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Identification and autonomous replication capability of a chromosomal replication origin from the archaeon *Sulfolobus solfataricus*

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Abstract Here, we describe the identification of a chromosomal DNA replication origin (*oriC*) from the hyperthermophilic archaeon *Sulfolobus solfataricus* (subdomain of Crenarchaeota). By means of a cumulative GC-skew analysis of the *Sulfolobus* genome sequence, a candidate *oriC* was mapped within a 1.12-kb region located between the two divergently transcribed *MCM*- and *cdc6*-like genes. We demonstrated that plasmids containing the *Sulfolobus oriC* sequence and a hygromycin-resistance selectable marker were maintained in an episomal state in transformed *S. solfataricus* cells under selective pressure. The proposed location of the origin was confirmed by 2-D gel electrophoresis experiments. This is the first report on the functional cloning of a chromosomal *oriC* from an archaeon and represents an important step toward the reconstitution of an archaeal in vitro DNA replication system.

Keywords Archaea · DNA replication · GC-skew analysis · Replication origin · *Sulfolobus solfataricus*

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Introduction

Eubacteria, bacteriophages, and animal viruses possess a single replication origin (*oriC*), whereas Eukarya, whose genomes comprise many chromosomes, have evolved a complex mechanism to initiate DNA replication at multiple sites in a highly coordinated fashion. Eubacterial initiation of DNA replication was investigated in great detail in *Escherichia coli* and an in vitro system was established for this organism by means of plasmids bearing the *oriC* and purified enzymes and protein factors (Kornberg and Baker 1992). The best-studied eukaryotic *oriC*s are those of the budding yeast *Saccharomyces cerevisiae*, which were originally isolated from chromosomes and naturally occurring plasmids as autonomously replicating sequences (ARS) (for a review see Diffley 1996). ARS elements were also identified in other unicellular eukaryotes (Kelly and Brown 2000). In Metazoa, although a considerable number of chromosomal regions were identified where initiation takes place, only in a few cases were sequences critical for origin function finely mapped (DePamphilis 1999).

Analysis of the genome sequence of several species and preliminary biochemical studies have recently suggested that quite likely archaea duplicate their single circular chromosome by using a replication machinery similar to that of eukaryotes, but considerably simpler (Olsen and Woese 1997; Tye 2000). Therefore, studying DNA replication in archaea is not only interesting in its own right from an evolutionary standpoint, but is also believed to provide a useful model to dissect key conserved aspects of replication initiation, as well as of replisome assembly and progression.

Several components of the replisome and primosome of the crenarchaeon *Sulfolobus solfataricus* were identified and biochemically characterized (Pisani et al. 1998, 2000; De Felice et al. 1999; Carpentieri et al. 2002; Dionne et al. 2003). This organism, originally isolated from an acidic hot spring near Naples (Italy), grows optimally at about 87°C and pH 3.5 under aerobic conditions

(Grogan 1989). Herein we describe the identification of a chromosomal DNA *oriC* from *S. solfataricus* and, for the first time in an archaeon, its functional cloning.

Materials and methods

Computational analysis

The complete sequence of *Sulfolobus solfataricus* (P2 strain) was downloaded from the Web page http://niji.imb.nrc.ca/sulfolobus/super_pub/supers.html and the cumulative GC-skew analysis was performed in nonoverlapping windows of 3 kb, as previously described (Grigoriev 1998).

Construction of plasmids

The *oriC* sequence (1,120 bp) was amplified by polymerase chain reaction from *S. solfataricus* P2 genomic DNA, using the following oligonucleotides: 5′Ssori: 5′-ggttctcgagatcaccatataattccttaataagttgttt-3′ and 3′Ssori: 5′-ggttctcgagaccccaatctaaattaagtggtattacctt-3′ and cloned into the *Sma*I-linearized *Escherichia coli* pUC28 vector (Benes et al. 1993) to create the plasmid named p*oriC*. The hygromycin-phosphotransferase (hph^T) gene cassette was excised from the pEXSs vector (Cannio et al. 1998) by *Bgl*II/*Nsi*I digestion and cloned into *BamH*I/*Pst*I-linearized pUC28 and p*oriC* plasmids to obtain the constructs designated phph^T and p*oriC*-hph^T, respectively.

Cultivation and transformation of *S. solfataricus* cells

S. solfataricus Gθ cells (Cannio et al. 1998) were grown aerobically in Brock's basal salt medium containing 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, and 0.1% (w/v) glucose and buffered at pH 3.5 at 80°C. Cells were made competent for electroporation and transformed with the plasmid constructs as previously described (Cannio et al. 1998). Recombinant clones (transformation efficiency: 5×10^2 colonies/μg of plasmid DNA) were selected on the same medium containing hygromycin B (150–200 μg/ml, the amount depending on the specific batch) in Gelrite plates (Kelco), propagated under selective growth conditions, and harvested at the mid-log growth phase.

Characterization of *S. solfataricus* transformed cells

Hygromycin-resistant clones were cultivated in 200 ml of selective medium and harvested at mid-log phase of the growth curve. The extrachromosomal DNA was prepared from 10 ml of each culture, using the NucleoSpin Plus Plasmid Miniprep kit (Clontech, Palo Alto, Calif., USA). Plasmid DNA was digested with the *Hin*dIII enzyme and subjected to Southern blot analysis, using the ^{32}P-radiolabeled *oriC* sequence as the probe under standard blotting and hybridization experimental conditions. Total DNA was extracted from *Sulfolobus*-transformed cells as described by Cannio et al. (1998). The DNA preparations were digested with the *Eco*RI enzyme and subjected to Southern blot analysis, as described above, using the ^{32}P-radiolabeled hph^T gene sequence as the probe. To determine the copy number of the p*oriC*-hph^T, total DNA extracted from two *Sulfolobus*-resistant clones was digested with *Hin*dIII and subjected to Southern blot analysis, using the *oriC* sequence as the probe.

Stability of the plasmid p*oriC*-hph^T in the *S. solfataricus* cells

The stability of the plasmid p*oriC*-hph^T was determined by calculating the rate at which the plasmid is lost per generation during nonselective growth, according to Huberman (1999). Two parameters were determined: P_s (ratio of plasmid-bearing cells during exponential growth in selective medium) and P_n (proportion of plasmid-bearing cells after G generation in nonselective medium). The plasmid loss rate (L) was then calculated according to the formula: $L=1-(P_n/P_s)^{1/G}$. To calculate the P_s value, cells from two independent p*oriC*-hph^T-transformed clones were grown up to the mid-log phase under selective conditions, and then, approximately 200 cells were plated onto selective and nonselective plates. To determine the P_n parameter, cells were grown up to midlog phase under selective conditions and then diluted by a factor of 2^6 in nonselective liquid medium, where six is the planned number of generations of growth in nonselective medium. After three and six generations of growth, approximately 200 cells were plated onto selective and nonselective plates.

Neutral/neutral 2-D agarose gel electrophoresis

S. solfataricus cells were cultivated aerobically in 100 ml medium, as described above. Cells were harvested when the absorbance at 600 nm of the culture reached a value of 0.3. The cell pellets (about 10^{10} cells) were washed and resuspended with 1 ml buffer, 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 10 mM EDTA. Cells were encapsulated into agarose plugs and lysed with proteinase K. The chromosomal DNA was digested with *Hin*dIII and *Pst*I and extracted from the plugs essentially as described by Matsunaga et al. (2001). Fifteen micrograms digested DNA were subjected to neutral/neutral (N/N) 2-D agarose gel electrophoresis, as described by Friedman and Brewer (1995). Agarose gels were run in standard TBE buffer in a cold room (first dimension: 0.4% agarose, 0.7 V/cm, 60 h; second dimension: 1% agarose, 3 V/cm, 24 h). Southern blot analysis was carried out

using the ^{32}P-labeled *oriC* sequence as the probe. Filters were exposed to autoradiographic films for 2 weeks.

Results

Identification of a putative *Sulfolobus solfataricus* chromosomal *oriC* by cumulative GC-skew diagram

Computational analysis of bacterial genomic sequences revealed in several instances a biased distribution of the nucleotide content between the two DNA strands. In particular, the replication-leading strand contains more Gs than Cs. This bias was proposed to be a consequence of unequal exposure of the two strands to damage and repair during "asymmetric" biochemical processes such as DNA replication and gene transcription (Grigoriev 1998; Mràzek and Karlin 1998; Frank and Lobry 1999). Based on this observation, cumulative GC-skew diagrams (Fig. 1; other related statistical methods, such as oligomer and codon skew plots) have been recently utilized to identify putative *oriC*s in completely sequenced chromosomes of several eubacterial and archaeal species (Grigoriev 1998; Salzberg et al. 1998; Lopez et al. 1999). The cumulative GC-skew diagrams plot numerically integrated skew $(G-C)/(G+C)$ calculated in adjacent windows over the whole genome and would have a global minimum at the start of the leading strand, or *oriC*, and a global maximum at the end of the leading strand, or replication terminus. We applied this approach to the genome of *Sulfolobus solfataricus* (P2 strain) consisting of a single circular chromosome of about 3 Mbp, whose sequence has been recently completed (She et al. 2001). Thus, a region was identified potentially containing an *oriC* in a minimum of the cumulative GC-skew diagram of the *Sulfolobus* chromosome around position 0.65 Mbp. This region lies between the two divergently transcribed *cdc6* and *MCM* genes that code for putative homologues of the eukaryotic initiation factor Cdc6 [open reading frame (ORF) no. SSO0771] and the replicative MCM-like DNA helicase (ORF no. SSO0774, Carpentieri et al. 2002), respectively (Fig. 2, plain arrow). It is also interesting to observe that just 1 kb downstream from the *cdc6* gene there are two ORFs coding for the large and small subunit of the *S. solfataricus* replication factor C [(*Sso* RFC), Pisani et al. 2000]. The minimum in the *Sulfolobus* GC-skew diagram is located approximately in the middle of the above intergenic region, in close proximity to two long AT-rich elements. The 1.2-kb *Sulfolobus oriC* sequence contains two short predicted ORFs (no. SSO0773, 516 bps, and no. SSO0772, 558 bps). These may be false predictions, having no homologues in other sequenced archaeal genomes, and thus could "create space" for allocating the *oriC* within a large intergenic region. The two small ORFs located in the putative *oriC* are not transcribed (data not shown).

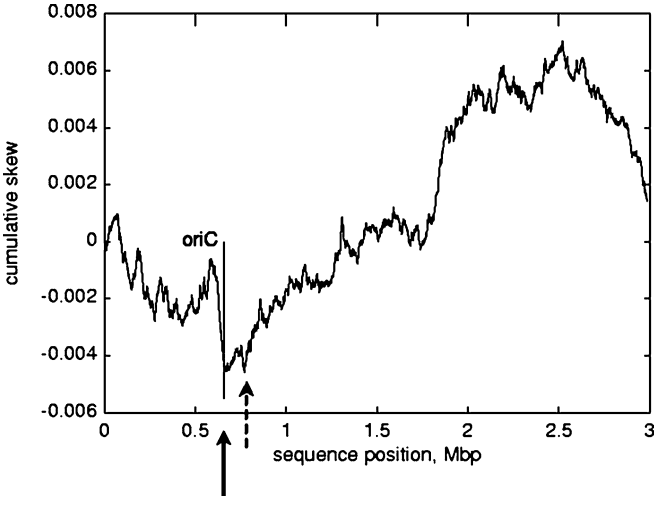

Fig. 1 Cumulative GC-skew diagram of the *Sulfolobus solfataricus* genome. The origin location in the minimum of the plot is indicated by a *plain arrow*. The *dashed arrow* indicates the prominent minimum farthest away from the replication origin (*oriC*)

Analysis of the autonomous replication capability of *oriC* plasmids in *S. solfataricus*

In order to analyze in vivo the autonomous replication capability of the *S. solfataricus oriC*, sequence we constructed the plasmid p*oriC-hph*^{T}, which is schematically shown in Fig. 3a. It contains the *Sulfolobus oriC* sequence (1,120 bp) and a thermoadapted mutant of the *Escherichia coli* hygromycin-phosphotransferase gene (*hph*^{T}) under the transcriptional control of the *S. solfataricus* aspartate-aminotransferase gene promoter and terminator sequences, as an antibiotic-resistance marker (Cannio et al. 1998). *S. solfataricus* competent cells were transformed with the p*oriC-hph*^{T} plasmid and plated on solid medium containing hygromycin B. Control experiments were carried out with the plasmids pUC28

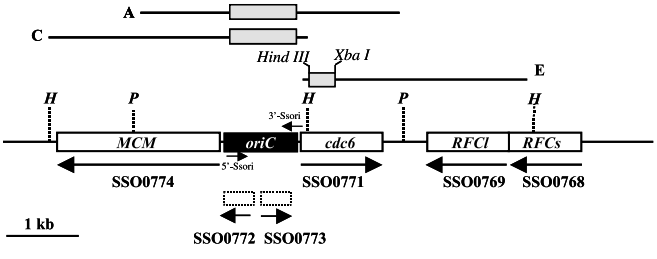

Fig. 2 Schematic representation of the *S. solfataricus* chromosomal *oriC* region. Genes coding for proteins putatively involved in DNA replication are shown, including the homologues of the eukaryotic factor Cdc6 and MCM DNA helicase (Carpentieri et al. 2002) and the large (*RFCl*) and small (*RFCs*) subunit of the replication factor C (Pisani et al. 2000). The numbers reported are those assigned to each open reading frame in the Web page http://www-archbac.u-psud.fr/projects/sulfolobus/. Locations of *Hin*dIII (*H*) and *Pst*I (*P*) restriction sites are shown. The restriction fragments analyzed and the probes utilized in the neutral/neutral (N/N) 2-D gel electrophoresis experiments are shown in the *upper part* of the figure

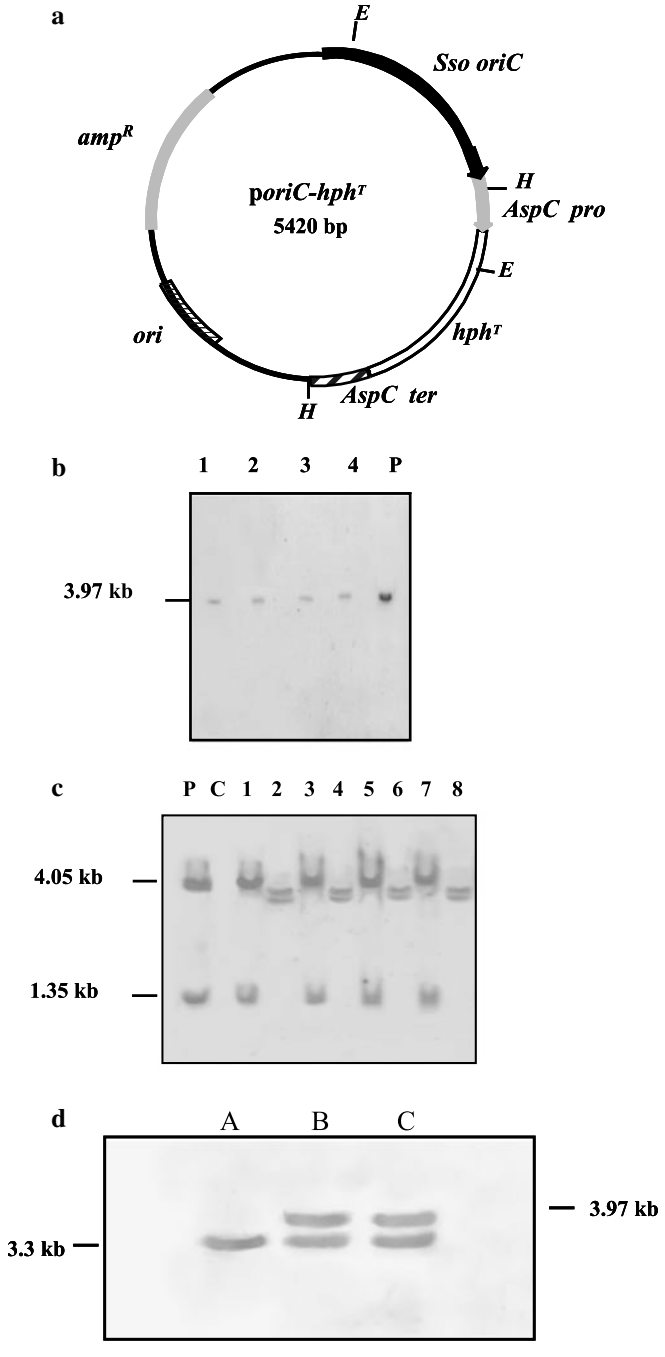

Fig. 3 **a** Map of the p*oriC-hph*^{T} plasmid. *AspC pro* and *AspC ter* are the promoter and terminator sequences of *S. solfataricus* aspartate-aminotransferase gene, respectively. *Hph* is the hygromycin-phosphotransferase gene randomly mutagenized for the thermoadaptation of the resistance phenotype. *amp*^{R} and *ori* represent the *β*-lactamase and the *oriC* of the pUC28 vector, respectively. The locations of *Hin*dIII (*H*) and *Eco*RI (*E*) restriction sites in the plasmid are shown. **b** Southern blot analysis of *Hin*dIII-digested extrachromosomal DNA extracted from cultures (10 ml) of *S. solfataricus* clones transformed with the p*oriC-hph*^{T} plasmid (*lanes 1–4*). The p*oriC-hph*^{T} plasmid (about 10 ng) propagated in *Escherichia coli* was loaded in *lane P* as the control. The ^{32}P-labeled *Sso oriC* sequence was used as the hybridization probe. **c** Southern blot analysis of total DNA preparations from various *Sulfolobus* transformant clones (clone A, *lanes 1–2*; clone B, *lanes 3–4*; clone C, *lanes 5–6*; clone D, *lanes 7–8*). The p*oriC-hph*^{T} plasmid propagated in *E. coli* and, total DNA from *S. solfataricus* nontransformed cells were loaded in *lanes P* and *C*, respectively, as the controls. The ^{32}P-labeled *hph*^{T} gene sequence was used as the hybridization probe. **d** Determination of the copy number of the p*oriC-hph*^{T} by Southern blot analysis. Total DNA from untransformed cells (*lane A*) and from two of the *Sulfolobus* transformant clones (*lanes B* and *C*) was digested with *Hin*dIII and hybridized with the *ori*C sequence

and p*hph*^{T}. The latter is identical to p*oriC-hph*^{T}, but does not contain the *oriC* sequence. Colony growth was observed when the *Sulfolobus* cells were transformed with p*oriC-hph*^{T}, but not with the control constructs. These results indicated that the *oriC* sequence and the *hph*^{T} marker gene were necessary for plasmid maintenance and the hygromycin-resistance phenotype, respectively.

In order to verify the presence of the p*oriC-hph*^{T} plasmid in the *S. solfataricus* transformants, Southern blot experiments were carried out on the extrachromosomal DNA extracted from cells grown in liquid medium supplemented with hygromycin B. The results of a typical experiment are shown in Fig. 3b: the radioactive probe hybridized specifically to a 3.97-kb DNA fragment in the lanes loaded with the *Hin*dIII-digested extrachromosomal DNA from four *Sulfolobus* hygromycin-resistant clones (lanes 1–4). Southern blot experiments were also carried out on *Eco*RI-digested and non-digested total DNA preparations from a set of *Sulfolobus* transformants, as shown in Fig. 3c. The ^{32}P-labeled *hph*^{T} gene, used as the probe, was found to hybridize only with the plasmid *Eco*RI-restriction fragments of 4.05 and 1.35 kb (lanes 1, 3, 5, and 7). In the lanes loaded with the undigested total DNA, hybridization signals were also observed that corresponded to the uncut p*oriC-hph*^{T}. These results indicated that the plasmid is present in the transformed cells in an episomal, nonintegrated, state. As evidence of the *S. solfataricus*/*E. coli* shuttle capability of the p*oriC-hph*^{T}, we observed unaltered restriction-enzyme patterns of the plasmid after transfer into and recovery from *E. coli* (not shown). A plasmid copy number of about one per *S. solfataricus* transformed cell was estimated by Southern blot. The intensity signals of the p*oriC-hph*^{T} and of the chromosomal DNA fragment, using the *oriC* as probe, was compared (Fig. 3, panel d). Moreover, the plasmid was found to be stably maintained either in selective or in nonselective medium. The efficiency of the *S. solfataricus* chromosomal *oriC* in conferring autonomous replication capability to the plasmid p*oriC-hph*^{T} was determined by calculating the plasmid loss rate parameter (*L*), i.e., the rate at which the plasmid is lost per generation during nonselective growth. Cells from two independent clones, named p*oriC-hph*^{T}-A and p*oriC-hph*^{T}-B, were cultivated as described in the experimental procedures, and parameters P_n and P_s, were determined. The plasmid loss rate values were found to be 0.05 for p*oriC-hph*^{T}-A and 0 for p*oriC-hph*^{T}-B after six generations of growth in nonselective

medium. This result suggests that the chromosomal *oriC* analyzed here is also able to support efficiently the autonomous replication of the plasmid p*oriC-hph*^{T} in the absence of selection conditions for several generations.

Analysis of replication intermediates by 2-D gel electrophoresis

Initiation of replication within a DNA restriction fragment can be analyzed in vivo by N/N 2-D gel electrophoresis (Friedman and Brewer 1995). *S. solfataricus* total DNA was extracted from asynchronous exponential growth phase cells after they were incorporated into agarose plugs to preserve the integrity of the replication intermediates (RIs). The total DNA was digested with *Pst*I or *Hin*dIII restriction enzymes, which produced a restriction fragment containing the *Sso oriC* sequence in the central third or at one end, respectively (Fig. 2). The RIs were separated by 2-D gel electrophoresis and detected by Southern blot analysis, using the ^{32}P-labeled *Sso oriC* sequence as the hybridization probe. The 7.2-kb *Pst*I fragment produced a "bubble arc" (Fig. 4a), whereas in the case of the digestion with *Hin*dIII, a signal was detected, which begins as a bubble arc and ends on the simple "Y arc" (Fig. 4c). As control, the *Hin*dIII–*Xba*I restriction fragment of the *cdc* gene (Fig. 2) was used as a hybridization probe: in this case, the *Hin*dIII fragment formed only a Y-shaped arc (Fig. 4e). These results are consistent with our proposed location of the *Sulfolobus oriC*.

Discussion

Analysis of base composition and oligomer distribution along the chromosome was utilized to predict the origin of DNA replication in the archaea *Methanobacterium thermoautotrophicum* and various *Pyrococcus* species (Grigoriev 1998; Lopez et al. 1999). However, only in the case of the euryarchaeon *P. abyssi* was the computational prediction experimentally verified by radioactive labelling of an early replicating chromosomal segment (Myllykallio et al. 2000) and by 2-D gel electrophoresis (Matsunaga et al. 2001). By the combination of the in silico and in vivo analyses described here, we were able to map the genomic *oriC* of the crenarchaeon *Sulfolobus solfataricus* within a chromosomal region of only 1.12 kb (*oriC*) and to demonstrate that plasmids harboring this DNA fragment were stably maintained in an episomal state in *Sulfolobus*-transformed cells.

The cumulative GC-skew plot of the *S. solfataricus* genome shows a sort of "jagged" appearance, very much like the diagrams of some of the bacterial species with

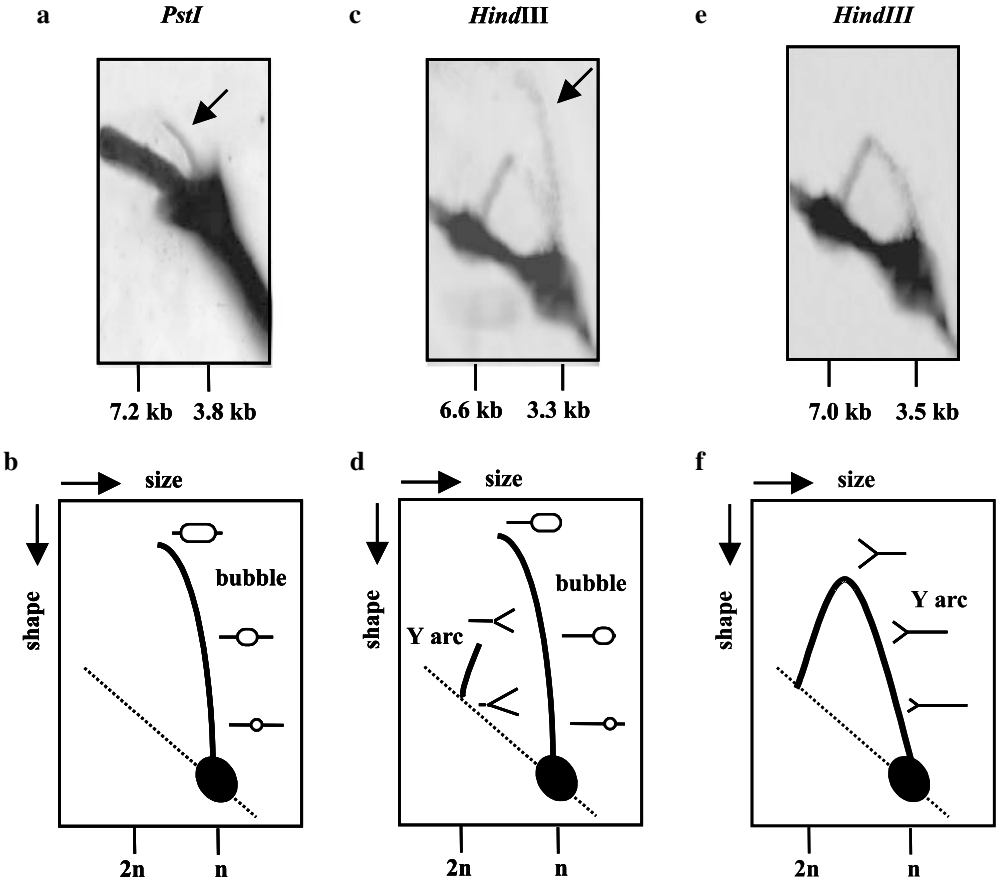

Fig. 4a–f N/N 2-D gel electrophoresis. **a** Replication intermediates were analyzed by Southern blot hybridization after digesting *Sulfolobus* total DNA with *Pst*I and *Hin*dIII restriction enzymes. The ^{32}P-labeled *Sso oriC* sequence and the *Hin*dIII–*Xba*I of the *cdc6* gene were used as the hybridization probes (see Fig. 2). Autoradiographic films are shown. The bubble arcs are indicated by *arrows*. In **b**, **d**, and **f**, a schematic representation of the expected 2-D gel pattern is reported for the experiments shown in **a**, **c**, and **e**, according to Friedman and Brewer (1995)

elevated levels of genomic rearrangements (inversions and translocations) and horizontal DNA transfer (Grigoriev 1998). By comparing the chromosomal sequences of two different strains of *Helicobacter pylori*, such rearrangements were shown to be responsible for numerous distortions on the cumulative skew plot of this species (Grigoriev 2000). Genome rearrangements have thus also likely led to multiple local peaks on the *S. solfataricus* skew diagram, with a few minima on the plot (in the vicinity of the origin and pointing to full and truncated transposase and integrase genes), rivaling the minimum corresponding to *oriC*. So the region near the *oriC* of *S. solfataricus* appears to have undergone multiple rearrangements, as is the case with *H. pylori*. The prominent minimum farthest away from *oriC* is near ORF no. SSO0907, encoding DNA topoisomerase I family A and ORF no. SSO0909, encoding a homologue of Cdc48 (Fig. 1, dashed arrow). These two genes are also divergently transcribed, but there is no space between them to accommodate a possible origin and no homology with the *oriC* sequence.

As frequently reported for the *oriC* of other organisms, the *S. solfataricus oriC* maps in a region where genes are clustered coding for various DNA replication factors. Quite interestingly, in the recently published genomic sequence of *Sulfolobus tokodaii* (Kawarabayasi et al. 2001), the genes encoding the homologues of MCM, Cdc6, and RFC are arranged in a way similar to the one found in *S. solfataricus*. Even more strikingly, in the *S. tokodaii* genome, the GC-skew diagram minimum is also located in the vicinity of these genes (Grigoriev et al., unpublished data). It is worth noting that in other archaea, candidate *oriC*s were mapped immediately upstream of the open reading frame coding for the homologue of the eukaryotic initiation factors Orc1p and Cdc6p by the computational skew analysis and, analogously, in several eubacteria, the *oriC* sequences are in close proximity to the gene for the initiator protein DnaA. Interestingly, the crystallographic analysis of the Orc1p/Cdc6p factor from the archaeon *Pyrobaculum aerophilum* revealed that this protein contains at its C-terminal end a winged-helix structural motif that is likely to be responsible for a sequence-specific DNA-binding activity (Liu et al. 2000). It should be observed that *S. solfataricus* possesses two other genes that code for putative homologues of eukaryotic Orc1p/Cdc6p; therefore, the presence of multiple *oriC*s in the chromosome of *S. solfataricus* can be hypothesized, as recently suggested by Zhang and Zhang (2003), for the genomic locations around the three *cdc6* genes identified. Moreover, in the genome sequence of other archaea, such as *M. thermoautotrophicum*, *Archaebacteria fulgidus*, *Thermoplasma acidophilum*, *Aeropyrum pernix*, and *S. tokodaii*, multiple potential Orc1p/Cdc6p-like factors have also identified.

The demonstration of the autonomously replicating capability of the p*oriC-hph*^{T} as well as the detection of RIs of the expected shape by 2-D gel analysis confirm our proposed location of this *oriC* in the genome of *S. solfataricus*. Nevertheless, these results are not exhaustive to exclude the possible presence of multiple chromosomal *oriC*s and/or their differential activation.

The fine mapping of a chromosomal *oriC* and the establishment of a minichromosome for the crenarchaeon *S. solfataricus* opens up the possibility of studying in vitro and in vivo the archaeal mechanisms of DNA replication initiation and its cell cycle-dependent regulation.

Moreover, the insertion of this *oriC* into a vector could allow the construction of minichromosome useful to carry out physiological and genetic studies that require expression of genes in single copy as the limiting factor.

Note added

While this paper was under review, Robinson et al. published the identification of two *oriC*s located upstream the *cdc6*1 and *cdc6*3 genes in *S. solfataricus* genome and relatively, no activity for the sequence close to *cdc6*2, described in this paper. This discrepancy could be only apparent. Laboratory *S. solfataricus* type strains can differ in their phenotypes both because of different growth conditions (for example, medium composition) and because of intrinsic instability of *S. solfataricus* genome. We believe that under our conditions, this genomic region was fired according to the hypothesis of a "secondary" *oriC*. In fact, our approach was aimed at checking the functionality of this specific genomic region.

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