An Origin of Replication and a Centromere Are Both Needed To Establish a Replicative Plasmid in the Yeast *Yarrowia lipolytica*

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Two DNA fragments displaying *ARS* activity on plasmids in the yeast *Yarrowia lipolytica* have previously been cloned and shown to harbor centromeric sequences (P. Fournier, A. Abbas, M. Chasles, B. Kudla, D. M. Ogrydziak, D. Yaver, J.-W. Xuan, A. Peito, A.-M. Ribet, C. Feynerol, F. He, and C. Gaillardin, Proc. Natl. Acad. Sci. USA 90:4912–4916, 1993; and P. Fournier, L. Guyaneux, M. Chasles, and C. Gaillardin, Yeast 7:25–36, 1991). We have used the integration properties of centromeric sequences to show that all *Y. lipolytica ARS* elements so far isolated are composed of both a replication origin and a centromere. The sequence and the distance between the origin and centromere do not seem to play a critical role, and many origins can function in association with one given centromere. A centromeric plasmid can therefore be used to clone putative chromosomal origins coming from several genomic locations, which confer the replicative property on the plasmid. The DNA sequences responsible for initiation in plasmids are short (several hundred base pairs) stretches which map close to or at replication initiation sites in the chromosome. Their chromosomal deletion abolishes initiation, but changing their chromosomal environment does not.

Although the nature of higher eukaryotic replication origins is not yet clear (12), that of origins in the yeast *Saccharomyces cerevisiae* is relatively well understood. It has been possible to clone *S. cerevisiae* genomic sequences that confer on a plasmid the ability to replicate extrachromosomally in this yeast. These sequences are called *ARS* (autonomously replicating sequence) elements. Most *ARS* elements are active as origins in their chromosomal context, as shown by two-dimensional (2D) gel replicon mapping studies (8, 44, 62). *ARS* elements are usually smaller than 150 bp, and they contain an essential motif that matches the 11-bp *ARS* consensus sequence (ACS; WTT TAYRTTTW) in at least 9 of 11 positions (61, 72). The ACS is essential for initiation of replication, both on plasmids and in the chromosome (17). Also essential is domain B, located at the 3′ end of the ACS (54, 55, 76). Depending on the *ARS* element, domain B can be subdivided into two or three sub-domains with variable sequences but conserved roles (42, 66). Finally, a DNA unwinding element is frequently present in *ARS* elements and is important for replication of plasmids (60) and chromosomes (41). Initiation at *ARS* elements is under strict cell cycle control (reviewed in reference 18).

In contrast to the defined origin sequences of *S. cerevisiae*, the degree to which specific sequences are employed as origins in the differentiated cells of higher organisms is not yet clear. Most studies employing labeling techniques suggest that initiation takes place at specific sites or in very small (a few kilobases or less) initiation zones (15, 19, 35). In contrast, all studies employing 2D gel replicon mapping techniques—and some studies using labeling techniques (31, 71)—suggest that initiation can take place at any of numerous locations within large initiation zones of 50 kb or more (reviewed in reference 36). Furthermore, in contrast to the discrete *ARS* elements of

S. cerevisiae, most human DNA pieces larger than ~10 kb permit extrachromosomal plasmid replication (38, 51). In this case, initiation does not start at a preferred locus, a result which is also observed in plasmid transformation in *Xenopus* eggs (46, 53). Demonstrations that the choice of initiation sites in vertebrate cells is influenced by nuclear and chromatin structure (31, 45) provide a rationale for these apparently disparate observations. They suggest that, in higher eukaryotic organisms, initiation sites may be selected primarily on the basis of chromatin and nuclear structure. Local nucleotide sequences may be important only as a result of their effect on chromatin structure.

A third view of eukaryotic replication origins is provided by the fission yeast *Schizosaccharomyces pombe*. In this organism, as in *S. cerevisiae*, initiation takes place at discrete sites in the chromosome and each of these sites corresponds to an *ARS* element (13, 22, 73, 77). *S. pombe ARS* elements are more than fourfold larger than those of *S. cerevisiae*, and they are characterized by an abundance of asymmetric AT-rich sequence motifs (14, 21, 80). The larger sizes of *S. pombe ARS* elements suggest that a greater number of protein-DNA contacts are required to initiate replication in this organism than are required in *S. cerevisiae*.

In several other yeasts, replicative plasmids have been described but the actual roles of the sequence elements have rarely been studied (69, 74). An *ARS* consensus has been described for *Kluyveromyces lactis* (23); this consensus is also present in *ARS* sequences from *Kluyveromyces marxianus* (47). In *K. lactis* the consensus is dispensable for the function of a large *ARS* fragment but stimulates the replication efficiency of a smaller *ARS* fragment (24).

To obtain additional information about the properties of eukaryotic replication origins, we are studying *ARS* elements and replication origins in the industrial yeast *Yarrowia lipolytica*, which is only distantly related to *S. cerevisiae* and *S. pombe*. We previously cloned on plasmids two sequences (*ARS18* and *ARS68*) able to promote extrachromosomal replication (27). These two *ARS* plasmids displayed a behavior

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different from that of traditional *ARS* elements in *S. cerevisiae*: low copy number, higher mitotic stability, and scarcity in a genomic DNA library. These unusual properties were later attributed to the presence of a centromere within each of the two *ARS* elements (26). At the same time another group also described the isolation of two *ARS* sequences from this yeast (57). One of them (so-called *ARS1*) displayed the same restriction map as *ARS68*, and a comparison of the 1-kb sequences revealed a difference of only a few base pairs, which may be due to some strain polymorphism in this region (data not published). The other replicator was a new sequence called *ARS2*, which shared with *ARS18* and *ARS68* the features of relative stability and low copy number. A centromeric property had not yet been associated with it. We present here evidence that all *Y. lipolytica ARS* elements so far isolated are organized in the same way, with the centromere and the putative replication origin being located at opposite ends of a DNA stretch of about 1 kb. As no simple *ARS* element was able to promote extrachromosomal replication in this yeast without the presence of a centromere, we wondered if the putative origins were the actual sites of initiation. We show here by 2D gel analysis that initiation takes place at discrete loci that map at or close to *ARS* elements in both plasmids and chromosomes.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* strains used were either DK1 [F⁻ *hsdR2* (r$_K$⁻ m$_K$⁺) *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 galU galK rpsL mcrA mcrB1* Δ(*srl-recA*)*306*] or XL1-Blue MRF′ [Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac l-*(F′::Tn*10 proA*⁺*B*⁺ *lacI*q*ZΔM15*)]. The *Y. lipolytica* recipient strain used for transformation was INAG33122 (*MatB lys2-5 leu2-35 ade1 xpr2*), and the reference strain for chromosome separation was E150 (*MatB his-1 leu2-270 ura3-302 xpr2-322*). Both are deposited at the Collection de Levures d'Intérêt Biotechnologique (CLIB) collection (Grignon, France) under the numbers CLIB118 and CLIB122, respectively. Media for cultivation have been described by Barth and Gaillardin (3). The mitotic stability of plasmids was measured qualitatively by cultivating the yeast transformants in complete medium for about 15 to 20 generations, followed by streaking on complete medium and replica plating on selective and nonselective minimal media. To measure the percentage of plasmid loss per generation quantitatively, we followed the procedure already described (27).

Plasmid pSL30-DN is a generous gift from M. Matsuoka. It consists of a 2.1-kb *Dra*I-*Nru*I *Y. lipolytica* genomic fragment cloned at the *Sma*I site of plasmid pSL14 as described by Matsuoka et al. (57). Plasmids containing entire or deleted versions of *ARS18* or *ARS68* have been described by Fournier et al. (26), and the integrative *LEU2*-pBR322 plasmid pINA62 has been described by Gaillardin and Ribet (28).

In order to study replication initiation in plasmids, the two sequences *ORI3018* and *ORI1068* were cloned in a *Sal*I-linearized pINA732 vector (a centromere-containing plasmid; see Fig. 2), giving rise, respectively, to pINA1223 and pINA1224. The *ORI3018 Sal*I-ended fragment was obtained by PCR amplification from pINA206 (which contains the entire *ARS18* cloned at *Bam*HI in pBLUESCRIPT) with two oligonucleotides, each of them carrying a *Sal*I site at its 5′ end: a 28-mer extending from position 2 to position 19 of the top strand of *ARS18* and a 27-mer extending from position 284 to position 268 of the bottom strand of *ARS18* (GenBank accession number, M91600). The *ORI1068 Sal*I-ended fragment was similarly obtained by PCR amplification from pINA736 (see Fig. 2) with two oligonucleotides also carrying *Sal*I sites at their 5′ ends: a 27-mer extending from position 737 to position 753 of the top strand of *ARS68* and a 27-mer extending from position 1071 to position 1055 of the bottom strand of *ARS68* (GenBank accession number, M91601).

In order to specifically delete the *ORI1068* sequence from its chromosomal locus, we constructed plasmid pINA888 by cloning the 0.8-kb *Not*I-*Nhe*I fragment of pINA758 (see Fig. 2; the *Not*I site belongs to a polylinker located a few base pairs from the left *Bam*HI site) into the plasmid pINA731 digested by *Not*I and *Nhe*I (*Nhe*I and *Not*I are two vector sites, *Not*I being located close to the *Bgl*II site of *ARS68*, which is cloned in the reverse direction in this plasmid [see reference 26]). Transformation of yeast INAG33122 was achieved with *Not*I-linearized pINA888 by selecting for Leu⁺ clones. Transformants were checked by Southern hybridization of *Bgl*II-digested genomic DNA with a *LEU2* probe. Gene conversion events gave rise only to the 2.8-kb *LEU2* band, whereas plasmid integrations displayed an additional 4.7-kb band (data not shown).

In order to move *ORI1068* apart from its 3′ chromosomal sequences, we constructed the plasmid pINA1231 by cloning a *Not*I-*Pvu*II *CEN*-bearing fragment from pINA731 (*Pvu*II and *Not*I are vector sites) into plasmid pINA758 digested by *Not*I and *Nru*I (*Nru*I is a vector site). Transformation of the yeast

strain INAG33122 by pINA1231 allowed integration of vector sequences and of the *LEU2* gene at the right end of *ORI1068* (base 1057). Transformants were checked by Southern blotting.

The cloning of chromosomal origins that are not normally positioned near centromeres was achieved by ligating *Sau*3A-digested genomic DNA into the plasmid pINA732 (see Fig. 2) digested by *Bam*HI.

Molecular techniques. *Y. lipolytica* transformation has been described elsewhere (3). Chromosome separation was achieved according to published protocols (26, 81). The preparation of replication intermediates and 2D gel analyses followed methods already described by Huberman (43). Gene cloning in *E. coli* and DNA hybridization employed standard procedures (70). Sequence comparisons were performed with the Genetics Computer Group package (University of Wisconsin, Madison, Wis.) or Macaw software (G. Schuler, National Center for Biotechnology Information, Bethesda, Md.) by using the Gibbs algorithm.

RESULTS

***ARS2* harbors a centromere.** On the basis of the genetic behavior in *Y. lipolytica* of plasmids bearing the *ARS18* and *ARS68* elements, we had concluded that a centromere was present in each of these sequences (26). We decided to investigate if this was also the case for *ARS2*, another *ARS* element described for this yeast (57). Plasmid pSL30-DN contains the *Y. lipolytica LEU2* gene and the *ARS2* sequence and thus transforms a *leu2* recipient strain of this yeast at high frequency. Matsuoka et al. (57) have demonstrated that the 2.1-kb *Dra*I-*Nru*I fragment present in pSL30-DN contains a 1.6-kb region essential for *ARS* function (Fig. 1A). We performed two deletions in this essential region, one at its *Nru*I-proximal end (*Sal*I deletion) and one in its central part (*Cla*I deletion). The resulting plasmids were tested for their ability to transform *Y. lipolytica*. As shown in Fig. 1A, the *Sal*I deletion was unable to transform at high frequency, suggesting that a sequence essential for *ARS* function had been eliminated. Surprisingly, the *Cla*I deletion was able to transform at high frequency, indicating that the central region of *ARS2* is dispensable.

If a centromere is present in *ARS2* and is required for autonomous replication, it should be located on one side or the other of the nonessential *Cla*I fragment. If the centromere is still present in the *Sal*I deletion plasmid, this plasmid should integrate at the *leu2* locus at a lower frequency than that of a plasmid that does not contain a centromere, because the introduction of an additional centromere on a *Y. lipolytica* chromosome leads to chromosome breakage (26). This may occur in essential genes and should therefore reduce transformation efficiency. We transformed *Y. lipolytica* by the lithium acetate procedure (78) with plasmids linearized at the *Xho*I sites within the *LEU2* gene in order to target integration to this locus. It is known from previous results that the 30-bp deletion occurring between the two *Xho*I sites is repaired in vivo against the chromosomal copy (26). The pSL30-DN and *Cla*I-deletion plasmids transformed at high frequency after linearization by *Xho*I (Fig. 1A). Most transformants tested were unstable, suggesting that the plasmids had recircularized by in vivo ligation and were replicating autonomously as previously described (26). In contrast, transformants obtained with the integrative vector pINA62 (pBR322 plus *LEU2*) were stable (data not shown). The *Sal*I deletion plasmid transformed at lower frequency, consistent with the presence of a centromeric sequence (Fig. 1A).

Chromosomes from some transformants obtained with the *Sal*I deletion of pSL30-DN were analyzed by pulsed-field gel electrophoresis. Additional smaller bands, consistent with the breakage of a larger chromosome, were generally observed (Fig. 1B). Surprisingly, the wild-type *LEU2*-bearing chromosome was still present. As shown in Fig. 1D, plasmid integration may occur before (I) or after (II) chromosome replication. The outcome of chromosome segregation may then differ: (i) if both centromeres of the dicentric chromosome resulting from
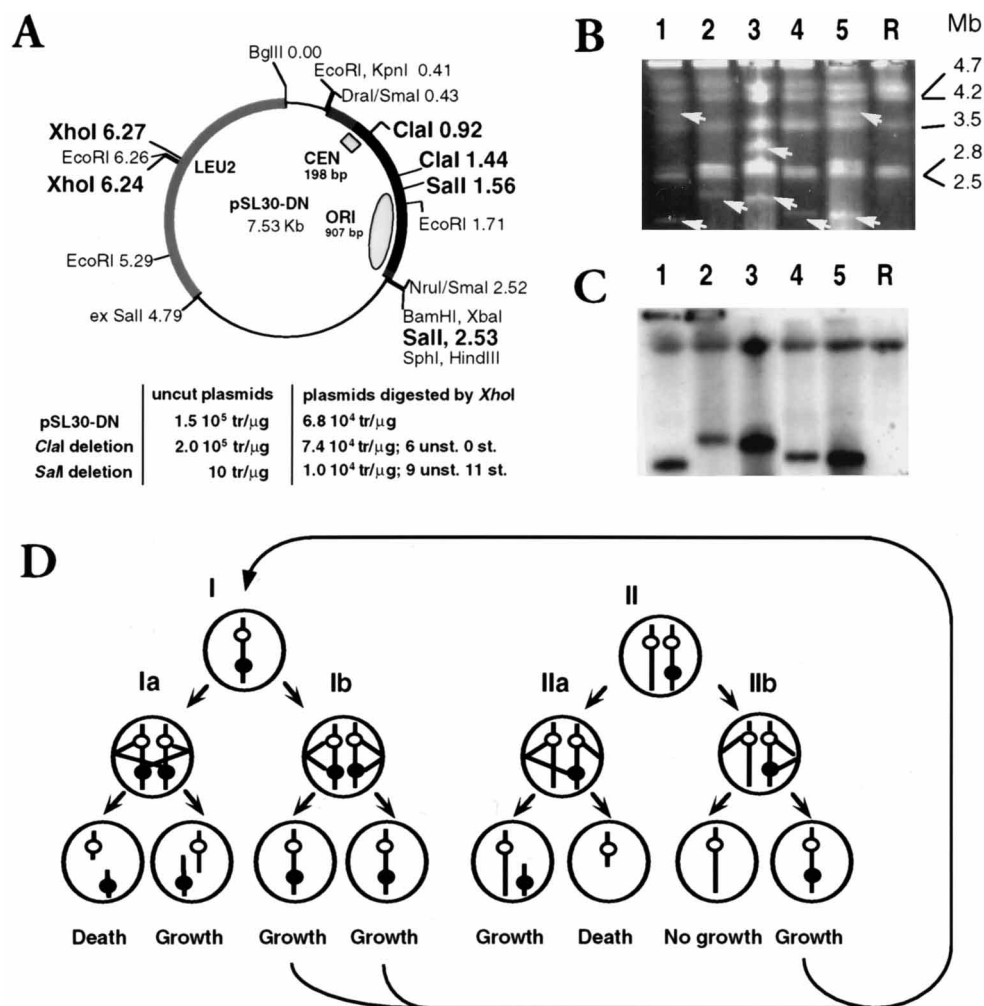
FIG. 1. Mapping of the centromere within *ARS2*. (A) On the map of plasmid pSL30-DN, the restriction sites used in this study are shown in boldface and the essential part of *ARS2* is shown in black. The *Xho*I sites within the *LEU2* gene were used to target plasmid integration. The results of the transformation assays are expressed as transformants (tr) per microgram of DNA. unst., unstable; st., stable. (B) Ethidium bromide staining of a pulsed-field gel of chromosomes of the recipient strain INAG33122 (R) and of five transformants obtained with the *Sal*I-deleted version of pSL30-DN plasmid digested with *Xho*I prior to the yeast transformation. Lane R shows the five *Yarrowia* chromosomes (the smallest band is actually a doublet which is best separated in the case of the transformants displayed in lanes 1, 2, and 5). In lanes 1 to 5, one extra band can be seen in the range of 800 kb to 2 Mb, and another one is visible in lanes 1, 3, and 5 at variable positions between 2.5 and 3.7 Mb. Extra bands are identified by arrows. The sizes of the chromosomal bands of the recipient strain are in megabases. (C) The gel shown in panel B was transferred to a membrane and hybridized with a *LEU2* probe. (D) Schematic drawing of the potential fates of an integrated centromeric vector (as described by Jäger and Philippsen [48]). Empty circles, native centromere. Plasmid integration (solid circles) may occur before (I) or after (II) chromosome replication. Mitotic segregation can take place with a *cis* (Ib, IIb) or a *trans* (Ia, IIa) attachment. Loss of genetic material leads to cell death, whereas absence of the marker gene stops growth on selective medium.

plasmid integration segregated to the same pole during the initial mitosis of the transformed cell, the transformant colonies (Fig. 1B, lanes 1, 3, and 5) would be mosaics of cells still harboring the dicentric chromosome (Fig. 1D, Ib and IIb) and of cells in which the dicentric chromosome has been broken into two pieces during subsequent mitosis (Fig. 1D, Ia); (ii) if integration occurred after chromosome duplication and the dicentric centromeres segregated to opposite poles of the cell (Fig. 1D, IIa), the surviving daughter cell would contain an intact chromosome and only one additional broken piece (Fig. 1B, lanes 2 and 4). We confirmed that, in each case, one of the shorter chromosomal bands contained the *LEU2* gene sequence, as did the intact 4.2-Mb chromosome (Fig. 1C). We conclude that a centromere is present in the pSL30-DN insert. These observations, combined with observations of the *ARS* activity of the *Cla*I deletion plasmid, suggest that the centro-

meric sequence is included within the 198 bp present between the *Dra*I-proximal end of the essential *ARS2* region and the first *Cla*I site. The replication origin would presumably be contained within the 907-bp portion extending from the second *Cla*I site to the other end of the essential region.

We have already shown (26) that the *ARS18* replicator is organized in similar fashion, the putative *ORI* and *CEN* sequences being separated by a dispensable region and being located at opposite ends of a 1.3-kb fragment. We wanted to check if this was a general feature of the *Y. lipolytica* replicators cloned so far, so we analyzed *ARS68* in detail.

ORI and CEN sequences are at opposite ends of the 1-kb *ARS68* sequence. We used exonuclease III deletions at both ends of *ARS68* (26) to determine the locations of *CEN* and *ORI* sequences. The following properties were expected for transformation with plasmids containing or lacking *CEN* se-

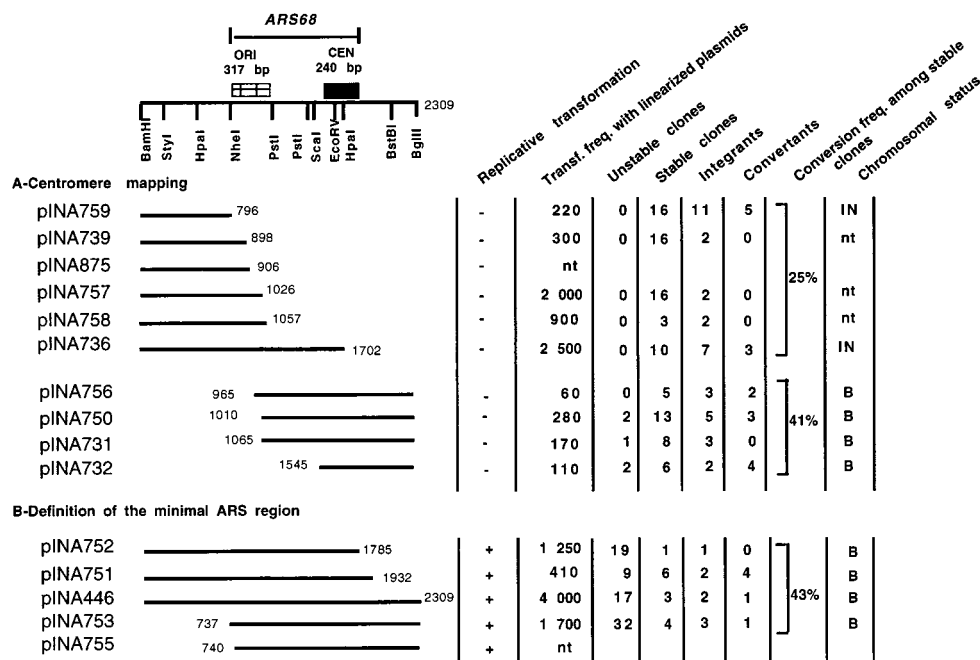|	Replicative transformation	Transf. freq. with linearized plasmids	Unstable clones	Stable clones	Integrants	Convertants	Conversion freq. among stable clones	Chromosomal status
A-Centromere mapping								
pINA759 (796)	−	220	0	16	11	5		IN
pINA739 (898)	−	300	0	16	2	0		nt
pINA875 (906)	−	nt						nt
pINA757 (1026)	−	2 000	0	16	2	0	25%	nt
pINA758 (1057)	−	900	0	3	2	0		nt
pINA736 (1702)	−	2 500	0	10	7	3		IN
pINA756 (965)	−	60	0	5	3	2		B
pINA750 (1010)	−	280	2	13	5	3	41%	B
pINA731 (1065)	−	170	1	8	3	0		B
pINA732 (1545)	−	110	2	6	2	4		B
B-Definition of the minimal ARS region								
pINA752 (1785)	+	1 250	19	1	1	0		B
pINA751 (1932)	+	410	9	6	2	4	43%	B
pINA446 (2309)	+	4 000	17	3	2	1		B
pINA753 (737)	+	1 700	32	4	3	1		B
pINA755 (740)	+	nt						

FIG. 2. Structural analysis of *ARS68* by deletion. Exonuclease III deletions were performed as described previously (26), and the remaining sequences are represented by horizontal lines. The different circular plasmids were transformed into yeast by electroporation (replicative transformation): a positive result (+) indicates the presence of a functional *ARS*, as integrative transformation by electroporation does not occur with our strains (3). Plasmids linearized at the *Xho*I site within the *LEU2* gene were transformed by the lithium acetate procedure. The frequency of transformation (transf. freq.) with linearized plasmids is expressed in transformants per microgram of DNA. The mitotic stability of these transformants was analyzed on complete medium, and the number of stable and unstable clones is indicated. DNA of some of the stable clones was extracted and analyzed by digestion with appropriate restriction enzymes to distinguish between plasmid integration and gene conversion. The average ratio of conversion among stable clones was calculated separately for each of the three groups of transformants: (i) those transformed with non-*ARS* plasmids lacking a centromere, (ii) those transformed with non-*ARS* plasmids bearing a centromere, and (iii) those transformed with *ARS* plasmids. Chromosomes of integrants were separated on pulsed-field gels in order to check if they displayed an intact pattern (IN) or broken chromosomes (B). Nucleotide position numbers are according to the sequence deposited in GenBank, M91601. nt, not tested. A control integrative plasmid (pINA62) linearized by *Apa*I gave 2,000 transformants per μg of DNA, and all 20 clones tested displayed a stable Leu⁺ phenotype.

quences: (i) the transformation efficiency of *CEN*-containing plasmids (which also lack *ORI* sequences) should be lower due to genomic rearrangements caused by centromere integration (see above); (ii) all integrative transformants obtained with plasmids lacking centromeres should be stable, whereas some *CEN* transformants should be mitotically stable and some should be unstable due to possible loss of duplicated material (if integration takes place after chromosome duplication and if chromosome breakage occurs, the broken half bearing the *LEU2* gene may segregate with the intact chromosome, as depicted in Fig. 1D, IIa, and such a partial aneuploid is slightly unstable on complete medium); (iii) among the stable transformants, the percentage of *LEU2* gene conversion should be higher in the case of transformation by *CEN*-containing plasmids due to selective death of many of the integrative transformants; (iv) for *CEN* transformants, chromosome breakage should be detectable by pulsed-field gel electrophoresis.

As illustrated in Fig. 2A, all deletions extending from the right border of the minimal *ARS* region share properties expected for fragments lacking a *CEN* sequence: after linearization within the *LEU2* gene some of them transformed as efficiently as the control integrative plasmid, and all transformants were mitotically stable. The number of *LEU2* gene conversions versus plasmid integrations was determined by Southern hybridization of total DNA digested with appropriate enzymes. The frequency of conversion among the stable clones was 25% (8/32) for this group of deletions. No alterations of chromosome sizes were observed for any of the transformants tested. In contrast, all deletions extending from the left border shared

features expected for *CEN*-containing fragments: lower frequency of transformation yielding both stable and unstable clones and a frequency of conversion of 41% (9/22). As controls, we used a set of plasmids capable of autonomous replication (Fig. 2B). These plasmids, which should contain both *ORI* and *CEN* sequences, transformed at high frequency even after linearization within the *LEU2* gene, mostly yielding unstable autonomously replicating transformants by in vivo plasmid repair and ligation. However, a few stable clones were produced, and among these the frequency of conversion was 43% (6/14), which is higher than the value observed for the transformants lacking the centromere but similar to the value (41%) obtained for the group of non-autonomously-replicating plasmids that appear to retain centromere function (Fig. 2A). The integrants derived from autonomously replicating plasmids similarly displayed new chromosomal bands due to breakage of dicentric chromosomes (Fig. 2B).

The data in Fig. 2 permit us to position the functional centromere between nucleotides 1545 and 1785, as defined by pINA752 and pINA732. The left border of the *ORI* sequence is at nucleotide 740, as determined by plasmid pINA755. Definition of the right border of *ORI* required construction of chimeric plasmids, which we describe below.

The association of *ORI* and *CEN* sequences is not specific. To find out if *ARS* activity can be generated by the physical linkage of a given centromere to one or another origin, we constructed a set of chimeric plasmids with regions from *ARS18* and *ARS68* (Fig. 3). Results obtained with plasmids pINA877, pINA876, pINA885, and pINA889 show that the
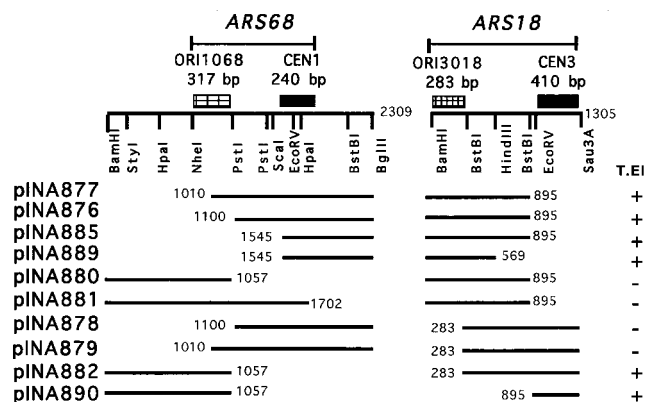
ARS68   **ARS18**

ORI1068 317 bp   CEN1 240 bp   ORI3018 283 bp   CEN3 410 bp

ARS68 restriction sites: BamHI, StyI, HpaI, NheI, PstI, PstI, ScaI, EcoRV, HpaI, BstBI, BglII — 2309

ARS18 restriction sites: BamHI, BstBI, HindIII, BstBI, EcoRV, Sau3A — 1305

Plasmid	ARS68	ARS18	T. El.
pINA877	1010	895	+
pINA876	1100	895	+
pINA885	1545	895	+
pINA889	1545	569	+
pINA880	1057	895	−
pINA881	1702	895	−
pINA878	1100	283	−
pINA879	1010	283	−
pINA882	1057	283	+
pINA890	1057	895	+

FIG. 3. The association between *ORI1068* and *CEN1* and between *ORI3018* and *CEN3* is not specific. *LEU2*-based vectors were constructed by cloning the indicated restriction fragments from *ARS18* within the *ARS68* exonuclease III deletion plasmids described in the legend for Fig. 2. *ARS* activity (T. El.) was tested by electroporation.

centromere of *ARS68* can cooperate with *ORI*-containing sequences from *ARS18* to produce *ARS* activity (transformation, as tested by electroporation). The left border of the *ARS68* centromere so defined corresponds to that deduced from the study summarized in Fig. 2. Reducing the size of the *ARS18* piece from 895 to 569 bp does not abolish *ARS* activity (compare pINA885 and pINA889).

ARS activity can also be generated by linking the centromere of *ARS18* to a 1,057-bp fragment encompassing the left end of minimal *ARS68* (pINA882 and pINA890). We conclude that *ORI1068* is contained within the 317-bp stretch between nucleotides 740 (pINA755; Fig. 2) and 1057 (pINA882 and pINA890). The mitotic stability of these hybrid *ARS* constructs was checked quantitatively; there were no significant differences between them and the original, nonhybrid *ARS* elements (data not shown). These data therefore demonstrate that the tested *ORI*s and *CEN*s can be interchanged and that the sequences lying between them do not play a detectable role in plasmid replication.

We have also built plasmids with two putative *ORI*s (pINA880 and pINA881). They did not transform, showing that the failure of *ORI⁺ CEN⁻* plasmids to transform is not due to poor initiation efficiency at *ORI* but to the requirement for a *CEN*. An absolute requirement for *ORI*s was also observed, as bi-*CEN* plasmids were unable to transform (pINA878 and pINA879).

Chromosomal localization and improved nomenclature. We hybridized each of the *ARS* elements to chromosomal blots of reference strain E150 and showed that *ARS68*, *ARS18*, and *ARS2* map to chromosomes 1, 3, and 4 respectively (data not shown). For this reason we decided to adopt the following identification rules. (i) Each centromere will be numbered according to its chromosome, namely, *CEN1*, *CEN3*, and *CEN4* (instead of *CEN68*, *CEN18*, and *CEN2*). (ii) Each origin will be assigned an *ORI* name instead of an *ARS* one, as origins alone are unable to promote replicative transformation. This *ORI* name will be followed by a four-digit number, the first digit corresponding to the chromosome number and the three following ones to a unique identification number. Thus, the *ORI*s from *ARS68*, *ARS18*, and *ARS2* will be named *ORI1068*, *ORI3018*, and *ORI4002*. We will keep the names *ARS68*, *ARS18*, and *ARS2* for the initially cloned, physically linked *CEN*s and *ORI*s which display an *ARS* phenotype.

***ORI3018* and *ORI1068* colocalize with initiation sites in plasmids.** From the results presented above it was obvious that

*Y. lipolytica ORI*s behave differently from *ARS* elements in *S. cerevisiae*. One could ask if, like *S. cerevisiae ARS* elements, they colocalize with the actual initiation sites of plasmid replication or if the initiation sites are located somewhere else in the plasmid. To answer this question, we analyzed replication intermediates by neutral/neutral (N/N) 2D gel electrophoresis, as described by Brewer and Fangman (8). For this procedure, total DNA is first digested with a restriction enzyme. After enrichment for replicating molecules, the restriction digest is fractionated by 2D gel electrophoresis under conditions that separate molecules primarily by mass (first dimension) and partly by shape (second dimension). After being blotted to a membrane, the restriction fragment of interest is visualized by hybridization with an appropriate radioactive probe. Initiation within the fragment of interest gives rise to a family of molecules containing replication bubbles of increasing size. During 2D gel electrophoresis, this family migrates along a so-called bubble arc (Fig. 4A). If replication starts at an origin located

A   Double Ys   Bubbles   Delocalized termination   Simple Ys

B   *Xho*I   *Xho*I   ORI1068   LEU2   CEN1   *Not*I   3.3 kb   4.4 kb
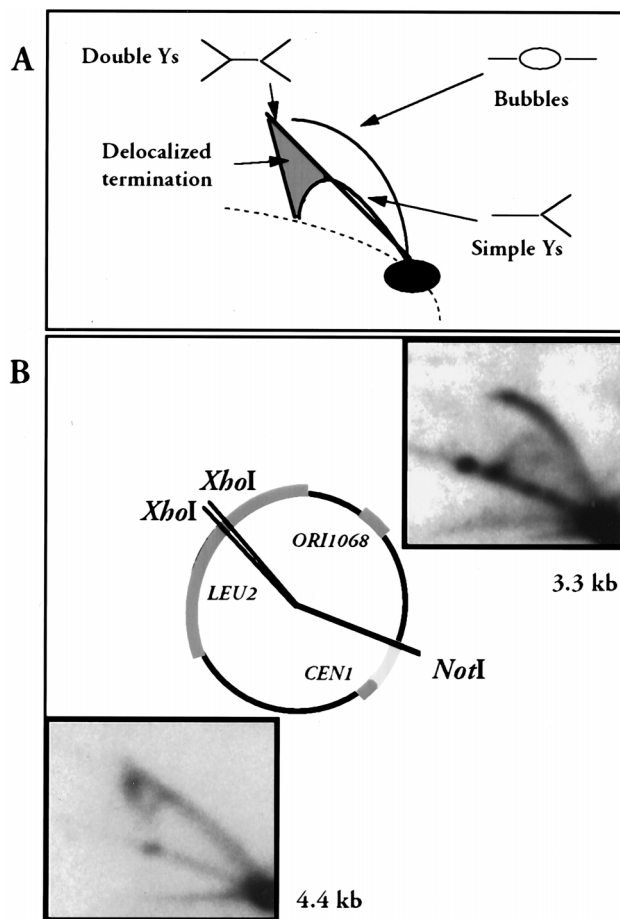
FIG. 4. *ORI1068* colocalizes with an initiation site within a plasmid. (A) Sketch showing the patterns of migration of various types of replication intermediates during N/N 2D gel electrophoresis. Bubbles, bubble arc; Simple Ys, Y arc migration by Y-shaped intermediates; Double Ys, straight-line migration by double-Y-shaped intermediates; Delocalized termination, triangular signal due to random termination. (B) Total DNA from a strain containing pINA1224 (construction is described in Materials and Methods) was digested by *Xho*I and *Not*I and run on an N/N 2D gel. The two fragments were detected with probes obtained by PCR from vector sequences (bases 251 to 2061 from pBR322 for the *ORI*-containing fragment and bases 773 to 2563 from pBluescript for the *CEN*-containing fragment). This plasmid contains bacterial parts, which are in black, and *Y. lipolytica* fragments (*LEU2* gene, *ORI1068*, and *CEN1*), which are in gray. The *CEN1* 3′ genomic environment is in light gray.

outside the fragment of interest, the replication intermediates will be Y-shaped and will migrate as a so-called Y arc (Fig. 4A). If termination takes place at a discrete locus in the fragment, the double-Y-shaped molecules migrate as a straight line, whereas if it occurs at random within the fragment, a triangular signal is produced (Fig. 4A) (79).

We have analyzed the replication intermediates of the two *CEN1*-based plasmids, pINA1223 and pINA1224, which, respectively, carry *ORI3018* and *ORI1068*. In these plasmids the *ORI*s are separated from *CEN* by vector sequences. We checked that the yeast transformants were mitotically unstable, as expected for autonomously replicating plasmids. For isolation of DNA replication intermediates, it was possible to grow these cells on nonselective medium because the plasmid loss per generation is low with centromeric plasmids and an overnight culture represents only a few generations. Total DNA was isolated and digested with *Xho*I and *Not*I and then was resolved by 2D gel electrophoresis. The double restriction divided each plasmid into three fragments: a 4.4-kb *CEN1*-containing fragment, a 3.3-kb fragment containing either *ORI3018* or *ORI1068* (the sizes of the two *ORI*s differ by only 34 bp), and a 29-bp *Xho*I fragment. In both cases, the results were similar: the *ORI*-containing fragment showed a strong bubble arc (Fig. 4B, upper right), indicating that initiation sites are located close to or at *ORI3018* and *ORI1068* in a plasmid.

Part of a Y arc was also revealed (Fig. 4B, upper right), which can be explained either by the fact that the *ORI* sequence is not absolutely centered in the fragment, or by the possibility that forks do not move at the same speed in both directions, or by the possible existence of plasmid multimers, or by the existence of another sequence acting as an origin in the other half of the plasmid, possibly resulting in initiation preference, as described for *S. cerevisiae* (7). To check this last possibility, the *CEN1*-containing restriction fragment was revealed by hybridization with an appropriate probe. It displayed only a Y arc and termination signals but no bubble arc (Fig. 4B, lower left). This led us to conclude that initiation of replication does not occur at random positions on plasmids in *Yarrowia* but rather at discrete loci corresponding to the *ORI* sequences required for *ARS* function.

Some peculiar features were observed in the 2D gel signals from the *CEN1*-containing fragment (Fig. 4B, lower left). The Y arc was incomplete, and there was an accumulation of signal in the upper left portion of the triangular termination region. These features could be explained by the existence of a region causing replication forks to pause in this part of the molecule, maybe in the centromere, as described for *S. cerevisiae* centromeres (32). Replication forks entering on the *CEN1* side would be so much slowed down that a random termination zone would be created around *CEN1*, and replication intermediates would accumulate there. Nevertheless, this incomplete Y pattern could also result from broken double-Y-shaped intermediates, and stalling of the replication fork at the termination point would result in a relative accumulation of signal, as described by Martin-Parras et al. (56). Anyway, further investigations are needed to clarify the exact nature of this pattern.

Because it is known from studies of *S. cerevisiae* that sequences which function as *ARS* elements on plasmids may not correspond to active replication origins in chromosomes (20, 62), we decided to check if this was the case for *ORI3018* and *ORI1068* in *Y. lipolytica*.

***ORI3018* is active in the chromosome.** A *Bgl*II digest of total DNA of an untransformed strain was run on an N/N 2D gel and hybridized with an *ORI3018* probe. *ORI3018* is located near the center of a 4.6-kb *Bgl*II fragment. After hybridization, a complex pattern was revealed (Fig. 5, top right), including a
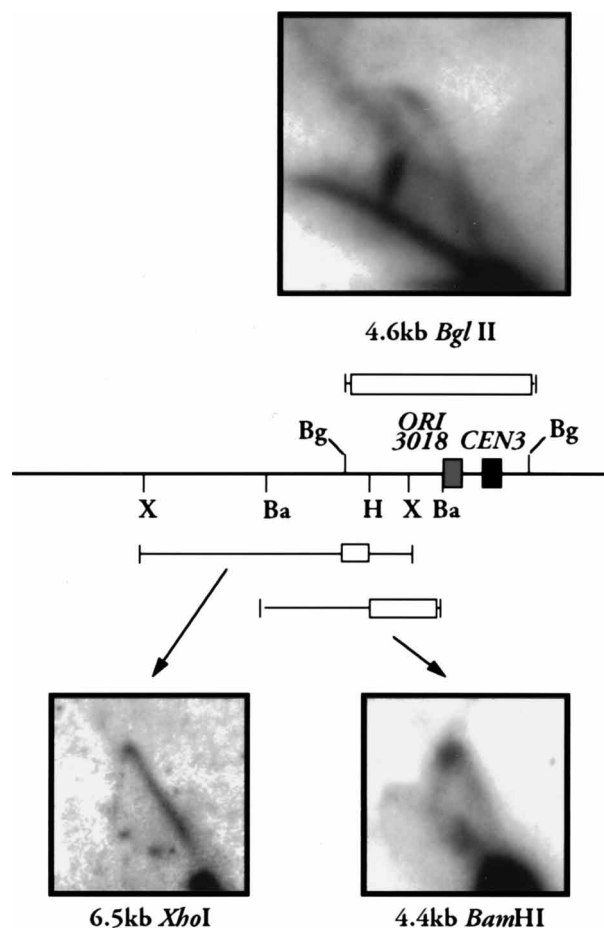

FIG. 5. Replication activity of *ORI3018* in the chromosome. Total DNA from reference strain INAG33122 was digested by *Bgl*II and run on an N/N 2D gel. The cloned 4.6-kb *Bgl*II fragment was used as a probe. Blots of the other genomic restriction digests (*Xho*I and *Bam*HI) were hybridized with the probes indicated. Ba, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; X, *Xho*I.

bubble arc, the late portion of a Y arc, and a diffuse line from lower right to upper left. The diffuse line was not observed on all gels and probably corresponds to an artifact. Such unexplained signals have already been observed on N/N 2D gels (79). The bubble arc and late Y arc are the signals expected if *ORI3018* is active as an origin. Because *ORI3018* is not precisely in the center of the *Bgl*II restriction fragment, replication forks would be expected to reach one end of the fragment before the other, generating late Y structures.

We also hybridized *Xho*I and *Bam*HI blots with appropriate probes as indicated in Fig. 5, lower portion. No bubble arc was detected in either of the detected fragments. Since bubble arcs are evident when origins are located within the central thirds of restriction fragments (52), we may conclude that *ORI3018* is probably the only origin within the 6.5-kb genomic region extending from 2.8 kb to the left of the left-most *Bgl*II site to the right end of *CEN3*.

***ORI1068* is necessary and sufficient for initiation in the vicinity of *CEN1*.** We used the same strategy to analyze the chromosomal region of *ORI1068*. A *Bgl*II digest was run on an N/N 2D gel, and the 3.8-kb fragment which contains *ORI1068* in its central part was revealed by hybridization (Fig. 6A). The pattern observed is similar to that associated with *ORI3018*,
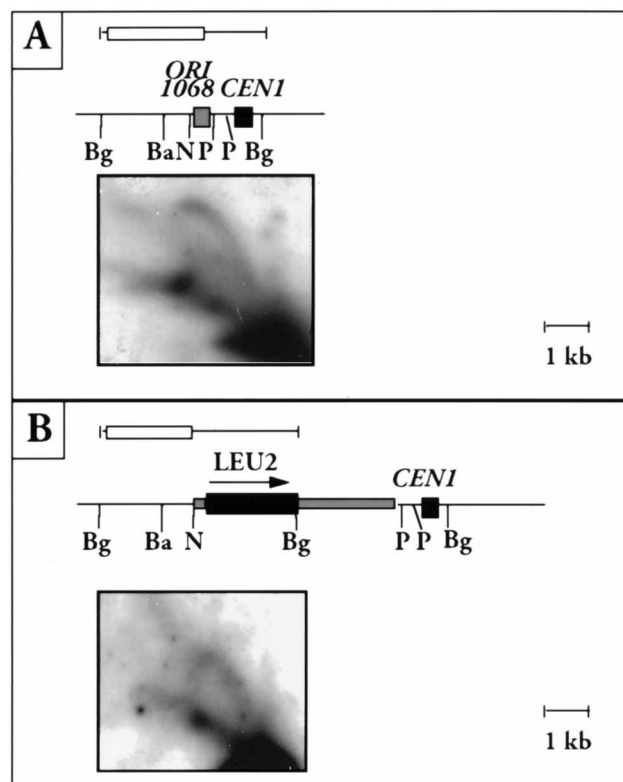
FIG. 6. Chromosomal activity of *ORI1068*. (A) A *Bgl*II digest of total DNA from strain INAG33122 was electrophoresed on an N/N 2D gel and hybridized with the probe shown as an open box in the diagram of the restriction fragment. (B) pINA888 (see Materials and Methods) linearized by *Not*I was used to transform strain INAG33122. Plasmid integration generated a deletion of the chromosomal copy of *ORI1068* (confirmed by restriction analysis). Integrated bacterial vector sequences are shown by gray boxes, and the *LEU2* gene and *CEN1* are shown by black boxes. The arrow indicates the direction of *LEU2* transcription. The DNA of this strain was digested by *Bgl*II, electrophoresed on an N/N 2D gel, and hybridized with the probe represented by an open box. Ba, *Bam*HI; Bg, *Bgl*II; N, *Nhe*I; P, *Pst*I.

the *Bgl*II fragment with *ORI1068* near its center was used as a probe. It revealed a weak but complete bubble arc and, probably, a complete Y arc (Fig. 7, left panel). A portion of a termination signal may also be present. The Y arc signal seems stronger than that of the bubble arc in the region of early replication intermediates, which was not the case in the chromosomal wild-type situation (Fig. 6A). This may indicate that, after introduction of flanking vector sequences (Fig. 7), *ORI1068* does not initiate replication as efficiently as in the absence of vector sequences. However more precise quantitation is obviously needed to ascertain this point. The 4.7-kb *Bgl*II fragment containing most of the *LEU2* gene and *CEN1* displayed a simple Y arc, suggesting the absence of additional origins in this fragment (Fig. 7, right panel). In conclusion, this pattern shows that *ORI1068* is capable of initiating replication even in an altered chromosomal locus.

Chromosomal origins can be cloned on a centromeric vector. We have confirmed our previous model (26) that two genomic fragments are needed in a plasmid to establish extrachromosomal replication. One fragment must contain a centromere, and the other must contain an origin. From these data we inferred that one should be able to clone any genomic origin of replication in a centromere-containing vector. We therefore performed a shotgun origin-cloning experiment with pINA732 (plasmid depicted in Fig. 2), which contains *LEU2* and *CEN1*. A *Sau*3A digest of total genomic DNA was cloned into the *Bam*HI site of this vector. The mean size of the inserts, checked on 20 *E. coli* clones, was about 1.0 kb (extreme values, 0.18 to 3.1 kb). The DNA of five pools of about 1,500 clones each was extracted and used to transform *Yarrowia*. The replicative control (*LEU2* plus *ORI1068* plus *CEN1*) gave rise to 2,000 transformants per µg of DNA, the integrative control (*LEU2* vector) gave rise to 10 transformants per µg, and 100 transformants were obtained per µg of the DNA from the pools. We were able to show that a replicative plasmid was present in the 53 yeast transformants which we have analyzed and that the sizes of the inserts varied from 0.1 to 2 kb. These
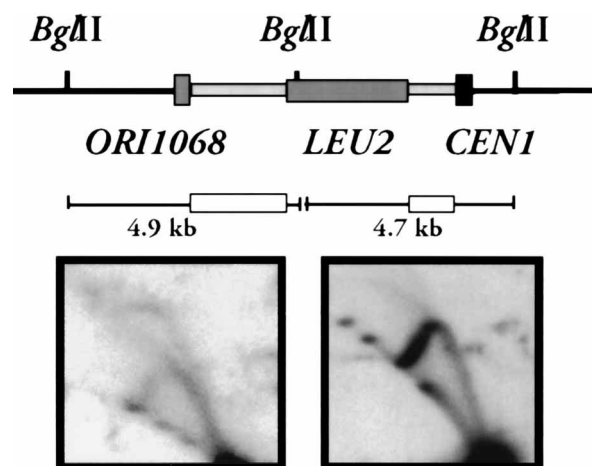
with a bubble arc and the late portion of a Y arc. Thus *ORI1068* is probably also active in the chromosome.

To check whether *ORI1068* is responsible for the bubble arc seen in Fig. 6A, we deleted *ORI1068* from its chromosomal location by replacing it with the *LEU2* gene (Fig. 6B). It is known from studies of *S. cerevisiae* that deletion of a single origin has no effect on the stability of an entire chromosome (16, 49). As *ORI1068* is close to *CEN1*, we also confirmed that the deletion construct was mitotically stable and that the chromosome pattern detected by pulsed-field gel electrophoresis was not altered (data not shown). N/N 2D gel electrophoresis of the *Bgl*II fragment from which *ORI1068* had been deleted showed that the bubble arc had disappeared and that a complete Y arc was now present (Fig. 6B). As a bubble arc would be detected if an origin were located within the central third of the restriction fragment, we can conclude that there is no additional origin between the *Bam*HI site and the *Nhe*I site and that the sequence required for initiation in a plasmid is also required for origin activity in the chromosome.

Another strain was constructed in which *ORI1068* was moved further away from *CEN1*. For this purpose we cloned the *LEU2* gene between *ORI1068* and *CEN1*, thereby placing bacterial vector sequences immediately downstream of *ORI1068* (Fig. 7). After *Bgl*II restriction and N/N 2D gel electrophoresis,

FIG. 7. Insertion of a foreign sequence at the 3′ end of *ORI1068* does not prevent initiation. Plasmid pINA1231 (see Materials and Methods) contains *ORI1068* and *CEN1* separated by the *LEU2* gene and by bacterial vector sequences. Opening the plasmid with *Not*I puts *ORI1068* and *CEN1* at opposite ends of the linearized DNA molecule, which was subsequently transformed into the INAG33122 *leu2* recipient strain. Double crossing-over resulted in the chromosome map shown in the upper part of the figure. Bacterial parts of the vector are shown by light gray boxes. *ORI1068* and *LEU2* are shown by darker gray boxes, and *CEN1* is shown by a black box. The probes shown by open boxes were sequentially hybridized with a blot of *Bgl*II-digested DNA which had been subjected to N/N 2D gel electrophoresis.

plasmids were able to retransform yeast at the same frequency as the original *ARS* (*ORI* plus *CEN*) vectors. We hybridized five (randomly chosen) cloned fragments to blots of chromosomes obtained by contour-clamped homogeneous electric field electrophoresis and observed that they hybridized to different *Yarrowia* chromosomes. Moreover, they belong to different DNA regions, which display specific genomic restriction maps (76a). These data suggest that many other putative origins coming from various chromosomal loci can be cloned and can function on a plasmid with a given centromere. From these data one can also calculate that putative origins of DNA replication are dispersed along the *Yarrowia* chromosome at intervals of roughly 20 kb.

DISCUSSION

Structure of a *Y. lipolytica* centromere. From a *Y. lipolytica* gene library, we had previously isolated the *ARS18* and *ARS68* sequences that are able to promote extrachromosomal replication in this yeast (27). We had shown that both *ARS* elements also have the ability to confer centromere-like segregation properties on yeast plasmids, and we had localized these characteristics to a 410-bp region in the case of *ARS18* (26). We show here that centromere activity is contained within a 240-bp DNA stretch in the case of *ARS68* and a 198-bp stretch in the case of *ARS2*, another *Y. lipolytica* replicator independently isolated by another group (57). The sizes of these *Y. lipolytica* centromeres are similar to those of the yeasts *S. cerevisiae*, *K. lactis*, and *Candida maltosa* but much smaller than those of *S. pombe* (40 to 100 kb). The *Y. lipolytica* centromeric sequences are AT rich (70.7% for *CEN1*, 70.5% for *CEN3*, and 71.5% for *CEN4*), which contrasts with 49.5% for the whole *Y. lipolytica* genome (59). This feature is shared by the small centromeres of several other yeasts (39, 47, 63). The *S. cerevisiae*, *K. lactis*, and *K. marxianus* centromeres display some similar sequences (37, 40, 47), called the *CEN* consensus. This feature is not found in *C. maltosa* (63), *S. pombe* (75), or *Y. lipolytica* (this work). The *Yarrowia* centromeric regions display several stretches of similarity (not shown), but further mutagenesis studies are needed to decipher the structure of a functional *Y. lipolytica* centromere. In any case, the results presented here illustrate that the centromeres of different yeast genera are not characterized by ubiquitously conserved sequence elements.

Mapping of centromeres by plasmid integration. When *Y. lipolytica* is transformed with linearized integrative plasmids, homologous recombination leads to either plasmid integration or marker gene conversion. Gene conversion was observed here in about one-fourth of the cases when the plasmids used did not harbor a centromere (8 cases out of 32; see Fig. 2). This frequency is higher than usual in *Y. lipolytica* (3), perhaps due to the position of the restriction enzyme site at which the plasmid was linearized. In *S. cerevisiae* the linearized plasmid is first degraded by nucleases and is then repaired with information from the chromosomal copy during the integration process (64). Perhaps the *Xho*I site is close to the position of the *leu2*-35 mutation in the chromosomal allele, thereby reducing the efficiency of repair.

Because some plasmids gave rise to new chromosomal bands upon integration, it was inferred that these plasmids contained centromeres. Because the creation of dicentric chromosomes leads to chromosome breakage and reduced cell viability, these centromere-containing plasmids displayed reduced (about 10-fold) transformation frequency and elevated frequency of gene conversion (9 convertants out of 22 stable transformants analyzed, i.e., 41%). In most transformant colonies obtained with centromeric plasmids, we observed the simultaneous presence of the intact chromosome and of two new smaller bands (Fig. 1, lanes 1, 3 and 5). In a minority of cases (2 out of 23 integrants), we detected a single broken chromosome plus an intact chromosome (Fig. 1, lanes 2 and 4). Both patterns can be explained by the fact that integration of a centromeric plasmid has different consequences depending on whether integration occurs before or after chromosome duplication and depending on the directions of segregation of the centromeres in the resulting dicentric chromosomes, as depicted in Fig. 1D (48). The results we observed mean that in most cases integration takes place either (i) before DNA replication, which is consistent with the late log stage of the competent yeast cells; or (ii) after replication, but with more frequent *cis* than *trans* segregation of the two centromeres of dicentric chromosomes, so that most surviving colonies are mixtures of cells carrying an intact chromosome with cells carrying two fragments.

In *S. cerevisiae* the healing of broken chromosomes can take place in two ways (33, 34): through recombination between a broken chromosome end and a homologous region of the original chromosome, which regenerates a dicentric chromosome, or by acquisition of new telomeres. These telomeres can be initiated in *S. cerevisiae* when the break site is close to some classes of TG_{1-3} repeats (50). For *Y. lipolytica*, the fact that all transformants resulting from centromeric plasmid integration contained new small chromosome bands implies that healing of broken chromosomes is mostly achieved by telomere acquisition. Because the new chromosome bands varied in size, it seems that numerous possible breakage and healing sites exist in this yeast. Possibly interaction between the sequence required by the telomerase and the telomerase itself is less stringent in *Y. lipolytica* than in *S. cerevisiae*, or possibly the recruited telomerase can migrate along the broken chromosome end. Efficient telomere healing has also been described in *Tetrahymena thermophila* (25), where the location of new telomeres near chromosome breakage sites is rather independent of DNA sequence. For further investigation of this phenomenon in *Y. lipolytica*, one would need to know the nucleotide sequences near the breakage sites and the sequences of the newly added telomeres.

***Y. lipolytica* ARS elements require an origin of replication and a centromere.** In the three cases described here, *ARS* activity required the simultaneous presence on the same plasmid of a centromere and of a sequence that, according to 2D gel electrophoresis, is located at or near initiation sites. In other words, *Y. lipolytica ARS* elements seem to require an *ORI* sequence and a *CEN* sequence. The fact that no *Y. lipolytica* sequence has yet been found that has *ARS* activity and does not have both *ORI* and *CEN* sequences suggests that this model is likely to prove general for this yeast. It is possible that this model may extend to multicellular fungi and even to mammalian cells, thus explaining previous failures to obtain stable extrachromosomal replication with origin-containing plasmids in these organisms, whose very large centromeres cannot be cloned in such a way.

Let us consider three explanations for the inability of *Y. lipolytica ORI* sequences to promote extrachromosomal replication in the absence of a centromere. First, this inability could be due to extremely inefficient initiation at *ORI* sequences, leading to extreme plasmid instability. In *Candida albicans* (65) and *S. pombe* (10), such instability can be reduced by creating vectors with two origins. This is not the case for *Y. lipolytica*, as pINA880 and pINA881 do not transform (Fig. 3). Furthermore, we confirmed that our *ARS* plasmids are present as covalently closed circular monomers in transformed yeast cells (data not shown). We conclude that, for the *ARS* elements we

have investigated, the presence of two origins on the same plasmid does not significantly enhance replication efficiency and does not compensate for the absence of a centromere.

A second possible reason for the requirement for a *CEN* sequence could be that the origins we isolated are not strikingly AT rich: 52% for *ORI1068*, 64% for *ORI3018*, and 54% for *ORI4002*. However, *ORI4002* has not been precisely mapped, and the sizes of both *ORI1068* and *ORI3018* could probably still be reduced. It is known that *S. cerevisiae* and *S. pombe ARS* elements are scaffold-attached regions (SARs) (1, 2). SARs have been identified in several eukaryotes as AT-rich regions with short homopolymeric runs of T or A (9, 67), although the chicken and *Drosophila melanogaster* nuclear matrices may also contain GC-rich clusters (11, 30). If origins were unable to attach to the nuclear scaffold, initiation might not occur. Thus, the only role of *CEN* in *Y. lipolytica ARS* elements might be to bring an AT-rich region capable of scaffold attachment to the vicinity of *ORI*. We are presently testing this hypothesis by trying to replace *CEN* with other sequences and by testing attachment to the nuclear matrix.

Finally the need for a *CEN* sequence could be explained by an extremely high plasmid segregation bias, making it impossible for a centromere-less replicated molecule to enter the bud. If this proves to be correct, *Yarrowia* could offer an excellent model for studying plasmid segregation.

An origin is close to the centromere in three chromosomes. We have found that an efficient origin of replication lies less than 1 kb from the centromere of at least three of the five *Y. lipolytica* chromosomes. The presence of an origin near a centromere has also been reported for *S. cerevisiae*, but so far only in the vicinity of two of the sixteen centromeres: *CEN3* (61) and *CEN12* (29). In the first case the origin is barely active in its chromosomal locus, whereas in the second it is only known that it is a weak *ARS* element in a plasmid. In the case of *S. pombe* (73), the centromere II, has a high density of *ARS* elements. The so-called K and L repeats are sites of occasional initiation in the chromosome, but the central core seems to be repressed. In *Y. lipolytica*, the significance of the close localization of *ORI* and *CEN* is not clear; it may prove to be fortuitous. However, we are now trying to clone the other two centromeres of this yeast to check if origins also lie in their vicinities.

Features of the three centromeric origins. Plasmid deletions have allowed us to carry out preliminary localization of the *ORI* sequences essential for *ARS* function. In two cases, these short sequences (240 bp for *ORI3018*, 317 bp for *ORI1068*) have been shown to map near or at initiation regions in plasmids and chromosomes. We can therefore rely upon the transformation assay to define *ORI* boundaries more precisely.

The *ORI* sequences of *Y. lipolytica* are not strikingly AT rich, at least within the fragments we considered in this study; this is contrary to the situation in *S. cerevisiae* (61, 72), *K. lactis* (24), *Hansenula polymorpha* (6, 68), and *S. pombe* (58, 80). A similar situation exists in the slime mold *Physarum polycephalum*, for which the efficient bidirectional replication origins so far described are actually relatively GC rich, with no striking sequence similarities between the four sequenced initiation sites (4, 5). However, in *Y. lipolytica* the three origins described here do contain short subregions with stretches of AT-rich DNA whose roles remain to be established.

None of the known consensus stretches described in some *ARS* elements (*S. cerevisiae ARS* consensus, Abf1-binding site, topoisomerase II-binding site, or SAR box) have been found in any of the three centromeric origins of *Y. lipolytica*. The establishment of the sequences of the cloned noncentromeric origins (in progress) will shed additional light upon the characteristics of replication origins in this yeast, which presents an alternative and independent model to study the requirements for the initiation of DNA replication in eukaryotic organisms.

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