DNA replication initiates at domains overlapping with nuclear matrix attachment regions in the xenopus and mouse c-myc promoter

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Abstract

Only a very few origins have been mapped in different multicellular organisms, and they do not share detectable consensus sequence elements. Moreover, it is not clear if origins are localized at similar positions in the corresponding locus in genomes of different organisms. Here, we have mapped DNA replication origins in the c-myc locus both in Xenopus and mouse, allowing a comparison of the corresponding sites in three different animal species (Xenopus, mouse, human). An origin of DNA replication is present in the three homologous c-myc loci. In Xenopus, a main DNA replication origin was located 3 kilobases (kb) upstream of the active c-myc promoter, whereas, in mouse, we detected an origin 1 kb upstream of the promoter, as previously mapped in human c-myc. We also identified a nuclear matrix attachment region in both Xenopus and mouse, which is localized to two different regions of the c-myc promoter region. However, in both cases, the nuclear matrix attachment sites are close to the DNA replication origin mapped in the locus. These data suggest that global features of chromatin organization in different organisms may contribute to DNA replication origin localization.

Keywords: Origin of DNA replication; Chromatin structure; P19 teratocarcinoma cells; Nuclear matrix

1. Introduction

In multicellular organisms, the nature of initiation sites of DNA replication is more complex than in simple genomes such as those of yeast or virus, and no clear consensus sequences have been defined. Metazoan origins often effect initiation at multiple sites distributed over larger distances, up to several dozens of kilobases (kb), with different frequency. As an example, ori β and γ are major sites of initiation in the DHFR locus, but initiation can also take place at many minor sites scattered along 55 kilobases (Dijkwel et al., 2002). The localization and efficiency of DNA replication origins may be influenced by epigenetic factors. Initiation site usage is submitted to developmental regulation (Hyrien et al., 1995; Sasaki et al., 1999; Lunyak et al., 2002) and seems to be affected by higher order of chromosome structure (DePamphilis, 1999; Mechali, 2001). In some cases, co-localization of DNA replication foci and origins with anchorage sites to the nuclear matrix has been described. Lamin B2, DHFR and globin origins coincide or are located in the vicinity of matrix attachment regions (Razin et al., 1991; Lagarkova et al., 1998; Djeliova et al., 2001; Mesner et al., 2003). Very few metazoan replication origins have been mapped so far, around 20, and only a small subset of them have been characterized in detail (Todorovic et al., 1999 for a review). In human and hamster, less than 10 origins have been identified whereas in the mouse, only four origins have been mapped, namely rDNA, adenosine deaminase (ADA), β-globin and Ig heavy chain loci (Carroll et al., 1993; Gogel et al., 1996; Aladjem et al., 2002; Zhou et al., 2002). In Xenopus laevis, a single DNA replication origin has been mapped, that was shown to be specifically localized in the rDNA domain in tail bud embryos, when the locus is active (Hyrien et al., 1995). A preferential attachment to the nuclear matrix could therefore be the rule in Xenopus.
matrix occurs at the same region at the same period of development (Vasssetzky et al., 2000).

The rDNA and β-globin origins are the only origins that have been described in more than one multicellular organism (Kitsberg et al., 1993; Hyrien et al., 1995; Gogel et al., 1996; Aladjem et al., 1998; Aladjem et al., 2002). However, the complexity of the rDNA locus does not permit a clear comparative analysis and the β-globin locus shows different initiation patterns in human and mouse. Therefore, it is not clear whether origin specification at a given locus is conserved throughout evolution. The replication of the human c-myc gene has been also extensively studied and was shown to initiate at multiple sites throughout a 12.5 kb locus (Vassilev and Johnson, 1990; Trivedi et al., 1998). A 2.4 kb segment located just upstream of the promoter confers autonomously replicating sequence (ARS) activity to a plasmid transfected in Hela cells (McWhinney and Leffak, 1990), and a major initiation site was shown to be located 0.5 kb upstream of the promoter in the endogenous c-myc locus in the same cells (Tao et al., 2000). An origin might be also present in the chicken c-myc promoter, although it has not been mapped precisely (Phi-van et al., 1996; Aladjem et al., 1998; Aladjem et al., 2002). However, the c-myc gene has been also extensively studied and was shown to initiate at multiple sites throughout a 12.5 kb locus (Vassilev and Johnson, 1990; Trivedi et al., 1998). A 2.4 kb segment located just upstream of the promoter confers autonomously replicating sequence (ARS) activity to a plasmid transfected in Hela cells (McWhinney and Leffak, 1990), and a major initiation site was shown to be located 0.5 kb upstream of the promoter in the endogenous c-myc locus in the same cells (Tao et al., 2000). An origin might be also present in the chicken c-myc promoter, although it has not been mapped precisely (Phi-van et al., 1998).

Here, we investigated whether the c-myc DNA replication origin is specifically localized in Xenopus embryos at the neurula stage, when transcription of c-myc is activated, and we compared its localization in mouse teratocarcinoma cells. We show that a DNA replication origin is detected 3 kb upstream of the c-myc gene whereas in mouse P19 cells, a DNA replication origin is detected 3 kb upstream of the c-myc gene. Interestingly, in both organisms, we found that the initiation sites correspond to a region of c-myc that is specifically attached to the nuclear matrix. Therefore, although two different origins and nuclear matrix sites can be observed within the c-myc domain in Xenopus and mouse, both of them co-localize with the anchorage sites to the nuclear matrix.

2. Materials and methods

2.1. Plasmid and DNA sequencing

The pXlmyc3 plasmid contains a 6.5 kb EcoRI fragment from −5400 to +1100 bp of the P2 X. laevis c-myc promoter. The region from −4482 up to +1100 was sequenced for this study (EMBL, accession number: AJ605770). The region from −1900 to +1100 was previously reported (Principaud and Spohr, 1991). The EG3 repeats found between −4482 and −3240 are framed by PstI cleavage sites and are inverted in the c-myc promoter. They are flanked in the 3′ region by 60 nucleotides of an incomplete EG3 sequence, which ends 3360 pb upstream to the P2 transcription start site.

2.2. DNA isolation

Xenopus eggs and embryos were collected as described (Menut et al.). Embryos were allowed to develop up to the neurula stage for 22 to 25 h at 18 °C. 2000 to 3000 neurula embryos were homogenised in 20 µl per embryo of extraction buffer (10 mM Tris pH 8.0, 0.1 M EDTA, 0.5% sarkosyl, 600 µg/ml protease K) and treated for 3 h at 37 °C. DNA was extracted three times with phenol–chloroform, once with chloroform and precipitated; 10⁴ exponentially growing P19 cells were lysed in DNAzol (Invitrogen) and DNA purified following manufacturer instructions.

2.3. Purification of nascent DNA strands

DNA resuspended in 10 mM Tris pH 8.0, 1 mM EDTA, 20 mM NaCl (TEN 20) was heat denatured, layered on a 38 ml 5–30% neutral sucrose gradient in 10 mM Tris pH 8.0, 1 mM EDTA, 500 mM NaCl (TEN 500), and size fractionated by centrifugation in a Beckman SW28 rotor at 26,000 rpm for 20 h at 4 °C. Thirty five fractions of 1 ml were collected, precipitated with ethanol, and resuspended in TEN 20. One tenth of each fraction was analysed by alkaline agarose gel electrophoresis and visualised either by ethidium bromide (EtBr) staining or Southern hybridization with 32P labelled genomic DNA. Fractions of chosen size were pooled and treated by T4 polynucleotide kinase and then by λ-exonuclease as described (Gerbi and Bielinsky, 1997). The nascent strand populations were used for quantitative PCR analysis.

2.4. Oligonucleotide primers and PCR

The purified nascent strands were analysed by real-time quantitative PCR at different locations on the c-myc gene locus. Primers used are described in Fig. 1B. PCR reactions were carried out in 15 µl with 5 µl of 10 fold diluted samples using Lightcycler capillaries and the Lightcycler FastStart DNA master SYBR Green I mix from Roche Molecular Diagnostics. The PCR mix contained 3 mM MgCl₂, 1 µM primers and 1.5 µl of master mix. Mouse P19 genomic DNA or X. laevis genomic DNA isolated from erythrocytes was used for the standard curve reactions. An initial denaturation of 8 min at 95 °C was followed by 45 cycles with denaturation for 15 s at 95 °C, annealing for 5 s at 60 °C, and polymerisation for 10 s at 72 °C. Specificity of the PCR products was assessed by a melting curve analysis and agarose electrophoresis.

2.5. Purification of nuclear matrices

Nuclei were purified from Xenopus embryos (Gorski and Schibier, 1986) or P19 cells (Gasser and Vasssetzky, 1998). Nuclear matrices were prepared by treatment of the isolated nuclei with deoxyribonuclease (DNase) I followed by ex-
traction with Lithium 3,5-diiodosalicylate (LIS) essentially as described (Gasser and Vassetzky, 1998). Digestion buffer (100 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, pH 7.5, 0.25 mM spermidine, 1 mM CaCl₂) was added to 10⁵ nuclei to a final volume of 400 µl. The nuclei were digested with 100 µg/ml DNasel for 3 h at 4 °C. The digestion was
followed by a stabilisation step, the addition of CuCl$_2$ to a final concentration of 1 mM and incubation for 10 min at 4°C. The nuclei were then extracted with five volumes of LIS extraction buffer containing 10 mM Tris–HCl, pH 7.5, 0.25 mM spermidine, 2 mM EDTA-KOH, pH 7.5, 0.1% digitonin, and 25 mM LIS for 5 min at room temperature. The histone-depleted nuclear matrices were recovered by centrifugation and the nuclear matrix pellet was washed three times in a buffer containing 20 mM Tris–HCl, pH 7.5, 0.25 mM spermidine, 0.05 mM spermine, 100 mM NaCl, and 0.1% digitonin. Nuclear matrices were digested with proteinase K, extracted with phenol–chloroform, and treated with ribonuclease (RNase) A. The DNA associated with the nuclear matrix/skeleton was used as substrate for real-time

Fig. 2. Origin of replication mapping in *X. laevis* c-myc promoter. (A) Map of *X. laevis* c-myc promoter region. Positions are indicated with respect to P2 promoter. Primer couples used for quantification are indicated. EG3 repeats are shown by shaded boxes; 5’ part of the c-myc gene is shown by an open box, with exons in black. (B) Nascent DNA strands of 0.5–1 kb were isolated as described in Fig. 1A and Materials and methods, then quantified by quantitative real-time PCR with indicated primers pairs. Similar results were obtained with two independent nascent strands preparations. Enrichment factor is obtained with respect to amount of DNA obtained for primers couple C. (C) Nascent DNA strands of indicated size were isolated and quantified by real-time PCR with indicated primers pairs. Arbitrary units (a.u) correspond to standards range used for quantitative real-time PCR.

![Diagram](image-url)
PCR quantification or as a probe on a slot blot with PCR fragments covering the 5’-end of the *X. laevis* c-mycI gene domain.

3. Results

3.1. Mapping initiation of DNA synthesis in the Xenopus c-mycI gene

Initiation of DNA replication has been extensively analysed in the human c-myc locus and a main replication initiation site was localized 0.5–2 kb upstream of the active P2 promoter (McWhinney and Leffak, 1990; Vassilev and Johnson, 1990; Tao et al., 2000; Liu et al., 2003). We have addressed the question of the initiation of replication along the c-myc locus in *X. laevis*, in neurula embryos, when cells divide actively and the c-myc gene is transcriptionally active (Taylor et al., 1986). We completed the sequence of the promoter cloned in the pXlmyc3 plasmid up to /C0 4.482 kb upstream of the active P2 c-myc promoter (AJ605770, Materials and methods). Interestingly, we observed that a region localized /C0 4482 to /C0 3420 bp contains eight repeats of a repetitive EG3 DNA sequence. These 133 bp elements represent 1.5% of the total *Xenopus* genome (Meyerhor et al., 1987).

To map DNA replication initiation sites in the *Xenopus* c-myc locus, we used the method of nascent strand relative abundance described in Fig. 1A (see also Materials and methods). Initiation of DNA synthesis occurs by the synthesis of RNA-primed DNA at replication origins. These fragments can be purified by denaturation of the DNA and sucrose gradient fractionation (Gerbi and Bielinsky, 1997; Giacca et al., 1997). A λ-exonuclease treatment of the nascent strand DNA population was further performed to remove any broken pieces of DNA that could contaminate the preparation (Gerbi and Bielinsky, 1997). The presence of a preferential site of initiation of DNA replication was analysed by real-time quantitative PCR, using primers described in Fig. 1B. We could identify in the *Xenopus* c-myc domain two regions (A and B) upstream of the promoter where short nascent 0.5–1 kb DNA were more abundant than the background value observed in c-myc exon 2 region (C) (Fig. 2B,C). The major site A was found 3 kb upstream of P2 and gave a 20-fold enrichment in the short nascent strand synthesis. Enrichment factor of 10 with this method is considered highly significant (Kobayashi et al., 1998; Kamath and Leffak, 2001; Aladjem et al., 2002). To confirm this result, we took a different primer pair in the same region (A2), with which we obtained a similar value. A minor site, B, gave an 8-fold enrichment and was located close to the promoter. We obtained similar data with independent preparations. To confirm the presence of an initiation site, we selected populations of nascent DNA of a larger size from the sucrose gradient. As the size of nascent DNA is longer in these populations, regions located further

Fig. 3. Origin of replication mapping in murine c-myc promoter. (A) Map of murine c-myc promoter region. Positions are indicated with respect to P2 promoter. Primer couples used for quantification are indicated. SINE repeats are shown by shaded boxes; 5’ part of the c-myc gene is shown by an open box, with exons in black. (B) P19 cells nascent DNA strands of 0.5 –1 kb were isolated as described in Fig. 1A and Materials and methods, then quantified by real time PCR with indicated primers pairs. Arbitrary units (a.u) correspond to standards range used for quantitative real-time PCR. Similar results were obtained with three independent nascent strands preparations.
Fig. 4. Matrix attachment site mapping. (A) Principle and major steps of matrix associated regions (MARs) isolation. Nuclei from embryos or P19 cells were isolated and treated by DNaseI. The nuclear matrix was then prepared using LIS extraction (see Materials and methods). DNA fragments remaining on the matrix were purified as matrix associated region and used for quantification. (B) Primers used for MAR mapping name, 5′ end position and sequence for each primer are indicated.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5′-3′)</th>
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</thead>
<tbody>
<tr>
<td><strong>Xenopus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 f:</td>
<td>-3254</td>
<td>cattccatcgttgacagcagtg</td>
</tr>
<tr>
<td>A3 r:</td>
<td>-3068</td>
<td>agggcatttactcgttac</td>
</tr>
<tr>
<td>B2 f:</td>
<td>-2765</td>
<td>ccaccaacccccaaag</td>
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<tr>
<td>B2 r:</td>
<td>-2523</td>
<td>cagagagcttgagactggagcag</td>
</tr>
<tr>
<td>C2 f:</td>
<td>-2363</td>
<td>ggccgcaatgaggttttac</td>
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<tr>
<td>C2 r:</td>
<td>-2087</td>
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</tr>
<tr>
<td>D2 f:</td>
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</tr>
<tr>
<td>D2 r:</td>
<td>-867</td>
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</tr>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A f:</td>
<td>-3800</td>
<td>cggcatgatgttgacgttaa</td>
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<td>A r:</td>
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<td>C f:</td>
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<tr>
<td>D r:</td>
<td>+3906</td>
<td>agccgcactcgcagcctt</td>
</tr>
</tbody>
</table>

* f: forward r: reverse  
† 5′ end position indicated with respect to P2 promoter
away from the origin are now expected to be enriched. Indeed, we observed that the amount of DNA for primers B and C increased progressively in 1.6–3 kb and more than 3 kb populations. As expected, this result is associated with a loss of relative enrichment between origin and nonorigin regions (Fig. 2C).

Fig. 5. Matrix attachment site mapping in Xenopus and mouse c-myc promoter. (A) Map of X. laevis c-myc promoter region. Positions are indicated with respect to P2 promoter. Primer couples used for quantification are indicated. EG3 repeats are shown by shaded boxes; 5' part of the c-myc gene is shown by an open box, with exons in black. (B) Tail bud embryos nuclei were isolated and matrix associated sequences purified as described in Fig. 4A and in Materials and methods. These sequences (MAR) or pXlmyc3 plasmid (control DNA) were used as a probe to hybridize on slot blotted PCR products of different indicated regions of the c-myc locus. Band intensities were quantified by scanning with a PhosphorImager. (C) Map of murine c-myc promoter region. Positions are indicated with respect to P2 promoter. Primer couples used for quantification are indicated. SINE repeats are shown by shaded boxes; 5' part of the c-myc gene is shown by an open box, with exons in black. (D) Matrix associated P19 DNA was isolated as described Fig. 4A and in Materials and methods. Quantification by real-time PCR analysis was realized with indicated primers for MAR or genomic DNA as control. Similar results were obtained with two independent measures of two independent MAR preparations.
3.2. Initiation of DNA replication in the c-myc locus from mouse P19 cells

Analysis of DNA replication initiation in human c-myc allowed to map a replication origin 1 kb upstream of the active promoter (Trivedi et al., 1998; Tao et al., 2000), therefore, at a location which appears different from the major Xenopus c-myc origin. To investigate whether the main replication origin might localize at different places according to the animal species analysed, we mapped the c-myc origin in mouse. Nascent strands where isolated from proliferative unsynchronized P19 cells following the same procedure as for Xenopus embryos and their enrichment along the c-myc locus was analysed by real-time PCR. Fig. 3 shows that in mouse P19 cells, c-myc origin localizes at a position similar to that identified in human cells, 1 kb upstream of the transcription site P2. However, we did not detect an origin in the more upstream region. Interestingly, human and mouse sequences are relatively conserved in the upstream promoter region, as opposed to the Xenopus corresponding region.

3.3. The origins of DNA replication in Xenopus late embryos and mouse P19 cells are both close to nuclear matrix attachment regions

The observation of a different localization of the c-myc origin in a domain of two species (mouse and Xenopus) where the sequences are quite divergent, led us to investigate if structural elements may be common in these two regions. Different origins have been localized close to or at matrix attached regions (Lagarkova et al., 1998; Djeliova et al., 2001; Mesner et al., 2003). Therefore, one question that we investigated was a possible correlation between the localization of the origin and the nuclear matrix attachment site in the c-myc locus from both species. We treated Xenopus embryonic nuclei salt buffer with lithium-3,5-diodosalicylate to remove histone and non-histone proteins but maintain the interactions of matrix associated regions (MARs) with the resulting nuclear matrix (Gasser and Vassetzky, 1998). We used this method to isolate the nuclear matrix after a DNaseI treatment to detach the DNA loops from the matrix (see Materials and methods and Fig. 4A). The DNA remaining on the matrix was radiolabelled and used as a probe in order to examine the organization of the nuclear matrix attachment sites at different positions of the Xenopus c-myc gene. PCR products of different c-myc regions between −3332 and +1199 were amplified (Fig. 4B) and slot blotted onto a nylon membrane. They were then quantified after hybridization with either total c-myc (control DNA), or with the nuclear matrix DNA fraction (Fig. 5A and B). A similar signal was detected when the complete c-myc domain was used as a probe, but when the nuclear matrix DNA was used as a probe, a preferential attachment site was detected between −3330 and −2500 from the c-myc promoter. This region also corresponds to the main initiation site of replication in the Xenopus c-myc.

To investigate if the DNA replication origin was also a nuclear matrix attachment site in mouse, we isolated matrix sequences from growing P19 cells using the same method, and quantified their enrichment along the mouse c-myc locus by quantitative PCR (Fig. 5C and D). We found that the preferential nuclear matrix attachment site was not localized at a similar 2.5–3 kb upstream position from the promoter, as in Xenopus, but at a position close to the promoter site, overlapping the mouse c-myc initiation site of DNA replication (Fig. 5D).

We conclude that both in Xenopus and mouse P19 cells, the major DNA replication origins, do not localize at a similar position in the locus, but that in both cases they localize close to the nuclear matrix attachment site.

4. Discussion

We have mapped origins of DNA replication in the c-myc domain in both Xenopus and mouse allowing a first comparison of origin specification in three different animal species (Xenopus, mouse and human; Fig. 6). Until now, searches for consensus sequences of origins have generally considered different origins in different organisms. We extend the observation of lack of a clear consensus sequence to a single locus. This lack of conserved sequences is in Fig. 6. Comparison of c-myc promoter organization in Xenopus, mouse and human. Positions are indicated related to P2 transcription promoter. Origin and MAR position are indicated according to results presented in this study. Major human origin is indicated according to previous mapping (Waltz et al., 1996; Tao et al., 2000). Repeats (EG3 in Xenopus, SINE for mouse and human) are shown by shaded boxes; 5′ part of the c-myc gene is shown by a filled box. DNaseI hypersensitive sites (HS) are indicated.
contrast with conservation of transcription factor binding sites in this promoter. We also found no correlation between the position of origin relative to the promoter or transcription factor binding sites. The proximity of the *Xenopus* c-myc replication origin to EG3 repeated sequences is intriguing. These repeated elements were isolated from a *X. laevis* gastrula and stage embryos cDNA library prepared from polyadenylated RNA (Meyerhor et al., 1987), and are thus likely to be transcribed. These sequences are not present in the mouse or human c-myc DNA locus, but other repeated sequence elements are also present at a similar distance from the P2 promoter in both mouse and human. Five mouse short interspersed repetitive (SINE) elements are present 2 kb upstream of the mouse c-myc promoter and five human SINE/Alu elements are detected 2 kb upstream of the human c-myc promoter (Fig. 6). The presence of A-T rich elements has been reported in several eukaryotic replication origins (Todorovic et al., 1999). Although c-myc upstream region can be considered enriched in A-T nucleotides, we could not detect any clear correlation with initiation sites of DNA replication in this domain.

Our observations also show that the localization of a replication origin tested with the same method, and within the same locus, may change according to the context of the cell including its species. These data extend to another locus the observation that the distribution of replication origins in the β-globin locus may be different according to species (Aladjem et al., 2002).

In *Xenopus*, we have also identified a DNase I hypersensitive site (HS) 2.6 kb upstream of P2, therefore, close to the major site of initiation of DNA replication (Prieoleau et al., 1995). DNase I HS often lie close to regulatory regions and are a hallmark of open chromatin regions. DNase I HS are located close to the human c-myc promoter as well as at 2 kb upstream of P2 (Michelotti et al., 1996), and they are conserved in the murine c-myc locus (Marcu et al., 1992). The main DNA replication origin in *Xenopus* and mouse are close to DNase I hypersensitivity sites. Dynamic changes in the chromatin structure of the DHFR β and γ origins were shown to be associated with micrococcal nuclease HS located near primary initiation sites. These HS sites were detected in cells collected at the G1/S boundary, and only in the chromatin fraction attached to the nuclear matrix (Pemov et al., 1998).

Although the nuclear matrix has not yet been well characterized at the biochemical level, nuclear matrix-associated DNA has been found to be associated with replication origins in several cases, including the chicken lysozyme replication origin (Phi-van et al., 1998), the DHFR origins β and β′ (Dijkwel and Hamlin, 1988; Mesner et al., 2003) and the lamin B2 origin (Lagarkova et al., 1998). Moreover, the enrichment of DNA replication intermediates by nuclear matrix isolation was one of the first method used to map replication origins (Dijkwel et al., 1991). In human, c-myc replication origin is nested within a DNA loop anchorage region (Gromova et al., 1995). Here, we found one major nuclear matrix attachment site in both the c-myc promoter domain from *Xenopus* and mouse cells. In both cases, this site overlaps with the main replication origin mapped in this locus. This result emphasizes a role of chromosomal structure in origin recognition in pluricellular organisms.

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