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CRISP-3, a Protein with Homology to Plant Defense Proteins, Is Expressed in Mouse B Cells under the Control of Oct2

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The Oct2 transcription factor is expressed throughout the B-lymphoid lineage and plays an essential role during the terminal phase of B-cell differentiation. Several genes specifically expressed in B lymphocytes have been identified that contain a functional octamer motif in their regulatory elements. However, expression of only a single gene, the murine CD36 gene, has been shown to date to be dependent on Oct2. Here, we present the identification and characterization of a further gene, coding for cysteine-rich secreted protein 3 (CRISP-3), whose expression in B cells is regulated by Oct2. We show that CRISP-3 is expressed in the B-lymphoid lineage specifically at the pre-B-cell stage. By using different experimental strategies, including nuclear run-on experiments, we demonstrate that this gene is transcriptionally activated by Oct2. Furthermore, analysis of CRISP-3 expression in primary B cells derived from either wild-type or Oct2-deficient mice demonstrates the dependence on Oct2. Two variant octamer motifs were identified in the upstream promoter region of the *crisp*-3 gene, and Oct2 interacts with both of them in vitro. Cotransfection experiments with expression vectors for Oct1 and Oct2 together with a reporter driven by the *crisp*-3 promoter showed that transcriptional activation of this promoter can only be achieved with Oct2. The C-terminal transactivation domain of Oct2 is required for this activation. Finally, introducing specific mutations in the two variant octamer motifs revealed that both of them are important for full transcriptional activation by Oct2.

Oct2 belongs to the family of POU domain transcription factors (18, 35, 38). The POU domain is roughly 150 amino acids in length and represents a bipartite DNA-binding domain consisting of POU-specific and POU homeo-subdomains (39, 42). In addition to mediating specific DNA binding, the POU domain is also involved in multiple protein-protein interactions (for recent overview, see references 17 and 47). POU domain transcription factors have been shown to be critical regulators of various developing cell lineages (33).

Oct2 expression is mainly restricted to the B-cell lineage, where multiple alternatively spliced isoforms are present throughout B-cell development (36, 45). Oct2 binds to the octamer motif (consensus sequence ATGCAAAT), a motif originally described as a conserved element in all immunoglobulin (Ig) gene promoters and in the Ig heavy chain enhancer (11, 29). This octamer motif has been shown to be involved in the B-cell-specific expression of the Ig genes and to mediate B-cell-restricted gene activity when included in minimal promoter constructs (46). The same octamer motif, however, is also recognized by the ubiquitously expressed Oct1 protein, and both the B-cell-restricted Oct2 and the ubiquitously expressed Oct1 are able to activate an octamer element-driven reporter gene in a B-cell-specific manner, in association with B-cell-restricted coactivators (14, 24, 25, 30, 31, 37). Thus, there is a functional overlap between the Oct1 and the Oct2 proteins, and it was only recently that functional differences between Oct1 and Oct2 could be shown (2, 12, 30). Moreover, with Oct2-deficient ($Oct2^{-/-}$) mice generated by homologous recombination, a unique role for Oct2 during general development and in the course of B-cell differentiation has been demonstrated. $Oct2^{-/-}$ mice die soon after birth for reasons that are still unknown. Interestingly, they also show severe defects in the terminal phase of B-cell development, resulting in the dramatic reduction of antibody-secreting plasma cell numbers (7, 8). In addition, fluorescence-activated cell sorter (FACS) analyses of bone marrow B cells indicated that the lack of Oct2 affects the differentiation to IgD-IgM double-positive mature B cells. Therefore, developmentally relevant genes must exist which are dependent on Oct2 for their expression. However, none of the genes known to contain octamer motifs and originally thought to be regulated by Oct2 was affected in its expression in $Oct2^{-/-}$ B cells (7, 8). In an attempt to find such elusive Oct2 target genes, we performed subtractive and differential cDNA cloning based on an $Oct2^{-/-}$ B-cell line expressing a conditional allele of Oct2. By this approach, we were able to identify the mouse gene encoding the cell surface glycoprotein CD36 as the first gene critically dependent on Oct2 for its expression in B cells (21).

Here, we report on another gene dependent on Oct2 which we found by screening further cDNA clones. The gene codes for the cysteine-rich secretory protein 3 (CRISP-3), which has been previously isolated from a mouse salivary gland cDNA library by its homology to the rat acidic epididymal glycoprotein (AEG, or CRISP-1) (15). We show that CRISP-3 is expressed in murine pre-B-cell lines and in the bone marrow and spleens of mice under the control of Oct2. The function of CRISP-3 is unknown, but intriguingly it shows homology to pathogenesis-related (PR) proteins from plants, which are induced in response to pathogen infections. By its homology to these plant defense proteins and its expression in B lymphocytes, CRISP-3 could be a molecule involved in fighting pathogens in mice.

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MATERIALS AND METHODS

Library screening and RNA analyses. The generation of the subtracted cDNA library, the details of the screening procedure and conditions for Northern (RNA) blotting have been described previously (21). For reverse transcription (RT)-PCR, the following primers were used. The CRISP-3 primers (13 pmol each) were 5′ CCTGTATTGAATTACAGTGGCC 3′ (forward) and 5′ GTCA TTGGCTAGATTTGACTTCATG 3′ (reverse). The size of the amplified PCR product was 441 bp. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin PCR have been described before (21, 23). Magnetic separation of newborn splenocytes with anti-B220-coated magnetic beads and the analysis of separated fractions by FACS have been described previously (21).

Nuclei were prepared and conditions for nuclear run-on analysis were as described previously (22). CRISP-3 transcripts were detected by hybridization to the 1.3-kb *Hin*dIII-*Xba*I cDNA insert from one of the isolated clones immobilized on nitrocellulose filters.

EMSA. The octamer probe, the wild-type and mutant competitor fragments, and the conditions for electrophoretic mobility shift assays (EMSAs) have been described previously (3). Antibody supershift experiments were performed as outlined previously (30). The *crisp*-3 promoter probes used were Oct_p, a 67-bp *Hin*cII to *Pvu*II fragment extending from position −83 to position −16 relative to the major start site of transcription (34), and Oct_d. For Oct_d, the following oligonucleotides corresponding to positions −466 to −445 of the promoter were annealed: 5′ TTAAGCTTATGCTCATGTAAATATGTGT 3′ and 5′ TTACA CATATTTACATGAGCATAAGCTT 3′.

Plasmid constructions and DNA transfections. The plasmid containing the herpes simplex virus thymidine kinase (HSV-tk) promoter (−109 to +52) upstream of the luciferase gene was obtained from A. Hecht. For the construction of the *crisp*-3 promoter reporter, the tk promoter fragment was replaced by a PCR-amplified fragment extending from −467 to +23 of the *crisp*-3 promoter region. For generation of specific mutations within the octamer motifs of the CRISP-3 promoter, the appropriate PCR fragments were cloned into the pTATA vector, which contains the minimal HSV-tk promoter from positions −38 to +52 (3). Primers for mutagenesis were as follows:

P1, 5′ TTAAGCTTATGCTCATGTAAATATGTGT 3′
P1m, 5′ TTAAGCTTATGCTCA**GTAC**AATATGTGT 3′
Pr, 5′ CAGGATCCGCTTATGCAAACTGGTC 3′
Prm, 5′ CAGGATCCGCTTA**GTAC**AACTGGTC 3′

The position of the octamer motif is underlined, and mutations introduced in the two octamer motifs are shown as boldface letters.

The Oct1 and Oct2 expression plasmids have been described previously (2). In cotransfection experiments, 10 μg of reporter plasmid was mixed with 10 μg of expression vector and 1 μg of CMV.*lacZ* (a construct expressing the bacterial beta-galactosidase gene under the control of the cytomegalovirus promoter as control for transfection efficiencies) and transfected by the calcium-phosphate protocol. Conditions for transfections of Abl/Oct2-ER cells by electroporation were as described previously (30).

RESULTS

In order to identify Oct2 target genes, we applied the subtractive cDNA cloning-screening strategy which we have described recently (21). Briefly, this strategy depends on an Oct2-deficient B-cell line derived from fetal livers of mice bearing a homozygous mutation of the *oct2* gene (8). This cell line was stably transfected with an expression construct for an Oct2-ER fusion protein (30), and Oct2 function is only detectable upon treatment of the cells with estrogen. When 1.5×10^4 recombinant cDNA clones of a subtractive cDNA library from this conditional Oct2-positive B-cell line (Abl/Oct2-ER) were screened as described previously (21), five independent cDNAs derived from the same primary transcripts were isolated. These isolates contained overlapping inserts of the same cDNA, and sequence analysis revealed that these cDNAs encode the murine *crisp*-3 gene (15).

Expression of CRISP-3 was undetectable in the Oct2-deficient B-cell line (Abl1.1) regardless of whether the cells were treated with estrogen or not (Fig. 1A, lanes 1 and 2). In contrast, high levels of CRISP-3 RNA were present in the estrogen-induced Abl/Oct2-ER cell line bearing the conditional Oct2 allele. In this cell line, CRISP-3 expression was dependent on hormone induction (Fig. 1A, lanes 3 and 4). The hormone-binding domain present in the Oct2-ER fusion protein contains a hormone-dependent transactivation function

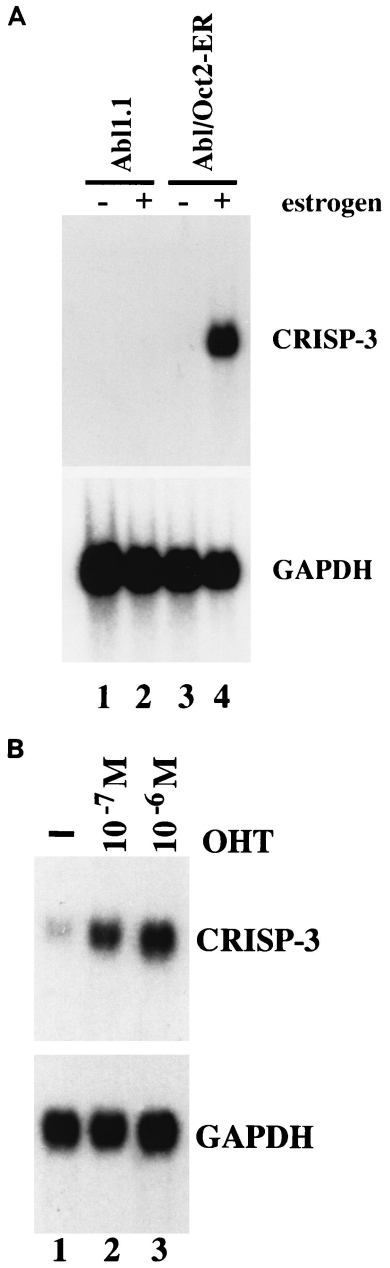

FIG. 1. Expression of CRISP-3 in Abl/Oct2-ER cells is induced by hormones. (A) Northern blot analysis with poly(A)$^+$ RNA from the parental Abl1.1 cell line and Abl/Oct2-ER cells either left untreated (−) or induced (+) with β-estradiol (1 μM) for 16 h, which was hybridized to CRISP-3 and GAPDH-specific probes as indicated. (B) Abl/Oct2-ER cells were induced with the indicated concentrations of the anti-estrogen hydroxy-tamoxifen (OHT), and RNA was analyzed by Northern blotting.

(43). To rule out the possibility that induction of CRISP-3 required the activation of this heterologous activation domain, Abl/Oct2-ER cells were treated with the anti-estrogen hydroxy-tamoxifen. This treatment does not induce the transactivation function in the ligand binding domain (43); however, it does activate the Oct2 protein (21, 30). As shown in Fig. 1B, hydroxy-tamoxifen was capable of inducing CRISP-3 expression in the conditional Oct2-ER cell line, albeit to a somewhat lesser extent than estrogen.

CRISP-3 had previously been isolated as an androgen-reg-

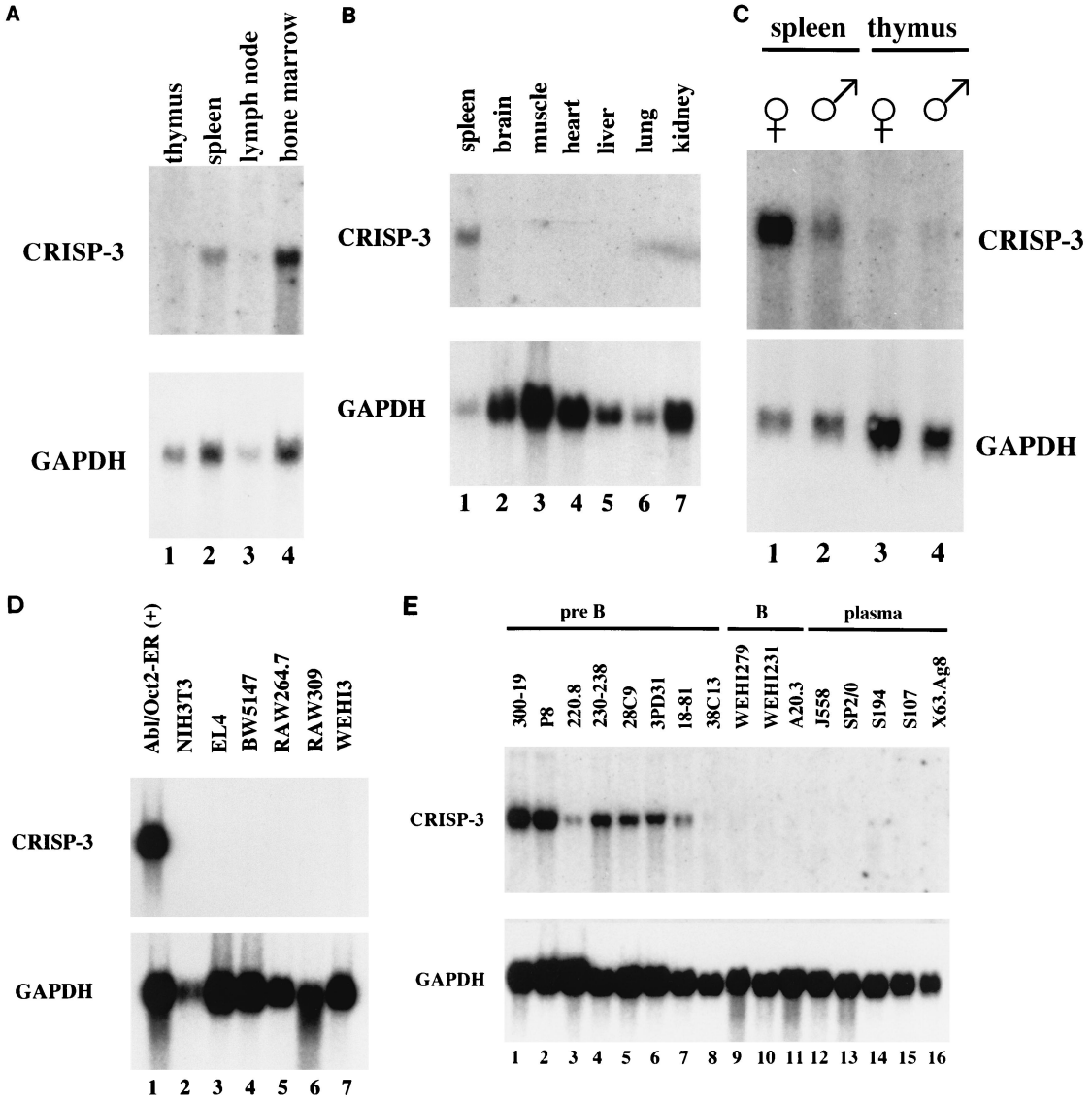

FIG. 2. Primary lymphoid tissues and pre-B-cell lines express CRISP-3. (A) Northern blot analysis with total RNA from lymphoid organs with the CRISP-3 and GAPDH-specific probes as indicated. (B) Tissue distribution of CRISP-3 RNA was determined by Northern blotting as described above. (C) CRISP-3 expression in the spleen and thymus is not affected by androgens. Total RNA from 8-week-old male and female littermates was analyzed for CRISP-3 expression by Northern blotting. (D) Northern blot analysis with cytoplasmic poly(A)$^+$ RNA from the indicated murine cell lines. NIH 3T3 represents a fibroblast cell line. EL4 and BW5147 are Oct2-positive and -negative thymoma cell lines, respectively. RAW264.7, RAW309, and WEHI3 are myeloid cell lines that do (RAW264.7 and RAW309) or do not (WEHI3) express Oct2. (E) Analysis of CRISP-3 expression within the B-lymphoid lineage. Cytoplasmic RNAs of the indicated cell lines were analyzed by Northern blotting as before.

ulated cDNA from the murine salivary gland (15). Since we found CRISP-3 in our screening expressed in a pre-B-cell line, we wondered whether CRISP-3 is also expressed in primary lymphoid organs. We therefore analyzed CRISP-3 expression in bone marrow, the thymus, the spleen, and the lymph nodes by Northern blotting. The strongest CRISP-3-specific signals were observed with RNA from bone marrow, somewhat reduced expression was evident in the spleen, and significantly lower expression levels were seen in the thymus and lymph nodes (Fig. 2A). No signal could be detected in various nonlymphoid organs (Fig. 2B). CRISP-3 expression in salivary glands has been shown to be androgen regulated and was much higher in male mice than in female mice (15). We therefore asked whether CRISP-3 expression in lymphoid organs was also androgen regulated. Interestingly, however, expression levels in the spleen and thymus were not higher in male mice than in female mice (Fig. 2C). Likewise, no difference in expression in the bone marrow was detectable between male and female mice (data not shown) suggesting that in the lymphoid lineage, CRISP-3 expression is not androgen regulated. The higher expression level in the female spleen than in the male spleen observed in this experiment was specific for this individual and was not reproducibly seen with other RNA samples.

Bone marrow and the spleen contain a variety of different cell types, such as B cells, T cells, macrophages, and erythrocytes. We therefore investigated whether CRISP-3 expression was confined to a specific hematopoietic compartment. When established cell lines representing the myeloid lineage or T cells were analyzed, no CRISP-3 expression was detected (Fig. 2D). Interestingly, several of these lines express endogenous

Oct2 proteins, such as the EL4 thymoma and the RAW264.7 and RAW309 macrophage cell lines (20, 35). This finding suggests that regulation of CRISP-3 expression is complex, and the mere presence of the Oct2 protein is not sufficient for its activation. This conclusion was further corroborated when we analyzed CRISP-3 expression within the B-lymphoid lineage. Whereas Oct2 is expressed throughout the B-cell lineage, CRISP-3 mRNA could only be detected in the different pre-B-cell lines tested (Fig. 2E, lanes 1 to 8). In contrast, no expression was seen in mature B cells or cell lines representing plasma cells (Fig. 2E, lanes 9 to 16). We therefore conclude that expression of CRISP-3 requires Oct2 and a further, pre-B-cell-specific activator. Alternatively, lack of expression of CRISP-3 in mature B cells and the Oct2-positive T cells and macrophage cell lines could be due to the action of a repressor.

To test whether in Abl/Oct2-ER cells CRISP-3 is regulated by Oct2 directly and at the transcriptional level, we analyzed CRISP-3 RNA induction by estrogen in the presence of an inhibitor of protein synthesis, anisomycin. CRISP-3 expression could still be induced efficiently by estrogen, whereas anisomycin alone did not significantly affect CRISP-3 expression (Fig. 3A). Furthermore, the direct measurement of transcription rates of the *crisp*-3 gene by nuclear run-on assay revealed a clear induction after addition of estrogen (Fig. 3B). Finally, in order to exclude that Oct2 induction might also affect the CRISP-3 RNA turnover, we measured RNA stability in the presence and absence of estrogen. The half-life of CRISP-3 mRNA was calculated to be 4 to 5 h, regardless of whether estrogen was present after gene induction (Fig. 3C). These results strongly suggest that Oct2 regulates CRISP-3 expression by directly inducing its transcription.

An important question to address was whether CRISP-3 expression in primary B cells would be affected by the absence of Oct2. We therefore established primary pre-B-cell cultures from day 15 fetal livers from the wild type, heterozygotes, and Oct2-deficient embryos. EMSAs with an octamer probe confirmed that no Oct2-specific complex was present in primary $Oct2^{-/-}$ pre-B-cell cultures (Fig. 4A). Expression of CRISP-3 was determined by RT-PCR analysis. The stroma cell line on which the pre-B cells were grown did not express CRISP-3 at any detectable levels (Fig. 4B). Interestingly, expression levels in heterozygous cells were already slightly reduced compared with that in the wild-type pre-B cells, and a much more striking reduction was evident in the Oct2-deficient pre-B cells (Fig. 4B). When the same cDNA samples were analyzed for actin mRNA expression, no differences were seen (Fig. 4B, lower panel). This analysis was then extended to neonatal splenocytes. Splenocytes from Oct2-deficient and heterozygous newborn mice were enriched for B cells with magnetic beads coated with antibodies against B220 (21). Analysis of CRISP-3 expression by RT-PCR again revealed a clear reduction in the sample from the Oct2-deficient cells (Fig. 4C). The main defect in B-cell physiology in Oct2-deficient mice was observed upon stimulation of mature splenic B cells with lipopolysaccharide (LPS) (7, 8). To test, whether CRISP-3 was expressed in primary B cells at this stage, splenic B cells were sorted with magnetic beads coupled with antibodies specific for B220 as described above, and RNA samples were again analyzed by RT-PCR. This analysis revealed that the primary splenic B cells still express CRISP-3 RNA (Fig. 4D).

The murine *crisp*-3 gene has recently been isolated, and the promoter region was sequenced (34). This analysis revealed the presence of two potential octamer motifs in the proximal promoter region. Both sites deviate from the octamer consensus by one nucleotide, however (Fig. 5A). To test whether these two motifs could interact with octamer transcription fac-

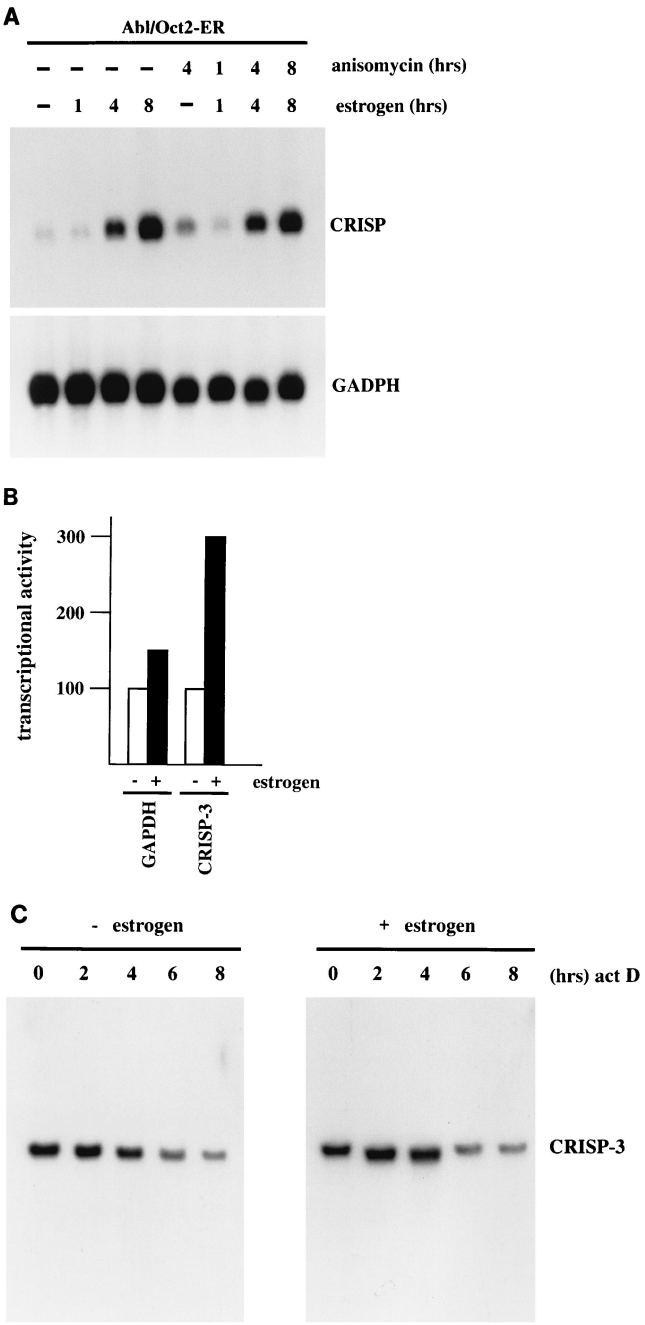

FIG. 3. Oct2 regulates CRISP-3 expression at the transcriptional level. (A) Hormone induction of CRISP-3 expression is independent of de novo protein synthesis. Northern blotting of RNA from Abl/Oct2-ER cells that were induced with β-estradiol (1 μM) for the times indicated, in either the presence or the absence of the protein synthesis inhibitor anisomycin (100 μM) as indicated. (B) Nuclear run-on analysis of CRISP-3 transcription in Abl/Oct2-ER cells. Run-on transcripts from cells either left untreated or induced by estrogen for 8 h were hybridized to filters containing CRISP-3- and GAPDH-specific probes. Signals were quantitated with a PhosphorImager; uninduced signals were set to 100. (C) Oct2 does not affect CRISP-3 mRNA stability. Abl/Oct2-ER cells were induced for 16 h with β-estradiol (1 μM) before addition of actinomycin D (act D [10 μg/ml]) to block further transcription. Incubation was continued for the indicated time periods in the presence or absence of β-estradiol. CRISP-3 RNA levels were determined by Northern blotting.

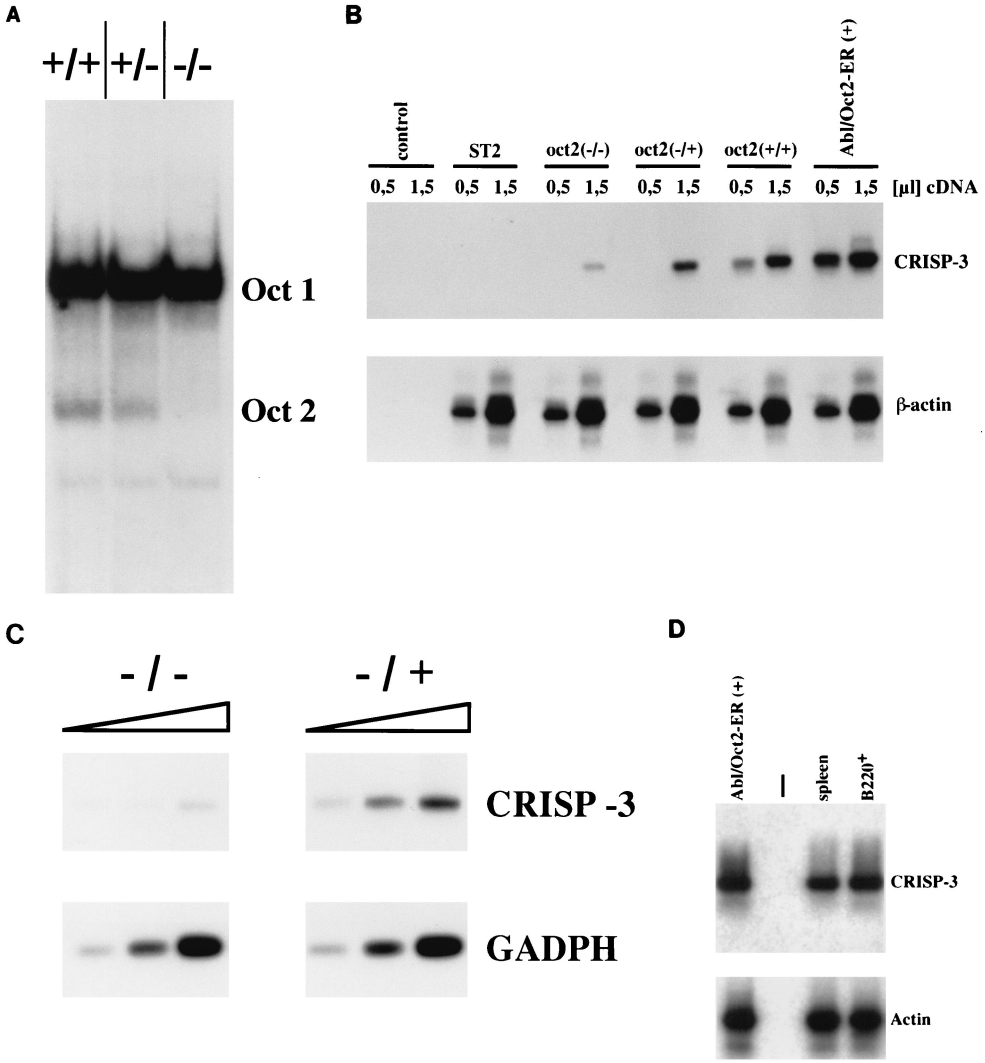

FIG. 4. CRISP-3 expression is regulated by Oct2 in primary B cells. (A) EMSA with nuclear extracts from wild-type (+/+), heterozygous (+/−), and Oct2-deficient (−/−) primary pre-B-cell lines with an octamer-specific probe. The positions of the Oct1 and Oct2 complexes are indicated. (B) RT-PCR analysis of CRISP-3 expression in primary pre-B-cell lines. Two concentrations (0.5 or 1.5 μl) of the cDNA synthesis reaction mixture (or a mock reaction mixture lacking RNA in the leftmost panel) were amplified with either CRISP-3-specific primers (upper panels) or β-actin-specific primers (lower panels). PCR products were resolved on agarose gels, blotted, and hybridized with radiolabelled CRISP-3 and β-actin-specific cDNA probes, respectively. The ST2 panel contains cDNA from the stroma cell line used as a feeder in these experiments. RT-PCR of β-estradiol-induced Abl/Oct2-ER cells is included as a positive control. (C) RT-PCR of B220-enriched neonatal splenocytes. The same cDNA preparation that was previously tested for CD36 (see Fig. 5 in reference 21) was used with CRISP-3- and GAPDH-specific primers in this experiment as indicated. (D) RT-PCR of B220-positive mature B cells from the spleens of normal mice. Total RNA from total spleen or B220-sorted cells was analyzed by RT-PCR with CRISP-3 and β-actin-specific primers as indicated. As a positive control, RNA from estrogen-induced Abl-Oct2-ER cells was used; no reverse transcriptase was added to the negative control (−).

tors, they were used as probes in EMSA experiments with nuclear extracts. Both the proximal motif and the distal motif yielded several complexes with nuclear extracts from the WEHI231 B-cell line (Fig. 5B). Inclusion of either a wild-type or mutated octamer competitor fragment revealed that two of the complexes formed with the Oct_p probe and that both Oct_d complexes were octamer specific (Fig. 5C). To unequivocally identify the proteins, we utilized antibodies that specifically recognize the Oct1 and Oct2 proteins. The Oct1-specific antibody removed the more-slowly migrating complex, whereas the Oct2-specific antibody specifically interacted with the faster-migrating complex (Fig. 5D). From this analysis, we conclude that both variant octamer motifs in the *crisp*-3 promoter can interact with the Oct1 and Oct2 proteins.

To directly assess the ability of Oct2 to activate the *crisp*-3 promoter, a reporter construct in which the luciferase gene is driven by this *crisp*-3 promoter region was generated (Fig. 6C). This construct was transfected into murine NIH 3T3 fibroblasts together with an Oct2 expression vector. Although the endogenous *crisp*-3 gene is not expressed in NIH 3T3 cells, the *crisp*-3 promoter reporter showed a measurable basal activity, which was roughly one-third of the HSV-tk promoter activity (Fig. 6A). However, cotransfection of the Oct2 expression vector specifically stimulated the *crisp*-3 promoter without affecting the HSV-tk promoter. This activation was specific for Oct2, because cotransfection of an Oct1 expression vector failed to yield a measurable stimulation. Cotransfection of Oct1 actually led to a reduction of the activity for both the control tk promoter and, to a larger extent, the *crisp*-3 promoter. The reasons for this repression are not understood.

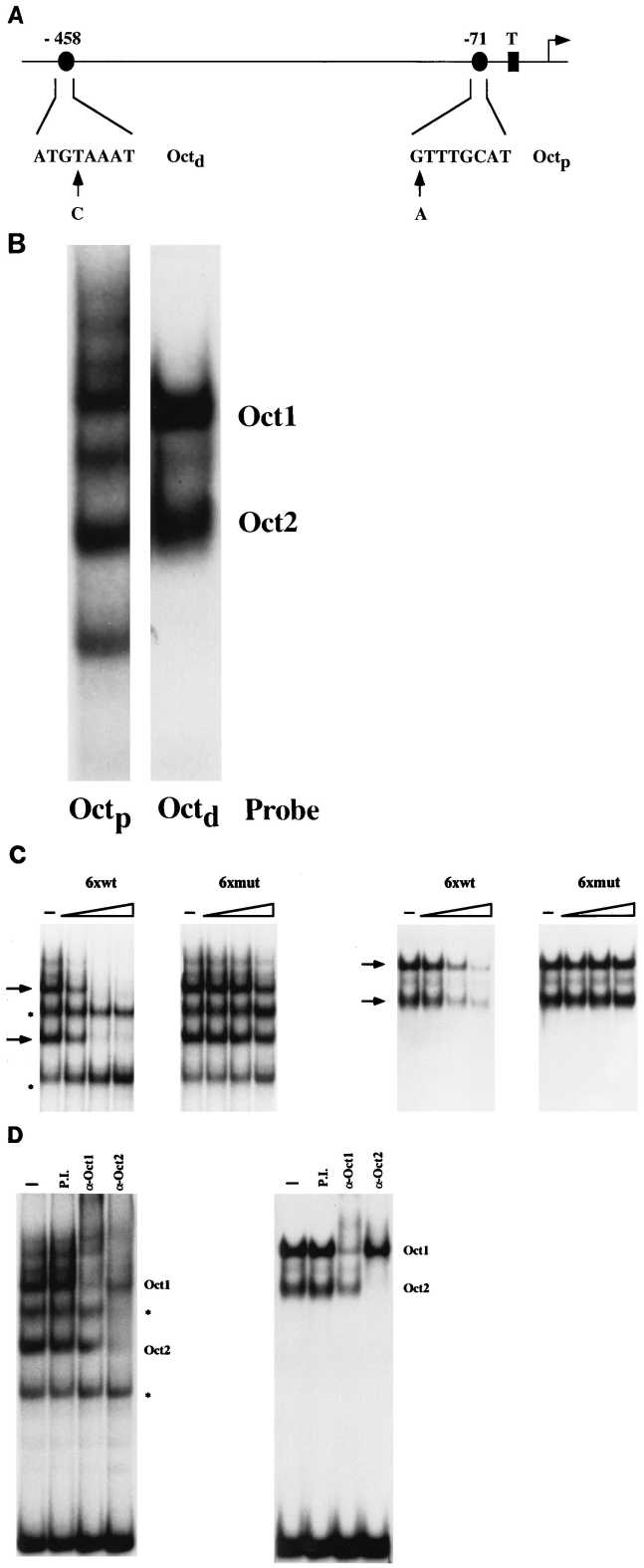

FIG. 5. Octamer transcription factors bind to two variant octamer motifs in the *crisp*-3 proximal promoter. (A) Scheme of the proximal *crisp*-3 promoter region that shows the position and sequence of the distal (Oct_d) and proximal (Oct_p) variant octamer motifs (the consensus nucleotides are indicated underneath). T, TATA box (the arrow indicates the major start site of transcription [34]). (B) EMSA with nuclear extracts and Oct_d or Oct_p probes, respectively. Nuclear extracts from WEHI231 cells were incubated with probes containing the proximal and distal octamer motifs, respectively. The positions of the Oct1- and Oct2-specific complexes are indicated. (C) EMSA competition experiment with a consensus wild-type (6xwt) and a mutant (6xmut) octamer motif fragment. Nuclear extracts from WEHI231 cells were used in EMSA with the Oct_d and Oct_p motifs. Increasing amounts of the wild-type and mutant competitor fragments were added as indicated. (D) EMSA with Oct_d and Oct_p probes and nuclear extracts from WEHI231 cells incubated with preimmune serum (P.I.) or antibodies (α) specific for the Oct1 and Oct2 proteins, respectively. Asterisks indicate non-octamer-specific complexes.

We had previously compared transcriptional activation by Oct1 and Oct2 and determined that the C-terminal transactivation domain of Oct2 is required for the Oct2-specific functions (2, 30, 44). We therefore analyzed whether this Oct2-specific activation of the *crisp*-3 promoter would be dependent on the Oct2 C-terminal transactivation domain. To this end, the ability of two deleted versions of Oct2, one lacking the N-terminal transactivation domain and the other lacking the C-terminal transactivation domain, were compared. Interestingly, deletion of the C terminus completely abolished activation of the *crisp*-3 promoter, whereas a deletion of the N terminus did not affect transactivation (Fig. 6B). The result that the N-terminal deletion in fact showed a higher activity than the full-length Oct2 expression vector is due to the fact that the two truncated proteins are expressed at a higher level than the full-length protein (reference 2 and data not shown).

Our previous results had suggested that proper regulation of the *crisp*-3 promoter is observed in pre-B-cell lines. To test the dependence of the proximal promoter region on Oct2 in pre-B cells, the conditional Oct2-positive cell line was transfected with the *crisp*-3 promoter reporter, and activity was determined in uninduced and estrogen-induced cells. Hormone addition resulted in an activation of the *crisp*-3 promoter, and again this induction was specific because the tk promoter remained unaffected (Fig. 6C). Thus, Oct2 can directly stimulate the *crisp*-3 promoter, both in pre-B cells and in fibroblasts. Finally, in order to investigate whether the two identified variant octamer motifs were responsible for the observed Oct2-specific transactivation of the *crisp*-3 promoter, specific mutations were introduced into the two motifs alone or in combination. Individual mutation of either the distal or proximal octamer motif already significantly reduced Oct2-specific transactivation, and the double mutant was not stimulated by Oct2 at all (Fig. 7A). These results definitely identify the two variant octamer motifs within the *crisp*-3 promoter as target binding sites for the Oct2-specific transactivation.

DISCUSSION

The Oct2 transcription factor is expressed throughout B-cell development and has been previously shown to be necessary for the late phase of B-cell differentiation in mice. However, all genes thought to be B-cell target genes for Oct2 like the Ig genes, B29, or CD21 showed no differences in expression when $Oct2^{-/-}$ B cells were compared with normal ones (7, 8). By a subtractive cDNA cloning strategy, we recently identified the gene encoding the cell surface glycoprotein CD36 as a target gene for Oct2 in B cells and macrophages (21). Here, we show that the *crisp*-3 gene is another gene dependent on Oct2 for its expression in mouse B cells.

Expression of CRISP-3 in Abl/Oct2-ER cells depends on a functional Oct2 protein, since the *crisp*-3 gene was expressed only when the Oct2-ER fusion protein had been activated by estrogen or hydroxy-tamoxifen. The induction by hydroxy-tamoxifen, which does not activate the transactivation function in the estrogen receptor hormone binding domain (43), suggests that the induction of the *crisp*-3 gene is caused by Oct2 trans-

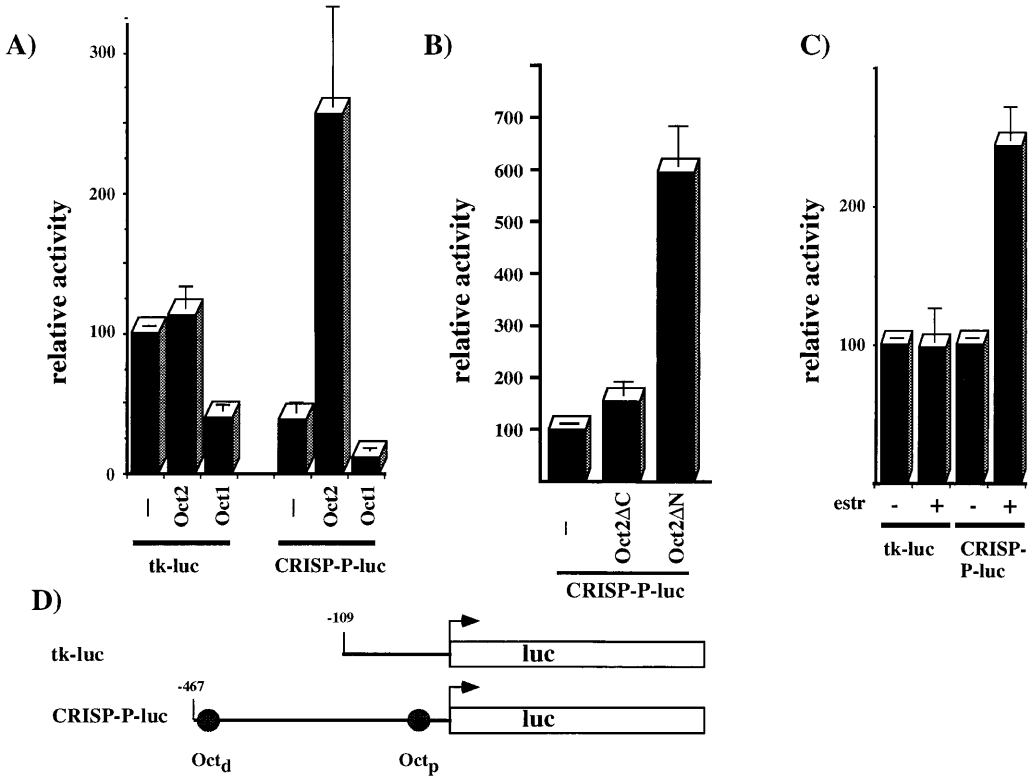

FIG. 6. Oct2 transactivates the proximal *crisp*-3 promoter. (A) Reporter plasmids containing either the HSV-tk promoter (−109 to +52) or the *crisp*-3 proximal promoter (−467 to +23) upstream of the luciferase (luc) coding region were transfected into NIH 3T3 murine fibroblasts. Expression vectors for Oct1 and Oct2 or the empty expression vector (−) were cotransfected as indicated. All reporter gene activities are expressed relative to the HSV-tk-luc activity in the absence of Oct2. This value was arbitrarily set to 100. (B) The *crisp*-3 proximal promoter reporter plasmid was cotransfected with the indicated expression vectors for truncated Oct2 proteins. Oct2ΔC contains amino acids 2 to 370 of Oct2 and completely lacks the C-terminal transactivation domain. Oct2ΔN contains amino acids 194 to 479 of Oct2 and lacks the entire N-terminal activation domain (2, 28). (C) *crisp*-3 promoter activity is estrogen (estr) inducible in Abl/Oct2-ER cells. Abl/Oct2-ER cells were transfected with the HSV-tk and *crisp*-3 promoter reporter constructs, and cells were either left untreated (−) or stimulated with estrogen after transfection as indicated. (D) Schematic representation of the reporter constructs used. The positions of the distal and proximal octamer motifs in the *crisp*-3 are indicated by solid circles.

activation. The increase in CRISP-3 mRNA expression appears to be due to induced transcription, as indicated by the lack of an effect of Oct2 activation by estrogen on the half-life of the CRISP-3 mRNA and the activation of CRISP-3 transcription in nuclear run-on analyses. Given the stability of the CRISP-3 RNA, the roughly 3-fold increase in transcription rates can explain the overall 10- to 30-fold increase in steady-state levels of mRNA observed after 24 h of induction seen in the Northern blot experiments. Moreover, induction of CRISP-3 by Oct2 takes place in the presence of protein synthesis inhibitors, suggesting that this process is independent of the expression of intermediate gene products. Finally, cotransfection of Oct2, but not of Oct1, resulted in a specific stimulation of *crisp*-3 promoter activity in NIH 3T3 fibroblasts. Surprisingly, the proximal promoter fragment used in these studies showed a detectable residual activity in fibroblasts, although the endogenous gene is not expressed at all in these cells. This result suggests that regulation of the endogenous gene is more complex and depends on additional regulatory elements either further upstream or downstream from the fragment used in our reporter constructs. This is further corroborated by the results in the pre-B-cell line bearing the conditional allele of Oct2. Even in the uninduced state, the promoter shows already significant activity which is, however, still increased upon induction of Oct2 function by hormone treatment. All of these findings argue in favor of a direct effect of Oct2 on *crisp*-3 gene transcription.

Characterization of transactivation by Oct1 and Oct2 had previously identified two transactivation domains within the Oct2 protein (4, 13, 28). Interestingly, whereas both domains are redundant with respect to activation of a typical octamer-dependent promoter in standard cotransfection experiments, the Oct2-specific functions, which distinguish Oct2 from Oct1, are localized in the C-terminal activation domain of Oct2 (2, 30, 40, 41, 44). This conclusion is further supported by our observation that the C-terminal activation domain is essential for stimulation of the *crisp*-3 promoter. Although the exact topology of Oct2-specific promoters is not understood in detail yet, our finding that both variant octamer motifs are required for efficient stimulation sheds some light on the potential mechanism. In fact, the requirement for the dual octamer motifs is in line with an earlier report from Oct2-deficient B cells, where some evidence for the specific importance of Oct2 on promoters carrying an array of octamer motifs was provided (12). Nevertheless, more work needs to be performed to elucidate whether or not other elements within the *crisp*-3 promoter also contribute to the Oct2 selectivity.

Expression of CRISP-3 is not a peculiarity of the Abl/Oct2-ER cell line. CRISP-3 mRNA was readily detectable by Northern blotting in a variety of murine pre-B-cell lines and in primary pre-B cells expanded from fetal livers in the presence of interleukin 7. However, we could not detect CRISP-3 transcripts in the mature B-cell and plasmacytoma cell lines tested, even though these cells express Oct2. This is in contrast to

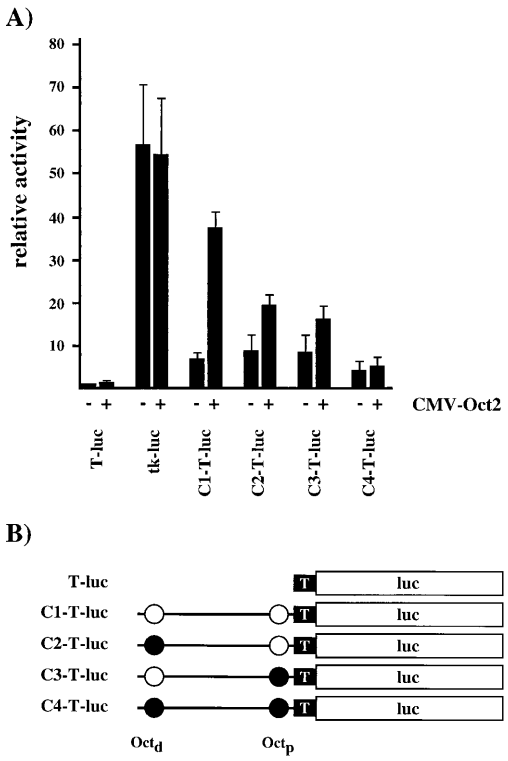

FIG. 7. The variant octamer motifs are required for Oct2-mediated transactivation. (A) Different versions of the *crisp*-3 promoter containing either wild type or mutated octamer motifs were inserted upstream of the minimal HSV-tk promoter. This minimal promoter (T-luc [−37 to +52]) contains only the TATA element. The reporter plasmids were transfected into HeLa cells together with the Oct2 expression vector or the empty expression vector as indicated. The activity of the T-luc reporter was arbitrarily assigned the value 1. (B) Schematic representation of the different constructs used in panel A. The *crisp*-3 promoter fragment used extends from −460 to −61 and encompasses both octamer motifs. Open circles denote wild-type octamer motifs (the variant sequences found in the *crisp*-3 promoter), and solid circles represent the mutant versions.

our previous findings for CD36, which is expressed in cell lines corresponding to all developmental B-cell stages. This suggests that further factors in addition to Oct2 are required for CRISP-3 expression in B cells. A more complex regulation of CRISP-3 expression is also suggested by the finding that EL4, as well as RAW309 and RAW264.7 cells, all of which express Oct2, fails to show detectable CRISP-3 RNA levels. The exact developmental stage within the B-lymphoid lineage at which CRISP-3 is turned off is not known, however. Analysis of the transformed cell lines suggested that downregulation coincides with the differentiation from the pre-B-cell stage to the mature B-cell stage, which is consistent with the stronger expression in the bone marrow than in the spleen. Nevertheless, CRISP-3 expression in the spleen, which contains largely mature, resting B cells and only small numbers of pre-B cells, was still detectable. Consistent with a preferential expression of CRISP-3 in more immature B cells, LPS treatment of splenic B cells, which induces terminal differentiation to plasma cells, resulted in a strong reduction of CRISP-3 RNA levels (29a). The expression pattern of CRISP-3 is consistent with CRISP-3 playing a role in the developmental defect seen in Oct2-deficient primary B cells. The main defect in these cells is apparent upon stimulation of mature splenic B cells, i.e., with LPS (7, 8). In addition, the earlier experiments with Oct2-deficient B cells also revealed a defect at the level of B-cell maturation from IgM surface-positive to IgM-IgD double-positive B cells.

CRISP-3 was originally isolated from a mouse salivary gland library by its homology to the *crisp*-1 gene. CRISP-1 is thought to be the mouse counterpart of the androgen-dependent rat AEG and is highly expressed in the epididymis, but it is also weakly expressed in the salivary gland of male animals (15). Albeit also regulated by androgen, CRISP-3 has been shown to be expressed in the mouse salivary gland but not in the epididymis (15). The functions of CRISP-3 and CRISP-1, which show about 77% amino acid identity, are not known yet. The rat CRISP-1 homolog, AEG, has been shown to be attached to the plasma membrane at the sperm head (5). Furthermore, CRISP-1 and CRISP-3 are 47% identical in their amino acid sequence to the deduced sequence of the mouse testis-specific gene-encoded protein Tpx-1 (CRISP-2), with which they share the conserved spacing of 16 cysteine residues in the carboxy-terminal half of the molecules (19). AA1, which is probably the guinea pig counterpart of CRISP-2 (Tpx-1), has been localized to the acrosomal region of spermatozoa, but nothing is known about the specific function there (16). Cysteine residues from the characteristic C-terminal domain of the CRISP family have been previously hypothesized to be involved in metal binding in analogy to cysteine motifs in metalloproteinases (6) and in the covalent attachment to the surface of sperm cells in the case of rat AEG (5). More recent data about mouse CRISP-1 indicate, however, that all of its cysteines are engaged in intramolecular disulfide bonds and that the C-terminal cysteine-rich region probably forms a discrete, compact domain (10). Helothermine, a toxin with hypothermic effects originating from the salivary secretions of the Mexican lizard *Heloderma horridum*, has recently been found to be another member of the CRISP family (26). Helothermine has been shown to block the ryanodine-sensitive sarcoplasmic calcium release channel in cell-free assays (27). Interestingly, the CRISP sequences show some stretches of complete identity and an overall 30% identity to two groups of nonmammalian proteins, certain venom proteins of vespids and ants, and perhaps even more intriguingly, to PR proteins (PR-1a, b, and c) of plants (for a detailed alignment of these proteins, see reference 27). All of these more distantly related proteins lack the cysteine-rich C-terminal region characteristic of the CRISP family. The vespid and ant venom proteins are major allergens, but no physiological function is known so far. PR proteins are known to be produced by many plant species in response to infection by pathogenic viruses, bacteria, and fungi (32). Several of these proteins possess antifungal activities in vitro and biochemical activities as chitinases, glucanases, and permatins. For the PR-1 proteins, no biochemical function has been demonstrated yet. However, like the PR proteins with enzymatic activities, they exist in both basic and acidic isoforms, which led to the suggestion that they also have some yet undefined enzymatic function (9). Most importantly, expression of PR-1a in transgenic tobacco mediates tolerance to certain fungal pathogens, demonstrating that PR-1a can act as a defense protein (1).

By its sequence homology to the pathogenesis-related proteins and its expression in B lymphocytes, it is tempting to speculate that CRISP-3 is also a defense-associated molecule in mammals. CRISPs might encode lytic enzymatic activities, which would be consistent with the observed association of AEG (CRISP-1) with the sperm head and presence of AA1 (CRISP-2) in the acrosom, where they could be involved in degrading egg structures during fertilization. In the case of CRISP-3 which is expressed in the salivary gland and in B cells, such lytic activities could be related to antifungal or antibacterial functions in saliva and in the blood or lymph.

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