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Nucleosome exclusion from the interspecies-conserved central AT-rich region of the *Ars* insulator

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The *Ars* insulator is a boundary element identified in the upstream region of the arylsulfatase (*HpArs*) gene in the sea urchin, *Hemicentrotus pulcherrimus*, and possesses the ability to both block enhancer–promoter communications and protect transgenes from silent chromatin. To understand the molecular mechanism of the *Ars* insulator, we investigated the correlation between chromatin structure, DNA structure and insulator activity. Nuclease digestion of nuclei isolated from sea urchin embryos revealed the presence of a nuclease-hypersensitive site within the *Ars* insulator. Analysis of micrococcal nuclease-sensitive sites in the *Ars* insulator, reconstituted with nucleosomes, showed the exclusion of nucleosomes from the central AT-rich region. Furthermore, the central AT-rich region in naked DNA was sensitive to nucleotide base modification by diethylpyrocarbonate (DEPC). These observations suggest that non-B-DNA structures in the central AT-rich region may inhibit nucleosomal formation, which leads to nuclease hypersensitivity. Furthermore, comparison of nucleotide sequences between the *HpArs* gene and its ortholog in *Strongylocentrotus purpuratus* revealed that the central AT-rich region of the *Ars* insulator is conserved, and this conserved region showed significant enhancer blocking activity. These results suggest that the central AT-rich nucleosome-free region plays an important role in the function of the *Ars* insulator.

Keywords: insulator/nuclease-hypersensitive site/nucleosome/sea urchin development.

Abbreviations: DEPC, diethylpyrocarbonate; DNase I, deoxyribonuclease I; MNase, micrococcal nuclease.

Thousands of genes are present on a single linear chromosomal DNA, and their expression is regulated by *cis*-regulatory elements, such as enhancers and silencers. In general, transcriptional activation by enhancer is independent of orientation and distance from the promoter. Inappropriate activation by enhancers is blocked, ensuring that expression of each gene is independent. Furthermore, two distinct chromatin environments are known. Decondensed euchromatin is permissive for gene expression, and highly condensed heterochromatin represses gene expression, although they are often adjacent to each other.

Insulators and boundary elements are DNA elements that are defined by two activities: an enhancer blocking activity that blocks enhancer–promoter communications when inserted between them, and a barrier activity that protects transgenes from the spreading of a silent chromatin environment. Insulators have been identified in several organisms and insulator-binding proteins have been found for each insulator (*1–3*).

A common feature of insulators is the presence of nuclease-hypersensitive sites. A constitutive hypersensitive site at the 5′-boundary of chicken β-globin domain (5′-HS4) serves as an insulator (*4*). In the 5′-HS4 insulator, basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factors (USF proteins) bind to the region that is responsible for the barrier activity (*5*) and a CCCTC-binding protein (CTCF) binds to the region responsible for the enhancer blocking activity (*6*). The *gypsy* insulator is a well- characterized insulator derived from the *gypsy* retrotransposon in *Drosophila* (*7*). The Suppressor of Hairy-wing [Su(Hw)] protein interacts with the *gypsy* insulator (*8*), and the Modifier of Mdg4 [Mod(mdg4)] protein directly associates with Su(Hw) proteins (*9*, *10*). Centrosomal Protein 190 (CP190) is recruited to the *gypsy* insulator through interactions with Su(Hw) and Mod(mdg4) (*11*). The *gypsy* insulator is also constitutively sensitive to nuclease (*12*). The scs (special chromatin structures) and scs' insulators that flank two divergently transcribed *hsp70* genes at the *87A7* heat shock locus are other well-characterized insulators in *Drosophila*. The Zeste-white5 (Zw5) protein that interacts with scs is essential for enhancer blocking activity (*13*). Boundary element-associated factor (BEAF) proteins, BEAF32A and BEAF32B bind to the scs' insulator and the BEAF-binding sites have been shown to be important for barrier activity (*14–17*). Each of the scs and scs' insulators is defined by two sets of closely spaced nuclease-hypersensitive sites arranged around a central nuclease-resistant segment (*18*). Thus, the open

chromatin structure of insulators may play an important role in their insulator function. Furthermore, the significance of nucleosome positioning in the insulator function was reported for the CTCF-mediated insulator in the *H19* imprinting control region (*19*)

The arylsulfatase (*HpArs*) gene of the sea urchin, *Hemicentrotus pulcherrimus*, is expressed in a developmental stage- (*20*) and tissue-specific manner (*21*). In searching for *cis*-regulatory elements that regulate expression of the *HpArs* gene, the *Ars* insulator was identified in the upstream region of the *HpArs* gene (*22*). The *Ars* insulator showed both enhancer blocking and barrier activities in sea urchin (*22*, *23*). The enhancer blocking activity is orientation-dependent, and functions not only in the sea urchin, but also in *Drosophila* (*22*). Furthermore, the barrier activity has been observed in HeLa cells (*22*), cultured tobacco BY2 cells (*24*), a human myeloid cell line, HL-60 and a murine embryonic stem cell line, CCF (*25*). We recently reported an *Ars* insulator-binding protein, Unichrom, which was originally identified as a G-stretch binding protein (*26*, *27*). Unichrom binds to the minor groove of the central AT-rich region of the *Ars* insulator (*27*). However, the molecular mechanism of the *Ars* insulator activity is poorly understood.

In this study, we investigated the correlation between chromatin structure, DNA structure and insulator activity of the *Ars* insulator. Our data suggest that a non-B-DNA structure in the central AT-rich region of the *Ars* insulator may interfere with nucleosomal formation and lead to nuclease hypersensitivity, and that such a nucleosome-free region may play an important role in *Ars* insulator activity.

Materials and Methods

Embryo culture

Gametes of the sea urchin *H. pulcherrimus* were obtained by coelomic injection of 0.55 M KCl, and fertilized eggs were cultured in artificial sea water Jamarin U (Jamarin Laboratory, Osaka, Japan), at 16°C with constant aeration and stirring.

Isolation of nuclei from sea urchin embryos

Hemicentrotus pulcherrimus embryos were grown at 16°C until they reached the unhatched blastula, hatched blastula, mesenchyme blastula or gastrula stages. A volume of 20 ml of packed embryos was harvested by centrifugation. Nuclei were isolated as described by Tung *et al.* (*28*) with minor modifications. Embryos were washed twice with 0.55 M KCl and suspended in 40 ml of washing solution [0.25 M sucrose, 0.1 M EDTA, 10 mM Tris–acetate (pH 8.0)]. After incubation on ice for 20 min, dissociated cells were collected by centrifugation at 3,000 rpm in a TS-7 swing rotor (Tomy Seiko, Tokyo, Japan). Cells were suspended in 20 ml of homogenization buffer [0.32 M sucrose, 5 mM Mg–acetate, 10 mM Tris–acetate (pH 8.0), 0.1 mM dithiothreitol, 1 mM EGTA, 1% aprotinin and 0.1 mM phenylmethylsulfonyl fluoride] and disrupted with ~20 strokes using a tight-fitting pestle in a Dounce homogenizer. After the final sucrose concentration was adjusted to 1.4 M by addition of homogenization buffer containing 2 M sucrose, the suspension was layered over 10 ml of homogenization buffer containing 2 M sucrose and centrifuged at 27,000 rpm for 30 min at 4°C in a P28S rotor (Hitachi, Tokyo, Japan). Pelleted nuclei were suspended in storage buffer [25% glycerol, 50 mM Tris–acetate (pH 8.0), 1 mM dithiothreitol, 1 mM EGTA, 1mM spermidine, 5 mM Mg–acetate and 0.1 mM phenylmethylsulfonyl fluoride], frozen quickly in liquid nitrogen and stored at −70°C.

Nuclease digestion of isolated nuclei

Nuclei were digested with nucleases as described by Fronk *et al.* (*29*). Isolated nuclei were suspended at 1 OD_{260}/ml in 1 ml buffer A [15 mM Tris–HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine] supplemented with 1 mM $MgCl_2$ for deoxyribonuclease I (DNase I) digestion or with 1 mM $CaCl_2$ for micrococcal nuclease (MNase) and endonuclease digestions. For DNase I digestion, samples were pre-incubated at 37°C for 5 min, followed by addition of 100 units of DNase I (TaKaRa Bio Inc., Otsu, Shiga, Japan), and incubation at 37°C for 0, 0.5, 1.0, 1.5, 2.0 and 2.5 min. For MNase digestions, after pre-incubation at 37°C for 5 min, samples were digested by addition of 1 unit of MNase (TaKaRa Bio) and incubation at 37°C for 0, 10 and 30 s and 1, 2, 4 and 8 min. For digestion by endogenous nuclease, isolated nuclei were incubated at 37°C for 0, 3, 5, 7, 9 and 13 min without pre-incubation and addition of nuclease. Reactions were terminated by addition of 250 µl 5% SDS/25 mM EDTA. After digestion by Proteinase K (50 µg/ml) at 45°C for overnight, genomic DNA was extracted with an equal volume of phenol/chloroform, precipitated with two volumes of ethanol, washed with 70% ethanol and dissolved in 300 µl of TE buffer [Tris–HCl (pH 7.4), 1 mM EDTA].

Indirect end labelling

Nuclease-digested genomic DNA was further digested with PstI (TaKaRa Bio), and purified by extraction with phenol/chloroform and subsequent precipitation with ethanol. Five micrograms of genomic DNA was electrophoresed on 11 cm 0.8% agarose gel in 1×TAE buffer (40 mM Tris–acetate, 1mM EDTA). Gels were stained with ethidium bromide, photographed and DNA denatured by treatment with 1 M NaCl/0.5 N NaOH for 1 h. DNA was transferred onto Nytran N nylon membranes (Schleicher & Schuell, Dassel, Germany) by capillary blotting and hybridized to the EcoT22I–PstI fragment (−101 to +167 bp) of the *HpArs* gene labelled with [α-^{32}P] dATP. After hybridization, filters were washed at room temperature in 1×SSC/1% SDS twice for 15 min each and at 65°C in 1×SSC/1% SDS twice for 20 min each. Filters were exposed to BioMax Ms X-ray film (Kodak, Rochester, NY, USA) at −70°C.

Nucleosome reconstitution with recombinant sea urchin histones

Nucleosome reconstitution on the *Ars* insulator was carried out with bacterially expressed core histones by the method described by Tanaka *et al.* (*30*) with some modifications.

The DNA-encoding sea urchin histones H2B and H3 were amplified by PCR and cloned into pET15b vector (Novagen, Gibbstown, NJ, USA). Since histone H2A and H4 were weakly expressed, designed histone H2A and H4, in which minor codons were replaced with major codons, were synthesized from chemically synthesized oligonucleotides by PCR-based method, and inserted into pET15b. Recombinant histones were expressed in *Escherichia coli* BL21(DE3)pLysS cells. Transformants were grown at 37°C in 3 ml of LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). After incubation for 16 h, cells were inoculated into 1 l of LB medium and incubated at 37°C for 1 h. Expression of recombinant histones were induced by addition of 5 ml of 0.1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG). Expression of H2B and the designed H4 were induced at 37°C, whereas expression of the designed H2A was induced at 30°C and that of H3 was induced at 30°C in LB medium supplemented with 1% glucose. After incubation for 10 h, cells were harvested and resuspended in 10 ml buffer P [50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol] and lysed by four rounds of sonications for 1 min each. Cell lysates were centrifuged at 9,800*g* for 20 min at 4°C and pellets were dissolved in 10 ml buffer U [50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 5% glycerol, 6 M urea]. After centrifugation at 9,800*g* for 20 min at 4°C, the supernatants were mixed with 1 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen, Tokyo, JP) and rotated at 4°C for 1 h. Beads were washed twice with buffer U containing 5 mM imidazole and His-tagged histones were eluted stepwise with buffer U containing 5, 10, 20, 50, 100, 200 and 300 mM imidazole. The purity was monitored by SDS–PAGE (polyacrylamide gel electrophoresis).

Purified His-tagged histone H2A and H2B (1 mg each) were mixed to reconstitute H2A/H2B dimers and purified His-tagged histone H3 and H4 (1 mg each) were mixed to reconstitute H3/H4 tetramers.

The mixtures were dialysed against 500 ml of buffer [50 mM Tris–HCl (pH 8.0), 10 mM dithiothreitol, 2 mM EDTA, 6 M urea, 2 M NaCl] at room temperature for 1 h and then overnight at 4°C. The mixtures were then dialysed against 500 ml of reconstitution buffer [20 mM Tris–HCl (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, 2 M NaCl] overnight at 4°C. The salt concentration was reduced by stepwise dialysis against reconstitution buffer containing 1 M NaCl for 4 h, 0.5 M NaCl for 4 h and 0.1 M NaCl overnight at 4°C. After centrifugation at 9,800*g* for 20 min at 4°C, His-tagged H2A/H2B dimers and H3/H4 tetramers were collected in the soluble fractions.

Nucleosome reconstitution on the *Ars* insulator fragment was carried out by salt-dialysis method. The *Ars* insulator fragment was PCR-amplified with 5′-biotin-labelled T7 primer and T3 primer using pARSSmBM as a template (*27*). PCR products were electrophoresed on 1% agarose and purified by electroelution (*31*). The purified *Ars* insulator fragment (10 μg) was mixed with H2A/H2B dimer (25 μg) and H3/H4 tetramer (25 μg) in 300 μl TE buffer containing 2 M NaCl and 9.6 mM β-mercaptoethanol, and dialysed against 500 ml TEMP buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.25 mM phenylmethylsulphonyl fluoride] containing 2 M NaCl. The salt concentration was reduced by stepwise dialysis against reconstitution buffer containing 2 M NaCl overnight, 1.5, 1.0 and 0.75 M NaCl for 1 h, 4 h and 0.75 for 4 h, respectively and without NaCl overnight at 4°C.

MNase digestion of reconstituted nucleosomes

Reconstituted nucleosomes containing 200 ng of *Ars* insulator fragment were dissolved in 20 mM Tris–HCl (pH 8.0), 70 mM NaCl, 10 mM $CaCl_2$, 1 mM dithiothreitol, 50 μg/ml BSA. After preincubation at room temperature for 5 min, MNase (TaKaRa Bio Inc.) was added at 0, 0.001, 0.0025 and 0.005 U/μl. Reactions were terminated by addition of an equal volume of 40 mM Tris–HCl (pH 8.0), 40 mM EDTA, 1% SDS. DNA was purified by Proteinase K digestion and phenol/chloroform extraction with subsequent precipitation with ethanol.

For low resolution mapping of MNase-digested sites, 10 ng of DNA was electrophoresed on 6% polyacrylamide gel and transferred onto a Nytran N nylon membranes by electroblotting. Streptavidin conjugated alkaline phosphatase (Roche, Mannheim, Germany) was used to probe membranes. Chemiluminescent signal produced by enzymatic dephosphorylation of CSPD (Tropix, Bedford, MA, USA) was detected by RX-U X-ray film (Fujifilm, Japan). For high-resolution mapping of MNase-digested sites, polymerase chain reaction was carried out using either IRD800-labelled T7 or T3 primers as described below.

Diethylpyrocarbonate reaction

pARSSmBM was used for modification by diethylpyrocarbonate (DEPC). To obtain linearized pARSSmBM, DNA was cleaved with ScaI. DEPC reactions were performed under physiological conditions according to the method described in Sakamoto *et al.* (*32*).

Negatively supercoiled or linearized pARSSmBM (3 μg) was dissolved in 100 μl of 0.3 M Tris–HCl (pH 7.5) containing 5 mM $MgCl_2$ and 0.25 M KCl. The DEPC reaction was started by adding 10 μl of DEPC, followed by vortexing for 1 min and incubation at 22°C for 30 min. DNA was precipitated with ethanol, rinsed in 70% ethanol and dissolved in TE buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA]. To determine sites of modification, polymerase chain reaction was carried out using either IRD800-labelled M13 forward or reverse primers as described below.

Primer extensions

For mapping of MNase-digested and DEPC-modified sites, plasmid pARSSmBM (200 ng) was mixed with 1 pmol of IRD800-labelled primer in 20 μl reaction mixture containing 1×LA PCR® buffer II (Mg^{2+} free), 250 μM dNTP, 2.5 mM $MgCl_2$ and 2.5 U of *TaKaRa LA Taq*® (TaKaRa Bio). Primer extension was carried out for 30 cycles: disassociation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min using a GeneAmp PCR System 9700 thermal cycler (Invitrogen, San Diego, CA, USA). Reactions were terminated by addition of 10 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). After denaturation, reaction products were analysed on LI-COR 4000 L DNA sequencer (Aloka, Tokyo, Japan).

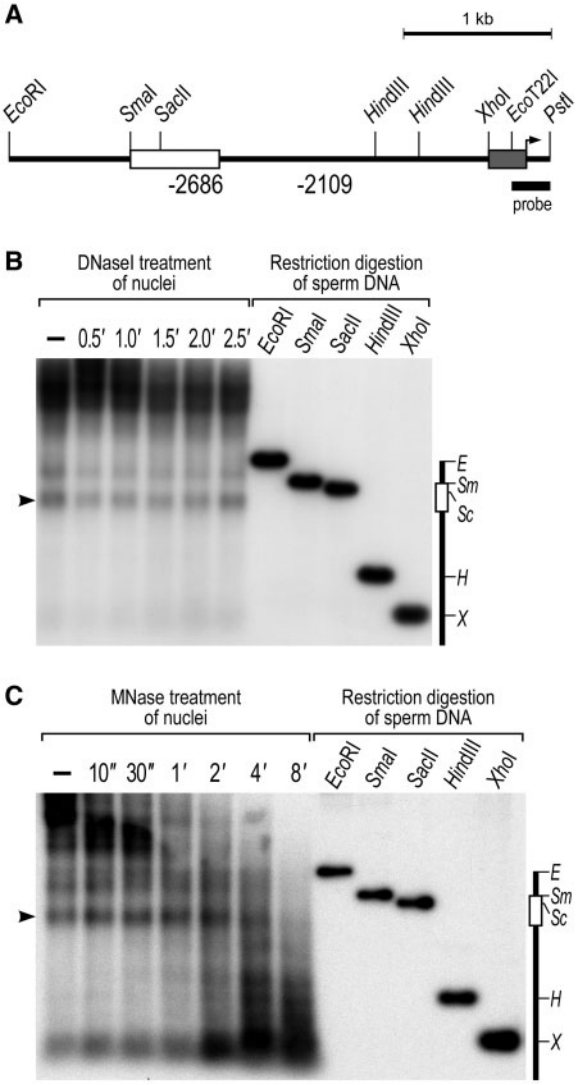

Fig. 1 Detection of nuclease-hypersensitive sites in the upstream region of *HpArs* gene at the gastrula stage. (A) Schematic structure of the upstream region of *HpArs* gene. *Ars* insulator and *Ars* promoter positions are indicated by open and shaded boxes, respectively. Recognition sites for restriction endonucleases are shown. Position of the probe used for indirect end labelling is denoted by the thick line. (B and C) Detection of DNase I- and MNase-hypersensitive sites in the upstream region of *HpArs* gene. Nuclease-hypersensitive sites within the *Ars* insulator are indicated by arrowheads to the left of gels. Time of nuclease digestion and restriction endonuclease used to estimate the cleaved position is indicated above gels. Schematic map of *HpArs* gene corresponding to the migration in the gels is shown on the right of gels. Thick vertical line, *HpArs* gene; *Ars* insulator, open box; E, EcoRI; Sm, SmaI; Sc, SacII; H, HindIII; X, XhoI.

For mapping of paused sites for *in vitro* DNA syntheses, TITANIUM™ *Taq* DNA Polymerase (Clontech-Takara Bio) and *TaKaRa Ex Taq*® (TaKaRa Bio) were used. Primer extension by TITANIUM™ *Taq* DNA Polymerase was carried out in 20 μl reaction mixture containing 200 ng of pARSSmBM, 1 pmol of IRD800-labelled primer, 1×TITANIUM™ *Taq* PCR buffer, 200 μM dNTP and 1×TITANIUM™ *Taq* DNA Polymerase for 30 cycles: disassociation at 95°C for 30 s and extension at 68°C for 1 min. Primer extension by *TaKaRa Ex Taq*® was carried out in 20 μl reaction mixture containing 200 ng of pARSSmBM, 1 pmol of IRD800-labelled primer, 1×*Ex Taq* buffer (2 mM Mg^{2+}), 250 μM dNTP and *TaKaRa Ex Taq*® for 30 cycles: disassociation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min.

Comparison of nucleotide sequences of sea urchin arylsulfatase genes

FamilyRelationsII software (*33*) was used for comparison of nucleotide sequences of arylsulfatase genes of *H. pulcherrimus* and *Strongylocentrotus purpuratus*. The 7 kb region of *HpArs* was compared with the *S. purpuratus* ortholog (*S. purpuratus* genomic contig, Accession No. NW_001468620: The complementary sequence of nucleotides 13533–20532).

Introduction of DNA by particle gun and luciferase assay

DNA constructs were introduced by particle gun (GIE-III IDERA, Tanaka Co. Ltd, Japan) and the luciferase assays were carried out as described by Akasaka *et al.* (*34*) with slight modifications. To normalize for luciferase activity, pRL-CMV (Promega, Madison, WI, USA) was co-introduced as a reference along with the luciferase reporter construct. Embryos were lysed in 0.1 M Tris–HCl (pH 7.5) and luciferase activities were measured on Turner Designs TD-20/20 Luminometer using Dual-Luciferase™ Reporter System (Promega).

Results

Nuclease-hypersensitive site within the *Ars* insulator

The presence of nuclease-hypersensitive sites is a common feature of chromatin insulators. To investigate whether the *Ars* insulator is hypersensitive to nuclease, nuclei were isolated from *H. pulcherrimus* embryos at the gastrula stage and treated with DNase I. To estimate the position of nuclease digestions, genomic DNA purified from *H. pulcherrimus* sperm was digested with restriction endonucleases known to cleave recognition sites within the upstream region of *HpArs* gene (Fig. 1A). All DNA samples were further cleaved with PstI, electrophoresed and transferred to nylon membrane. The upstream region of the *HpArs* gene was indirectly end-labelled by hybridization with the radioactive EcoT22I–PstI fragment of *HpArs* gene (−101 to +167 bp) (Fig. 1A).

When isolated nuclei were digested with DNase I, two hypersensitive sites were detected in the upstream region of *HpArs* gene and one of them located within the *Ars* insulator (Fig. 1B, arrowhead). This indicates that the *Ars* insulator contains a nuclease-hypersensitive site as observed in other chromatin insulators. To further examine the nuclease sensitivity of the *Ars* insulator, the isolated nuclei were digested with MNase. Although the promoter region was

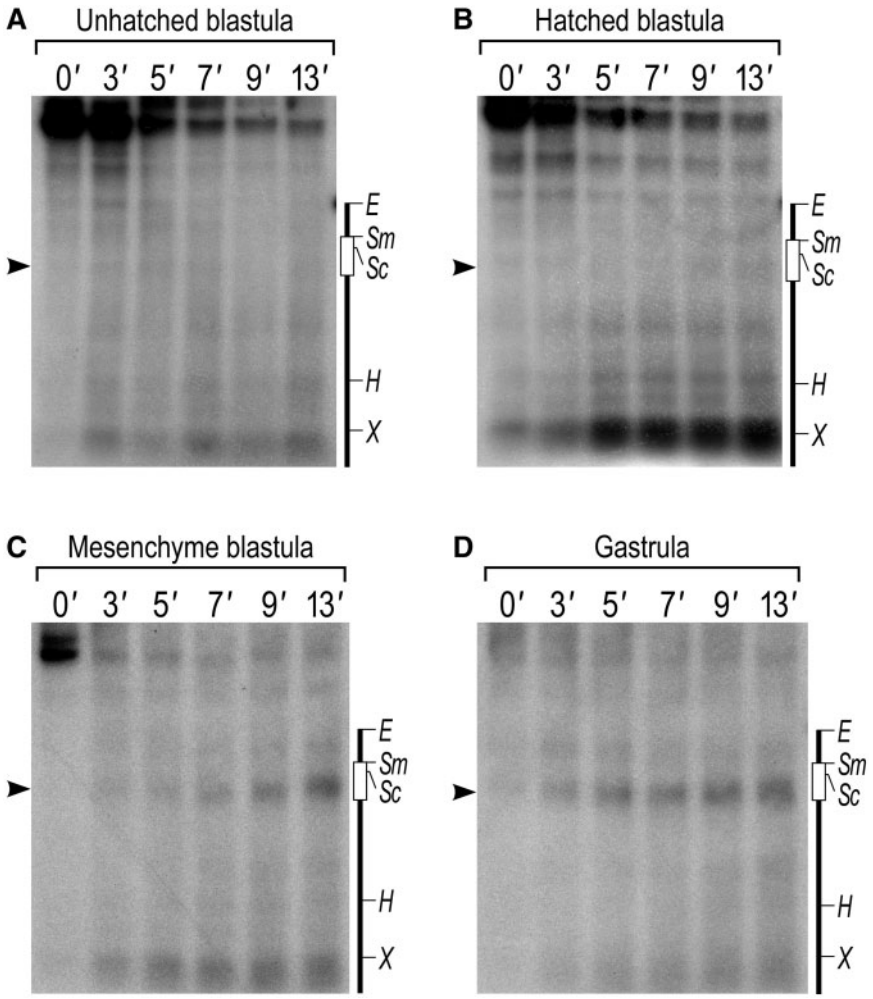

Fig. 2 Detection of endogenous endonuclease-hypersensitive sites in the upstream region of the *HpArs* gene during sea urchin development. (A) Nuclease sensitivity at unhatched blastula stage. (B) Nuclease sensitivity at hatched blastula stage. (C) Nuclease sensitivity at mesenchyme blastula stage. (D) Nuclease sensitivity at gastrula stage. Arrowheads to the left of gels indicate position of nuclease-sensitive sites within the *Ars* insulator. Time of endonuclease digestion is indicated above gels. Schematic map of the *HpArs* gene corresponding to the migration in the gel is shown on the right of gels. Thick vertical line, *HpArs* gene; *Ars* insulator, open box; E, EcoRI; Sm, SmaI; Sc, SacII; H, HindIII; X, XhoI.

cleaved by MNase treatment, digestion occurred preferentially in the *Ars* insulator over a shorter period (Fig. 1C, arrowhead). This confirms the nuclease hypersensitivity of the *Ars* insulator.

In these experiments, nuclease sensitivity of the *Ars* insulator was observed just after pre-incubation, without addition of any enzymes, suggesting that endogenous endonucleases can be used to detect the nuclease sensitivity of the *Ars* insulator. This is similar to results already reported by Anderson *et al*. (*35*). Therefore, we next analysed the sensitivity of the *Ars* insulator to endogenous endonuclease in nuclei isolated at various developmental stages. The *Ars* insulator showed very weak sensitivity to the endonuclease at the unhatched blastula and the hatched blastula stages (Fig. 2A and B). However, a remarkable increase in sensitivity to endogenous endonuclease was observed at the mesenchyme blastula and the gastrula stages (Fig. 2C and D) suggesting that chromatin structure of the *Ars* insulator may change during early sea urchin development.

Nucleosome positioning in the Ars insulator

Nuclease sensitivity in the isolated nuclei indicated the presence of a nuclease-hypersensitive site within the *Ars* insulator and that the surrounding region was protected from nuclease digestion. This suggests that nucleosome structures may be formed at fixed positions within the *Ars* insulator. Therefore, to examine the ability of the *Ars* insulator to form nucleosomes, the *Ars* insulator fragment was amplified by PCR and subjected to *in vitro* reconstitution of nucleosomes with bacterially expressed sea urchin core histones, and assayed for MNase sensitivity.

Nucleosomes were reconstituted with biotin-labelled PCR products of the *Ars* insulator (Fig. 3A) and MNase-sensitive sites were determined by PAGE. Although terminal regions of the *Ars* insulator were protected from MNase in reconstituted nucleosomes (Fig. 3B, asterisks), the central region showed MNase sensitivity similar to that of naked DNA (Fig. 3B). Three tandem repeats of the sea urchin 5S ribosomal DNA gene, known to have a nucleosome positioning sequence (*36*), was used as a control, and showed formation of three nucleosomes (data not shown). The nucleotide sequence in the central region of the *Ars* insulator is relatively AT-rich (Fig. 3C, AT contents are ~71% in shaded box) and a part of this region shows ~83% AT content in a 53 bp region (Fig. 3C, underlined).

To create a detailed map of nucleosomes formed on the *Ars* insulator, MNase-digested sites were determined by primer extension with *TaKaRa LA Taq*® (TaKaRa Bio). The region upstream from the G-stretch sequence (nucleotides −2,686 to −2,523) was protected from MNase digestion in reconstituted nucleosomes compared with naked DNA (Fig. 4A and B, asterisks), indicating nucleosome formation. In contrast, many nucleotides in the central AT-rich region of the *Ars* insulator and its vicinity (nucleotides −2,442 to −2,267) showed significant MNase sensitivity in the reconstituted nucleosome compared to naked DNA (Fig. 4A and B, filled triangles) indicating that nucleosomes may be excluded from this region. It is

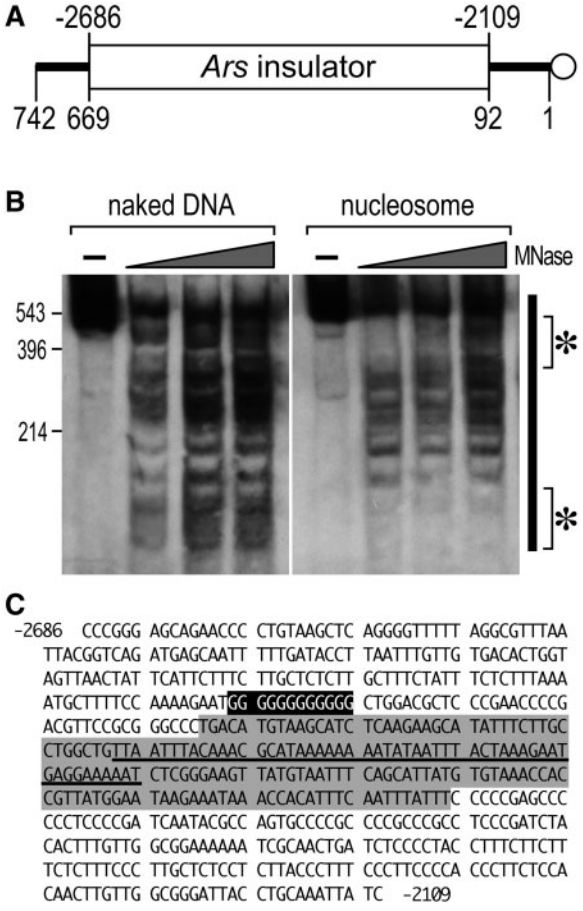

Fig. 3 Low resolution mapping of nucleosomes reconstituted *in vitro* on the *Ars* insulator. (A) Schematic map of the *Ars* insulator fragment used in nucleosome reconstitution. Thick horizontal line, DNA derived from pBluescript SK-vector; open box, *Ars* insulator; open circle, biotin-label. Length (bp) from biotin-label is shown below the picture and nucleotide number in the *HpArs* gene is shown on top. (B) Detection of MNase-sensitive sites on reconstituted nucleosomes. The size (bp) of HinfI-digested pUC119 is shown to the left of the gel. Position of the *Ars* insulator is shown to the right of the gel, and regions protected from MNase digestion are indicated by asterisks. (C) Nucleotide sequence of the *Ars* insulator. Tone is reversed at G-stretch. Shaded box indicates the nucleotide sequence of the central region and the highly AT-rich region is underlined.

noteworthy that a G-stretch sequence is present at the boundary between the nucleosomal region and excluded region (Fig. 4A and B, open triangles) suggesting that the G-stretch may define the boundary between nucleosomal and nucleosome-free regions. Furthermore, many nucleotides downstream from nucleotide −2,245 were protected from MNase digestion in the reconstituted nucleosome compared with naked DNA (Fig. 4B, asterisks).

DNA structure of the *Ars* insulator

Since nucleosome formation is affected by nucleotide sequence and unusual DNA structures (*37–40*), we sought to examine the structure of the *Ars* insulator. We used DEPC treatment to modify nucleotide bases in linearized and negatively supercoiled pARSSmBM plasmids that contain the *Ars* insulator. DEPC assays

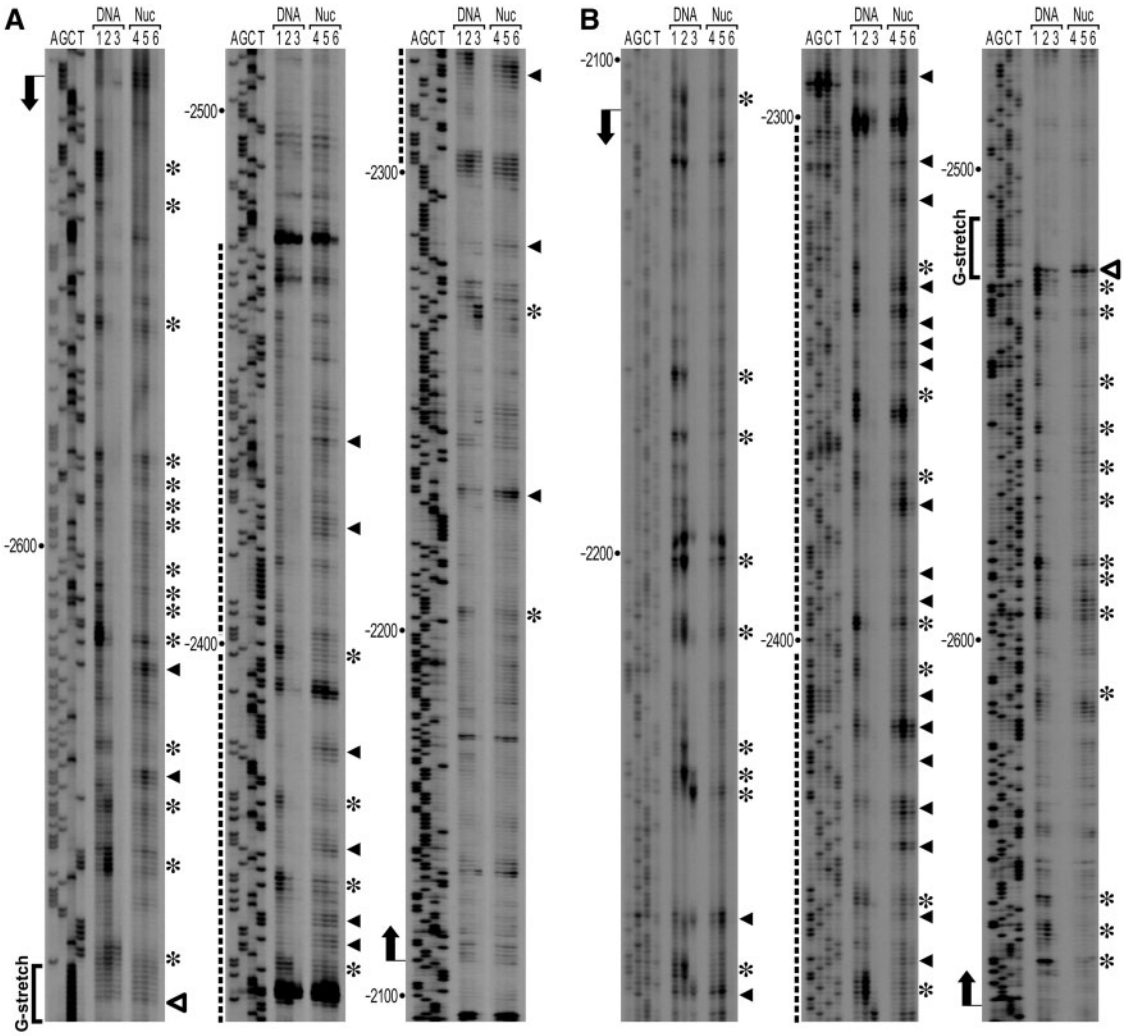

Fig. 4 High resolution mapping of nucleosomes reconstituted *in vitro* on the *Ars* insulator. (A) Detection of MNase-digested sites by primer extension from downstream of the *Ars* insulator. (B) Detection of MNase-digested sites by primer extension from upstream of the *Ars* insulator. Lanes A, G, C and T indicate sequence ladders of dideoxy chain termination chemistry. Naked DNA (lanes 1–3) and reconstituted nucleosomes (lanes 4–6) were digested for 5 min with concentrations of MNase as follows: 1 U/ml (lane 1), 2.5 U/ml (lane 2), 5 U/ml (lane 3), 10 U/ml (lane 4), 50 U/ml (lane 5) and 100 U/ml (lane 6). Nucleotide numbers in the *HpArs* gene and positions of the G-stretch sequence are shown to the left of picture. Areas enclosed by two vertical arrows represent the position of the *Ars* insulator and dotted lines indicate the position of the central AT-rich region. Sites protected from MNase digestions in reconstituted nucleosomes are marked by asterisks, and MNase-sensitive sites in reconstituted nucleosomes are marked by filled triangles. Open triangles indicate the boundary of nucleosomal DNA at G-stretch.

were conducted at neutral pH under physiological ionic strength and Mg^{2+} concentration. Bands detected in the DEPC-untreated samples indicate sites of pausing by unusual DNA structure as described below, and the base modifications by DEPC can increase the intensities of bands. In the primer extension mapping, *TaKaRa LA Taq*® was used because this enzyme was less affected by unusual DNA structure in our preliminary experiment (data not shown). Intense base modifications were observed within the central AT-rich region (nucleotides −2,418 to −2,384) in both linear and circular plasmids (Fig. 5A, asterisks), indicating that DNA structure is distorted in this region, and that nucleotide bases are accessible to DEPC in a negative supercoil-independent manner. Furthermore, nucleotide bases in two regions (nucleotides −2,230 to −2,223 and −2,314 to −2,310) were moderately modified by DEPC (Fig. 5A and B, filled triangles). Nucleotides upstream from the G-stretch also showed moderate sensitivity to DEPC (Fig. 5).

Since it has been reported that *in vitro* DNA synthesis may be stalled at a site of unusual DNA structure, such as a triplex (*41*), we tested the propensity of the *Ars* insulator to stall *in vitro* DNA synthesis by two thermostable DNA polymerases, TITANIUM™ *Taq* DNA polymerase (Clontech-TaKaRa Bio) and *TaKaRa Ex Taq*® (TaKaRa Bio). Strong pausing was detected at two regions (nucleotides −2,304 to −2,290 and −2,229 to −2,215) in an orientation-dependent manner (Fig. 6A and B, asterisks). These sites were close to those modified moderately by DEPC (Fig. 5A and B, filled triangles).

The results of high resolution mapping of MNase-digested sites in the reconstituted nucleosomes,

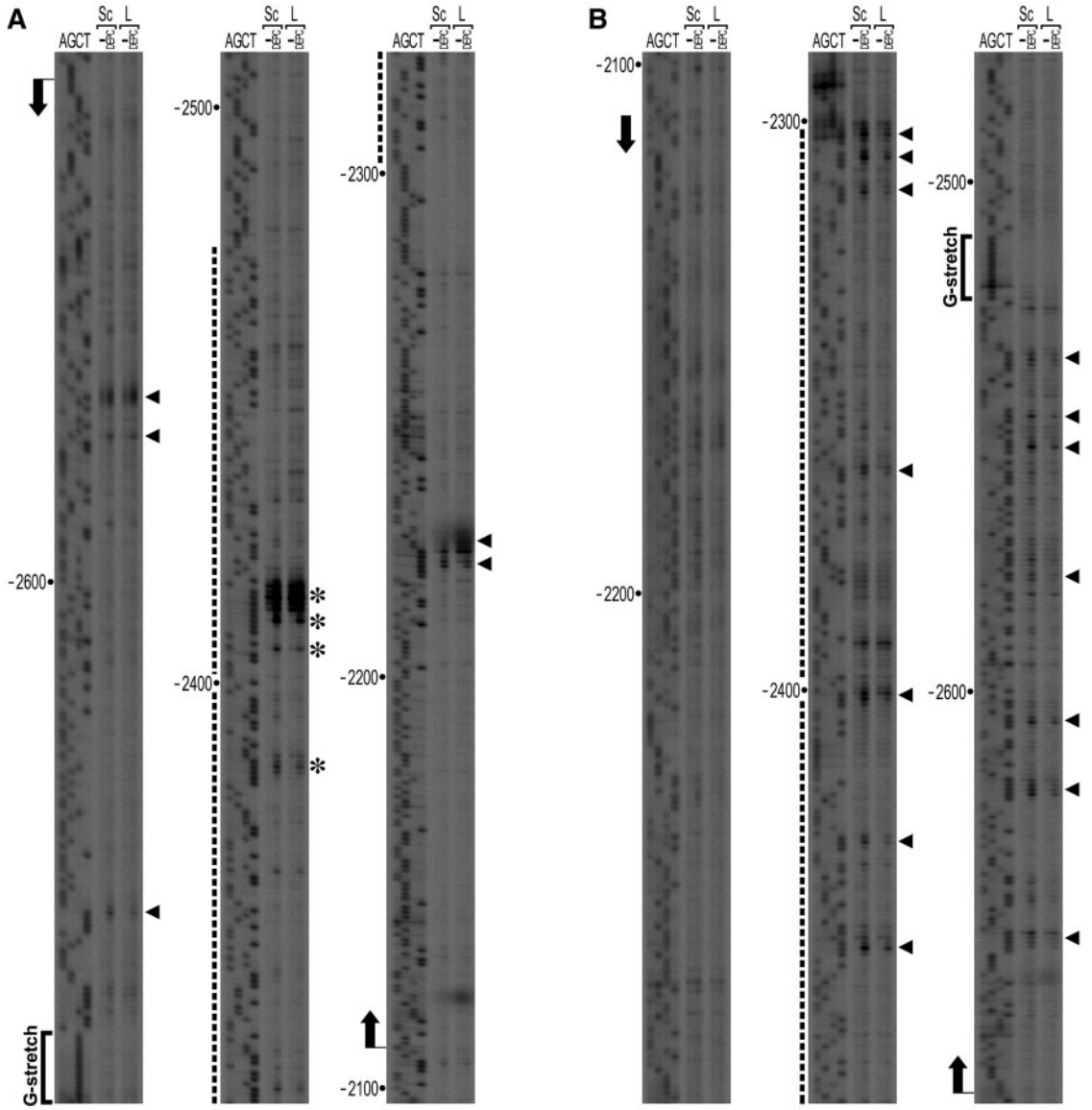

Fig. 5 Base modification of the *Ars* insulator in linear and negatively supercoiled plasmids by DEPC. (A) Detection of DEPC-modified sites by primer extension from downstream of the *Ars* insulator. (B) Detection of DEPC-modified sites by primer extension from upstream of the *Ars* insulator. Lanes A, G, C and T indicate sequence ladders of dideoxy chain termination chemistry. Negatively supercoiled (lanes labelled 'Sc') and linear (lanes labelled 'L') pARSSmBM plasmids were incubated at 22°C at 30 min with DEPC (lanes labelled 'DEPC') or without DEPC (lanes labelled '−'). Nucleotide numbers in the *HpArs* gene and positions of the G-stretch sequence are shown to the left of picture. Areas enclosed by two vertical arrows represent the position of the *Ars* insulator and dotted lines indicate the position of the central AT-rich region. Sites highly sensitive to DEPC are marked by asterisks to the right of the gel and those moderately sensitive to DEPC are marked by filled triangles.

DEPC-modified sites and DNA polymerase pausing sites are summarized in Fig. 7. Whereas the region upstream from the G-stretch sequence was well protected from MNase digestion in reconstituted nucleosomes, the central AT-rich region was highly sensitive to MNase in the reconstituted nucleosomes, indicating the exclusion of nucleosomes from the central AT-rich region. Nucleotides −2,418 to −2,401 in the highly AT-rich region were strongly modified by DEPC and showed pausing of *in vitro* DNA synthesis. Similar property was observed at nucleotides −2,314 to −2,283, suggesting that non-B-DNA structures in these regions may inhibit nucleosomal formation.

Enhancer blocking activity of the central AT-rich region of the *Ars* insulator

To study the functional significance of the central AT-rich region of the *Ars* insulator, we surveyed the core region for the enhancer blocking activity. Since it is widely accepted that the interspecies-conserved sequences are likely to be functionally important (*42*, *43*), the nucleotide sequence of the 7 kb region of the *HpArs* gene was compared with the corresponding region of the arylsulfatase gene ortholog in the closely related sea urchin species, *S. purpuratus*, using FamilyRelationsII software (*33*) (Fig. 8). When a 30 bp window size and a similarity of 75% were

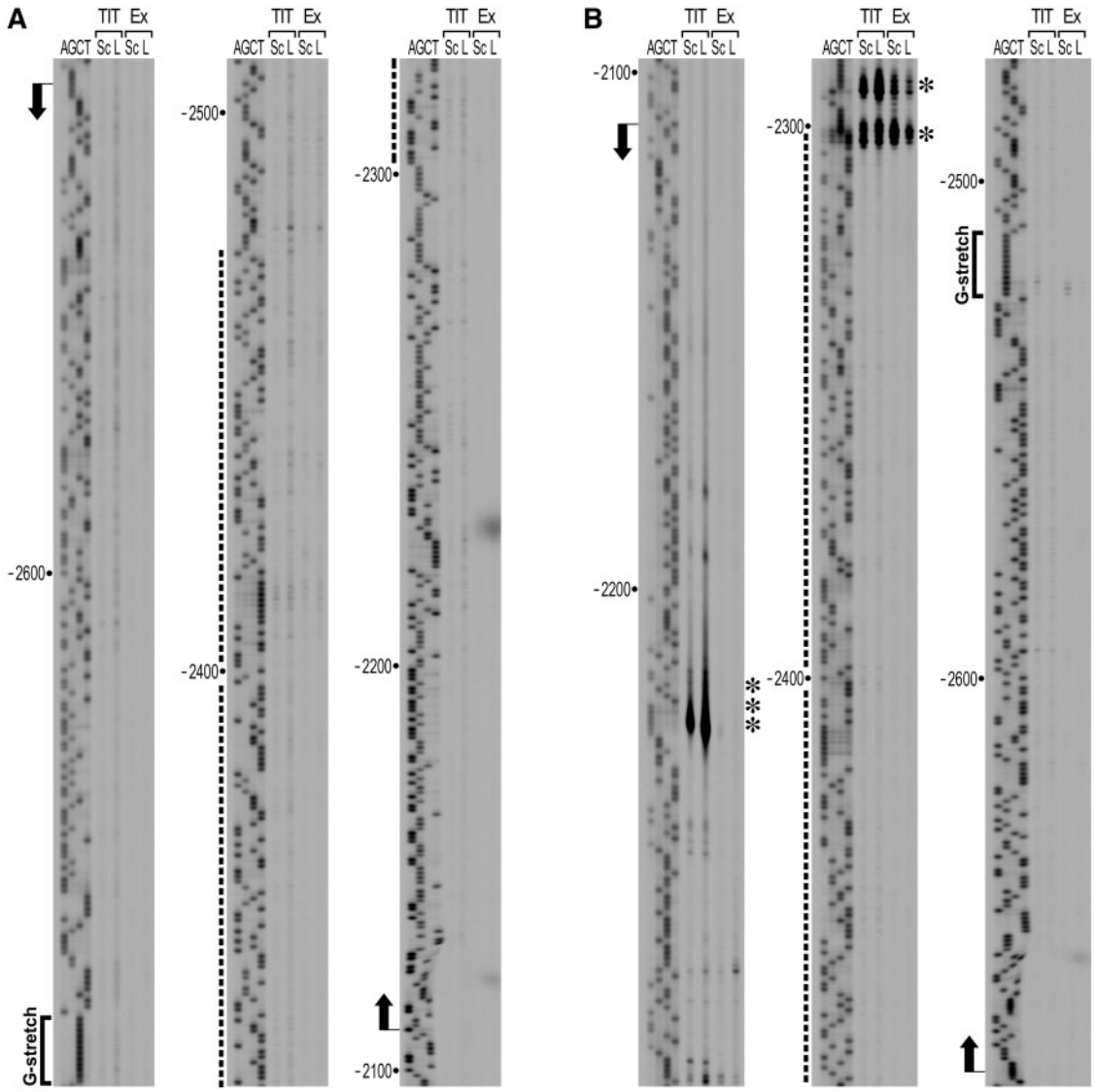

Fig. 6 Pausing of *in vitro* DNA synthesis in the *Ars* insulator in linear and negatively supercoiled plasmids. (A) Pausing of DNA synthesis by primer extension from downstream of the *Ars* insulator. (B) Pausing of DNA synthesis by primer extension from upstream of the *Ars* insulator. Lanes A, G, C and T indicate sequence ladders of dideoxy chain termination chemistry. Primer extension was carried out with two thermostable DNA polymerases, TITANIUM™ *Taq* DNA polymerase (lanes labelled 'TIT') and *TaKaRa Ex Taq*® (lanes labelled 'Ex'), using negatively supercoiled (lanes labelled 'Sc') and linear (lanes labelled 'L') pARSSmBM plasmids as templates. Nucleotide numbers in the *HpArs* gene and positions of the G-stretch sequence are shown to the left of picture. Areas enclosed by two vertical arrows represent the position of the *Ars* insulator and dotted lines indicate the position of the central AT-rich region. Sites showing strong pausing of DNA synthesis are marked by asterisks to the right of the gel.

used, interspecific sequence conservation was observed in the 1st exon, 1st intron, promoter and C15 enhancer (Fig. 8A). Although the upstream region from the *Ars* promoter showed relatively low homology to the *S. purpuratus* ortholog, conserved stretches were found in a restricted region within the *Ars* insulator (Fig. 8A). Significant homology was observed in the central AT-rich region and its vicinity (Fig. 8B), and we referred to this interspecies-conserved region as ArsInsC. Furthermore, the presence of the G-stretch was conserved upstream of the ArsInsC region.

To test the ability of the ArsInsC region to block enhancer–promoter communications, we prepared four ArsInsC-containing sequences: ArsInsC alone (nucleotides −2,473 to −2,292), ArsInsC flanked by the G-stretch (nucleotides −2,544 to −2,292), ArsInsC flanked by the downstream GC-rich sequence (nucleotides −2,473 to −2,230) and ArsInsC flanked by both G-stretch and GC-rich sequences (nucleotides −2,544 to −2,230). These sequences were inserted between the *Ars* promoter and the C15 enhancer, which drives the luciferase reporter gene, and constructs were introduced into fertilized eggs by particle gun. Luciferase activities were measured at the prism stage. The ArsInsC alone was able to block enhancer–promoter communication (Fig. 9). However, the *Ars* insulator with internal deletion of ArsInsC also retained a significant enhancer blocking activity comparable to ArsInsC alone (data not shown). Taken together, although the interspecies-conserved ArsInsC region is

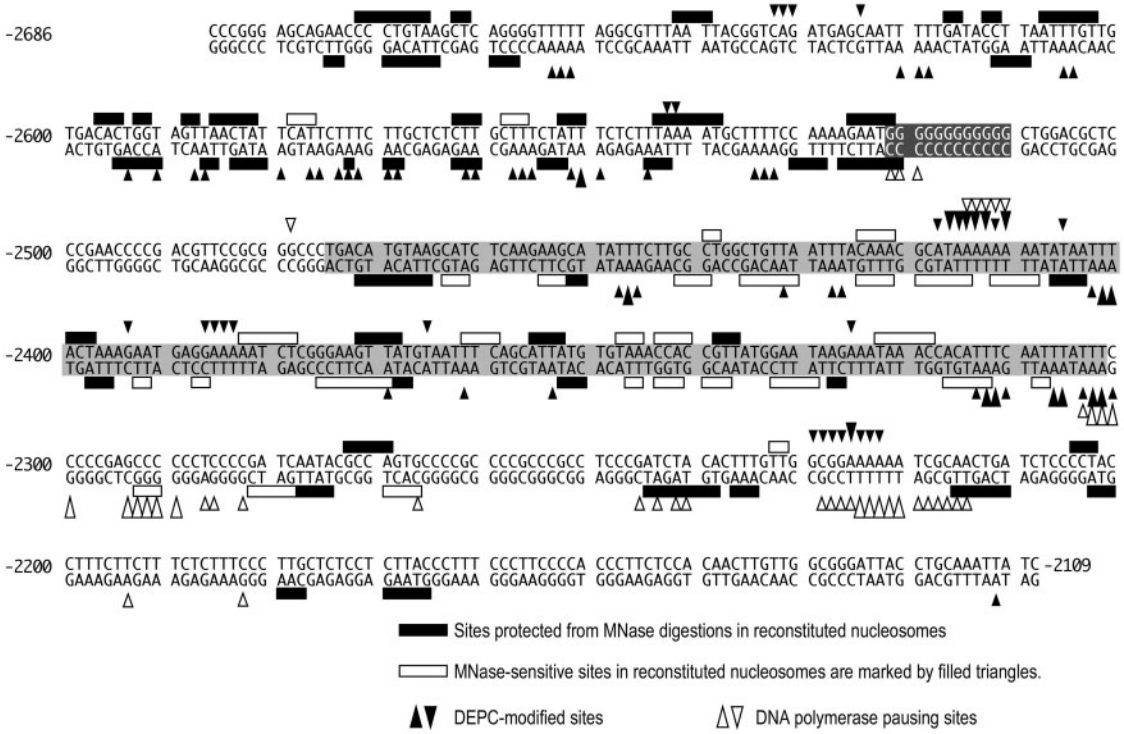

Fig. 7 Summary of sites of MNase-digestion, DEPC-modification and DNA polymerase pausing within the *Ars* insulator. The sites of MNase digestion determined by experiments in Fig. 4, DEPC-modified sites determined by experiments in Fig. 5 and sites showing pausing of DNA synthesis determined by experiments in Fig. 6 are mapped in the nucleotide sequence of the *Ars* insulator. Tone is reversed at G-stretch. Shaded box indicates the nucleotide sequence of the central AT-rich region. Filled boxes indicate the sites protected from MNase digestions in reconstituted nucleosomes and open boxes indicate MNase-sensitive sites in reconstituted nucleosomes. Open triangles indicate the DEPC-modified sites and filled triangles indicate the DNA polymerase pausing sites.

likely to be functionally important and it possess a significant enhancer blocking activity, this region seems not to be essential for the enhancer blocking activity of the *Ars* insulator.

Discussion

Function of the interspecies-conserved region of the *Ars* insulator

Interspecific conservation of the nucleotide sequences of the central AT-rich region indicates that this region is likely to be functionally important (Fig. 8) and this region showed significant enhancer blocking activity (Fig. 9). However, the ArsInsC-deleted *Ars* insulator also retained a significant enhancer blocking activity comparable to ArsInsC alone (data not shown). Therefore, the surrounding region of the ArsInsC may also possess the enhancer blocking activity to ensure the complete insulation of inappropriate signals from enhancers.

Alternatively, it is possible that the ArsInsC may be the core region for the barrier activity of the *Ars* insulator. The chicken β-globin 5′-HS4 insulator is highly enriched in acetylated histones and methylated H3-K4 that represent permissive histone modifications (*44*, *45*). Deletion of a single USF-binding site disrupts the recruitment of histone acetylation and the barrier activity, and these permissive histone modifications of 5′-HS4 insulator are proposed to interfere with the spread of silencing factor (*5*, *46*). Thus, the nucleosome exclusion property of the ArsInsC may facilitate the binding of proteins that can recruit histone modification activities.

Nucleosome structure and non-B-DNA structure in the *Ars* insulator

We have demonstrated that the *Ars* insulator contains a nuclease-hypersensitive site and that nucleosomes are excluded from the central AT-rich region within the *Ars* insulator in reconstituted nucleosomes *in vitro*. In addition, since the naked *Ars* insulator was not hypersensitive to DNase I without nucleosome reconstitution (data not shown) it is likely that the exclusion of nucleosomes in the central AT-rich region confers the nuclease hypersensitivity in the *Ars* insulator.

We observed the presence of unusual superhelical torsion-independent DNA structures in the central AT-rich region of the *Ars* insulator. This observation is coincident with our previous finding showing that the central region has a base-unpairing property (*27*). Since the base-unpairing property of the AT-rich sequence is important for biological function of matrix attachment regions (*47*), the non-B-DNA structure in the *Ars* insulator may be involved in attachment of the *Ars* insulator to the nuclear matrix.

Non-B-DNA structures such as Z-DNA and triplexes have been known to disrupt nucleosome formation (*48*–*50*). Furthermore, AT-rich sequences such

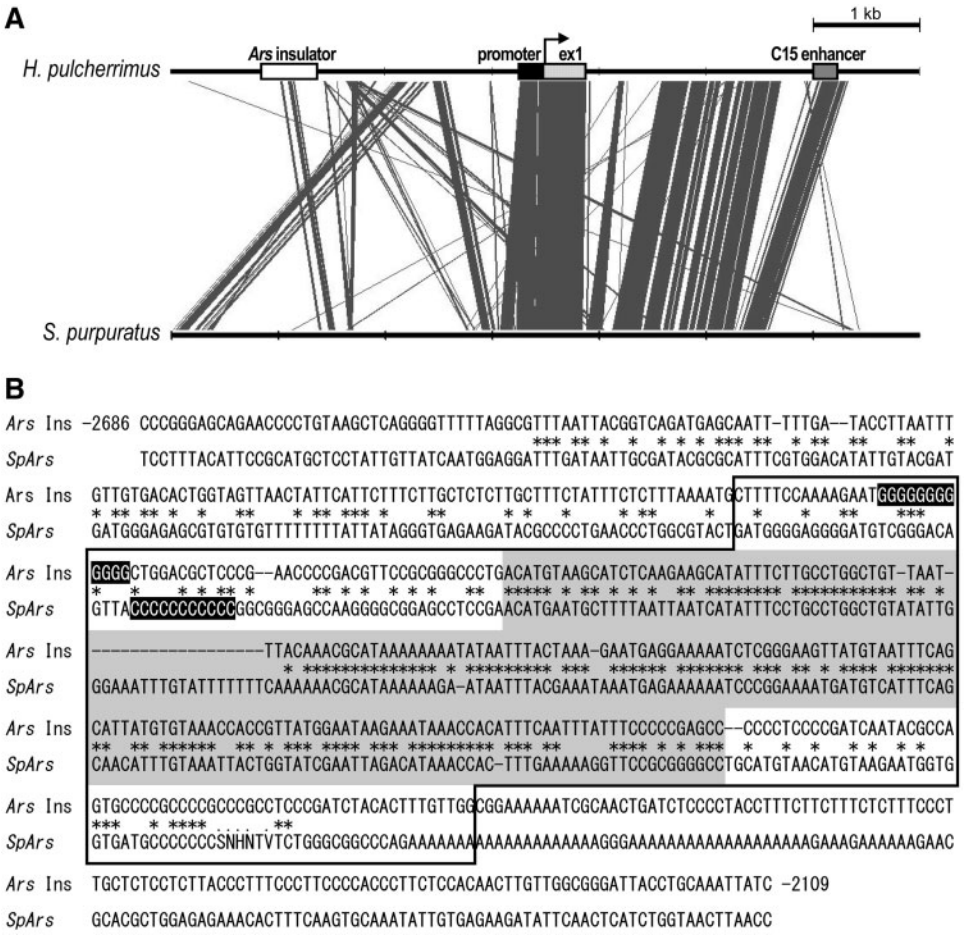

Fig. 8 Interspecies conservation of the *Ars* insulator sequence. (A) Pairwise comparison of the *arylsulfatase* gene between *H. pulcherrimus* (*Hp*) and *S. purpuratus* (*Sp*) using FamilyRelationsII software. Horizontal black lines represent the genomic sequences of each *arylsulfatase* gene. Grey lines were drawn between the conserved regions that showed 75% identity in the 30 bp of sliding window. The white box represents the *Ars* insulator, filled box *Ars* promoter, dotted box 1st exon and slashed box C15 enhancer, respectively. (B) Comparison of nucleotide sequences in the *Ars* insulator region. Asterisks indicate identical nucleotides between two species. Shaded box indicates the highly conserved region (ArsInsC) that contains the central AT-rich sequence. Boxed region was used for analysis of insulator activity in Fig. 9.

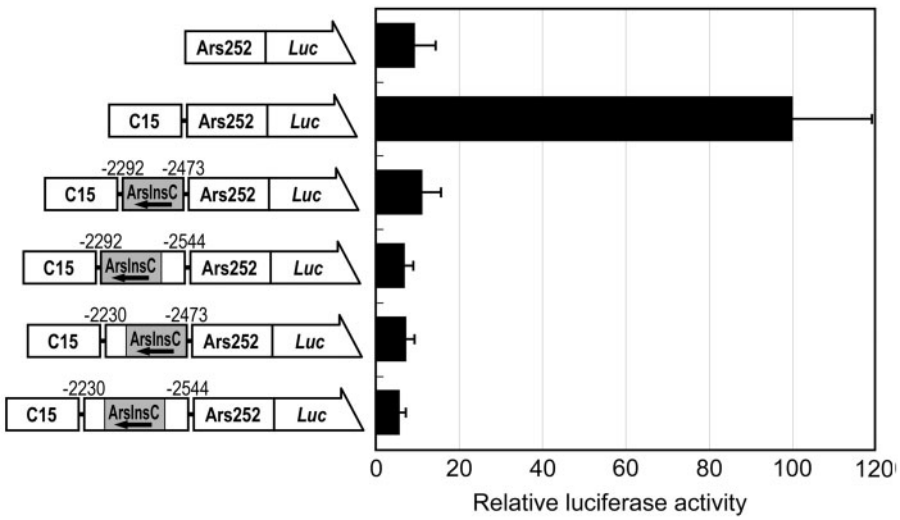

Fig. 9 Enhancer blocking assay of interspecies-conserved region of the *Ars* insulator. Conserved regions that include ArsInsC are inserted between the C15 enhancer and the *Ars* promoter. The relative luciferase activities were represented based on that of C15-Ars252-Luc, measured in the same experiment and assigned a value of 100. Bars indicate standard errors of nine independent experiments.

as poly(dA)•poly(dT) and $A_{15}TATA_{16}$ have been reported to destabilize nucleosomes (*51*). Thus, non-B-DNA structures in the central AT-rich sequence of the *Ars* insulator may interfere with nucleosomal formation resulting in hypersensitivity to nucleases.

Several functions of the nucleosome-free central AT-rich sequence of the *Ars* insulator are conceivable. One possibility is that the nucleosome-free region may expose a target sequence for regulatory proteins. In the CTCF-mediated insulator of the *H19* imprinting control region, nucleosome positionings set the CTCF target sites to nucleosomal linkers to maintain availability of target sites (*19*). We previously identified Unichrom, which is a nuclear matrix protein that can bind to the *Ars* insulator (*27*). Unichrom can recognize the minor groove of the AT-rich region within the *Ars* insulator that has a base-unpairing property. Thus it is plausible that Unichrom can bind to the *Ars* insulator *in vivo*. Another possibility is that the disruption of nucleosomal context may interrupt the spread of silent chromatin. Nucleosome-excluding sequences (CCGNN)n and poly(dA-dT) can also act as silent chromatin barriers (*52*).

Constitutive nuclease hypersensitivity was observed in the chicken β-globin 5′-HS4 insulator (*4*) and the *Drosophila gypsy* insulator (*12*). However, the nuclease sensitivity of the *Ars* insulator seemed to change during the sea urchin development (Fig. 2). Hence, the chromatin structure of the *Ars* insulator may change during sea urchin development. It is possible that endogenous endonuclease activity may change during development. However, when nuclei isolated from the unhatched blastula were digested with MNase, we could not detect any hypersensitive sites within the *Ars* insulator (data not shown). This suggests that the developmental changes of the nuclease sensitivity of the *Ars* insulator observed in Fig. 2 is due to the changes in chromatin structure rather than those in the endogenous endonuclease activity. It is also possible that nuclease sensitivity of the *Ars* insulator might be undetectable due to changes in the whole chromatin structure during early development. In fact, sea urchin contains four distinct sets of histone variants and three of them are sequentially expressed during development (*53*, *54*). Furthermore, nucleosomal repeat length changes during early development (*55*).

Biological roles of G-stretch

Since the presence of the G-stretch is conserved across species, this sequence is likely to play an important role in *Ars* insulator function. Analysis of genomic DNA sequences has revealed that poly(dG)•poly(dC) sequence of 12 bp and longer are frequently found in the non-coding regions of unrelated genes of various organisms (*56*). We showed that the G-stretch is positioned at the boundary between nucleosome-containing and nucleosome-free regions in reconstituted nucleosomes on the *Ars* insulator. Therefore, it is possible that the G-stretch may be involved in determination of nucleosome positioning. Although it was recently suggested that CTCF binding to its target site provides precise positioning of nucleosomes (*57*), the nucleosome positioning in the *Ars* insulator may be a feature of nucleotide sequence rather than binding proteins. The G-stretch has the ability to form unusual DNA structures, such as the intra-molecular triplex (*58*, *59*), and triplex structure can prevent nucleosome assembly (*47*). However, the G-stretch in the *Ars* insulator seems to be too short to form a triplex (*58*) and therefore the triplex structure is unlikely to be involved in determining nucleosome position in the *Ars* insulator.

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Conflict of Interest

None declared.

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