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Role of the RepA1 Protein in RepFIC Plasmid Replication

RENATA MAAS* AND CHI WANG

Department of Microbiology, New York University Medical Center, New York, New York 10016

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Using a sensitive primer extension technique, we have carried out studies to localize the start site of replication of the replicon RepFIC. In the course of these studies, we have found evidence that supports the hypothesis that transcription is an integral component of the initiation of replication. On the basis of our findings, we suggest that the transcript is processed to act as a primer, and therefore we propose that the transcript has a dual role as primer of replication and mRNA for the RepA1 protein. We present a model, based on our evidence, for the initiation of replication of the replicon RepFIC. This model provides as well an alternative explanation for what has been called the *cis* action of RepA1, and we show that RepA1 may act in *trans* as well as in *cis*.

Plasmid EntP307 is a large (110-kb) conjugative plasmid that was isolated from the porcine *Escherichia coli* strain P307. This plasmid codes for two enterotoxins (heat labile and heat stable) as well as those functions necessary for its own maintenance and transmission. EntP307, like many plasmids of the IncF group, contains two replication regions or basic replicons (16), which are representative of the two types of replication control found in plasmids, namely, the iteron type and the antisense type. One (RepFIB), which we have used extensively in these studies as a cloning vector, is of the iteron type and is similar in its manner of control to the replicon RepFIA (mini-F) of F and to the basic replicons of R6K, RK2, and P1 (19). The other, RepFIC, which is the object of this study, is of the antisense type and is similar in its manner of control to the replicons of plasmids R1 and R100 in *E. coli*, and ColIb-P9 in *Shigella*. The latter plasmids, including RepFIC, do not contain origin iterons and may therefore control their replication by a mechanism different from that used by iteron-type plasmids.

Work on antisense replicons from several laboratories has led to some general conclusions that are illustrated in Fig. 1 and summarized below (6, 15, 18, 24, 25). In their wild-type state, for instance, all of these plasmid replicons have been reported to occur in a low copy number of two to three per cell. They consist of a promoter followed by three cassettes, one (cassette I) which is dispensable and two (cassettes II and III) which are absolutely required (Fig. 1). The dispensable cassette consists of the repressor protein RepA2 and its target, the promoter Pa. This cassette is present in the wild-type replicons of R1 and R100 and in RepFIC but is absent in the cloned replicon of ColIb-P9. Cassette II includes a regulatory countertranscript characteristic of antisense replicons and the second open reading frame, uORF (upstream ORF) (see below). Cassette III comprises the structural gene of the Rep protein proper (called RepA1) and the minimal origin (cassette III will be referred to as the RepA-ori cassette). The RepA1 proteins of different antisense replicons have specificity for the origin, since each known type of Rep protein occurs with its own type of origin, always in the 5′→3′ order RepA1-origin (3).

The summary that follows is intended to put our findings in the context of what is known about plasmid replication. In the replicon RepFIC, like in R1 and R100, the promoter Pc gives rise to a transcript that in turn can be translated into three polypeptides, all of which have been shown to be expressed, while the promoter Pa gives rise to a shortened version of the transcript and codes only for the two downstream polypeptides uORF and RepA1. RepA2, which is translated only from the longer transcript, is a small protein of an approximate molecular weight (MW) of 11,000 and is a classical repressor of transcription. Its function is to switch off the downstream promoter Pa. The transcripts which are generated from either promoter terminate in the mid region of the indicated origin. The significance of transcription of the part of the origin immediately following the *repA1* structural gene will become apparent as we present our findings. Regulatory cassette II, as mentioned above, includes the second ORF (uORF) and the countertranscript shown in Fig. 1. The uORF codes for a 24-amino-acid polypeptide which precedes and overlaps by two amino acids the absolutely required initiator of replication RepA1 (for a review see reference 23). The promoter Pe transcribes on the opposite strand the countertranscript RNA-e. RNA-e has been shown to be a regulatory RNA whose target is the complementary RNA that is transcribed from one of the two upstream promoters, Pc and Pa. This regulatory cassette controls the coupled translation of the uORF and RepA1. It has been proposed by others that the relative amounts of transcript and countertranscript set the copy number of the plasmid by setting the level of translation (and hence the cellular concentration) of the initiator RepA1. We show in this report that the control of copy number, although dependent on the availability of the RepA1 protein, is more intricate than has been proposed partly because it is not uniquely dependent on the RepA1 concentration.

The fact that the Rep protein and origin occur in a cassette implies that they interact and, furthermore, that the origin is a target for the RepA1 protein. The fact that the cassette is always organized in the order structural gene-origin raises the possibility that the function of RepA1 and the origin is such that it requires continuity at the genetic level. We will present evidence for both the RepA1-origin interaction and the requirement for continuity at the genetic level. The in vivo Rep protein-origin interactions as studied in this work have led us to a model that might lead to a more precise understanding of the events that occur at the start site of replication.

Our first studies were performed in vitro, and they involved the overexpression of the RepA1 protein as well as its purification to homogeneity (9). We showed that RepA1 is highly soluble, has a predicted and actual MW of 40,000, and has an

* Corresponding author. Mailing address: 550 First Ave., New York, NY 10016. Fax: (212) 263-8276. E-mail: maasr01@mcrcr6.med.nyu.edu.

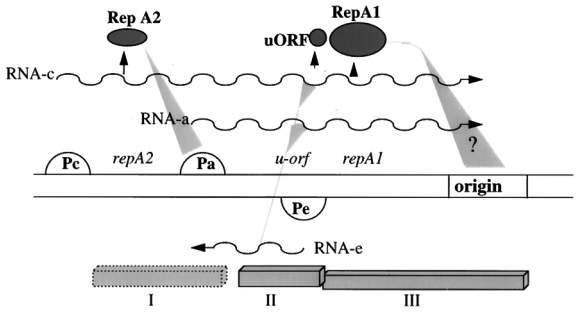

FIG. 1. Organization of a class of antisense replicons. I, II, and III indicate interchangeable genetic cassettes. Each cassette consists of a product and its target. I is strictly regulatory and dispensable for replication; II regulates translation and is absolutely required; III (RepA-ori cassette), which is also absolutely required, is involved in the initiation of replication.

isoelectric point of 8.3 to 8.4, the latter suggesting that RepA1 is a DNA binding protein. RepA1 crystallizes as a dimer (21), and although we concluded from molecular sieve chromatography studies that RepA1 may exist as a monomer in solution, these conclusions have to be viewed with caution, particularly since the shape of the molecule is such as to make the molecular sieve studies unreliable (15a). The crystal structure is currently being solved at 2-Å resolution in the laboratory of Simon Phillips at the University of Leeds, and the shape of the molecule leaves little doubt that RepA1 functions as a dimer (15a). We used the above-mentioned highly purified preparations of RepA1 for gel shift assays and DNase footprints. We were not able to demonstrate the expected in vitro interaction, and although the reasons for our failure are not apparent yet, we have gained some insight from our in vivo studies regarding the location of one of the inferred interactions of RepA1 and the origin. Another type of interaction, not related to initiation, will be presented in a subsequent publication.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* host C600 was used, except in those cases where the host N99cI857 (obtained from Nigel Godson) was required for controlled expression of the RepA1 protein (9). Plasmids are described in the text and in the pertinent legends. The replicon RepFIC (the object of this study) was cloned in pUC and pBR vectors for the purpose of inactivating the absolutely required initiator protein. In those cases where the RepA1 initiator protein was supplied in *trans* (itself cloned in pBR), RepFIC fragments were cloned in RepFIB miniplasmids, which are fully compatible with pBR derivatives.

Growth of bacteria. Strains were grown in tryptone-yeast extract medium (Difco) supplemented with the appropriate antibiotic for continued plasmid selection. Cultures were grown at 37°C except for expression of the RepA1 protein, when they were pregrown at 30°C to a concentration of 10^8 cells per ml and then shifted for induction to 37°C (time of induction = 90 min or as indicated).

Preparation of DNA. Unless otherwise indicated, cells were grown to an optical density at 590 nm of 0.45. Plasmid DNA was prepared with a Qiagen Plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.) as recommended by the manufacturer. We usually carried out an extra alcohol precipitation of the DNA at the end of the procedure. Concentrations were estimated after electrophoresis in 0.8% agarose gels and ethidium bromide staining.

Transformation. Bacteria were transformed by electroporation with an *E. coli* Pulser (Bio-Rad).

Cycle sequencing. Sequencing ladders were used as MW markers in all primer extension experiments. A Perkin-Elmer Amplicycle sequencing kit was used as recommended by the manufacturer. The radioactive precursor was [α-^{33}P]dATP (2,000 Ci/mmol; New England Nuclear), and the resulting fragments were resolved in 6% denaturing sequencing gels. Circular plasmids (always the same as the ones being tested) were used directly, and the ladders were generated with the same primers as those radiolabeled for the experiment.

Primer extensions to determine 5′ ends. Since we were studying a plasmid of low copy number, and furthermore trying to identify replication intermediates in a relatively small plasmid pool, it was necessary to enhance the signals obtained by primer extension. To this end, primer extensions were repeated as indicated with the help of a thermal cycler and the thermostable enzyme *Taq* polymerase. Single-stranded DNA primers (about 20-mer) were labeled either with [γ-^{33}P]ATP (2,000 Ci/mmol) or [γ-^{32}P]ATP (6,000 Ci/mmol) and polynucleotide kinase (New England Biolabs). Primer extensions were carried out in a Perkin-Elmer thermal cycler under the following conditions (per PCR tube): 20 fmol of template; 100-fold molar excess of labeled primer; 1 U of *ampli-Taq* polymerase (Perkin-Elmer); deoxynucleoside triphosphate precursors, 25 μM; buffer, as provided and recommended by the manufacturer; total volume, 8 μl; loading dye-stop solution, 4 μl. One-fifth volume was loaded per lane. Cycling was done as follows: 94°C for 1 min; annealing temperature [calculated from the formula 4(G + C) + 2(A + T) − 5]) for 1 min; 72°C extension for 2 min with 20-s increments. The cycle was repeated 10 times (unless indicated otherwise).

Nucleotide sequence accession number. The sequence of RepFIC has been assigned GenBank accession number M16167.

RESULTS

Experimental design. Electron microscopy studies carried out with plasmid R100 by Miyazaki et al. showed that the replication of this plasmid is unidirectional and proceeds from the origin region in the direction away from the Rep protein ORF (14). Our first step in studying the proposed RepA1-origin interaction was to map precisely the start site of replication on the leading strand. We assumed that the start site and direction of replication (the latter is shown in Fig. 3) were analogous to those of R100.

We reasoned that cycled primer extensions along the leading DNA strand, carried out with labeled reverse primers complementary to regions downstream of the origin, should produce a fragment length that is indicative of a 5′ end in the leading strand (1). For such an experiment to work, it is important to use as templates plasmid DNA preparations that have a sufficient number of replication intermediates and also to use as negative controls plasmids that contain the region being probed but which do not utilize this region for replication. The choice of negative controls was not difficult. RepFIC plasmids replicate in *polA* mutant hosts, while pBR derivatives do not. RepFIC-pBR chimeras also replicate in *polA* hosts, while origin clones in pBR and chimeras where RepA1 has been inactivated by mutation do not (9). This enabled us to use plasmids pRM4052 (pBR-ori) and pRM4061 (pBR-RepFIC RepA1*) as negative controls. Signals that were obtained by primer extension as described above had to meet the following three criteria before they could be considered start sites of replication: (i) they must not occur in the negative controls, (ii) they must persist after pretreatment of the template with ligase, and (iii) they must persist after pretreatment of the template with alkali (the last would preclude RNA templates).

Nick site *nis*. The results of a prototype of the first series of experiments that we carried out are shown in Fig. 2. The primer was a reverse primer downstream of the minimal origin, marked in Fig. 3 as bp 2368 to 2389 reverse. The salient features of this experiment are that there are (i) a very definite band at the top of the gel and (ii) a family of bands approximately 80 bases downstream. However, the bands occur for all plasmids tested, namely, the positive controls 3930 and 4071 and the negative controls 4052, 4067, and 4061. A brief description of these plasmids follows: 3930, mini-RepFIC, replicates in *polA* strains; 4071, pUC-RepFIC chimera, replicates in *polA* strains; 4052, pBR vector–carboxy-terminal RepA1 structural gene–ori, does not replicate in *polA* strains; 4067, 4071 with the transcriptional terminator Ω inserted between the RepA1 structural gene and origin, thus aborting the transcript at the end of the RepA1 structural gene, does not replicate in *polA* strains, described previously (9); and 4061, pUC-RepFIC chimera with RepA1 inactivated, does not replicate in *polA* strains. Therefore, our first criterion for start site of replication was not met. When we pretreated the templates with ligase, the lanes were almost completely blank (results not shown). These

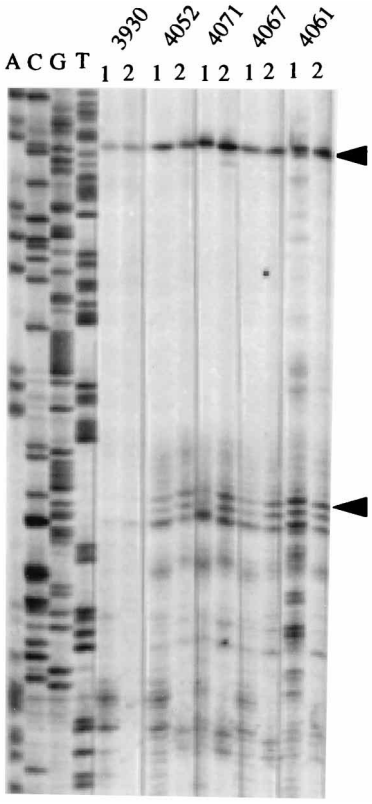

FIG. 2. Primer extensions along the leading strand downstream of the origin region in functional and nonfunctional RepFIC plasmids. Lanes: 1, 10× primer extensions; 2, 25× primer extensions. *E. coli* C600 was used as the host for plasmids 3930 (mini-RepFIC), 4052 (a pBR derivative containing half of the total RepA1 ORF [carboxy terminal] and the entire origin), 4071 (a pUC clone of functional RepFIC), 4067 (Ω was inserted in 4071 between *repA1* and *ori*, making RepFIC nonfunctional), and 4061 (an internal deletion within *repA1* was made in 4071, rendering RepFIC nonfunctional). The upper and lower arrowheads indicate bp 2202 (*nis*) and about bp 2282 (a G-C-rich region), respectively.

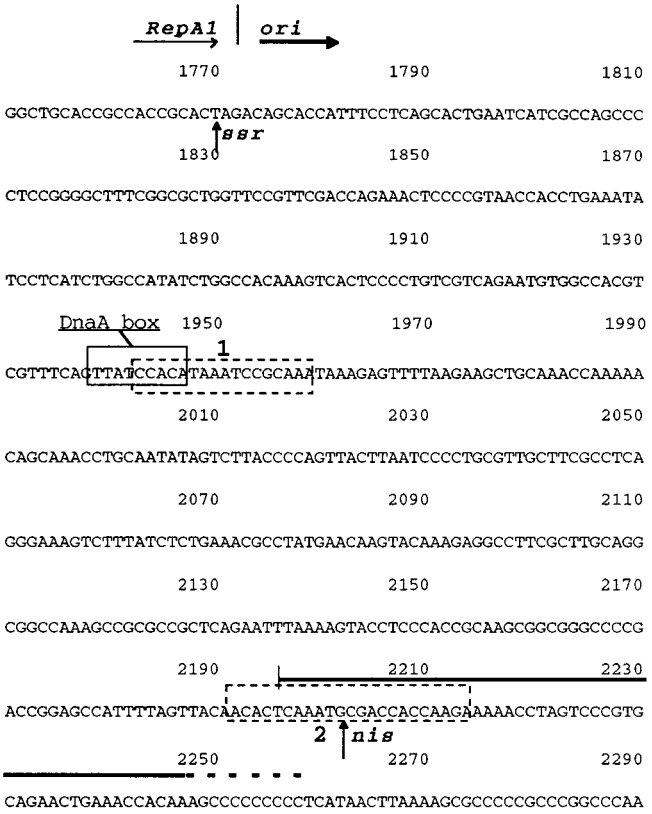

FIG. 3. Sequence of the origin region of RepFIC. The broken-line rectangles indicate the positions of primers used for primer extension. 1 and 2 are reverse primers. Signals detected by primer extension are indicated by vertical arrows. The heavy line starting at bp 2197 indicates the deletion made to confirm that *nis* is not part of the minimal origin. Coordinates of primer used to detect *nis* are bp 2368 to 2389 reverse. The leftmost horizontal arrow indicates the direction of transcription; the adjoining heavy horizontal arrow indicates the direction of replication.

templates were of course electrophoresed along with the untreated templates. Thus, the second criterion was not met. Alkali pretreatment did not affect the signals; therefore, they were DNA 5′ ends. Their possible identities are addressed below and in Discussion.

A few more features are worth noting. Since these were our first exploratory experiments, we compared the results of doing 10× and 25× primer extensions. A close scrutiny of the signals closest to the primer length (bottom of the gel) reveals that increasing the number of cycles is counterproductive for the shorter lengths, where signals present in the 10× lanes disappear in the 25× lanes for every template tested. This result signifies that extended primers can be further extended with each cycle of extension (and this may result in the loss of some signals where the signals are weak to begin with). In all subsequent experiments, we thus used 10× primer extensions. Second, a true start site signal would be expected to be strongest for the mini-RepFIC plasmid pRM3930, but most of the signals were weakest when 3930 DNA was used as a template. All template concentrations were carefully matched by the criteria described in Materials and Methods. Finally, to get an idea about the characteristics of the upper signal (at bp 2202), we carried out primer extensions along the opposite (lagging) strand with a forward primer (coordinates 1993 to 2016). Since no signal appeared in the entire 2200 region (results not shown), we concluded that the signal represented a single-stranded nick in the leading strand. We designate this site *nis*, for nick site (Fig. 3).

To test whether *nis* is part of the minimal origin, we made a deletion of the mini-RepFIC plasmid 3930 by PCR amplification, using a set of divergent primers that excluded the entire region downstream of and including bp 2196. The location of the deletion is shown in Fig. 3. We obtained a viable plasmid, which proved to us that *nis* is outside the minimal origin of replication.

Start site, *ssr*. Preliminary primer extensions designed to find 5′ ends within the minimal origin did not reveal signals that met our criteria for the start site. Reasoning that the nick at *nis* possibly occurs after the plasmid has replicated, we chose a primer that straddled the nick signal, with the expectation that such an approach might eliminate a proportion of plasmid molecules that are not replicating. Using this approach, we found a clear band that indicated what we thought was the start site of replication, because it met our first and most important criterion: it appeared only for our positive-control templates (results not shown). The signal was unchanged when the templates were pretreated with ligase or alkali. Again results are not shown for the reasons discussed below. We concluded that the straddling primer (primer 2 [Fig. 3]) was instrumental in preferentially utilizing early and presumably unfinished molecules as templates by favoring DNA molecules that had not

been nicked. However, the signal was approximately 400 bp upstream from the labeled primer and therefore could not at this point be mapped with precision.

We reasoned that if we could increase the level of initiation, we could select a primer closer to the initiation signal, because at any one time, there would be enough replicating template molecules among the total pool to be detected by our assay. Our approach was to stimulate replication by transforming into the same host mini-RepFIC and an inducible plasmid which overexpresses the RepA1 protein (9). The plasmid yields of mini-RepFIC in the presence of additional RepA1 protein were considerably higher than in the absence of an additional source of the initiator. We were therefore confident that replication was occurring at a stimulated level and proceeded with our cycled primer extensions using this strain. The primer used for exploring the start site of replication (primer 1) is indicated in Fig. 3. The results obtained by using this method, which has become our standard assay for locating the start site of replication, are shown in Fig. 4. The arrow indicates the signal that conforms to the first and most important criterion for the start site of replication. The two negative controls, 4052 and 4061 (described above), show no signal. Mini-RepFIC (3930) showed a faint shadow in the original X rays at the indicated position when fresh radioactivity was used. We propose that the start site of replication (*ssr* in Fig. 3) is located where the indicated prominent band appears in the primer extension lanes of 4110 (a strain that contains both mini-RepFIC and the plasmid that overproduces RepA1). Since the start site signal maps precisely within the translational stop codon TAG of the RepA1 protein, we considered the possibility that the transcript was being processed and utilized as a primer. We already had supportive evidence for the transcriptional requirement of *polA*-independent replication of RepFIC (9). We had shown that insertion between *repA1* and the origin of the above-mentioned fragment Ω (plasmid 4067) eliminates replication of this RepFIC derivative in a *polA* strain. Removal of Ω and its transcriptional stops restores normal replication.

We then tested the effect of RepA1 on the origin region alone and on the RepA-ori cassette (Fig. 1) in the absence of transcription. To test the ability of the origin and the RepA-ori cassette to serve as replication templates, each had to be cloned in an independent vector (other than RepFIC and compatible with pBR, since our RepA1 source was a pBR derivative). Each clone could then be tested by primer extension in the presence of RepA1 supplied in *trans*. To eliminate transcription of the cloned fragments, they were cloned as follows: 4121, RepFIB vector—transcriptional terminator fragment Ω—ori, in the orientation 5′→3′ (See the introduction for RepFIB and reference 17 for description of Ω); 4125, RepFIB vector—transcriptional terminator fragment Ω—Rep-ori cassette, in the orientation 5′→3′. The DNAs from these clones were transformed into a strain that contained the RepA1-overproducing plasmid. Total DNAs from the strains (with and without RepA1) were isolated and primer extended with primer 1 (for the start site assay). The results are shown in Fig. 5 along with those for the positive-control strain 4110 (containing mini-RepFIC and an additional source of RepA1). The origin clone results are shown in Fig. 5A, and the RepA-ori cassette results are shown in Fig. 5B. These results are consistent with the hypothesis that RepA1 does not initiate replication at *ssr* in the absence of transcription.

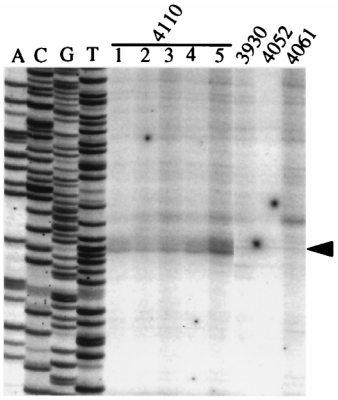

FIG. 4. The start site of replication. Primer extensions were carried out with primer 1 (Fig. 3). The arrowhead indicates the signal for the 5′ end of the extension product (*ssr* signal). This experiment demonstrates the requirement for RepA1 and stimulation of replication by RepA1. Lanes: 1, no induction of RepA1; 2 to 5, 30, 60, 90, and 120 min of induction, respectively. 4110 = 3930 plus RepA1-overproducing plasmid. 3930 = mini-RepFIC. 4052 = pBR clone of the origin of RepFIC. 4061 = pUC-RepFIC chimera with RepA1 inactivated by mutation.

DISCUSSION

With the help of a sensitive primer extension technique, we have explored replication intermediates of the replicon RepFIC. Our results are consistent with the occurrence of an in vivo start site of replication at the junction of the RepA1 coding region and the origin. Previously, using an in vitro replication system, Masai and Arai showed for R1 that replication starts 400 bp downstream of the equivalent of *ssr* (11). A reexamination of their data revealed a signal identical to ours in their Fig. 3, upper part of the gel, which they ignored. The signal that they chose, which is much weaker than the one we agree with, corresponds roughly to the triple signal shown in Fig. 2, at about bp 2280. One possible explanation for the signals seen in the lower part of the gel in Fig. 2 is that they are nonspecific breakdown products, and their accumulation at about bp 2280 reflects that this region is totally G-C, as shown by the adjoining sequence lanes in Fig. 2. The advantage of our in vivo replication system is that we could design replication-negative controls. This is not possible for an in vitro system, because replication-negative controls do not incorporate precursors.

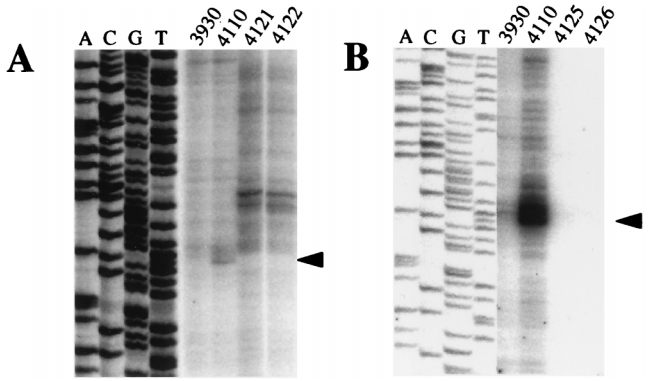

FIG. 5. Requirement for transcription across the *repA1*-ori junction for initiation of replication. The host used was *E. coli* N99cI857. (A) Origin clones where there is no transcription do not initiate in the presence or absence of RepA1. 4121 = Ω—ori clone in mini-RepFIB; 4122 = Ω—ori plasmid plus REPA1-overproducing plasmid. (B) Clones that have the entire *repA1*-ori region (but no transcription) do not initiate. 4125 = Ω—Rep-ori cassette clone in RepFIB. 4126 = Ω—Rep-ori plasmid plus RepA1-overproducing plasmid. The arrowheads indicate *ssr*.

Bernander et al. (1) performed cycled primer extensions on isolated R1 DNA in a manner analogous to ours. They saw the same clear signal that we see at *nis* and attributed it to a stem-loop structure. In our hands, stem-loop structures do not show in cycled primer extensions where one performs repeated 94°C melts; second, there is no signal at this location on the opposite strand. We think that they also were looking at a single-stranded nick. The signal that they call the start site occurs roughly 80 bp downstream of *nis*. The broadness of their band and the fact that they cycled 30 times, compared to our 10 times, suggest that a family of bands was being consolidated into one by the repeated primer extensions. In fact, when they carried out single-round primer extensions, they obtained many bands in this region, which, like us, they interpret as breakdown products. We can only guess that the start site as determined by us was too weak under their conditions (no additional initiator was supplied in *trans*) and/or was being lost by repeating the primer extensions 30 times.

On the basis of our determined position of the start site of replication, namely, the stop codon TAG of the RepA1 protein (Fig. 3 and 4), and the fact that transcription of the *repA1*-origin junction appears to be absolutely required, we formulated the hypothesis that the initiation event in replication is a priming event, and we postulate that the function of RepA1 in initiation is either enzymatic or accessory to an enzyme and that this function resembles the role of RNase H in ColE1 replication (7). We prefer to think that RepA1 is an accessory to an enzyme for reasons that will become apparent below. We propose that the processing of replicon transcripts gives rise to a replication primer, which then primes the polymerase to start adding deoxynucleotides at the start site of replication. The requirement for transcription implicates the upstream replicon promoters in functions additional to supplying RNA messengers. Also, RepA1 is then not the only participant in the initiation reaction: the reaction would depend as much on the supply of appropriate transcript as on the initiating function of the RepA1 protein.

This hypothesis could explain why under some conditions, when the origin is separated from the upstream replicon promoters, the separated origin fails to provide a functional start site of replication (10, 13). As expected, the soluble protein product RepA1 appears to be equally active in *trans* and in *cis* (as shown by the stimulation of replication of RepFIC in strain 4110 [Fig. 4]), provided that there is appropriate transcription of the *repA1*-origin junction. Transcript processing could also provide an explanation for the unexpectedly low yield of RepA1 under some conditions (when an active origin is present), as shown by Dong et al. (4). Equally supportive is the in vitro work of Masai et al. (12), who used a coupled transcription-translation system to demonstrate the dependence of replication on RepA1.

As for the requirement of a supply of appropriate transcript, it is interesting that the regulatory cassette II exerts its negative control at the level of translation, not transcription. Numerous studies have additionally shown that the translation of the initiator Rep protein appears to be coupled to the translation of the uORF (2, 20, 24, 26), although 10 to 20 times as much uORF as RepA1 is synthesized (2, 26). Many uORFs, both eukaryotic and prokaryotic, are involved in the attenuation of translation (5), and it therefore seems likely that the mentioned uORFs, as well as the RepFIC uORF, are involved in attenuation. We propose that the RNA-e–uORF cassette ensures that (i) RepA1 is synthesized as a result of its translation being coupled to the translation of the uORF, (ii) the coupled translation is sufficiently inhibited for the continued presence of untranslated transcript, and (iii) the uORF further attenuates the translation of the RepA1 protein, thus explaining why the uORF is translated at a higher level than RepA1. The regulatory cassette would thus become a modulator of the priming function by providing sufficient transcript, and the uORF or leader sequence of RepA1 would be a functional accessory to the action of RepA1. The cassette would still regulate the frequency of initiation, albeit in a more intricate manner than occurs in ColE1 (22). Our model for the regulation of replication in RepFIC is illustrated in Fig. 6.

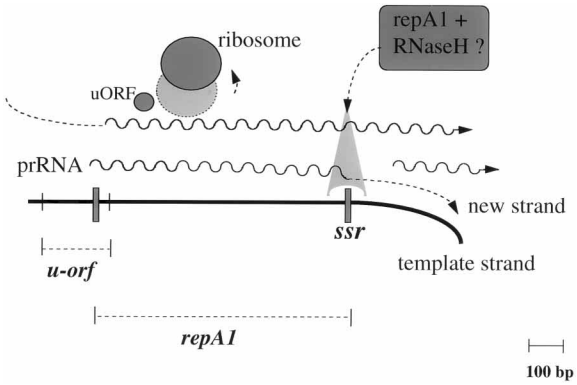

FIG. 6. A transcriptional model for the initiation of replication of the replicon RepFIC.

As mentioned above, we prefer not to assign an enzymatic function to RepA1 but rather to postulate an ancillary and possibly topological function (for which we have very preliminary evidence) because a comparison of the sequences of RepA1 and RNase H has not shown significant similarities. Recent experiments have provided support for the hypothesis that a host RNase H is directly involved in RepFIC replication. Using an RNase H mutant isolated in our laboratory by Lima and Lim (8), we were able to show that the RepFIC miniplasmid is transformed into this mutant at 31% efficiency (compared to 66% efficiency for RepFIB miniplasmids), and the copy number in early log phase is at least fivefold less than in the wild type. For RepFIB, the copy number in the RNase H mutant is approximately two-thirds of the copy number in the wild type.

In summary, genetic manipulation and in vivo functional studies have provided evidence consistent with the utilization of messenger for the synthesis of primer as being an integral part of the replication process of the replicon RepFIC. Transcription across the start site *ssr* appears to be a requirement, followed by a postulated processing of the transcript. The organization of the replicon is such, then, that a much larger region than the origin itself is involved in the initiation function. Further experimentation is required to define what length of transcript is needed for the specific processing postulated at *ssr*. Once this requirement is defined, the appropriate sequence can be cloned with a suitable promoter, and initiation can be tested in the presence of RepA1 protein supplied in *trans*. Such a cloned fragment may then be defined as the functional origin of the replicon, and *ssr* would be the specific locus where replication begins.

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