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Investigation of the Mechanism of Meiotic DNA Cleavage by *VMA1*-Derived Endonuclease Uncovers a Meiotic Alteration in Chromatin Structure around the Target Site

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***VMA1*-derived endonuclease (VDE), a homing endonuclease in *Saccharomyces cerevisiae*, is encoded by the mobile intein-coding sequence within the nuclear *VMA1* gene. VDE recognizes and cleaves DNA at the 31-bp VDE recognition sequence (VRS) in the *VMA1* gene lacking the intein-coding sequence during meiosis to insert a copy of the intein-coding sequence at the cleaved site. The mechanism underlying the meiosis specificity of *VMA1* intein-coding sequence homing remains unclear. We studied various factors that might influence the cleavage activity in vivo and found that VDE binding to the VRS can be detected only when DNA cleavage by VDE takes place, implying that meiosis-specific DNA cleavage is regulated by the accessibility of VDE to its target site. As a possible candidate for the determinant of this accessibility, we analyzed chromatin structure around the VRS and revealed that local chromatin structure near the VRS is altered during meiosis. Although the meiotic chromatin alteration exhibits correlations with DNA binding and cleavage by VDE at the *VMA1* locus, such a chromatin alteration is not necessarily observed when the VRS is embedded in ectopic gene loci. This suggests that nucleosome positioning or occupancy around the VRS by itself is not the sole mechanism for the regulation of meiosis-specific DNA cleavage by VDE and that other mechanisms are involved in the regulation.**

Homing endonucleases are site-specific DNA endonucleases encoded within mobile intein-coding sequences or introns and are found in each of the biological kingdoms (1–3). They promote the transfer of intein-coding sequences/introns from an allele containing an intein-coding sequence/intron to an allele lacking an intein-coding sequence/intron, in a process known as homing (1–3). Homing is initiated by endonuclease binding and cleavage at its recognition sequence embedded in the allele lacking an intein-coding sequence/intron. The role of the homing endonuclease is limited to the production of a double-strand break (DSB) at the recognition sequence. The DSB is then repaired, using the allele containing an intein-coding sequence/intron as a template, by the host recombination machinery, leading to the conversion of an allele lacking the intein-coding sequence/intron to an allele containing the intein-coding sequence/intron (16, 23, 30). Mobile intein-coding sequences and introns thus spread throughout the population by homing.

Mobile intein-coding sequences and introns, as genetic parasites, have evolved several strategies to minimize their impact on host fitness to ensure their persistence in the host genome (2). They are removed at the protein or RNA level by a splicing reaction so as not to interrupt the functional products encoded by their intervening sequence. The lengths of the recognition sequences of their coding endonucleases are 14 to 40 bp, preventing extra digestion of the host genome, because the sequences are expected to be present in a single or a few copies (17). They are usually located in the middle of their own recognition sequence, interrupting the sequence, and chromosomes bearing them are therefore protected from cleavage by their coding endonucleases. In addition, they and their recognition sequences are often inserted in critical regions of the genome for host viability. This appears to prevent their excision from the host genome, preserve their splicing activity, and restore target sequence degenerations (2, 20, 31). Thus, mobile intein-coding sequences and introns establish a parasitic relationship with the host organism.

An intein-coding sequence inserted within the nuclear gene *VMA1* encodes a homing endonuclease, *VMA1*-derived endonuclease (VDE), in several species of *Saccharomyces cerevisiae* (12, 20, 29). Phylogenetic analyses have demonstrated that intra/interspecies transfer and degeneration of intein-coding sequences have occurred among yeasts during evolutionary history (20, 29). In *Saccharomyces cerevisiae*, homing of the *VMA1* intein-coding sequence occurs frequently in diploid cells heterozygous for the allele containing an intein-coding sequence, *VMA1*$^+$, and the allele lacking an intein-coding sequence, *VMA1*$^-$. In *VMA1*$^+$/*VMA1*$^-$ cells, a DSB is introduced in the *VMA1*$^-$ allele by VDE (also called PI-SceI) and is repaired by homologous recombination using *VMA1*$^+$ as a donor (12). The *VMA1* intein-coding sequence has developed a unique strategy for self-propagation in that the introduction of the DSB by VDE occurs largely during meiosis and hardly at mitosis (8, 12). This period specificity distinguishes VDE from other homing endonucleases, which produce a DSB once the allele containing

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TABLE 1. Yeast strains

Strain	Genotype	Reference or source
YOC2813	*MAT***a** *VMA1-101 ho*::*hisG leu2 ura3 lys2 TRP1*	26
YOC2968	*MAT*α *VMA1-101 ho*::*LYS2 leu2 ura3 lys2 trp1*	9
NKY899	*MAT***a** *VMA1-105 ho*::*hisG ade2 leu2 ura3 lys2 trp1*	N. Kleckner
YOC3632	*MAT***a**/α *VMA1-201/VMA1-202 ho*::*LYS2/ho*::*LYS2 leu2/leu2 ura3/ura3 lys2/lys2 trp1/TRP1 ade2/ADE2*	This study
YOC3777	*MAT***a**/α *VMA1-101/VMA1-101 ho*::*LYS2/ho*::*LYS2 leu2/leu2 ura3/ura3 lys2/lys2 ADE2/ade2 TRP1/trp1*	This study
YOC3787	*MAT***a**/α YOC3777 but *ura3*::*FLAG-VDE*::*URA3/ura3*	This study
YOC3803	*MAT***a**/α *VMA1-207/VMA1-201 ho*::*hisG/ho*::*LYS2 leu2/leu2 ura3/ura3 lys2/lys2 TRP1/trp1*	8
YOC3804	*MAT***a**/α YOC3803 but *ade3*::*VDE*::*TRP1/ADE3*	This study
YOC3805	*MAT***a**/α YOC3803 but *ade3*::*NLS-VDE*::*TRP1/ADE3*	This study
YOC3784	*MAT***a**/α YOC3777 but *ura3*::*VDE*::*URA3/ura3*	This study
YOC3785	*MAT***a**/α YOC3777 but *ura3*::*VDE(D326V)*::*URA3/ura3*	This study
YOC3786	*MAT***a**/α YOC3777 but *ura3*::*VDE(R90A)*::*URA3/ura3*	This study
YOC3708	*MAT***a**/α *VMA1-101/VMA1-101 ho*::*LYS2/ho*::*LYS2 leu2/leu2 ura3/ura3 lys2/lys2 arg4-bgl/arg4-nsp*	This study
YOC3709	*MAT***a**/α YOC3708 but *VMA1/VMA1*	This study
TFY100	*MAT***a**/α YOC3708 but *MCM4*::*VRS/MCM4*::*VRS*	This study
YOC3792	*MAT***a**/α *VMA1-101/VMA1-101 ho*::*hisG/ho*::*LYS2 leu2/leu2 ura3/ura3 lys2/lys2 TRP1/trp1*	This study
YOC3794	*MAT***a**/α YOC3792 but *ADE2*::*VRS/ADE2*::*VRS*	This study
YOC3681	*MAT*α *VMA1-101 ho*::*LYS2 leu2 ura3 lys2 arg4-nsp*	This study
YOC3694	*MAT*α YOC3794 but *sir2*::*KanMX4*	This study
YOC3778	*MAT***a**/α YOC3777 but *ime1*::*KanMX4/ime1*::*KanMX4*	This study
YOC3779	*MAT***a**/α YOC3777 but *ime2*::*KanMX4/ime2*::*KanMX4*	This study
YOC3781	*MAT***a**/α YOC3777 but *ndt80*::*KanMX4/ndt80*::*KanMX4*	This study
YOC3782	*MAT***a**/α YOC3777 but *clb5*::*KanMX4/clb5*::*KanMX4 clb6*::Cg*URA3/clb6*::Cg*LEU2*	This study
YOC3675	*MAT***a**/α YOC3632 but *ime1*::Cg*LEU2/ime1*::Cg*LEU2*	This study
YOC3676	*MAT***a**/α YOC3632 but *ime2*::Cg*LEU2/ime2*::Cg*LEU2*	This study
YOC3649	*MAT***a**/α YOC3632 but *clb5*::*KanMX4/clb5*::*KanMX4 clb6*::Cg*URA3/clb6*::Cg*URA3*	This study
YOC3678	*MAT***a**/α YOC3632 but *ndt80*::Cg*LEU2/ndt80*::Cg*LEU2*	This study
YOC3878	*MAT***a**/α YOC3784 but *ime1*::*KanMX4/ime1*::*KanMX4*	This study
YOC3879	*MAT***a**/α YOC3784 but *ime2*::*KanMX4/ime2*::*KanMX4*	This study
YOC3880	*MAT***a**/α YOC3784 but *clb5*::*KanMX4/clb5*::*KanMX4 clb6*::Cg*URA3/clb6*::Cg*LEU2*	This study
YOC3881	*MAT***a**/α YOC3784 but *ndt80*::*KanMX4/ndt80*::*KanMX4*	This study

an intein-coding sequence/intron is accompanied by the allele lacking an intein-coding sequence/intron (23, 39, 42), and is assumed to be beneficial for reliable repair, which is mediated by the meiotic recombination pathway (9).

Gimble and Thorner first reported meiosis-specific homing and discussed several possible explanations for it (12). It has been revealed that VDE is excluded from the nucleus in vegetatively growing cells and that its nuclear localization is induced in response to nutrient limitation (24). In addition, blocking premeiotic DNA replication has been shown to affect the formation of DSB by VDE (9). These findings implicated host factors in the regulation of VDE-mediated DSB formation at meiosis. However, besides these insights, little has been known about the mechanism of the regulation. In this study, to explore the mechanism of the meiosis-specific DSB by VDE, we examined several factors likely to regulate meiotic DSB formation and imply that meiotic DNA cleavage by VDE is regulated by the accessibility of VDE to the VDE recognition sequence (VRS). To search for the determinant of this accessibility, we investigated the chromatin structure around the VRS and found that it changes during meiosis. We present examinations of this chromatin alteration and discuss possible mechanisms for meiosis-specific DNA cleavage by VDE.

MATERIALS AND METHODS

Strains. The yeast strains used in this study are listed in Table 1. All strains were derivatives of the SK1 strain, which enters meiosis in a highly synchronous manner (18). Strains were constructed by standard genetic crosses, transformation, and other genetic procedures. The *VMA1-101* and *VMA1-201* alleles have a complete deletion of the *VMA1* intein-coding sequence (*VMA1*$^-$). The *VMA1*, *VMA1-202*, and *VMA1-207* alleles contain the *VMA1* intein-coding sequence (8). *VMA1-207* has a mutation in the VDE coding region that results in a loss of DNA-binding activity. The BamHI site, which is located 866 bp upstream of the *VMA1* start codon, is replaced with a SalI site in the *VMA1-202* and *VMA1-207* allele, enabling us to distinguish the *VMA1*$^+$ homing product from the *VMA1*$^+$ donor in Southern analysis (9).

For the expression of VDE at the *ADE3* or *URA3* gene locus under the *VMA1* promoter, cells were constructed as described previously (24, 26). For the expression of Flag-tagged VDE, the synthetic oligonucleotides 5′-CAT GGA CTA CAA GGA CGA TGA CGA TAA GGG TAC-3′ and 5′-CCT TAT CGT CAT CGT CCT TGT AGT C-3′ were annealed and cloned into the NcoI-KpnI site of plasmids harboring the *VMA1* promoter-fused VDE coding region (26).

To construct the *ADE2*::*VRS* allele, the HindIII-digested fragment of pYO2411, which contains the VRS at the XbaI site near the ATG start codon of the *ADE2* gene in pASZ10 (34), was transformed into the predeleted *ADE2* gene locus. For the construction of the *MCM4*::*VRS* allele, the XbaI restriction site was inserted adjacent to the ATG start codon by a two-step PCR method (15) using primer pairs 5′-AGT CCT CGA GAC TGG GAG AAT CTT CGA GCC AAT GC-3′ and 5′-GTT GGA GAG CTA GAC TGT TGT CTA GAC ATT TTA AGT TCT TGA G-3′ and 5′-CTC AAG AAC TTA AAA TGT CTA GAC AAC AGT CTA GCT CTC CAA C-3′ and 5′-AGT CGC GGC CGC CCA GAA GCG CTC GCA GAA GAA CG-3′, and then a PCR product was cloned into pRS306 (33). The synthetic oligonucleotides 5′-CTA GCT ATG TCG GGT GCG GAG AAA GAG GTA ATG AAA-3′ and 5′-CTA GTT TCA TTA CCT CTT TCT CCG CAC CCG ACA TAG-3′ were annealed and cloned into the XbaI site to produce pRS306MCM4VRS. After SphI digestion and integration at the *MCM4* gene locus, pop-out of the *URA3* marker and a part of the gene were performed on 5-fluoroorotic acid plates, resulting in the generation of the *MCM4*::*VRS* allele.

Deletion mutations of each gene were constructed by the PCR-mediated gene disruption method (32) and confirmed by PCR.

Synchronous sporulation, physical analysis of intein-coding sequence homing, immunoblotting of VDE, endonuclease activity assay, and immunostaining of VDE. Synchronous sporulation, physical analysis of homing, and the preparation of protein samples and immunoblotting were carried out as described previously

(9). For the endonuclease assays of VDE, cells expressing Flag-tagged VDE were sampled at various time points, frozen, and resuspended in lysis buffer (200 mM Tris-HCl [pH 8.0], 400 mM ammonium sulfate, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol) containing protease inhibitors. After we vortexed the cell extracts with glass beads, they were clarified by sonication and centrifugation. Protein concentrations were estimated by a bicinchoninic acid assay (Pierce), and aliquots of cell extracts were incubated with anti-Flag-Sepharose resin (Sigma) overnight at 4°C, washed, and then eluted in elution buffer (25 mM Tris-HCl [pH 8.5], 100 mM KCl) with 150 μg/ml 3× Flag peptide (Sigma). Aliquots of the eluates were incubated with 0.5 μg of pBSΔVDE linearized with XmnI (19) in 25 mM Tris-HCl (pH 8.5), 100 mM KCl, and 2.5 mM $MnCl_2$ at 30°C. Nuclear staining and immunofluorescence microscopy were performed as described previously (24), with some modifications. Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin antibody was used as a second antibody.

Chromatin preparation, digestion by MNase, and hybridization. The preparation of crude chromatin fractions from yeast cells and the treatment of chromatin with micrococcal nuclease (MNase) were performed as described previously (27, 28), with the minor modification that spheroplasts be prepared by incubation with a higher concentration (0.1 mg/ml) of Zymolyase 100T (Seikagakukogyo Co. Ltd) for 5 min on ice, due to the recently improved solubility and activity of the commercially available Zymolyase. DNA was digested with the indicated restriction endonucleases, separated in agarose gels, and transferred to Biodyne B membranes (Pall) by alkaline transfer. Radioactive probes were prepared by random priming of DNA fragments purified by agarose gel electrophoresis from appropriate PCR products. The membranes were hybridized according to the method of Church and Gilbert (5).

ChIP assay. Chromatin immunoprecipitation (ChIP) was performed basically as described previously (36). Aliquots of cells taken from a synchronous sporulation culture were fixed in 1% formaldehyde for 35 min at room temperature. Immunoprecipitations were carried out with affinity-purified anti-VDE antibody (24) bound to protein G-coated Dynabeads (Dynal). After treatment with RNase A and proteinase K, samples were purified using a QIAquick PCR purification kit (QIAGEN). Multiplex PCR amplification by AmpliTaq Gold (Perkin-Elmer) was performed with primer pairs, the sequences of which are described elsewhere (10). PCR products were separated on 2% agarose gels containing ethidium bromide and analyzed with FLA3000 (Fujifilm).

RESULTS

VDE is constitutively present and exhibits endonuclease activity in both mitotic and meiotic cell extracts. The simplest explanation for the meiosis specificity of DSB formation by VDE is that DNA cleavage is controlled by the amount or activity of VDE. However, as reported previously (9, 24), VDE is expressed abundantly in mitotic cells as well as in meiotic cells. We consequently examined the endonuclease activity of VDE during mitosis and meiosis. Cells expressing Flag-VDE were removed and harvested at various time points from synchronously sporulating cultures. The Flag-VDE was then recovered with anti-Flag antibody and incubated with linear double-stranded DNA containing the VRS. Immunoprecipitated VDE obtained from mitotic and meiotic cells exhibited equivalent levels of endonuclease activity (Fig. 1A). Therefore, we conclude that VDE is constitutively expressed and active during both mitosis and meiosis, ruling out the possibility that VDE-mediated DNA cleavage is regulated by quantitative or qualitative differences in VDE during meiosis.

Meiosis-specific DNA cleavage by VDE is not simply due to nuclear import. VDE is reported to be excluded from the nucleus in vegetatively growing cells, and its nuclear localization is induced in response to nutrient limitation (24). It has also been revealed that the attachment of a nuclear localization signal (NLS) to VDE causes the accumulation of VDE in the nucleus and induces homing events to some extent even during mitosis (24). To determine the precise contribution of the nuclear localization of VDE to meiosis-specific DSB, we conducted Southern analyses to examine the effects of NLS addition to VDE on homing. We prepared a heterozygous *VMA1-207/VMA1*$^-$ diploid as a tester strain. *VMA1-207* is an allele containing the intein-coding sequence, but its VDE has an R90A mutation that abolishes the DNA binding and cleavage activity of VDE (14, 26). As shown in Fig. 1B, we confirmed that neither cleavage nor homing products could be detected in the tester strain. VDE or NLS-fused VDE was expressed under the *VMA1* promoter in this tester strain. The expression of wild-type VDE resulted in DSB formation at the VRS during meiosis (Fig. 1B). Homing products are faintly observed in premeiotic cells resulting from mitotic leak of homing, as reported elsewhere (9, 24), but greatly accumulate in the later meiotic stages (Fig. 1B). When the NLS-fused VDE was expressed, the kinetics of the VDE-mediated DSB formation and appearance of the homing products were generally similar to those observed for normal VDE expression (Fig. 1B). These results suggest that meiotic induction of DSB formation and homing are not necessarily consequences of meiotic nuclear localization of VDE and implicate the existence of other factors that regulate the meiosis-specific actions of VDE.

Entry into the meiotic program is necessary for DSB formation by VDE. In yeast, meiosis is induced in diploid cells under certain conditions of nutrient limitation. After the induction of meiosis, premeiotic DNA replication, two successive nuclear divisions, and sporulation take place in sequential order. Previously, it was shown that full activation of VDE-mediated DSB formation requires premeiotic DNA replication since blocking premeiotic DNA replication reduces and delays DNA cleavage by VDE (9).

To examine detailed requirements of meiotic progression for VDE-mediated DSBs, we introduced a mutation in genes of key regulators of meiotic events and observed homing by Southern analysis. In the *ime1*Δ and *ime2*Δ mutants, which are defective in the activation of early events of meiosis, including entry into the meiotic program and the induction of premeiotic DNA replication (6, 7, 37), VDE-mediated DSBs and homing products were not observed (Fig. 1C). In contrast to the case of the *clb5*Δ *clb6*Δ double mutant, in which the progression of premeiotic DNA replication is prevented (35), VDE-mediated DSBs were hardly observed at later time points in these mutants (Fig. 1C). These results suggest that entry into the meiotic program is indispensable for the activation of VDE-mediated DSB formation. As shown in Fig. 1D, VDE was localized both in the cytoplasm and in the nucleus in each mutant after 4 h of incubation in sporulation medium (SPM). Thus, it is suggested that a nutritional limitation only, which is sufficient for nuclear entry of VDE, is insufficient for the induction of homing, consistent with the results for NLS-fused VDE (Fig. 1B). On the contrary, the *ndt80*Δ mutant, which leads to a failure to induce the middle sporulation genes and a subsequent arrest before nuclear divisions (4, 37), exhibited an induction of DSBs and intein-coding sequence homing (Fig. 1C), indicating that VDE-mediated DSB formation is an event which occurs before nuclear divisions. Thus, these results lead us to hypothesize that the nuclear-localized VDE, which was induced by nutritional limitation, is subjected to further regulation that inhibits VDE-mediated DSB formation until mei-

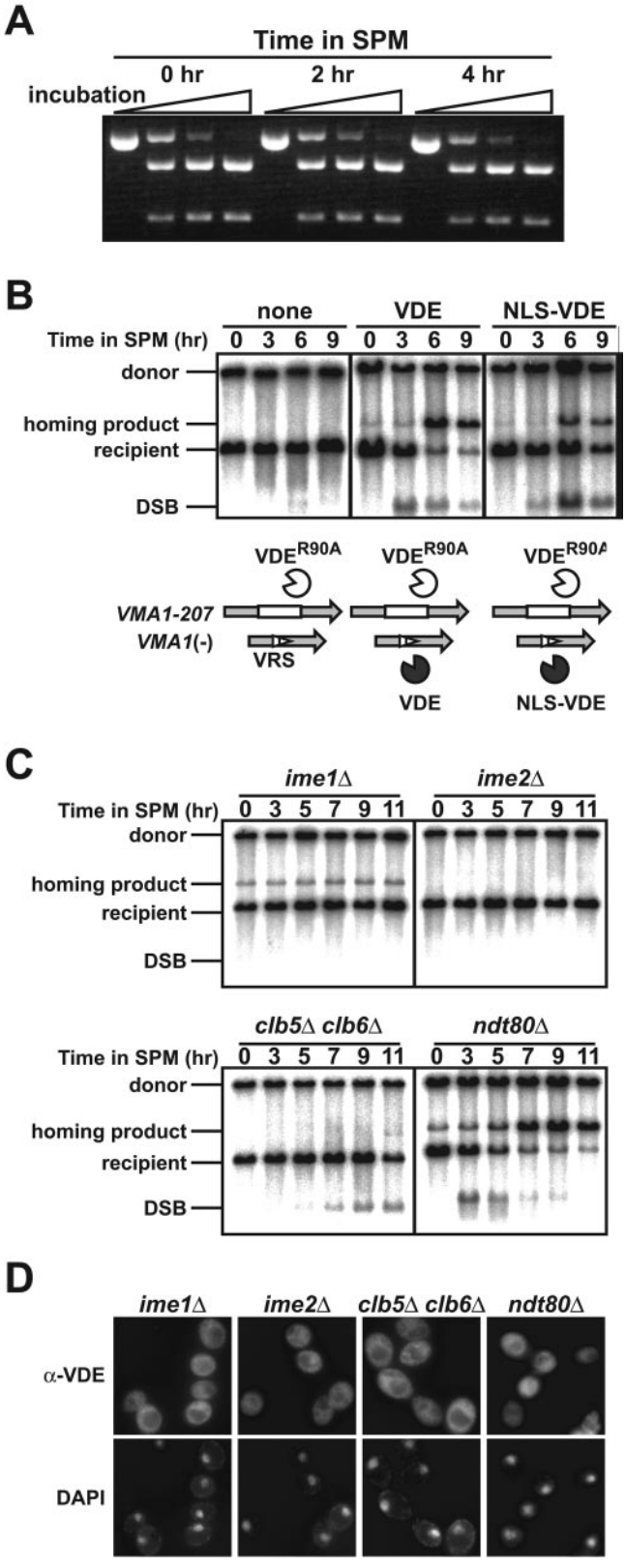

FIG. 1. Examination of endonuclease activity and effects of nuclear localization or meiotic progression on meiosis-specific homing. (A) Endonuclease activity of immunoprecipitated VDE. Cells expressing Flag-tagged VDE were subjected to synchronous sporulation and sampled at various time points in SPM. Using an anti-Flag antibody, VDE was immunoprecipitated and assayed by cleavage of the 3.3-kb linearized DNA containing the VRS that produces 2.2- and 1.1-kb fragments after digestion at the VRS. Substrates were incubated for 0, 15, 30, and 60 min at 30°C. (B) Physical detection of homing by Southern blotting. *VMA1-207*/*VMA1*$^-$ heterozygous diploid cells ectopically expressing no VDE, wild-type VDE, or NLS-fused VDE were subjected to synchronous sporulation. Since the *VMA1-207* allele contains an intein-coding sequence encoding inactive VDE (R90A), only VDE that is ectopically expressed can produce DSBs and the cleaved recipient is repaired using a *VMA1-207* donor as a template, resulting in the production of a *VMA1*$^+$ homing product. DNA was isolated from cells at the indicated times after incubation in SPM and subjected to Southern analysis after BamHI digestion. The BamHI site polymorphism makes it possible to distinguish *VMA1*$^+$ homing products from *VMA1-207* donors. (C) Physical analysis of homing in mutants deficient in progression of meiotic program. Southern analysis was performed on the *VMA1*$^+$/*VMA1*$^-$ heterozygous diploid cells in which meiotic entry, DNA replication, or nuclear divisions were blocked by the introduction of each mutation. (D) VDE localization in meiotic mutants at 4 h in SPM. The upper panels show localization of VDE, and the lower panels show the nuclei. DAPI, 4′,6′-diamidino-2-phenylindole.

otic entry or that activates DSB formation during meiotic progression.

VDE binding to the VRS occurs during meiosis. To test whether VDE can bind to its target DNA before digestion, we examined the in vivo association of VDE to the VRS by a ChIP assay. Cells homozygous for *VMA1*$^-$ expressing VDE were cross-linked with formaldehyde at various time points to generate protein-DNA cross-links. After fragmentation of the chromatin, VDE was immunoprecipitated with an anti-VDE antibody, and the precipitated DNA was purified. Purified DNAs were analyzed by PCR using primer sets for the sequence around the VRS (VRS3 and VRS5). Compared to the signals in the control input samples or the signals by the primer set for telomeric sequences, the anti-VDE-precipitated fraction exhibited a significant enrichment of the sequence detected by the VRS3 and VRS5 primer sets (Fig. 2A and B). These enrichments were not detected in cells expressing VDE^{R90A}, which is defective in DNA binding, or in the *ime1*Δ, *ime2*Δ, or *clb5*Δ *clb6*Δ mutants, whereas the *ndt80*Δ mutant showed meiotic enrichment of the signals for the sequence near the VRS (Fig. 2C). These results indicate that VDE binding to the VRS is closely correlated with DSB formation by VDE and that VDE can be detected at the target site only when DSB formation takes place. Taken together, it is implied that meiosis-specific DNA cleavage by VDE is regulated by an accessibility of VDE to the target site.

Chromatin configuration changes around the VRS during meiosis. The results of the ChIP assay led us to explore the determinant of the accessibility of VDE to the VRS. We examined the possibility of cleavage regulation by the substrate, namely, the chromatin structure around the VRS, because regulations through chromatin structure are involved in many DNA metabolic processes, including DNA replication, transcription, and recombination. We prepared chromatin fractions from diploid strains homozygous for *VMA1*$^-$ at various time points in meiotic culture. The isolated chromatin was digested with various concentrations of MNase. We further digested the MNase-digested DNA with BamHI and SphI. The

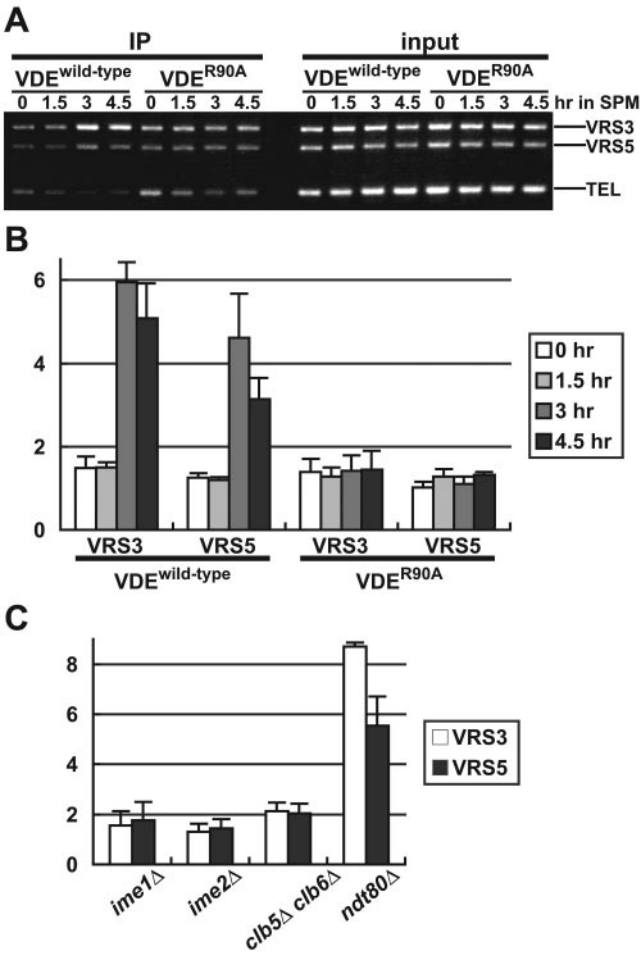

FIG. 2. Detection of VDE binding to the VRS. (A) Diploid cells homozygous for *VMA1*$^-$- expressing VDE or DNA-binding-deficient VDE (R90A) were sampled at the indicated times during meiosis. Cells were treated with formaldhyde, and then the cell extract was prepared, sonicated to shear the chromatin, and immunoprecipitated with anti-VDE antibody. DNA from input samples and in the immunoprecipitates (IP) was amplified by multiplex PCR with primers for the 3′ region adjacent to the VRS (VRS3), the 5′ region adjacent to the VRS (VRS5), and the telomeric region of chromosome VI (TEL). (B) The ratios of the VRS signals to the TEL signal were normalized to those of the input samples at each time point. Error bars denote the standard deviations among three independent experiments. (C) A ChIP assay with anti-VDE antibody was performed for the indicated mutants. The signals obtained for the 4.5-h samples are shown as in panel B.

DNA fragments were then analyzed by Southern hybridization using a short probe for the sequence adjacent to the BamHI site (indirect end-labeling). Figure 3A shows the MNase-sensitive sites in chromatin of the *VMA1*$^-$ locus in cells at various time points exhibiting a ladder-like pattern of sensitive sites in the open reading frame of *VMA1*$^-$. The VRS was mapped at a position adjacent to the sensitive site on the 5′ side. We detected a prominent MNase-sensitive site appearing immediately at the 5′ site adjacent to the VRS after 3 and 4.5 h of meiotic culture, whereas MNase sensitivity at this site was very faint in MNase-digested chromatin from mitotic and early meiotic cells (Fig. 3A, arrowheads). Importantly, this MNase-sensitive site was not detected in naked DNA partially digested with MNase (Fig. 3A), indicating that the appearance of this site reflects changes in local chromatin structure around the VRS during meiosis. In this experiment, most of the cells completed premeiotic DNA replication by 3 h and started the first meiotic division at 6 h (Fig. 3B and C), indicating that chromatin alteration near the VRS occurs around the period of VDE-mediated DSB formation.

We next examined the effect of VDE on chromatin structure. We analyzed chromatin configuration in a diploid strain homozygous for *VMA1*$^-$ but expressing VDE under the *VMA1* promoter at the *URA3* gene locus. At 0 h, the patterns of the MNase-sensitive sites were very similar to those observed in the strain lacking VDE (Fig. 3A and 4A). Unfortunately, robust VDE-mediated DSBs made it impossible to detect the chromatin structure around the VRS after 4 h of incubation in SPM (Fig. 4A). Thus, we prepared strains expressing VDE^{D326V}, which is defective in endonuclease activity, and VDE^{R90A}, which is defective in DNA binding (11, 26). In each case, we observed chromatin alteration around the VRS during meiosis as in the wild-type cells (Fig. 4B), suggesting that the chromatin change is not affected by the expression or target binding of VDE.

Chromatin alteration around the VRS takes place in correlation with DNA binding and cleavage by VDE. It is possible that the observed chromatin alteration occurs in response to metabolic changes caused by starvation, as in the case of nuclear entry of VDE. We consequently examined whether haploid cells undergo chromatin changes around the VRS during culture in SPM. As shown in Fig. 4C, the MNase-sensitive site near the VRS remained very faint even after 4 h of incubation in SPM, indicating that chromatin transition does not take place in starved haploid cells in SPM. In addition, we introduced a *sir2*Δ mutation into the haploid strain, which resulted in the derepression of the normally silent mating type loci *HMR* and *HML*, leading to the coexpression of **a** and α mating types and permitting haploid meiosis. After 4 h of incubation in SPM, the *sir2*Δ haploid cells exhibited the same chromatin structure as that observed in diploid cells (Fig. 4C). These data suggest that the chromatin alteration around the VRS is coupled with meiotic induction, not with metabolic changes. To confirm this notion, we further examined the dependence of the chromatin alteration on a set of meiotic events. In the *ime1*Δ and *ime2*Δ mutants, chromatin alteration around the VRS was barely detected even after 4.5 h of meiotic culture (Fig. 4D), indicating that the chromatin changes are dependent on entry into meiotic programs. The *clb5*Δ *clb6*Δ double mutation also severely affected the chromatin alteration around the VRS. In contrast, the *ndt80*Δ mutant exhibited normal chromatin alteration (Fig. 4D). Thus, the dependency of chromatin alteration on meiotic events is very similar to the case of DNA binding and cleavage by VDE.

The VRS element does not necessarily cause chromatin alteration. To test the sequence dependency of the chromatin alteration around the VRS, we next studied chromatin configuration in a *VMA1*$^+$ homozygous strain. In the *VMA1*$^+$ allele, insertion of the intein-coding sequence interrupts the VRS element and VDE is no longer able to cleave the sequence. In this strain, the site where meiotic MNase sensitivity was observed in the *VMA1*$^-$ strain showed high MNase sensitivity even at 0 h, and little change in the pattern of sensitive

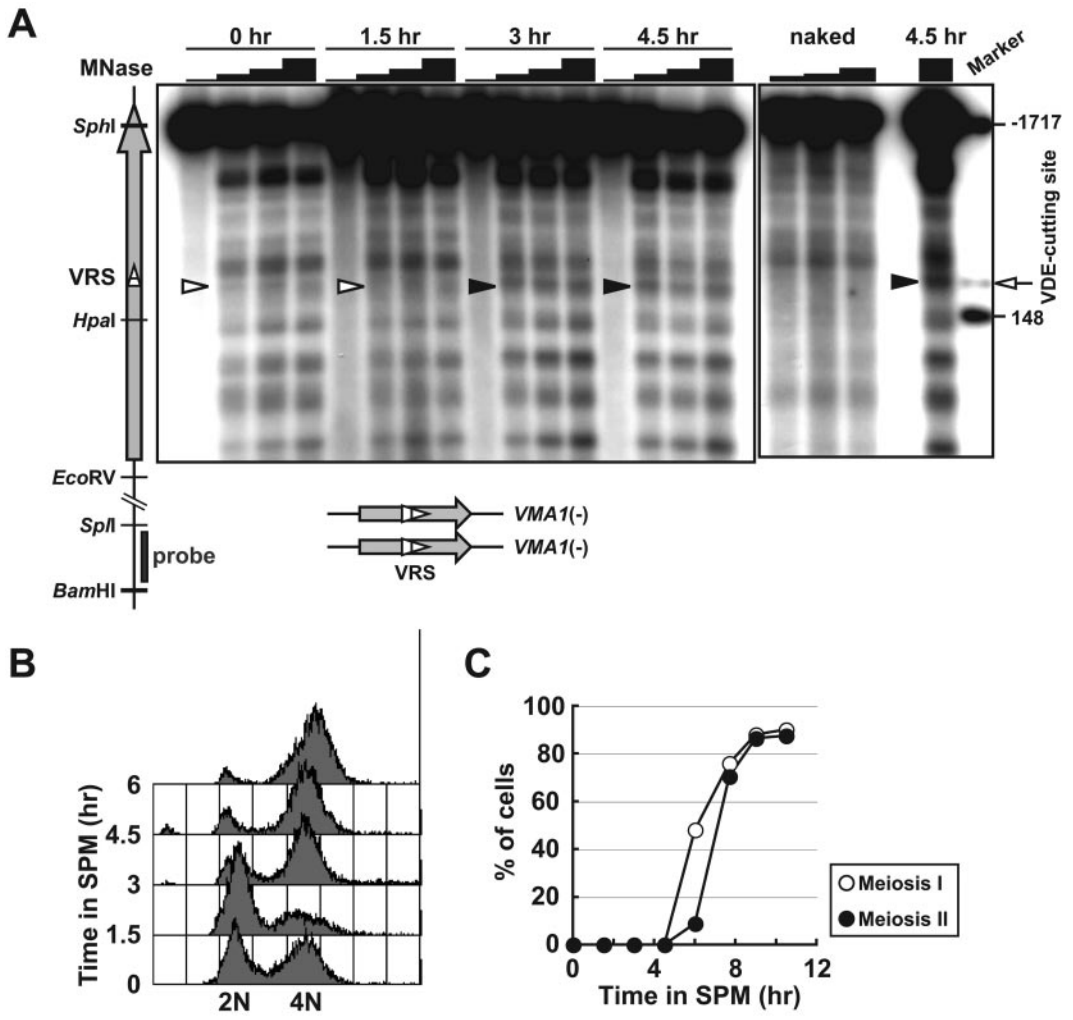

FIG. 3. Chromatin configuration at the *VMA1*$^-$ locus during meiosis. (A) Chromatin was prepared in diploid cells homozygous for *VMA1*$^-$ at various times of incubation in SPM, treated with 0, 5, 10, or 20 U/ml of MNase, and redigested with BamHI and SphI. Naked DNA was also treated with 0.2, 0.4, or 0.8 U/ml of MNase. MNase-sensitive sites were detected by indirect end-labeling using a probe for the sequence adjacent to the BamHI site. The vertical gray arrow indicates the position of the coding region of the *VMA1*$^-$ locus. The arrowheads show MNase-sensitive sites near the VRS that became prominent during meiosis. Numbers indicate the positions in nucleotides relative to the VDE cutting site that is shown by the horizontal arrow. Meiotic progression was monitored by DNA content, which was analyzed by a fluorescence-activated cell sorter (B), and by nuclear division, which was analyzed by microscopy of 4′,6′-diamidino-2-phenylindole-stained cells (C).

sites was detected after 4 h of incubation (Fig. 5A). This was not due to the presence of VDE in the *VMA1*$^+$ cells, since the chromatin structure at 0 h was not affected regardless of the presence or absence of VDE (Fig. 4A). Thus, intein-coding sequence insertion at the VRS affects chromatin configuration around the VRS during mitosis, implying that a complete VRS element is required for proper chromatin changes to occur around the VRS.

It has been shown that insertion of the VRS is sufficient to introduce a meiotic DSB by VDE and a recombination event at the site (25, 26). If the observed meiotic chromatin alteration plays a critical role in the regulation of meiosis-specific action of VDE, the insertion of the VRS at an ectopic site causes a chromatin alteration at the site. To study whether the chromatin alteration is specific to its original chromosomal position, we analyzed chromatin structure around the VRS that is inserted at a different chromosomal location. We constructed a diploid strain in which the VRS is inserted in frame near the ATG start codon of the *ADE2* gene or the *MCM4* gene and investigated premeiotic and meiotic chromatin structures around the gene loci with or without VRS. In each case, meiotic DSBs were observed when VDE was expressed from *VMA1*$^+$ (Fig. 5D). In the VRS-embedded *ADE2* allele, a prominent MNase-sensitive site appeared during meiosis, while the sensitive site was ambiguous at 0 h of meiosis (Fig. 5B, arrowheads). No such meiotic chromatin alteration was observed in the wild-type *ADE2* allele, in which a distinct MNase-sensitive band was detected at the corresponding site at 0 h (Fig. 5B, arrowheads). Although meiotic alteration in chromatin was observed, the MNase-sensitive site locates relatively apart from the VRS insertion site. In the case of VRS integration into the *MCM4* locus, meiotic change in chromatin was not significantly observed around the VRS, while the VRS insertion brought some alterations in the chromatin configu-

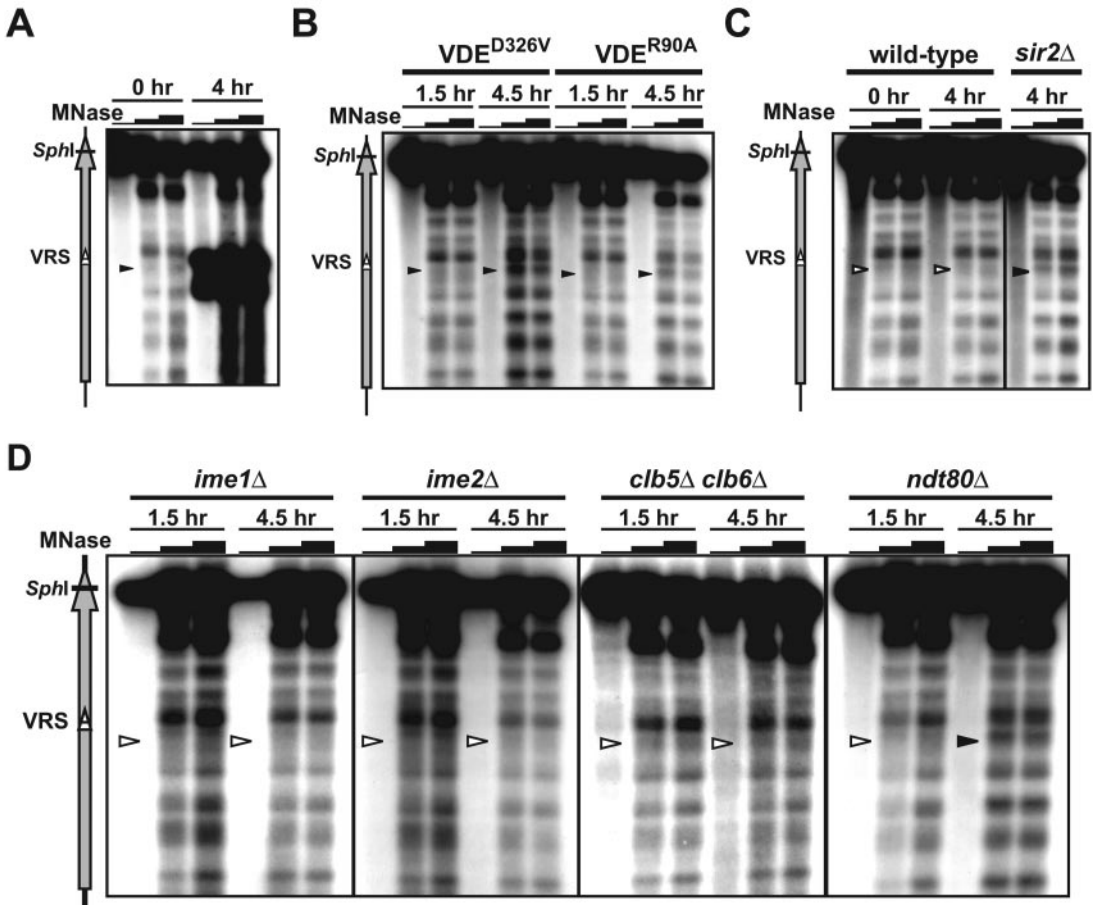

FIG. 4. Chromatin alteration during meiosis occurs independently of VDE expression and is dependent on meiotic progression. Results of chromatin analysis of *VMA1*$^-$ homozygous cells expressing wild-type VDE (A), endonuclease-defective VDE (D326V) (B), and VDE defective in DNA binding (R90A) (B) are shown. Samples were treated with 0, 5, or 10 U/ml of MNase. Indirect end-labeling was performed, and MNase-sensitive sites were analyzed as described in the legend to Fig. 3. The arrowheads indicate the chromatin alteration site. (C) Chromatin structure of the haploid cell in SPM. Chromatin was prepared in *VMA1*$^-$ haploid cells sampled at the indicated times of incubation in SPM and treated with 0, 5, or 10 U/ml of MNase. The *sir2*Δ mutation permits haploid cells to enter the meiotic program. (C) Chromatin structure around the VRS in mutant cells defective in meiotic progression. Chromatin was prepared from *VMA1*$^-$ homozygous diploid cells after 1.5 and 4.5 h of incubation in SPM and subjected to indirect end-labeling.

ration along the gene locus. Thus, VRS does not necessarily determine the chromatin structure around itself. Therefore, it is suggested that chromatin structure per se is not the sole mechanism for meiosis-specific homing of the intein-coding sequence and that another factor(s) exists and regulates the meiosis-specific DNA binding and cleavage of VDE.

DISCUSSION

VDE is a unique homing endonuclease that introduces a DSB in a meiosis-specific manner, while other homing endonucleases, such as I-SceI and I-SceII encoded by mobile introns in *S. cerevisiae* mitochondria, cleave DNA at their recognition sequences whenever they exist (39, 42). Hence, host factors are assumed to be involved in meiosis-specific DNA cleavage by VDE. This notion is supported by the previous and present results showing that meiosis-specific DNA cleavage by VDE is not simply due to modulation in the quantitative or qualitative properties of VDE: both mitotic and meiotic cells display a sufficient expression of VDE (9, 24), and the immunoprecipitated VDE shows similar levels of endonuclease activity in both of the cells (Fig. 1). In addition, VDE can be purified from vegetative cells to near homogeneity based on its endonuclease activity (13).

Although a large portion of DSB formation by VDE occurs during meiosis, according to the development of a method to detect homing products, it has been revealed that there is a minor pathway by which VDE can form subtle levels of DSBs and homing products in a meiosis-independent manner (Fig. 1C) (9, 24). The regulation of the subcellular localization of VDE is likely to contribute to the prevention of such a mitotic leak of VDE-mediated DSBs. VDE is excluded from the nucleus in vegetatively growing cells, and forced localization of VDE into the nucleus increases mitotic homing products (24). Thus, the exclusion of VDE from the nucleus during vegetative stages enhances the meiosis specificity of homing by reducing the risk of VDE encountering its target sequence (8, 24). Interestingly, subtle homing products at 0 h of meiosis are

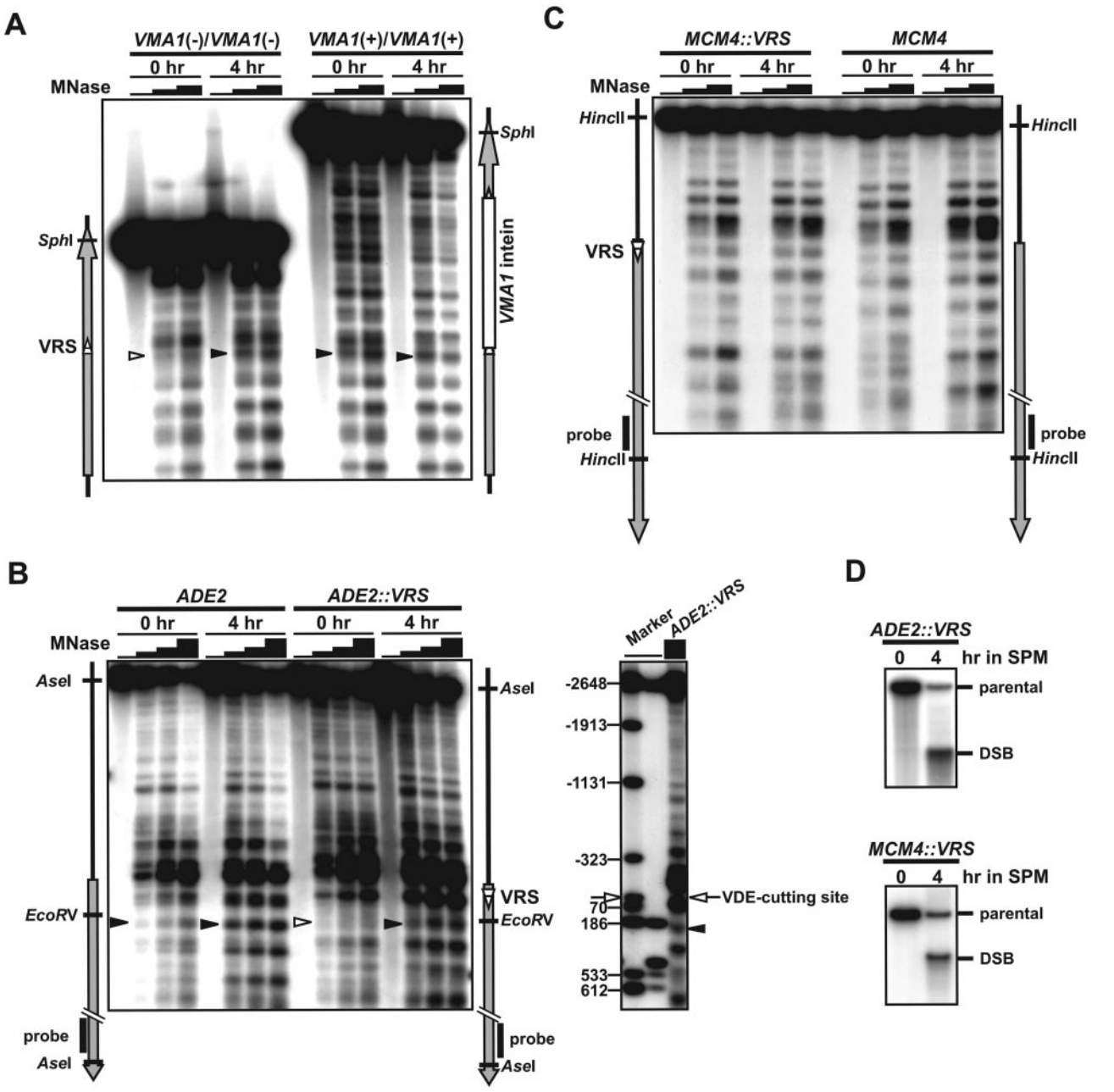

FIG. 5. (A) Chromatin structure around the VRS in diploid cells homozygous for *VMA1*$^-$ or *VMA1*$^+$. In the *VMA1*$^+$ allele, the VRS is interrupted by the insertion of an intein-coding sequence in the middle of the sequence. Samples were treated with 0, 5, or 10 U/ml of MNase. (B) Chromatin configuration at the *ADE2* gene locus. Mitotic (0 h) and meiotic (4 h) chromatins were prepared from diploid cells homozygous for *ADE2* or VRS-inserted *ADE2* (designated *ADE2*::*VRS*), treated with 0, 5, 10, or 20 U/ml of MNase, and redigested with AseI. MNase-sensitive sites were detected by indirect end-labeling with a probe for the sequence adjacent to the AseI site. The vertical gray arrow indicates the position of the coding region for the *ADE2* locus. The arrowheads show MNase-sensitive sites that became prominent in *ADE2*::*VRS* during meiosis. The right panel shows the positions of the VDE cutting site (horizontal arrow) and the chromatin alteration site (arrowhead). Numbers indicate the positions in nucleotides relative to the VDE cutting site. (C) Chromatin configuration at the *MCM4* gene locus. Mitotic (0 h) and meiotic (4 h) chromatins were prepared from diploid cells homozygous for *MCM4* or VRS-inserted *MCM4* (designated *MCM4*::*VRS*) and then analyzed by end-labeling after treatment with 0, 5, or 10 U/ml of MNase. (D) Physical detection of the VDE-mediated DSB by Southern blotting. *VMA1*$^+$ diploid cells homozygous for *ADE2*::*VRS* or *MCM4*::*VRS* were subjected to synchronous sporulation. DNA was isolated from cells at the indicated times after incubation in SPM and subjected to Southern analysis.

detectable in wild-type cells and *ime1*Δ and *ndt80*Δ mutants but hardly in *ime2*Δ and *clb5*Δ *clb6*Δ mutants (Fig. 1C). Ime1p stimulates expression of the Ime2p protein kinase, which activates premeiotic DNA replication and downstream transcription factors such as Ndt80p. Clb5p and Clb6p are S cyclins involved in the progression of S phase. In addition, it has been revealed that nuclear translocation of VDE is regulated by the TOR (target of rapamycin) path-

way (24). The regulation of the minor pathway of homing by those factors or signaling pathways would be an interesting subject for a future study.

The present ChIP study with anti-VDE antibody revealed that the binding of VDE to the VRS is above the detection level only during meiosis, implying that the meiosis specificity is regulated by the accessibility of VDE to the target site. This made it possible for DNA cleavage via increasing local DNA accessibility to VDE to be achieved by the regulation in chromatin configuration around the target site. This notion is supported by previous observations that DNA-related processes, such as transcription, replication, repair, and recombination, are regulated by chromatin structure, which governs the access of *trans*-acting factors to *cis*-acting DNA elements. Indeed, we observed meiotic alteration in chromatin structure around the VRS, which requires the induction of the meiotic program and full-length set of the VRS and shows a parallel correlation with VDE binding and cleavage. These results raised the possibility that chromatin around the VRS makes the VRS-inaccessible state to VDE during premeiotic stages but changes to produce a VDE-accessible state in response to meiotic induction. Such regulation has been revealed in the case of chromatin remodeling and DSB formation at the fission yeast meiotic recombination hot spot *ade6-M26* (22).

Meiotic recombination is initiated by the introduction of DSBs by Spo11p at meiotic recombination hot spots. In budding yeast, meiotic DSB sites are located in regions of chromatin that are hypersensitive to nucleases, suggesting that Spo11p-mediated DSBs occur at sites where DNA is particularly accessible (28, 40). Hypersensitivity to nuclease increases specifically at hot spots before the appearance of DSBs, which is thought to be a result of a modification that is necessary to provide a proper substrate for Spo11p or a consequence of the assembly of a DSB formation complex at the site. In the *ade6-M26* hot spot of the fission yeast, chromatin structure changes meiotically (22). Meiotic chromatin remodeling at *ade6-M26* is required for hot spot activity and involves the binding of transcription factors and the cooperative action of histone acetyltransferases and ATP-dependent chromatin remodeling factors (41).

The increased sensitivity to MNase reflects a change in chromatin structure at the site which may result from one of a variety of diverse mechanisms, involving changes in nucleosome positioning or occupancy, DNA unwinding, association of DNA-binding proteins, and higher-order chromatin folding. Therefore, the entity or the cause of chromatin alteration near the VRS remains unclear. Possibly, some host factors may be recruited at the VRS and may remodel chromatin structure at the site during meiosis. It is also possible that meiotic association or dissociation of host proteins at or near the VRS may influence the MNase sensitivity detected in our assay. Or, premeiotic DNA replication may rearrange nucleosome positioning at the region.

In spite of the good correlation among VDE binding and cleavage and chromatin alteration at the *VMA1*$^-$ locus, it is unlikely that chromatin configuration around the VRS per se is the sole mechanism for the regulation of meiosis-specific DNA cleavage by VDE. Meiotic DSB formation and recombination can be induced by an insertion of the VRS, even at ectopic loci (25, 26). On the contrary, the introduction of the VRS at gene loci other than *VMA1* is not necessarily accompanied with the meiotic chromatin alteration as seen in the authentic locus (Fig. 5). Based on these observations, we propose that a mechanism(s) other than the local change of chromatin structure is involved in the regulation of meiotic DNA binding and cleavage of VDE.

There are several possibilities for implications of the chromatin alteration around the VRS in VDE-mediated DSBs. First, the chromatin alteration may be a part of a series of the mechanism that regulates DSB formation by VDE. It may be prerequisite that a certain state of chromatin is established around the VRS for meiotic DNA cleavage by VDE. Therefore, meiotic chromatin alteration is not necessary when the VRS is located at the appropriate state of chromatin in advance of meiosis. Second, it is possible that the meiotic increase in MNase sensitivity near the VRS reflects the association and dissociation of *trans*-acting host factors that control the DNA cleavage reaction of VDE. Such an interaction might cause a DNA distortion or unwinding, leading to a change in MNase sensitivity that can be detected in some cases. Third, the meiotic alteration in chromatin may not have a critical function in meiosis specificity, but it might contribute to the cleavage efficiency to some extent, through the increased exposure of the underlying DNA sequence. Or, there might be multiple independent pathways, including chromatin structure, localization of VDE, and other factors, by which mitotic DSB formation is inhibited and meiotic DSB formation is activated, and then the contribution of each pathway to meiosis specificity might differ according to the location of the VRS.

According to the present study, there are several possibilities for the mechanism other than chromatin structure that regulates meiosis-specific DNA cleavage by VDE. One is that *trans*-acting factors interact with VRS to prevent loading or stable association of VDE on the VRS under premeiotic conditions. In this case, once meiosis is initiated, such factors may dissociate from chromatin, allowing VDE access to the VRS to introduce DSBs. A second possibility is that *trans*-acting factors binding to the VRS may help VDE load on the VRS during meiosis. Such a coactivating mechanism can be found in the case of HO endonuclease in budding yeast. An activity to bind to a sequence near the target site of HO endonuclease was detected previously, and it is suggested that the formation of HO-mediated DSB in vivo is regulated by this coactivating sequence (38).

Alternatively, it is possible that host factors interact with VDE and regulate DNA binding and cleavage. First, host factors may prevent VDE from binding to the VRS through the direct interaction during mitosis and release VDE in response to meiotic entry. Indeed, it has been reported that heat shock protein binds to the mitochondrial homing endonuclease and changes the sequence specificity (21). Second, host factors, which are meiotically expressed or activated, may facilitate the recruitment or stable binding of VDE to the target site. In any case, it might be effective to search for the factors that interact with VDE or the VRS in order to find a clue to the elucidation of the mechanism (7, 30).

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