182	R.Diaz (17)
P678-54	thr,leu,lacY,minA, minB,gal,str,thi	CloDF13	H.Nijkamp
BT1000	polA1,thy,str,end	F	F.Bonhoeffer

uble material determined as described previously (19).

RNA polymerase assay. Assay mixtures (25 µl) contained 20 mM Hepes-KOH pH 8.0, 100 mM KCl, 10 mM Mg-acetate, 0.2 mM each of ATP, CTP, GTP, and ^{3}H-UTP (60 cpm/pmol), 40 mg/ml plasmid DNA, and 0.4 mM K-phosphate pH 8.0. After 10 min incubation at 37°C the RNA synthesis was stopped by the addition of 0.5 ml of 5% trichloroacetic acid and the acid-insoluble radioactivity determined. One unit of enzyme incorporates 1 nmol of ^{3}H-UMP into acid-insoluble material in 10 min at 37°C.

RNAase H assay. Assay mixtures (25 µl) containing 20 mM Hepes-KOH pH 8.0, 100 mM KCl, 10 mM Mg-acetate, and 20 µM ^{3}H-poly(A)·poly(dT) (50 cpm/pmol) were incubated for 20 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 5% trichloroacetic acid and the remaining acid-insoluble radioactivity determined. One unit of enzyme produces 1 nmol of acid-soluble nucleotide from ^{3}H-poly(A)·poly(dT) in 20 min at 37°C.

RNAase III assay. Assay mixtures (25 µl) contained 20 mM Hepes-KOH pH 8.0, 100 mM KCl, 10 mM Mg-acetate, and 30 µM ^{3}H-poly(A)·poly(U) (20 cpm/pmol). After incubation for 20 min at 37°C the reaction was terminated by adding 0.5 ml of 5% trichloroacetic acid and the acid-insoluble radioactivity was determined. One unit of enzyme produces 1 nmol of acid-soluble nucleotide from ^{3}H-poly(A)·poly(U) in 20 min at 37°C.

Electron microscopy. Spreading of DNA was carried out in the presence of formamide as described by Morris et al. (20). DNA was picked up from the hyperphase on freshly prepared collodium coated copper grids. The grids were platinium-iridium shadowed and DNA molecules photographed with a Philips 301 electron microscope at a magnification of 11,000. Replicated and unreplicated regions of the molecules were measured with an electronic planimeter (Numonics graphics calculator). The total length of the DNA was taken as 100% and the variance in size did not exceed 5%.

Protein fractionation. Frozen cells (71 g) of E.coli BT1000 were lysed and a crude extract was prepared as described by Bouché et al. (21). All further operations were performed at 0 - 2°C. The KCl concentration of the extract was adjusted to 0.25 M and Mg-acetate was added to a final concentration of 5 mM. Ribosomes were removed by centrifugation at 100,000 x g for 4 hours. The

high-speed supernatant (fraction I, 90 ml, 1.88 g protein) was adjusted with ammonium sulfate (0.22 g/ml) to 33% saturation and the precipitate was removed by centrifugation. The supernatant was brought to 50% saturation with ammonium sulfate (0.14 g/ml) and the precipitate was collected by centrifugation and dissolved in buffer A (25 mM Hepes-KOH pH 8.0 — 50 mM KCl — 1 mM EDTA — 1 mM dithiothreitol — 10% (w/w) ethylene glycol). The ammonium sulfate fraction (33 - 50% saturation) was dialysed overnight against buffer A and diluted with the same buffer to a protein concentration of 10 mg/ml (fraction II, 137 ml, 1.37 g protein). Fraction II was applied to a Heparin-Sepharose column (2.6 x 6.5 cm) equilibrated with buffer A. The column was washed with 2 volumes of buffer A and then eluted with 0.8 M KCl. The eluate (fraction III, 40 ml, 296 mg protein) was dialysed against buffer B (0.1 M K-phosphate pH 6.8 — 0.1 M KCl — 1 mM dithiothreitol — 10% (w/w) ethylene glycol) and loaded onto a hydroxylapatite column (2.6 x 6 cm) equilibrated with buffer B. After washing the column with 2 volumes of buffer B bound proteins were eluted with 0.5 M K-phosphate pH 6.8 and dialysed against buffer A (fraction IV, 15 ml, 75 mg protein). Protein concentrations were determined by the method of Spector (22).

RESULTS

Fractionation of the replication system

Conrad and Campbell (23) have reported previously that the ColE1 replication system can be partially purified by precipitating the soluble proteins from crude extracts of E.coli with ammonium sulfate (0 - 75% saturation). In order to dissect the replication apparatus into proteins required for the initiation and elongation stage of plasmid replication respectively, we carried out a fractionated precipitation with ammonium sulfate. Confirming earlier observations (18,24) we found that DNA polymerase III as well as the other proteins involved in the elongation stage precipitated at low ammonium sulfate concentrations (0 - 33% saturation). On the other hand, all the enzymes required for the initiation stage remained in the supernatant, which was further divided into fractions precipitating at 33 - 50% and 50 - 75% satu-

ration of ammonium sulfate. The latter fraction, which contained the bulk of DNA polymerase I activity, could be replaced by the purified enzyme, since the corresponding fraction from a polA1 mutant had no stimulatory effect on plasmid DNA synthesis.

The replication proteins precipitated with ammonium sulfate at 33 - 50% saturation (fraction II) were further purified approximately 20-fold by step-wise elution from Heparin-Sepharose and hydroxylapatite columns. Upon complementation with DNA polymerase I this protein preparation (fraction IV) supported the synthesis of ColE1 DNA to a similar extent as fraction II indicating that no essential replication function had been removed by the purification procedure. The incorporation was characterized as replicative DNA synthesis distinct from DNA polymerase I catalyzed nick translation by its sensitivity to rifampicin and novobiocin (data not shown). Furthermore this enzyme system was found to be selective for ColE1-type replicons and could not initiate DNA synthesis on mini-R1 plasmids, the in vitro replication of which is tightly coupled to protein synthesis (17).

Fraction IV was further fractionated by chromatography on DEAE-Sephacel. As shown in Fig.1, a priming activity for DNA polymerase I dependent plasmid DNA synthesis coeluted from the column with RNA polymerase and DNA gyrase. Surprisingly, this "priming fraction" no longer discriminated between different groups of plasmids and was also active with mini-R1 DNA. It was found, however, that discrimination between ColE1 and R1 DNA could be restored by addition of the flow-through from the DEAE-Sephacel column containing both RNAase III and RNAase H activity. Taking advantage of their different molecular weights these two enzymes could be fully separated by passing the concentrated flow-through of the DEAE-Sephacel column through a Sephadex G-75 column. It can be seen from the gel filtration profile (Fig.2) that the discriminating activity copurified with RNAase H, whereas fractions containing RNAase III had no effect on plasmid DNA synthesis.

Although less than 0.03% of the total protein from the high-speed supernatant remained in the RNAase H fraction (specific activity 3,200 units/mg), the enzyme preparation was not electrophoretically pure and contained several polypeptides with mole-

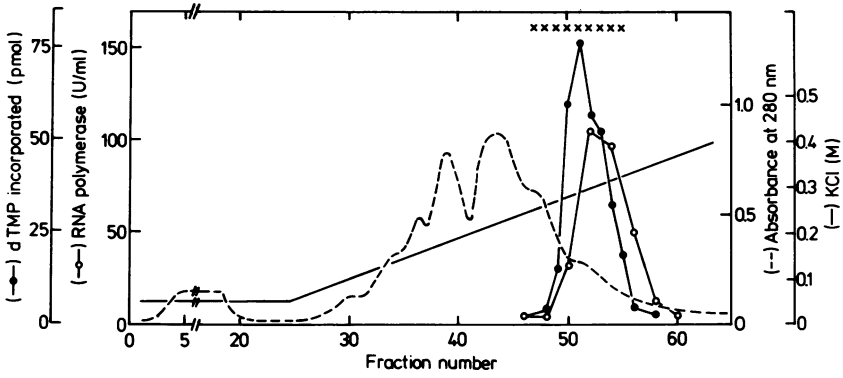

Fig. 1. DEAE-Sephacel chromatography. Protein fraction IV was applied to a DEAE-Sephacel column (0.9 x 8 cm) equilibrated with buffer A. The column was washed with 2 volumes of buffer A and then developed with a 40-ml gradient of 0.05 - 0.6 M KCl. Fractions (1 ml) were collected and assayed for priming of DNA synthesis (-•-) and RNA polymerase (-o-). Supercoiling activity (x x x) was assayed with relaxed ColE1 DNA as described previously (39). Fractions stimulating DNA synthesis were pooled and dialysed against buffer A (fraction Va = priming fraction, 4.7 ml, 2.8 mg protein). The flow-through was also collected and concentrated by dialysis against 20% (w/v) polyethylene glycol in buffer A (fraction Vb, 2.4 ml, 14.4 mg protein).

cular weights around 20,000 (data not shown). This RNAase H preparation was therefore designated as "discriminating fraction", since a function of the protein contaminants in the selective initiation of plasmid DNA synthesis cannot be excluded (11,12).

Analysis of ColE1 DNA synthesis

The discriminating fraction affected differently plasmid DNA synthesis (catalyzed by DNA polymerase I and priming fraction) depending on the amount of protein added to the system. Thus addition of small quantities of the RNAase H containing fraction had no selective effect but stimulated both ColE1 and R1 DNA synthesis (Fig.3). However, increasing the amount of discriminating fraction resulted in a complete suppression of R1 DNA synthesis, whereas the synthesis of ColE1 DNA was only slightly reduced.

ColE1 DNA synthesis occurring under discriminatory conditions continued at a nearly linear rate for more than two hours at 30°C. The incorporation was strongly inhibited by rifampicin and novobiocin indicating an involvement of RNA polymerase and DNA gyrase (Fig.4). The rifampicin-insensitive background synthesis

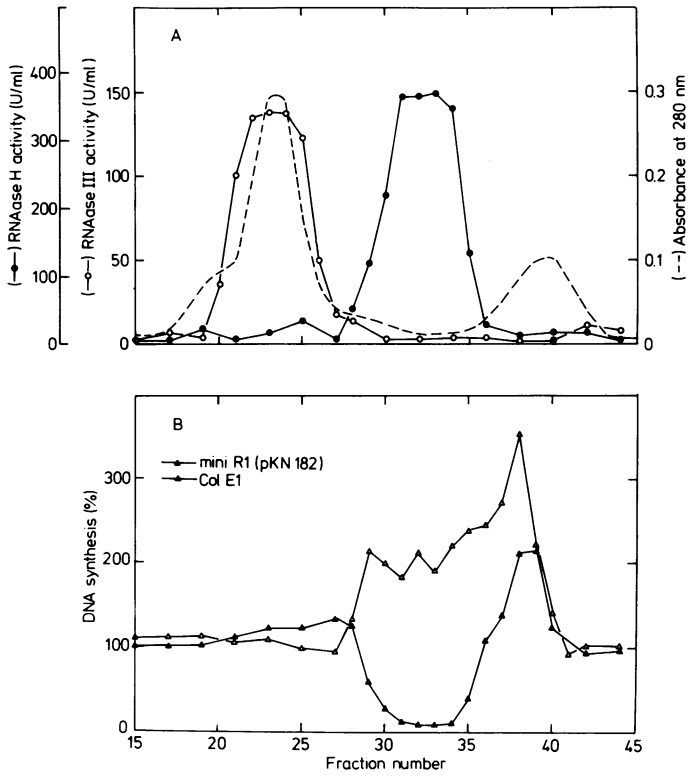

Fig. 2. Sephadex G-75 gel filtration. Fraction Vb was filtered at a flow rate of 6 ml/h through a Sephadex G-75 column (1.6 x 80 cm) equilibrated with buffer A and 3-ml fractions were collected. The elution profiles of RNAase III (-o-) and RNAase H (-●-) activities are plotted in panel A. Aliquots (2.5 µl) of the fractions were tested for their effect on plasmid DNA synthesis (panel B) in the presence of priming fraction (3 µg protein) with either ColE1 (-Δ-) or pKN182 DNA (-▲-) as template. 100% synthesis corresponds to a dTMP incorporation of 42 pmoles (ColE1) or 53 pmoles (pKN182) respectively. Fractions containing discriminatory activity (measured by the selective inhibition of pKN182 directed DNA synthesis) were pooled, concentrated, and dialysed against buffer A (fraction VI = discriminating fraction, 1.1 ml, 0.55 mg protein).

was increased if DNA ligase was omitted from the reaction mixture. It probably resulted from nick translation carried out by DNA polymerase I at single-strand interruptions in the template DNA. Omission of the discriminating fraction had little or no effect on the total incorporation and its sensitivity to antibiotics.

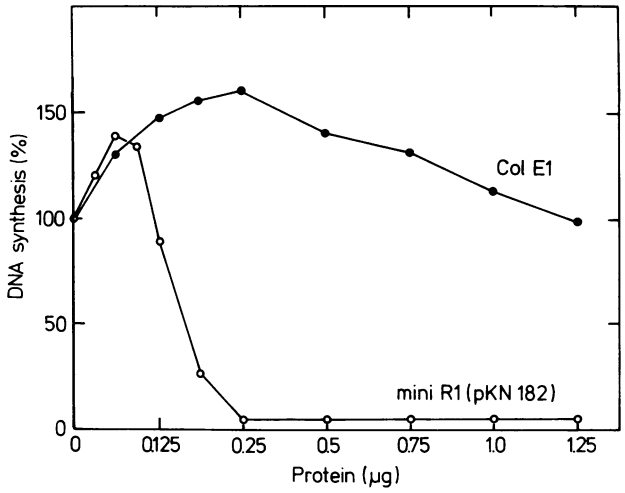

Fig. 3. Effect of discriminating fraction on plasmid DNA synthesis. Reaction mixtures (25 µl) contained priming fraction (3 µg protein) and either ColE1 DNA (-●-) or pKN182 DNA (-o-). Discriminating fraction was added as indicated. 100% synthesis corresponds to a dTMP incorporation of 48 pmoles (ColE1) or 55 pmoles (pKN182) respectively.

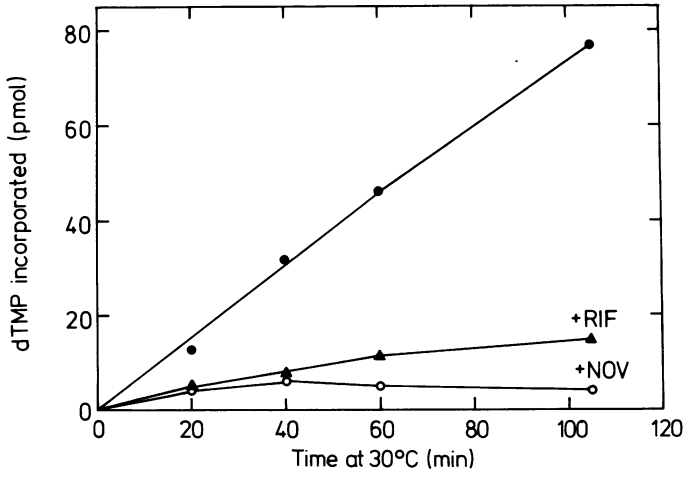

Fig. 4. Kinetics of ColE1 DNA synthesis. Reaction mixtures (125 µl) containing priming fraction (15 µg protein) and discriminating fraction (6 µg protein) were incubated without addition of inhibitors (-●-), with rifampicin (25 µg/ml) added (-▲-), or with novobiocin (25 µg/ml) added (-o-). At the times indicated aliquots (25 µl) were removed and the acid-insoluble radioactivity determined.

Product analysis in alkaline sucrose gradients indicated that the radioactive label was incorporated almost exclusively into DNA chains of less than unit length (data not shown). The size of the newly synthesized DNA chains increased with time but even after one hour of incubation no completely replicated closed-circular DNA molecules were detectable. In the absence of discriminating fraction the average length of the labeled DNA chains was considerably smaller than under discriminatory conditions (see also Fig.6) although the total amount of DNA synthesis was about the same. Apparently, the omission of RNAase H caused an increase in the number of growing points accompanied by a reduced rate of chain growth.

When the reaction products were examined in the electron microscope, up to 20% of the total DNA molecules appeared as θ-shaped replication intermediates. These structures were converted by cleavage with EcoRI into linear molecules containing a bubble of replicated DNA bound by two unreplicated arms. The replication bubbles consisted either of one double-stranded and one single-stranded branch (D-loop) or two double-stranded branches (eye-form). Both types of intermediates were observed with similar frequencies. If RNAase H had been omitted from the reaction mixture, the bubbles were rather small and located in different parts of the ColE1 DNA (Fig. 5a-c). Replication bubbles formed under discriminatory conditions were more heterogeneous in size but the distance between the EcoRI site and the proximal branch point of the bubble was approximately constant (Fig. 5d-f).

Around 90% of the replicative intermediates synthesized in the presence of RNAase H could be oriented with respect to the unreplicated arm of constant length and ordered in a sequence consistent with an origin located at a distance of 16.6 ± 3.7% genome length from the EcoRI site with replication proceeding unidirectionally away from the restriction site (Fig.6A). Under discriminatory conditions origin usage and directionality of replication are therefore in full agreement with the pattern observed previously in vivo (25,26) and in cell extracts (27). On the other hand, the partially replicated molecules formed in the absence of the discriminating fraction could not be arranged in a series compatible with a unique origin of DNA synthesis (Fig.6B).

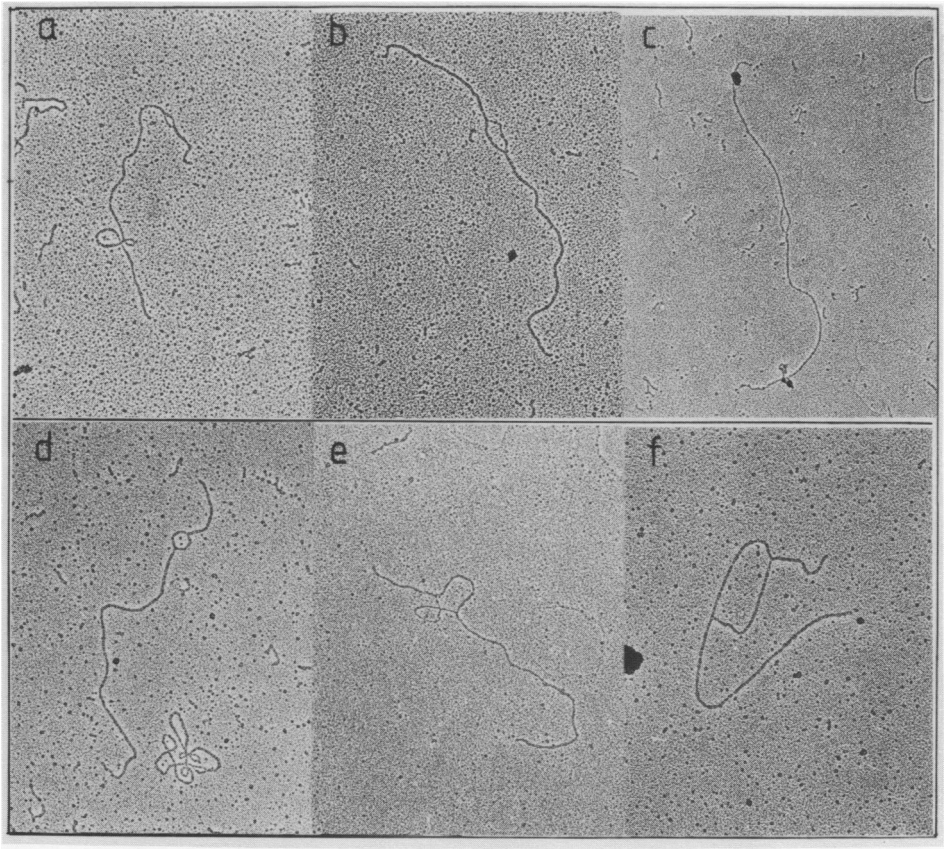

Fig. 5. Electron micrographs of partially replicated ColE1 DNA. DNA synthesis was carried out in reaction mixtures (125 µl) containing priming fraction (15 µg protein) in the absence (a - c) or presence (d - f) of discriminating fraction (6 µg protein). The incubation was terminated after 60 min at 30°C by addition of EDTA to a final concentration of 20 mM. Samples were extracted with phenol, precipitated with ethanol, and digested with EcoRI.

It can be seen from the histogram that under non-discriminatory conditions DNA synthesis occurred in three preferred regions of the plasmid DNA, none of which included the normal origin. It should be noted that these regions have only been located with respect to one reference point and thus cannot be unambiguously correlated with the physical map of ColE1 DNA.

Selectivity of DNA synthesis

The discriminating fraction was originally characterized by

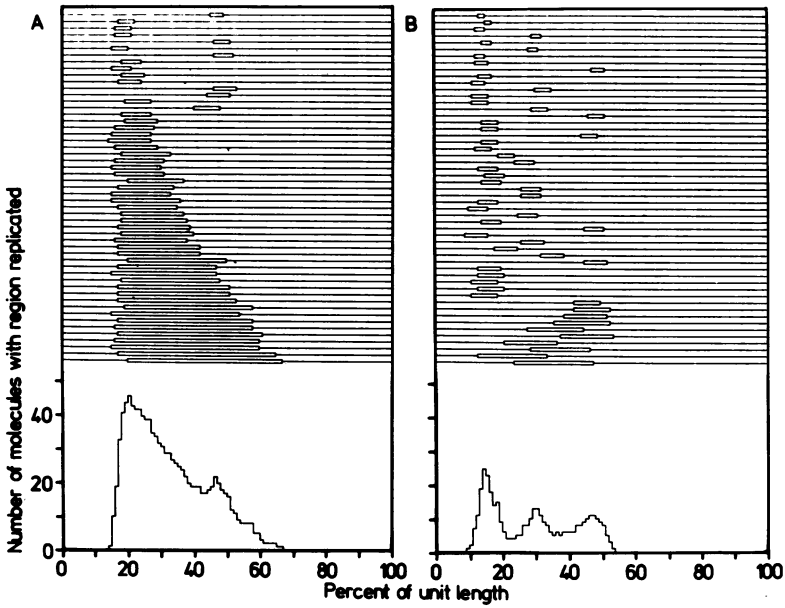

Fig. 6. Line drawings and histograms of partially replicated ColE1 DNA. DNA synthesis was carried out in the presence (A) or absence (B) of discriminating fraction as described in the legend to Fig. 5. The EcoRI cleaved DNA molecules are aligned in order of increasing extent of replication with the short unreplicated region to the left. Measurements for each molecule are presented in terms of percent of total molecular length.

its differential effect on ColE1 and R1 DNA synthesis (see Fig.3). It was therefore of interest to determine whether this protein fraction can in general differentiate between amplifiable and non-amplifiable plasmids. The data summarized in Table 2 indicate that this is the case. Except for ColE3, all plasmids tested supported a comparable amount of incorporation when incubated with DNA polymerase I and priming fraction. However, in the presence of discriminating fraction replicative DNA synthesis was observed with the amplifiable plasmids ColE1, pBR322, RSF1030, and CloDF13, but not with the non-amplifiable plasmids pSC101, pKN182, pSE014, and pSC138. (The residual incorporation into pSC101 and pSC138 DNA represented repair-type synthesis, since it could not be inhibited with rifampicin or novobiocin.) Unexpectedly, the mini-F plasmid pRE300, which is derived from an F inc$^-$ mutant

Table 2. Selective inhibition of plasmid DNA synthesis

Template DNA	^{3}H-dTMP incorporated (pmol)	
	Addition	
	None	Discriminating fraction
ColE1	50.8	86.5
ColE3	5.3	4.5
pBR322	48.3	60.6
RSF1030	49.9	62.1
CloDF13	54.0	24.2
pSC101	53.2	7.8
miniR1(pKN182)	60.0	2.4
miniR6-5(pSE014)	44.8	2.5
miniF(pSC138)	55.0	5.4
miniF(pRE300)	41.6	35.4

DNA synthesis was carried out for 60 min at 30°C in reaction mixtures (25µl) containing priming fraction (3µg protein) in the absence or presence of discriminating fraction (0.5µg protein).

(28), retained its template activity in the presence of RNAase H. Further characterization of this plasmid revealed, that pRE300 continues to replicate in the presence of chloramphenicol and may therefore be considered a novel ColE1-type replicon (R.Eichenlaub, unpublished).

Origin usage of the plasmids RSF1030 and CloDF13 was determined by electron microscopic analysis of partially replicated molecules after incubation under discriminatory conditions. For the introduction of reference points RSF1030 DNA was cleaved with HpaI (29) and CloDF13 DNA was cut with SalI (30). Examination in the electron microscope of DNA samples treated with the respective restriction enzyme showed numerous molecules with a replication bubble and two unreplicated arms. Electron micrographs of some of these molecules are presented in Fig.7. In case of RSF 1030 the shorter unreplicated arm of the linearized intermediates was of constant length (Fig. 7a-c), whereas for CloDF13 the length of the longer unreplicated arm was approximately constant

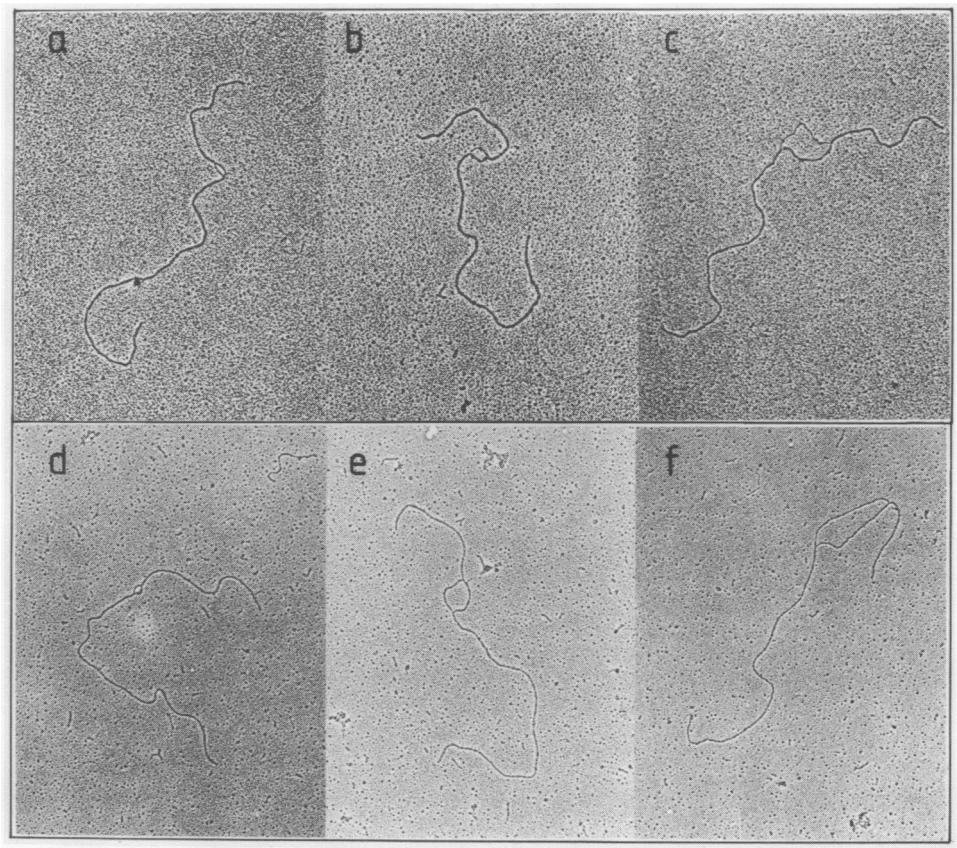

Fig. 7. Electron micrographs of partially replicated RSF1030 and CloDF13 DNA. DNA synthesis was performed in reaction mixtures (125 µl) containing priming fraction (15 µg protein) and discriminating fraction (6 µg protein) with either RSF1030 or CloDF13 DNA as template. The reaction product was isolated as described in the legend to Fig. 5 : (a - c) RSF1030 DNA cleaved with HpaI, (d - f) CloDF13 DNA cleaved with SalI.

(Fig. 7d-f). The replicating DNA molecules of both plasmids could thus be arranged in sequences compatible with a unidirectional mode of replication from a single origin as indicated by the line diagrams shown in Fig.8. The RSF1030 origin was located at 28.7 ± 1.6% genome length from the HpaI site with replication progressing away from the restriction site (Fig.8A). CloDF13 DNA synthesis started at a distance of 35.7 ± 1.4% molecular length from the SalI site and proceeded towards this site (Fig.8B). For both

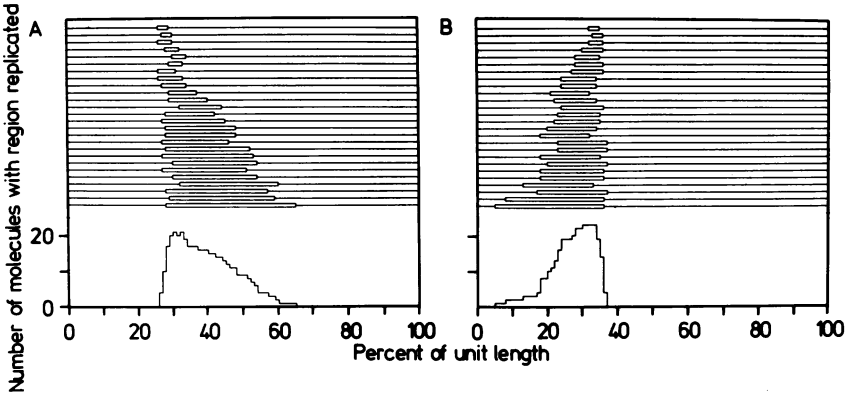

Fig. 8. Line drawings and histograms of partially replicated RSF1030 and CloDF13 DNA. DNA synthesis was carried out as described in the legend to Fig. 7. HpaI cleaved RSF1030 molecules (A) and SalI cleaved CloDF13 molecules (B) are arranged in order of increasing extent of replication with the short unreplicated region to the left. Measurements for each molecule are presented in terms of percent of total molecular length.

plasmids origin localization and directionality of replication were found to be fully consistent with the previously established mode of replication (29,30).

DISCUSSION

In this paper we have shown that the initiation stage of ColE1-type plasmid replication can be reconstituted with purified protein fractions. The reconstituted system consists of DNA polymerase I complemented with a priming and a discriminating fraction. The main components of the priming fraction are RNA polymerase and DNA gyrase, whereas the only enzymatic activity detected in the discriminating fraction is RNAase H.

RNA polymerase is obviously required for the rifampicin-sensitive synthesis of a primer RNA to be elongated by DNA polymerase I (9). However, in order to function as a primer for DNA synthesis, the 5'-terminus of the transcript must remain associated with the template DNA. According to the model of the ternary transcription complex proposed by Rohrer and Zillig (31), RNA polymerase unwinds 20 - 40 base pairs of the DNA double-helix forming a hybrid between the template strand and the nascent RNA while displacing

the nontranscribed strand. In a non-supercoiled DNA molecule the base pairing between the RNA and the transcribed DNA strand is unstable and the transcript dissociates from the template, restoring the DNA duplex as the RNA polymerase moves on. For a negatively supercoiled DNA, on the other hand, the torsional strain in the DNA molecule favours the disruption of the DNA base pairs and an RNA-DNA helix of appreciable length may form (32). Such hybrid structures could then be further stabilized as a result of intrastrand base pairing in the exposed single-stranded DNA. One might therefore expect the formation of RNA-DNA hybrids to occur preferentially in regions of supercoiled DNA with a short two-fold rotational symmetry, which allows the formation of stem-loop structures in the nontranscribed DNA strand.

Supercoiling by DNA gyrase not only stabilizes the template-primer complex but also facilitates the elongation of the primer by providing a driving force for the unwinding of the parental DNA strands (33,34). Since the unwinding of one turn of the double-helix is compensated by the removal of one negative superhelical turn, only a limited amount of DNA synthesis is possible in the absence of gyrase function. Thus Itoh and Tomizawa (8) observed that chain elongation by DNA polymerase I stopped near the point at which the plasmid DNA no longer contained superhelical turns. These authors concluded that gyrase activity increased only the length but not the number of initiated DNA chains. It must be kept in mind, however, that in the absence of relaxing topoisomerases, DNA gyrase increases the superhelix density of native form I DNA by a factor of about 1.5 (35). The correlated increase in superhelix energy of the template DNA should markedly stimulate the formation of RNA-DNA hybrids, and therefore increase the initiation frequency under reaction conditions in which the concentration of primed template is limiting.

In the presence of DNA gyrase the elongation of the primer RNA by DNA polymerase I resulted in DNA chains of up to one genome length. The newly synthesized DNA is therefore considerably longer than the 6 S pieces of the early intermediate DNA formed in crude extracts. This indicates that the size limitation of these initiation fragments is not caused by some intrinsic property of DNA polymerase I, but results from its interaction with other

proteins not present in the purified system (1). It should be noted, however, that even under optimal reaction conditions no fully replicated DNA strands which could be closed by DNA ligase were formed. Apparently, the purified system is deficient in the termination stage of plasmid replication.

RNAase H, a ribonuclease which degrades RNA specifically in RNA-DNA hybrid structures, is presumably the essential component of the discriminating fraction. It has previously been reported that this enzyme acts as a stimulatory factor for ColE1 DNA replication by carrying out the cleavage of the pre-primer RNA at the replication origin (8,9). In the present work a non-selective stimulation of RNA primed DNA synthesis was observed only at low RNAase H concentrations. Increasing the amount of RNAase H eliminated the stimulatory effect concomitant with the removal of all RNA primers except for those associated with the origin region of amplifiable plasmids.

This discriminatory effect of RNAase H must be based on some

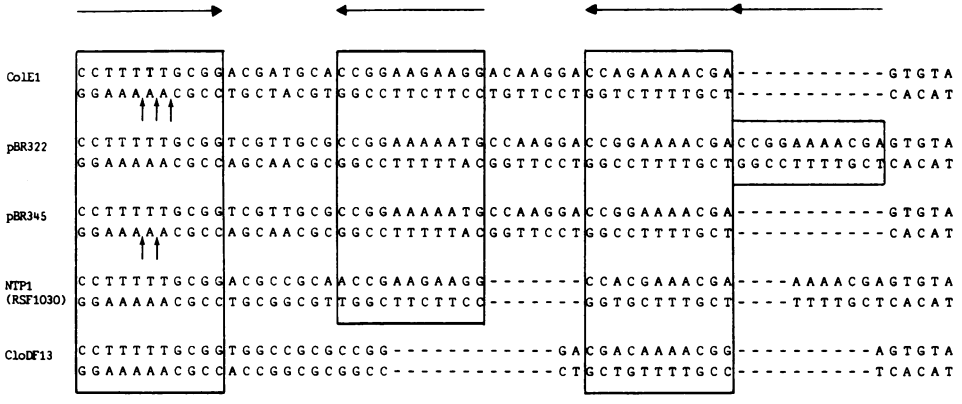

Fig. 9. Nucleotide sequences from the origin region of amplifiable plasmids. Corresponding sequences of ColE1 (40,41), pBR322 (36), pBR345 (42), NTP1 (43), and CloDF13 (44) are aligned for maximum homology. Plasmids pBR322 and pBR345 are both derived from pMB1 (37), NTP1 is considered to be identical with RSF1030 (43). The upper strand is 3' → 5' and the lower strand is complementary 5' → 3'. Horizontal arrows indicate the orientation of nucleotide sequences (in boxes) homologous to the origin sequence. The identified RNA/DNA transition points (41,42) are denoted by vertical arrows. The direction of replication is from left to right with the lower strand as leading strand.

structural peculiarity of the RNA-DNA hybrids formed at the origin of ColE1-type plasmids, which stabilizes them and protects them against degradation. Inspection of the origin sequences of amplifiable plasmids reveals that all of them contain within a short distance downstream from the origin an 11-bp sequence, which is of close homology but opposite orientation to the nucleotide sequence surrounding the primer RNA/DNA transition point (Fig.9). The replication origin of ColE1-type plasmids has therefore the potential of forming a stem-loop structure as previously suggested by Sutcliffe (36). Interestingly, these plasmids differ in containing either one (CloDF13), two (ColE1, pBR345, and NTP1), or three (pBR322) copies of the inverted origin sequence. This variation could arise from faulty replication and/or recombination provoked by base pairing between the origin and the inverted sequence. Furthermore, a large symmetrical sequence was noted by Backman et al. (37) in the region preceding the ColE1 origin. These authors have shown that the entire DNA segment between the origin and the proximal gyrase binding site (38) can form an extensive hairpin structure. This sequence is nearly completely conserved in the pMB1-derived plasmids and in NTP1 (RSF1030). An analogous stem-loop structure is also possible for CloDF13 with an additional DNA segment of about 40 nucleotides inserted in the loop region (see Fig.10).

Taking into account these symmetry elements of the origin DNA as well as the processing pattern of the primer RNA established by Itoh and Tomizawa (9), we propose the structure shown in Fig. 10A for the RNA-DNA hybrid at the origin region of ColE1. A similar secondary structure can also be drawn for CloDF13 in spite of extensive sequence alterations (Fig.10B). The proposed structure has two implications, which could help to explain the selective initiation of ColE1-type replication in the presence of RNAase H: (i) The association of the nascent primer RNA with the transcribed DNA strand is induced and/or stabilized by the formation of a stem-loop in the parental leading strand DNA at the origin; (ii) the protection of the processed primer against complete degradation by RNAase H is mediated by an extensive secondary RNA structure upstream from the origin. Such a nucleic acid structure could be sufficient by itself to stabilize the template-primer complex

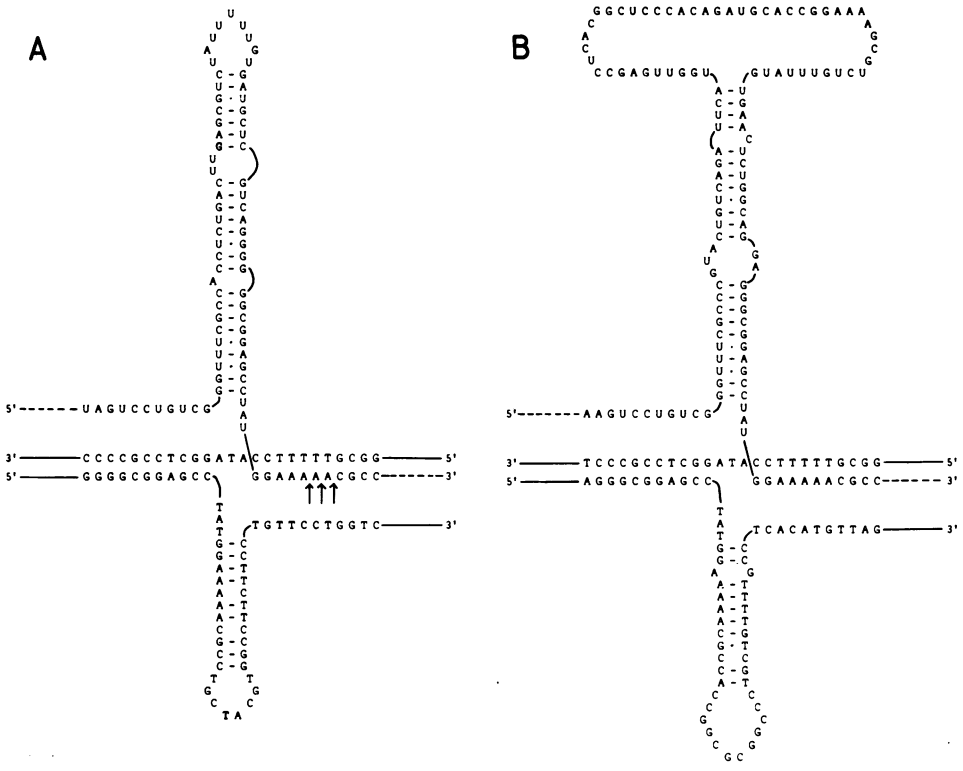

Fig. 10. Proposed structure of the template-primer complex at the origin region of ColE1 (A) and CloDF13 (B). DNA and RNA chains are presented as solid and dashed lines respectively. Vertical arrows indicate the RNA/DNA transition points.

or alternatively some specific protein factor(s) might also be required. As shown by Vicuna et al. (11,12) two additional discriminatory proteins are needed, together with RNAase H, for the selective initiation of filamentous phage complementary strand synthesis. The presence of these or analogous proteins in our enzyme fractions cannot be excluded. Finally we wish to point out that the secondary structure of the primer RNA (depicted in our model) should interfere with its removal by the 5' → 3' exonuclease function of DNA polymerase I. Termination of plasmid replication might therefore require additional nuclease activities.

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