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Close association of a DNA replication origin and an *ARS* element on chromosome III of the yeast, *Saccharomyces cerevisiae*

Joel A.Huberman, Jiguang Zhu, Leslie R.Davis and Carol S.Newlon[1]

Department of Molecular and Cellular Biology, Roswell Park Memorial Institute, Buffalo, NY 14263 and [1]Department of Microbiology, UMDNJ-New Jersey Medical School, 100 Bergen Street, Newark, NJ 07103, USA

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ABSTRACT

Two dimensional gel electrophoretic techniques were used to locate all functional DNA replication origins in a 22.5 kb stretch of yeast chromosome III. Only one origin was detected, and that origin is located within several hundred bp of an *ARS* element.

INTRODUCTION

Considerable evidence suggests that eukaryotic DNA replication is usually initiated at specific DNA sequences, or origins (reviewed in 1). Very little is known about the nature of these origin sequences, largely because, until recently, direct tests for identifying functional origin sequences were not available. Last year both Huberman et al. (2) and Brewer and Fangman (3) described methods for identifying replication origins based on two-dimensional (2D) gel electrophoresis of replication-fork-containing DNA restriction fragments. By using these 2D gel methods, they were able to localize the replication origins of the yeast 2 μm plasmid (2,3) and of another yeast plasmid (3) to short stretches of DNA which had previously been identified as *ARS* elements.

ARS elements (or *a*utonomously *r*eplicating *s*equences) are short (at most several hundred bp), *cis*-acting DNA segments which allow plasmids containing them to replicate autonomously in an appropriate host eukaryotic cell. *ARS* elements were first discovered in the chromosomal DNA of yeast (4), and preliminary reports suggest their occurrence in mammalian DNA as well (5-7). The coincidence of replication origins and *ARS* elements in yeast plasmids suggested that *ARS* elements might also serve as origins for chromosomal DNA replication. To test this possibility, we used 2D gel methods to map functional origins within a 22.5 kb stretch of DNA in yeast chromosome III. We report here that we found a single origin, located close to or at the single *ARS* element within this stretch.

MATERIALS AND METHODS

Cell Growth

Unsynchronized *S. cerevisiae* 4910-3-3 cells (provided by Leland Hartwell) were grown as previously described (8) to a final density of 1.8×10^7 cells/ml.

DNA Purification and Enrichment for Replicating DNA

Cells were harvested and chromosomal DNA was purified as previously described (2). The purified DNA was digested to completion with *Bam*HI, and then replication-fork-containing restriction fragments were enriched by fractionation on benzoylated naphthoylated DEAE-cellulose (BND-cellulose) as described previously (2). In a typical experiment, five hundred µl of packed BND-cellulose were used for fractionation of 230 µg of DNA. Most of the DNA (220 µg) emerged in the "flow-through" fraction (which contained fully double-stranded, nonreplicating DNA), and 2.5 µg were recovered in the "caffeine wash" fraction (which contained partially or fully single-stranded molecules, including replication-fork-containing restriction fragments).

Two-Dimensional Neutral/Alkaline Gel Electrophoresis

About 1 µg each of "caffeine wash" and "flow-through" DNAs were subjected to 2D gel electrophoresis as previously described (2), with the following modifications. The first (neutral) dimension gel was 0.4% agarose. Prior to completion of the first dimension gel run, second dimension gels consisting of 1% agarose in water were poured using a comb to form marker wells. The first dimension gel was run so that the restriction fragment size range of interest would be contained within 5 cm. After the first dimension run, the lanes of interest were visualized with 360 nm light, excised from the gel, and cut to a length of 5 cm which included the fragment size range of interest. The first dimension lanes were then placed into slots cut in the second dimension gels (the lower edges of the slots were in line with the marker wells), and the remaining space in the slots was filled with molten 1% agarose. The second dimension gels were soaked twice for 30 min in alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA), and (after loading marker DNAs and bromcresol green dye into the marker wells) subjected to electrophoresis at 12 V (1.2 V/cm) overnight, until the dye had migrated 6.5 - 7.0 cm. After electrophoresis, the DNA was transferred to nylon membranes by alkaline blotting as previously described (2).

Only results for the "caffeine wash" DNAs are shown in this paper. However, slight signals from replicating DNA molecules were also obtained from the 2D gels of "flow-through" DNAs, perhaps due to imperfect fractionation by the BND-cellulose.

Two-Dimensional Neutral/Neutral Gel Electrophoresis

We used the procedure of Brewer and Fangman (3) with the following modifications. First dimension gel electrophoresis of 250 ng samples of "caffeine wash" DNA was carried out as described above for neutral/alkaline electrophoresis (the lanes for both types of second dimension were run in parallel in the same gel), and 8-cm-long lanes were transferred, as above, into second dimension gels consisting of 1% agarose in 1 ×TBE buffer with 0.5 µg/ml ethidium bromide. After a 1-hr equilibration period in this buffer at 4°, second dimension electrophoresis was conducted at 6 V/cm at 4° for 3 h without buffer recirculation.

Preparation of Probe DNAs

The probe DNAs used are described in Fig. 1 and in the Results section. First, the B9G,

A6C, and A1G *Bam*HI fragments of yeast chromosome III were cloned into the *Bam*HI site of the yeast shuttle vector YIp5 (9). The shorter probe fragments used in this study were then obtained by digesting the cloned DNAs with the appropriate restriction enzymes (indicated in Fig. 1) and purifying the desired fragments by electrophoresis in low-gelling-temperature agarose. The fragments were labeled to high specific activity with [α-^{32}P]dTTP using the random oligonucleotide procedure (10).

Hybridization

Hybridization was carried out as previously described (2) with the following modifications. The freshly-labeled probe DNA was denatured by incubation in 0.25 M NaOH at 37° for 10 min, then mixed with the other components of the hybridization mix. Hybridization was carried out at 42° for 16-24 hrs. All post-hybridization washes were at room temperature. Old probe was stripped off the membranes by incubation at room temperature in 0.2 M NaOH (two washes, 20 min each) followed by a single 20-min wash in 0.1 M Trizma-HCl (Sigma), 1 mM EDTA.

RESULTS

The A6C Region of Chromosome III

In earlier work, Newlon et al. (9) cloned the *Bam*HI fragments from a 200 kb circular derivative of chromosome III and mapped the locations of all *ARS* elements in the 200 kb stretch. Determination of the locations of functional replication origins throughout the same 200 kb stretch should provide adequate data for a thorough evaluation of the extent of correlation between origins and *ARS* elements in yeast chromosomal DNA.

We have taken an initial step toward locating functional origins in the cloned stretch by

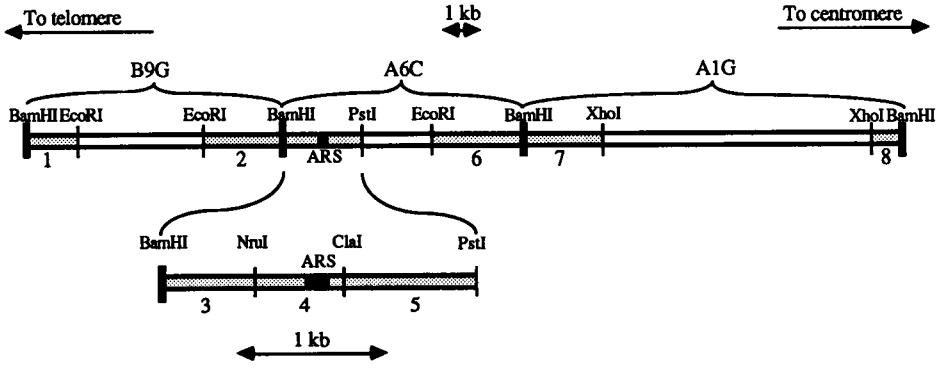

Figure 1. Map of a 22.5 kb portion of yeast chromosome III. The restriction endonuclease cutting sites and the *ARS* element shown in this map are described in previous published studies (9, 11) or were mapped more recently (Van Houten and Newlon, unpublished). The shaded numbered boxes represent the stretches used as hybridization probes in the present study.

mapping a single origin within a 22.5 kb subset of this stretch. The region we investigated is diagramed in Fig. 1. The three adjacent *Bam*HI fragments named B9G, A6C, and A1G are located between the *HIS4* and *HML* genes on chromosome III. The three fragments contain only a single *ARS* element, located in the position shown in Fig. 1 (9, 11, and Van Houten and Newlon, unpublished results).

Origin Mapping by 2D Neutral/Alkaline Gel Electrophoresis

The rationale for use of 2D neutral/alkaline gel electrophoresis in mapping functional replication origins has been described previously (2, 12), and will be summarized here. DNA is isolated from exponentially growing cells, then cut with a restriction endonuclease. Replication-fork-containing restriction fragments are enriched by BND-cellulose chromatography, then separated according to extent of replication in a neutral agarose gel. This first dimension gel is then rotated 90°, surrounded by higher-percentage agarose, incubated with alkaline electrophoresis buffer, and subjected to alkaline electrophoresis in the second dimension, during which individual strands are separated according to size. The two parental strands of fork-containing fragments are constant in size, regardless of extent of replication. Therefore, they form a horizontal line in the 2D gel. However, the two nascent strands vary in size according to extent of replication. Because they are always smaller than the parental strands, they migrate as an angled arc or line ahead of the parental strands.

The final 2D gel is blotted to a nylon membrane and then hybridized sequentially with short probes derived from different regions of the restriction fragment of interest. The short probes used in the present work are indicated in Fig. 1 by numbered and shaded boxes. The proximity of any probe to an origin or to the earliest replicated end of a restriction fragment can then be estimated by measurement of the minimum length of nascent strand detected by that probe.

Replication Forks Move Unidirectionally Through the B9G *Bam*HI Fragment Towards the Telomere

DNA from growing yeast cells was digested with *Bam*HI, and fork-containing restriction fragments were enriched by adsorption to BND-cellulose. The enriched fragments were subjected to 2D neutral/alkaline gel electrophoresis, then blotted to a nylon membrane. The autoradiogram obtained after hybridization of the membrane with probe 1 (Fig. 2, left panel) is denser, because it was exposed for a longer time, than the autoradiogram obtained after hybridization of the same membrane with probe 2 (Fig. 2, right panel).

The major spot at the upper left in each autoradiogram in Fig. 2 is due to intact nonreplicating B9G *Bam*HI fragments; despite the BND-cellulose fractionation, these are the most abundant species. The vertical streak downwards from the major spot is due to rare random single-strand nicks in the nonreplicating DNA; the nicked strand fragments migrate more rapidly than the intact strands in the alkaline dimension. The horizontal line extending rightwards from the major spot is due to the parental strands of replicating restriction fragments. The parental strands

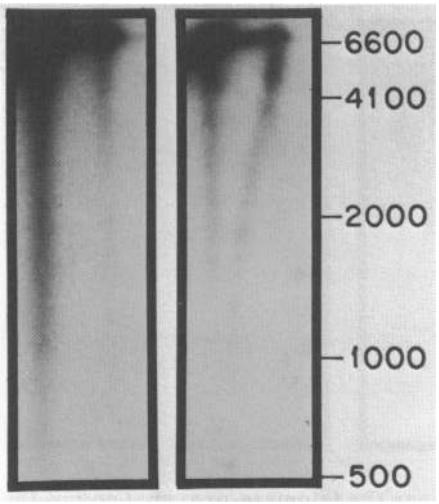

Figure 2. Unidirectional movement of replication forks through the B9G *Bam*HI fragment.. Left panel, hybridization with probe 1, exposure time 4 days. Right panel, hybridization with probe 2, exposure time 1 day. The numbers along the right show the lengths of marker DNA strands separated in the second (alkaline) dimension. First dimension (neutral) electrophoresis was from right to left; second dimension (alkaline) electrophoresis was from top to bottom. The procedures are described in Materials and Methods, and the significance of different portions of the autoradiograms is described in Results.

remain constant in size as extent of replication increases from left to right. The faint vertical streak downwards from the right end of the parental strand horizontal line in the probe 1 autoradiogram (Fig. 2, left panel) is due to rare nicks in the parental and nascent strands of the replicating restriction fragments which are more than 70% replicated and which [due to anomalous migration in the first dimension (3, 12)] accumulate at the right end of the parental strand line.

The nascent strands of replicating restriction fragments form an arc running upwards and rightwards from just to the right of the vertical streak due to nicked nonreplicating strands (in the lower third of the gel) to the right-hand end of the parental strand line. Probe 1 detects only the longest strands in this arc (obscured in Fig. 2 by the signal from the overexposed parental strand line) while probe 2 detects strands as short as 500 b (evident on longer exposure). Notice that, at any given strand length, the probe 2 signal from nascent strands is as strong as the probe 2 signal from nicked nonreplicating strand fragments. Because probe 2 lies at one end of the B9G fragment, it should detect nicked strand fragments of all sizes. The parallel signal intensity from nascent strands suggests that probe 2 detects nascent strands of all sizes. Since probe 1 detects only long nascent strands, replication forks must enter the B9G fragment at the probe 2 end (or originate near the centromere-proximal end of probe 2) and move unidirectionally through the fragment towards the probe 1 end. These results suggest that the origin responsible for replicating

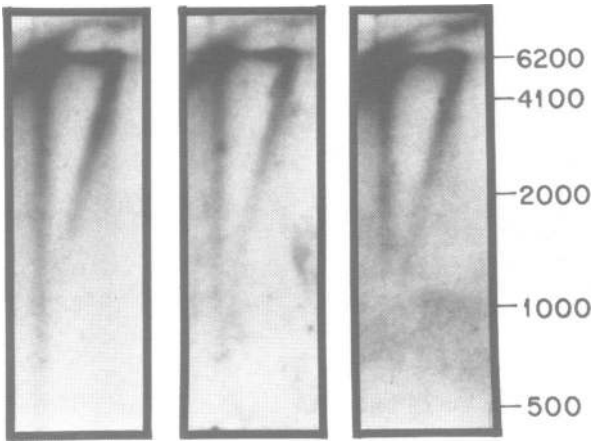

Figure 3. An origin near the telomere-proximal end of the A6C *Bam*HI fragment. Left panel, hybridization with probe 3, exposure time 5 days. Central panel, hybridization with probe 4, exposure time 4 days. Right panel, hybridization with probe 5, exposure time 1 day (higher specific activity probe). Other conditions as in Fig. 2.

the B9G fragment lies outside the fragment towards the centromere (or near the centromere-proximal end of probe 2).

Replication Forks Move Unidirectionally Through the A1G *Bam*HI Fragment Towards the Centromere

A blot from a 2D gel similar to that used in Fig. 2 was hybridized sequentially with probes 7 and 8 (see Fig. 1 for probe locations). The results (not shown) were similar in appearance to those shown in Fig. 2. Probe 7 detected nascent strands of all sizes, but probe 8 detected only the longest nascent strands even after long exposures. Thus replication forks move unidirectionally through the A1G *Bam*HI fragment, towards the centromere. This direction is opposite to the direction in which forks move through the B9G fragment; therefore, a replication origin must be located within the intervening fragment, A6C (or at the A6C-proximal end of the B9G or A1G fragment).

An Origin Is Located Near the B9G-Proximal End of the A6C *Bam*HI Fragment

The same blot used in Fig. 2 was hybridized sequentially with probes 3-6 (see Fig. 1 for probe locations). Probe 6 detected only very long nascent strands (not shown, but see Fig. 4), while probes 3-5 detected shorter nascent strands (Figs. 3 and 4).

Notice, in Fig. 3, that the shortest **nonreplicating nicked** strand fragments detected by the three probes become progressively longer from probe 3 to probe 5. That trend is due to the fact that, when nicks are rare (as they are in this case), so that a strand can contain at most just a single nick (which divides the strand into two strand fragments), the nick must be located within

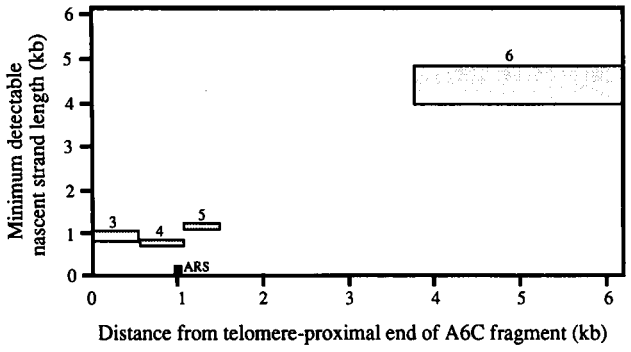

Figure 4. The shortest nascent strands are detected by probe 4. Measurements of minimum detectable nascent strand lengths from longer exposures of the autoradiograms in Fig. 3 (probes 3, 4, and 5) and from other autoradiograms not shown (probe 6) are plotted against the positions of the probes on the restriction map of the A6C *Bam*HI fragment (compare with Fig. 1). The vertical dimension of each shaded "data box" represents the estimated uncertainty in determination of minimum nascent strand length for that probe, and the horizontal dimension represents the position of that probe on the restriction map.

or distal to the probe complementary region in a strand fragment for the strand fragment to be detectable by the probe. Notice also in Fig. 3 (left panel) that the shortest **nascent** strands detectable by probe 3 (about 900 b) are distinctly longer than the shortest **nonreplicating nicked** strand fragments detected by the same probe (< 500 b). If replication forks traversed the A6C fragment from telomere end to centromere end, then the shortest nascent strands detected by probe 3 should be no longer than the shortest nicked strand fragments detected by the same probe. The length discrepancy suggests that the A6C fragment is replicated from an internal origin. The facts that (i) eukaryotic DNA replication is usually bidirectional and (ii) the shortest nascent strands detected by probe 3 are about 900 b suggest that the origin might be located about 450 b to the centromere side of probe 3. That expectation is consistent with the data obtained with probes 4 and 5.

Despite the higher background obtained during hybridization with probe 4, the autoradiogram (Fig. 3, center, and longer exposures, not shown) reveals that probe 4 can detect nascent strands somewhat shorter (about 750 b) than the shortest nascent strands detected by probe 3. Furthermore, the shortest **nascent** strands detected by probe 4 appear to be no longer than the shortest **nonreplicating nicked** strand fragments detected by the same probe.

Probe 5 (Fig. 3, right panel) detects both nicked and nascent strands of about the same length (about 1200 b), distinctly longer than the lengths detected by probes 3 and 4.

These findings are summarized in Fig. 4 where the vertical dimension of each data box represents uncertainty in measurement of minimum detectable nascent strand length and the hori-

zontal dimension represents the size of the probe segment. The minimum *detectable* nascent strand length is necessarily longer than the minimum length of a nascent strand containing a portion of the probe sequence, because (i) the logarithmic relationship between strand length and mobility during gel electrophoresis leads to increasing dilution of signal with decreasing strand length and (ii) the signal produced by nascent strands of a given length should be proportional to the fraction of the probe sequence contained within the nascent strands. Thus, the data in Figs. 3 and 4 suggest that there is an origin for bidirectional replication within the portion of the A6C fragment covered by probe 4, even though the minimum nascent strand length detected by probe 4 is about 750 b.

The fact that probe 6 detected only very long nascent strands (Fig. 4) while the adjacent probe 7 detected nascent strands of all sizes (not shown; see section on A1G above) is due to the location of probe 6 at the origin-**distal** end of the A6C fragment while probe 7 is at the origin-**proximal** end of the A1G fragment.

Confirmation of Origin Location by Neutral/Neutral 2D Gel Electrophoresis

Origins can also be mapped by the neutral/neutral 2D gel electrophoresis method of Brewer and Fangman (3). We have used this independent method to test whether an origin is located near the *ARS* element in the A6C fragment.

When an aliquot of the same *Bam*HI-digested DNA used for the neutral/alkaline gel of Figs. 2 and 3 was subjected to neutral/neutral 2D gel electrophoresis, blotted to a nylon membrane, and then hybridized with probe 1, the results shown in the left half of Fig. 5 were obtained. The black spot in the lower left is due to the overexposed nonreplicating B9G fragment. The faint spots indicated as "partials" are on the line formed by nonreplicating linear restriction fragments. Their mobilities in the first dimension are consistent with their being due to incomplete digestion by *Bam*HI at one end or the other of the B9G fragment. The arc which rises above the nonreplicating fragment and then falls back down to the line of linear fragments has the shape characteristic of arcs generated by restriction fragments containing single forks ("simple Y" structures; 3). This result is consistent with the conclusion obtained by neutral/alkaline electrophoresis: the B9G fragment is replicated by a single fork traveling from one end to the other.

When the same blot was hybridized with probe 6 to reveal the A6C *Bam*HI fragment, an arc with the same shape as that generated by the replicating B9G fragment was detected (result not shown). No deviation from the "simple Y" arc pattern was detected, even though the A6C fragment would be expected to contain an internal bubble for the first third of its replication. Brewer and Fangman have shown previously that it is difficult to detect origins located near the ends of restriction fragments by neutral/neutral electrophoresis (3).

To circumvent this problem, we decided to use *Eco*RI-digested DNA rather than *Bam*HI-digested DNA. As shown in Fig. 1, the *ARS* element is located near the center of a 5.8 kb *Eco*RI fragment (to be precise, the *ARS* element is located 3.05 kb from the left end and 2.75 kb from

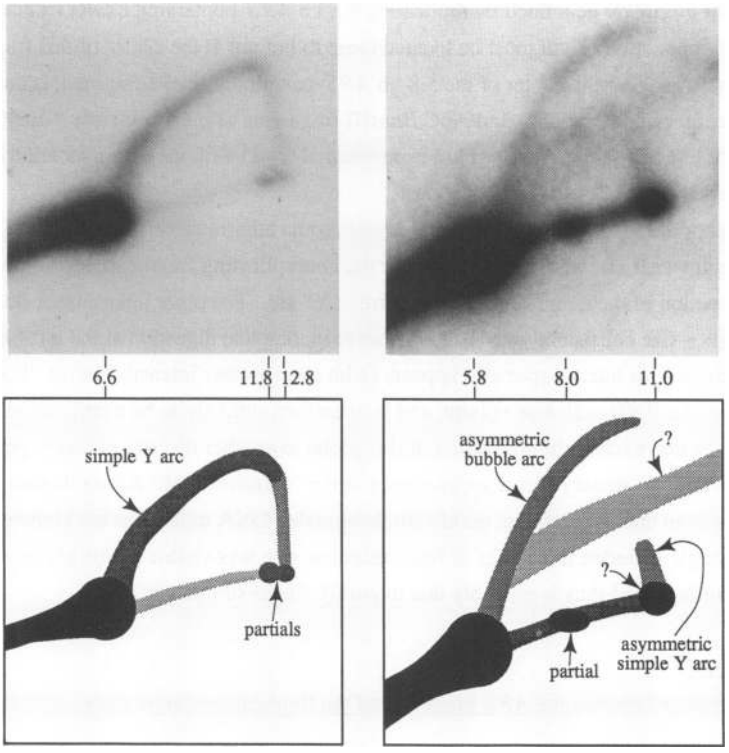

Figure 5. A replication origin near the B9G/A6C boundary detected by 2D neutral/neutral gel electrophoresis. Left half: *Bam*HI-digested DNA hybridized with probe 1 to reveal the 6.6 kb B9G fragment. Exposure time 4 days. Right half: *Eco*RI-digested DNA hybridized with probe 2 to reveal the 5.8 kb *ARS*-containing *Eco*RI fragment. Exposure time 7 days. The numbers between the diagrams and autoradiograms show sizes in kb determined from markers run in the first dimension. As in Figs. 2 and 3, first dimension electrophoresis was from right to left and second dimension electrophoresis was from top to bottom. The procedures are described in Materials and Methods, and the significance of different portions of the autoradiograms is described in Results.

the right end of this fragment). If an origin is located at the *ARS* element, then replicating 5.8 kb *Eco*RI fragments would contain internal bubbles until about 95% replicated. The bubble-containing molecules should produce a characteristic high curved arc (3) which would not return to the line of linears. However, after 95% replication, the rightwards-moving fork should run off the end of the fragment, generating "simple Y" fragments which should produce the final portion of a "simple Y" arc as they complete replication (see Fig. 1 in reference 3).

The autoradiogram in the right half of Fig. 5 shows that both the predicted high curved arc due to an internal origin and the predicted final-portion "simple Y" arc due to the asymmetric lo-

cation of that origin are generated by replicating 5.8 kb *ARS*-containing *Eco*RI fragments. Therefore, a replication origin must be located close to but not at the center of this fragment. Location of an origin near the center of the 5.8 kb *ARS*-containing *Eco*RI fragment is consistent with replication of both the B9G and A6C *Bam*HI fragments as predominantly "simple Y" structures (Fig. 5, left half) and agrees (within experimental error) with the origin location determined by neutral/alkaline 2D electrophoresis.

The autoradiogram (Fig. 5, right half) reveals some additional features: a faint smeary signal running upwards and rightwards from near the nonreplicating fragment, and an intense spot at the intersection of the linear line and the "simple Y" arc. The other intense spot (indicated as "partial") has a size consistent with its being due to incomplete digestion at the left-hand *Eco*RI site. The mysterious intense spot also appears (with even greater intensity) in the "flow-through" fraction from the BND-cellulose column and is, therefore, unlikely to be a replication intermediate. It may be due to cross-hybridization of this probe to another discrete genomic yeast *Eco*RI fragment. The faint smeary signal appears only in the "caffeine wash" autoradiogram. However, it occurs in an area where long, purely single-stranded DNA molecules are known to run (an arc due to single-stranded molecules of heterogeneous size was visible in this gel by ethidium bromide staining), and thus is probably due to purely single-stranded DNA.

DISCUSSION

The Relationship Between the *ARS* Element and the Replication Origin in the A6C *Bam*HI Fragment

In earlier work (9, 11, Van Houten and Newlon, unpublished), a single *ARS* element was found within the 22.5 kb stretch of yeast chromosome III diagramed in Fig. 1. In the present study, we have found a single replication origin within the same 22.5 kb stretch. Is it pure coincidence that the *ARS* element and the origin appear to be located within the same 550 bp *NruI/ClaI* restriction fragment? The facts that replication origins have previously been mapped to within several hundred bp of *ARS* elements in yeast plasmids (2,3) and that the replication origin for yeast rDNA is located within several hundred bp of an *ARS* element (Linskens and Huberman, manuscript submitted for publication) strongly suggest that the colocalization of *ARS* element and replication origin in the present study is not due to chance. Instead, the results presented in this paper strengthen the suggestion, raised by the earlier studies, that yeast replication origins are *ARS* elements.

If the A6C origin really corresponds to the A6C *ARS* element, then why does probe 3 detect shorter nascent strands than probe 5, suggesting that replication may initiate slightly (300-400 bp) to the telomere side of the *ARS* element (see Figs. 3 and 4)? There are three possible explanations for this apparent discrepancy. First, the data plotted in Fig. 4 may be misleading due to the lengths of the probe segments used. More accurate estimates of the map positions of the shortest detectable nascent strands would be provided by shorter probes (as in 2), because

shorter probes provide a truer "end labeling" effect. Second, replication may begin at the *ARS* element, but the initial rates of replication fork movement away from the *ARS* element may be unequal. If the movement of replication forks toward the telomere were faster than toward the centromere, probe 3 would detect shorter nascent strands than probe 5, as observed. Third, it is possible that all the DNA signals essential for initiation are contained in the short stretch identified as an *ARS* element in Figs. 1 and 4, but that initiation actually begins some distance away from these signals. Such displaced initiation might occur, for example, if the *ARS* element simply provided signals for initiation of unwinding of parental strands (13) with initiation actually taking place elsewhere within the unwound stretch.

A Note of Caution

Although the data obtained so far are consistent with a 1 to 1 correspondence between *ARS* elements and origins, it is important that final judgment be reserved until many more origins have been mapped. Because the only consistent features of *ARS* elements are one or more close matches to an 11 bp consensus sequence (14, 15) and a stretch of DNA which must be easily unwound (13, 14) or contain additional matches to the consensus sequence (15), or both, it is conceivable that these features might occur by chance in stretches of DNA which cannot serve as functional origins *in vivo* due to surrounding chromatin structure, or to heavy transcription, or for some other reason. Such stretches might well serve as *ARS* elements when their normal chromosomal surroundings are replaced by plasmid sequences. We predict, therefore, that it is likely that not all *ARS* elements will prove to be origins. Whether all origins will prove to be *ARS* elements is impossible to predict at this point. It will be exciting, however, if more extensive mapping reveals origins which do not function as *ARS* elements in plasmids.

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