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Differential transactivation potential of Oct1 and Oct2 is determined by additional B cell-specific activities

Petra Pfisterer, Arnd Annweiler[2], Christoph Ullmer[2], Lynn M.Corcoran[1] and Thomas Wirth[3]

Zentrum für Molekulare Biologie Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany and [1]The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria 3050, Australia
[2]Present address: Preclinical Research, Sandoz Pharma Ltd, 4002 Basel, Switzerland
[3]Corresponding author

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Cell type-specific transcriptional regulation is generally believed to be mediated by sequence-specific transcription factors that are specifically present in the corresponding cells. The interaction of the lymphoid-specific Oct2 transcription factor has been thought to be responsible for the B cell-specific activity of octamer-containing promoter and enhancer elements. Here we show that physiological concentrations of Oct2 do not suffice to generate octamer-dependent promoter activity in non-B cell lines. Furthermore, we have tested the activity of octamer-dependent promoter and enhancer elements in B cell lines that lack the endogenous Oct2 protein. Our results demonstrate that in these Oct2-deficient B cells the ubiquitous endogenous Oct1 protein is able to stimulate octamer-containing promoters to a level comparable with that of normal Oct2-positive B cells. However, reporter constructs bearing the octamer motif in a distal enhancer position are not stimulated by the Oct1 protein, but do require the presence of Oct2. The B cell-specific octamer-dependent promoter activity mediated by Oct1 correlates with the presence of a novel B cell-specific octamer-binding complex containing the Oct1 protein. From these results we conclude that B cells contain two different activities: one that interacts with both Oct1 and Oct2 and mediates promoter proximal activity of the octamer motif and a second that specifically interacts with Oct2 to confer function from a remote enhancer position.

Key words: cell type-specific transcriptional regulation/cofactors/Oct1/Oct2

Introduction

The octamer motif was originally identified as a conserved motif present in all immunoglobulin (Ig) heavy and light chain promoters, as well as the intronic heavy chain enhancer element (Falkner and Zachau, 1984; Parslow *et al.*, 1984). Subsequent mutational analyses revealed that this motif is an essential element for the B cell-specific expression of Ig promoters and it also contributes to the B cell-specific activity of Ig enhancers (Staudt and Lenardo, 1991). In addition, it has been shown that a single octamer motif is sufficient to confer B cell specificity to a minimal promoter element consisting of only a heterologous TATA-box (Dreyfus *et al.*, 1987; Wirth *et al.*, 1987). In experiments with transgenic mice, expression of a rearranged Ig transgene is strongly compromised when the octamer motif in the promoter element is mutated (Jenuwein and Grosschedl, 1991). The enhancer octamer also contributed to full B cell-specific activity of the enhancer element in transgenic mice (Jenuwein and Grosschedl, 1991; Annweiler *et al.*, 1992a). However, the octamer motif is also conserved in a variety of promoter and enhancer elements of genes that are not expressed in a B cell-specific fashion, such as the histone *H2B* gene or the *Usn* RNA genes. In the context of these regulatory elements, the octamer motif also contributes to transcriptional activity in non-B cell lines.

The different effects of the octamer motif are probably due to the presence of different octamer-binding proteins in different tissues. Several proteins have been identified that specifically interact with the octamer motif (Schöler *et al.*, 1989). All the octamer-binding proteins are members of a growing family of transcription factors that share the conserved POU domain (Herr *et al.*, 1988). The POU domain is a bipartite DNA-binding domain consisting of a POU-specific and a POU-homeo subdomain. Both subdomains interact with DNA via helix–turn–helix motifs and high-affinity binding of the octamer motif requires the presence of both domains. The two best characterized octamer-binding proteins are the Oct1 and Oct2 transcription factors. The Oct1 protein is ubiquitously expressed and is thought to be responsible for the ubiquitous activities of the octamer motif. In contrast, expression of the Oct2 protein is restricted to B cells and only a few other cell types. Octamer-dependent promoter and enhancer elements are active in B cells which contain both Oct1 and Oct2 proteins. In contrast, they are inactive in non-B cells, i.e. HeLa cells or NIH/3T3 fibroblasts, which only contain the Oct1 protein. These observations suggested that it is the Oct2 protein that is responsible for the B cell-specific activities of the octamer motif. This interpretation was strongly supported by the finding that transient cotransfection of Oct2 into non-B cells was able to stimulate octamer-containing promoters (Müller *et al.*, 1988). The cotransfection experiments allowed the identification of two distinct transactivation domains in the Oct2 protein (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). The functional difference between Oct1 and Oct2 was localized to the C-terminal transactivation domain and correlated to differential phosphorylation (Tanaka and Herr, 1990). Further evidence for the importance of Oct2 for B cell-specific octamer promoter function came from the study of somatic cell hybrids between fibroblasts and B cells. In such hybrids, the extinction of Ig gene transcription correlated with the loss of Oct2 protein expression (Bergman *et al.*, 1990; Junker *et al.*, 1990). Upon transfection of an

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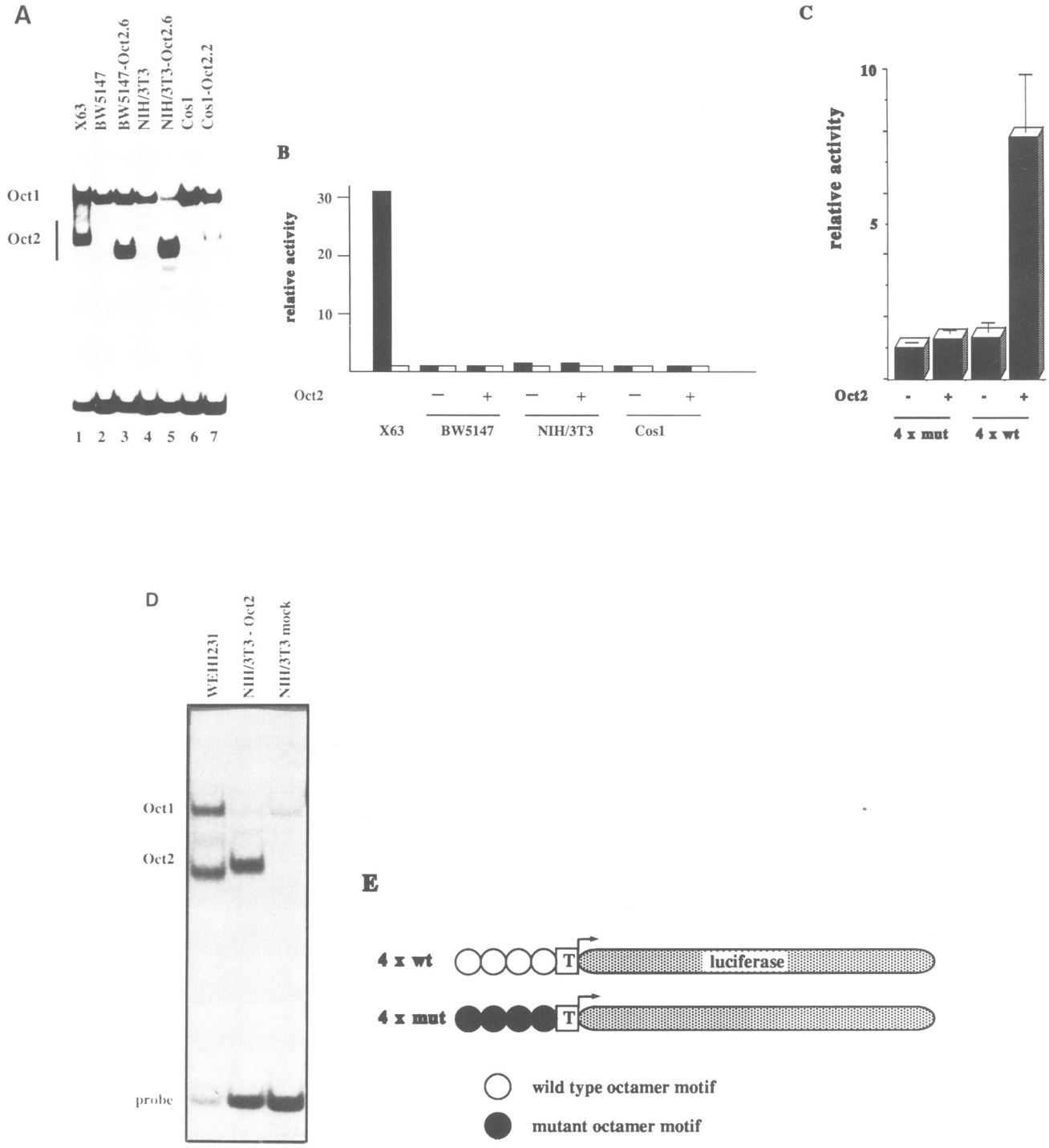

Fig. 1. Stably expressed Oct2 does not activate octamer-dependent promoters in non-B cells. (**A**) EMSA with an octamer-containing probe (Wirth *et al.*, 1991) and nuclear extracts (10 μg) from the indicated cell lines. X63 represents a 'normal' B cell line expressing several endogenous Oct2 isoforms (lane 1). BW5147−Oct2.6 and NIH/3T3−Oct2.6 represent stable transfectants of the respective parental BW5147 or NIH/3T3 cell lines with an Oct2.6 expression vector. Cos1−Oct2.2 are stably transfected Cos1 cells containing the Oct2.2 expression vector. (**B**) Activity of reporter constructs containing either wild-type (black bars) or mutant (white bars) octamer promoters in the cell lines shown in (A). Oct+/− indicates the presence or absence of stably transfected Oct2 in these cells. For all cell lines, the activity of the promoter-containing mutant octamer motifs was set to 1. Results from several independent transfection experiments varied by $<10\%$. (**C**) Transient cotransfection of Oct2 with the octamer-dependent reporter constructs into NIH/3T3 cells. The two bars on the left represent the promoter-containing mutant octamer motifs, the bars on the right contain the wild-type element. Oct2 −/+ indicates cotransfection of either the Oct2 expression vector or the parental vector DNA, respectively. The activity of the mutant promoter was set to 1. (**D**) EMSA experiment with 10 μg of either WEHI231 nuclear extract or 10 μg each of whole cell extracts from NIH/3T3 cells transiently transfected with either the Oct2 expression vector or the parental empty expression vector as indicated. (**E**) Schematic representation of the reporter constructs used in (B) and (C). The constructs contain four copies of either the wild-type octamer motif or a point mutant thereof immediately upstream of the HSV thymidine kinase TATA-box and the luciferase reporter gene (Annweiler *et al.*, 1993).

Oct2 expression vector, Ig promoter activity was partially regenerated (Junker *et al.*, 1990).

However, several experimental findings contradict the simple model that Oct2 alone is responsible for the B cell-specific activity of octamer promoters. (i) In somatic cell hybrids between B and T cells, Ig gene transcription is also extinguished but Oct2 expression is unaffected (Yu *et al.*, 1989). (ii) In some cotransfection experiments overexpression of Oct1 was also capable of transactivating an Ig promoter in non-B cells (Kemler *et al.*, 1991). Furthermore, efficient transactivation by ectopically-expressed Oct2 depended on very high levels of Oct2 protein (Gerster *et al.*, 1990). (iii) *In vitro* transcription experiments revealed that Oct1 is as potent an activator of Ig promoters as Oct2 (Scheidereit *et al.*, 1987; LeBowitz *et al.*, 1988; Johnson *et al.*, 1990; Pierani *et al.*, 1990; Luo *et al.*, 1992; Annweiler *et al.*, 1993). In some of these *in vitro* transcription experiments, an additional B cell-specific component has been identified which interacts with the octamer transcription factors and potentiates the activity of Ig promoters. The ubiquitously active histone H2B promoter is not affected by this additional B cell-specific component (Pierani *et al.*, 1990; Luo *et al.*, 1992). (iv) With respect to octamer-dependent enhancer activation, we could show that a further B cell-restricted activity is required in addition to Oct2 (Annweiler *et al.*, 1992b).

In an attempt to characterize the B cell-specific promoter activity of the octamer motif, we have studied the transactivation potential of Oct1 and Oct2 in B cells and non-B cells. We show that concentrations of Oct2 comparable with those occurring in B cells are insufficient to activate an octamer-dependent promoter in non-B cells. Furthermore B cell-specific octamer-dependent promoter activity is not dependent on Oct2. In contrast and in agreement with our previous observations, octamer-mediated activation from a remote enhancer position requires Oct2; Oct1 cannot supply this function in B cells. Finally, we show that the increased transcriptional potential of Oct1 in B cells versus non-B cells correlates with the occurrence of a novel Oct1-containing protein–DNA complex.

Results

To assess the transactivation potential of Oct2 in non-B cells at concentrations comparable with those in B cells, we generated stable transfectants of different parental non-B cell lines. NIH/3T3 fibroblasts were stably transfected with expression vectors for the six previously described Oct2 isoforms. Likewise, all Oct2 isoforms were also introduced into the BW5147 thymoma cell line. In addition, we generated a stable Cos1 transfectant containing the Oct2.2 isoform. A total of >20 stable cell lines was generated and they were analysed both as pools of transfected cells and as individual stably transfected clones. All transfectants were shown to express the respective isoform assayed by EMSA; however, the expression levels varied between different isoforms. The Oct2.6 isoform gave the highest and Oct2.4 gave the lowest levels of stably expressed Oct2 proteins. The other isoforms were expressed at intermediate levels (data not shown). A representative set of transfectants is shown in Figure 1A. The stable transfectants express levels of Oct2 comparable with the endogenous levels present in a B cell line (Figure 1A, lane 1). The Oct2.6 isoform migrates slightly faster than the bulk Oct2 complex in the X63 B cells because this isoform lacks a short region N-terminal of the transactivation domain due to differential splicing (Wirth *et al.*, 1991).

Octamer-dependent transcriptional activity was measured in these cell lines using a reporter construct containing four copies of the octamer motif upstream of the HSV-tk TATA-box and the luciferase gene (Annweiler *et al.*, 1993). When transfected into a B cell line, this reporter shows 30-fold higher activity compared with a control construct in which the octamer motifs bore point mutations (Figure 1B). When the activities of these reporters were analysed in the stable Oct2-positive transfectants and parental cell lines, no specific Oct2-dependent stimulation was seen (Figure 1B). Identical results were obtained for all stable transfectants, regardless of the Oct2 isoform or the cell line used. No difference was observed when pools of stable transfectants or isolated clones of transfected cells were analysed (data not shown). In NIH/3T3 cells, the wild-type octamer motif-containing reporter was reproducibly slightly more active than the mutated version. However, no additional stimulation was observed when Oct2 proteins were stably expressed in these cells. This lack of Oct2 responsiveness is not due to intrinsic features of the reporter constructs. Transient cotransfection of a CMV-based Oct2 expression vector into NIH/3T3 cells resulted in transactivation which was dependent on the integrity of the octamer motifs (Figure 1C). This result is in agreement with earlier findings of transient cotransfection experiments and it suggests that Oct2 has to be present in non-physiologically high concentrations to stimulate octamer-containing promoters in non-B cells.

This interpretation was confirmed when the amounts of Oct2 protein present after transient transfection were quantified by EMSA. The experiment was performed in triplicate to obtain controls for transfection efficiency and promoter stimulation (9.9-fold in this experiment) simultaneously. Although only 6% of the cells took up DNA, as measured by *in situ* staining of the cells for lacZ activity, the amount of Oct2 protein was greater than in a control WEHI231 B cell nuclear extract (Figure 1D). WEHI231 is known to be the mouse B cell line with the highest content of Oct2 protein. Thus, if the transfection efficiency is taken into account, transient transfections resulted in at least a 20-fold overexpression of Oct2 protein.

Clearly, transactivation by Oct2 in B cells must be mechanistically different from that observed in non-B cells because levels of Oct2 in the stable transfectants were comparable with those present in B cells, where they suffice to transactivate these reporter constructs. Consequently, it should also be considered whether Oct1 might also function differently in B cells versus non-B cells. To address this question we used Oct2-deficient B cell lines. An *oct2* null mutation in the mouse germline was generated recently (Corcoran *et al.*, 1993). In these mice, early B cell development in the fetal liver proceeds normally, and transformed pre-B cell lines were generated from these mice using the Abelson murine retrovirus. These lines fail to show any Oct2 protein detectable by both EMSA (Figure 2A) or Western immunoblot (Corcoran *et al.*, 1993). The activity of the octamer-dependent reporters was then assessed in these Oct2-deficient B cell lines. For comparison, we used either a plasmacytoma cell line (S194) or an Abelson transformed pre-B cell line from a normal *oct2*-positive mouse (PD31).

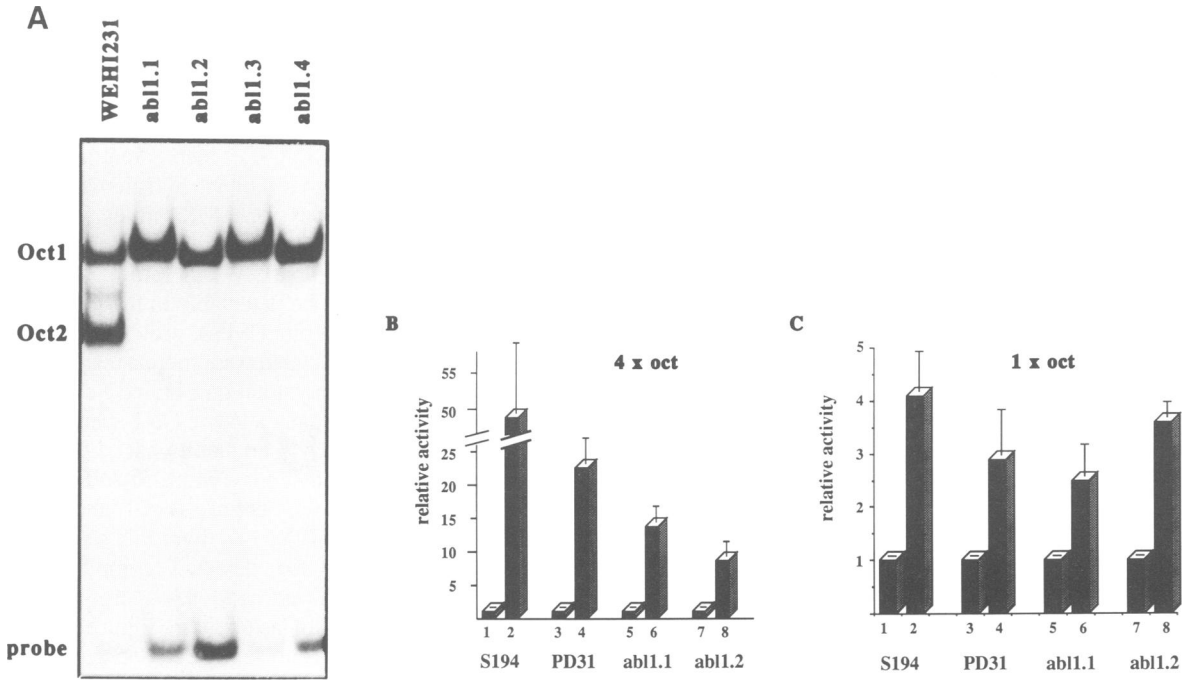

Fig. 2. Octamer-dependent promoter activity in B cells does not require the presence of Oct2 protein. (**A**) EMSA with an octamer-containing probe and nuclear extracts (10 μg) from either Oct2-positive B cells (WEHI231) or four different pre-B cell lines from Oct2-deficient mice (abl1.1−abl1.4). (**B**) Activity of reporter constructs with four copies of the octamer motif (Figure 1C) in Oct2-containing (S194 and PD31) and Oct2-deficient (abl1.1 and abl1.2) B cell lines. Odd numbered bars show the relative expression of the mutant reporter constructs which was arbitrarily set to 1 for each cell line. Even numbered bars show the activity of wild-type promoters. (**C**) Same as in (B) with reporters containing only a single octamer motif upstream of the TATA-box (Annweiler *et al.*, 1993).

The PD31 and the *oct2*-mutant pre-B cell lines are arrested at comparable stages of B cell development (data not shown). The reporter containing four copies of the octamer motif upstream of the TATA-box showed only a slightly reduced activity in the Oct2-deficient B cells compared with the PD31 control pre-B cell line (Figure 2B). We had previously observed that Oct1 is less efficient than Oct2 in stimulating *in vitro* transcription from promoters with only a single octamer motif upstream of the TATA-box (Annweiler *et al.*, 1993). We therefore compared the activity of such a promoter construct with a single octamer motif in normal and in Oct2-deficient B cells. Clearly, in a B cell background Oct1 is able to activate such reporters to a level indistinguishable from that in Oct2-positive B cells (Figure 2C). Thus, the B cell-specific activity of the octamer motif present in a promoter proximal position is not dependent on the Oct2 protein.

We have shown that octamer function from a distal enhancer position requires Oct2 together with an undefined B cell-restricted activity (Annweiler *et al.*, 1992b). As those experiments were performed in B cells that contained low levels of endogenous Oct2 proteins, we readdressed the question of octamer-dependent enhancer activity in the Oct2-deficient B cells. When reporter constructs containing octamer-dependent enhancer elements (Figure 3C) were transfected into the Oct2-deficient B cell lines, no octamer-dependent activity was observed (Figure 3A). This synthetic enhancer element (construct CLED) was barely active in the Oct2-negative cells and mutation of the octamer motif (CLEd) resulted in a slight increase in activity. The latter finding suggests that binding of Oct1 might interfere with the function of another positively acting factor, most likely a factor binding to the neighbouring E-box motif. Alternatively, the mutation in the octamer motif might increase the affinity for another transcription factor. Similar results were obtained with reporter constructs containing the enhancer upstream of the 600 bp-long lysozyme promoter element (constructs EDCL and EdCL in Figure 3A). When the synthetic enhancer element was moved into an immediate promoter proximal position next to the TATA-box (construct ED.TATA), it was active in an octamer-dependent fashion (Figure 3B). Thus, the failure to be active from a distal enhancer position is not due to the presence of specific repressor factors, but rather to the absence of the activating Oct2 transcription factor. This interpretation was confirmed by cotransfection of an Oct2 expression vector, as octamer-dependent enhancer activity was at least partially reconstituted in the presence of cotransfected Oct2 (Figure 3A). In accordance with our previous observations, the C-terminal transactivation domain of Oct2 suffices for this B cell-specific enhancer activation (data not shown).

As we had previously shown that transient cotransfections might result in aberrantly high levels of Oct protein, we wanted to ensure that physiological amounts of Oct2 suffice for this activation. Therefore we generated a stable transfectant of the abl1.1 cell line using an expression vector for a chimeric Oct2 protein whose activity can be regulated by oestrogen (Figure 4D). This chimeric protein was made by fusing the Oct2 coding region with the hormone-binding domain of the human oestrogen receptor (A.Annweiler, P.Pfisterer and T.Wirth, manuscript in preparation). A stable transfectant was obtained that expressed the appropriate fusion protein (Figure 4A). The amounts of protein present in this stable transfectant were comparable with the amount

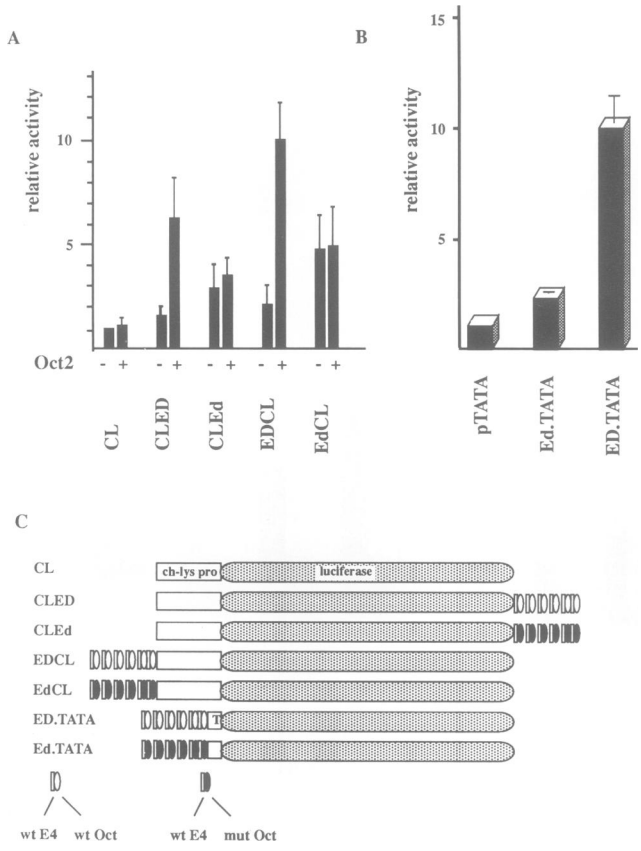

Fig. 3. Octamer-dependent enhancer activity requires the presence of Oct2 in B cells. (**A**) Activity of reporter constructs (5 μg) carrying the octamer-dependent enhancer element (outlined in **C**) in Oct2-deficient B cells (abl1.1). +/− Oct2 indicates whether or not an Oct2 expression vector or the parental expression plasmid (5 μg) was included in the transient transfections. (**B**) Activity in abl1.1 cells of reporter constructs (10 μg) containing the synthetic enhancer element proximal to the TATA-box. (C) Structure of the reporter constructs used in (A) and (B). CL, CLED and CLEd have been described (Annweiler *et al.*, 1992b). In EDCL, EdCL, ED.TATA and Ed.TATA the respective synthetic enhancer fragment was inserted upstream of the chicken lysozyme promoter or the minimal HSV-tk promoter (T).

of Oct2 protein in a typical B cell line (i.e. X63.Ag8, data not shown). Octamer-dependent promoter and enhancer reporter constructs were transfected into these cells. Upon stimulation of the transfected cells with oestrogen, activity of the octamer-dependent promoter reporter was only slightly (1.6-fold) increased (Figure 4B). This result is consistent with the interpretation that Oct1 suffices to generate almost maximal levels of activity from this position. In contrast, the activity of the reporter containing the octamer-dependent enhancer element was stimulated >8-fold (Figure 4C). This is in good agreement with previous mutational analyses which had shown that in normal Oct2-positive B cells mutation of the octamer motif in the enhancer element results in a 7- to 10-fold decrease in activity (Gerster *et al.*, 1987; Annweiler *et al.*, 1992b). No effect of the oestrogen treatment was apparent in the non-transfected parental abl1.1 cell line (Figure 4B and C, right panels).

From the results described so far we conclude that B cells must contain two different coactivating activities that distinguish the function of the octamer motif in promoter proximal and distal positions, respectively. The first of these two activities can functionally interact with both Oct1 and Oct2, and confers B cell-specific promoter activity to the octamer motif. The latter is specific for Oct2 and is required to confer octamer-dependent enhancer activity. Luo and colleagues have recently identified a B cell-specific factor in a human B cell line, OCA-B, which potentiates octamer-dependent promoter activity *in vitro* (Luo *et al.*, 1992). Using modified conditions for EMSA, they were able to show preferential interaction of this factor with Oct1, generating a retarded complex. We therefore asked whether such a factor might also be detectable in the Oct2-deficient cell lines. We modified our EMSA protocol by including glycerol in the gel and analysed increasing amounts of extract. In contrast to the situation obtained under normal EMSA conditions where only a single Oct1-specific complex was obtained (Figure 2A), an additional slightly slower migrating complex was evident (Figure 5A). This complex was present in all Oct2-deficient pre-B cell lines, but it was not detectable with the BW5147 thymoma extract. Using these conditions, we also detected complexes in the Oct2-deficient pre-B cell extracts with a similar migration to Oct2 (marked with an asterisk in Figure 5A). These complexes most likely represent degradation products of Oct1. They reacted with Oct1-specific antibodies but did not crossreact with an Oct2-specific antiserum (see Figure 6B and data not shown). To exclude the possibility that the additional complex might occur at higher extract concentrations in BW5147 cells, we increased the amounts of nuclear extract in the assay (Figure 5B). However, even when 10-fold more BW5147 nuclear extract was used, the complex could not be detected. We wanted to test whether the occurrence of the slower migrating complex correlated with the transcriptional activity. We therefore analysed nuclear extracts from different B cells and non-B cell lines for the presence of this slower migrating shift activity. In all B cell lines this additional complex was observed, albeit to somewhat variable extents. In contrast, none of the non-B cells shows detectable levels of this complex (Figure 5C). In addition to the B cell lines shown in Figure 5C, the complex was also detected in all other mouse B cell lines tested (WEHI231, X63.Ag8, SP 2/0), but not in other non-B cell lines like Cos1 or the WEHI3 monocytic cell line. This distribution is in complete agreement with the observed octamer promoter activity (data not shown). The B cell-specific octamer coactivator described by Luo *et al.* (1992) was isolated from the human Namalwa B cell line. We therefore analysed nuclear extracts from Namalwa cells for the presence of this novel shift activity. A retarded complex of identical migration behaviour is detected in these cells suggesting that this complex could in fact represent the Oct1−OCA-B complex (Figure 5D).

To characterize this protein−DNA complex, competition experiments with wild-type and mutant octamer competitors were performed. Increasing amounts of the wild-type competitor abolish the complex with kinetics similar to the bona fide Oct1 complex. The mutant competitor had no effect, however (Figure 6A). To determine whether the Oct1 protein is present in this complex, we raised polyclonal antibodies against the N-terminal domain of Oct1. These antibodies specifically recognize the Oct1 protein and do not crossreact with Oct2 at the concentrations used (data not shown). This antibody removed and supershifted both the normal Oct1 as well as the retarded complex, whereas pre-

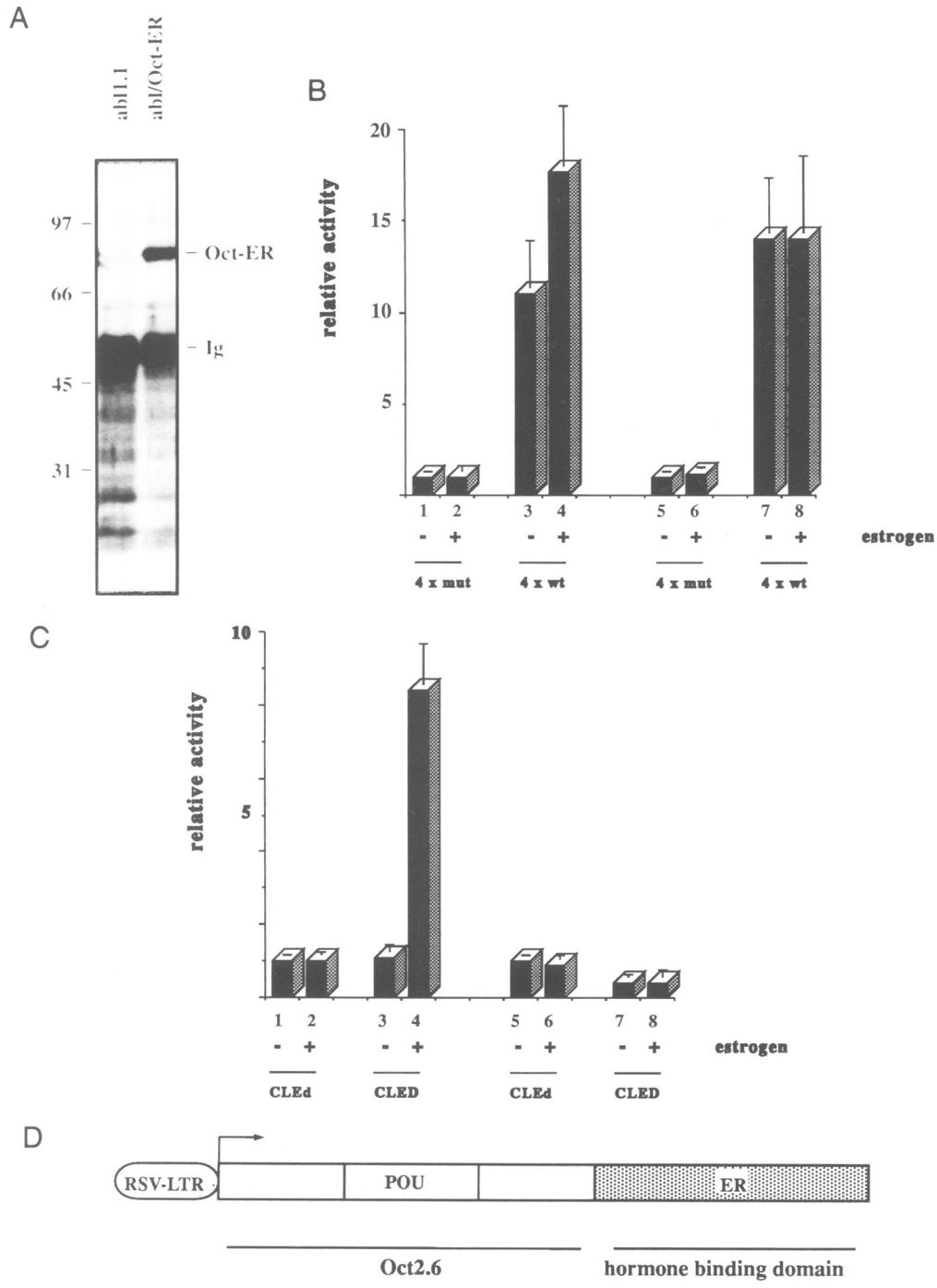

Fig. 4. Activity of octamer-dependent promoter and enhancer elements in stable Oct2−ER transfectants of the abl1.1 line. (**A**) Nuclear extracts from the parental abl1.1 cell line and stable Oct2−ER transfectants were immunoprecipitated with Oct2-specific antibodies and precipitates were analysed with the Oct2-specific antibodies in an immunoblot. The positions of the correctly sized fusion protein (Oct−ER), as well as Ig molecules from the immunoprecipitation reaction are indicated. Positions of molecular weight markers are given on the left side of the figure. (**B**) Activity of reporter constructs with four wild-type or mutant copies of the octamer motif (Figure 1E) in Oct2−ER transfectants (lanes 1−4) or the parental abl1.1 line (lanes 5−8). Odd numbered lanes, controls; even numbered lanes, + oestrogen. (**C**) Activity of reporter constructs with wild-type or mutant octamer-dependent enhancer elements (Figure 3C) in Oct2−ER transfectants (lanes 1−4) and the parental abl1.1 cell line (lanes 5−8). Odd numbered lanes, controls; even numbered lanes, + oestrogen. (**D**) Structure of the Oct2−ER expression vector. The position of the POU domain of Oct2 is indicated. The hormone-binding domain of the human oestrogen receptor is shown as a stippled box.

immune serum and the Oct2-specific antibody had no effect on either complex (Figure 6B and data not shown). From these results we conclude that Oct1 is present in this complex either in a modified form or associated with a second protein. To test whether the alteration resulting in the retarded migration affects the interaction with DNA, we performed chemical footprinting experiments on the two complexes (Kuwabara and Sigman, 1987). Figure 6C shows that the retarded complex does not protect additional nucleotides when compared with Oct1 alone.

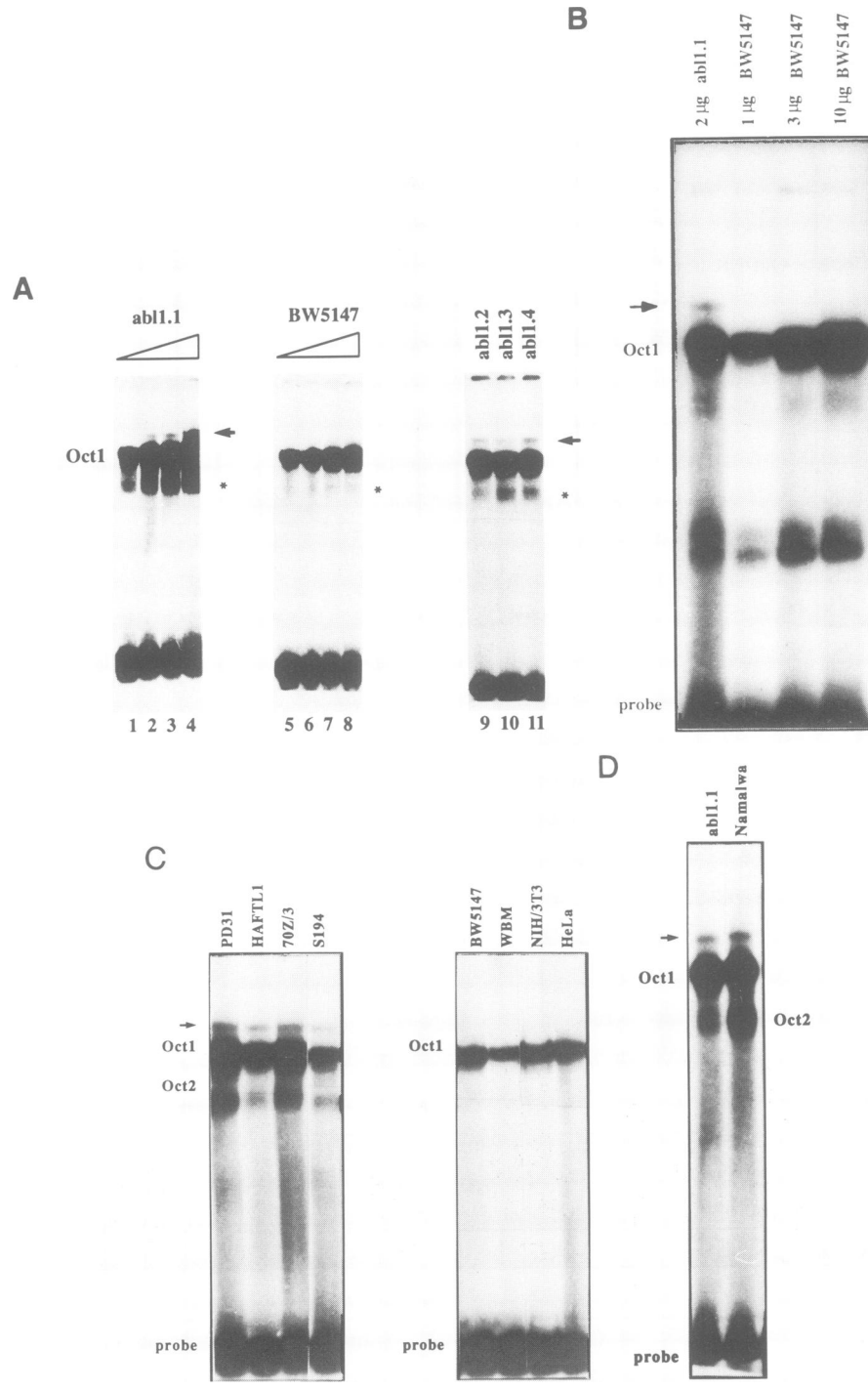

Fig. 5. A B cell-specific complex is detectable in modified EMSA conditions. (**A**) Increasing amounts (1−4 µg) of nuclear extracts from abl1.1 cells (lanes 1−4) or BW5147 cells (lanes 5−8), or 4 µg of nuclear extracts from the other Oct2-deficient B cell lines (lanes 9−11) were tested using the modified EMSA conditions. The position of the Oct1 complex is indicated. The arrow marks the position of a novel retarded complex, the asterisk indicates the position of proteolytic degradation products of Oct1. (**B**) Up to 10 µg of BW5147 were analysed for the presence of the retarded complex as indicated. (**C**) Between 2 and 5 µg of nuclear extracts from the indicated cell lines were tested. The positions of Oct1, Oct2 and the retarded complexes are indicated. The left panel shows different B cell lines, the right panel shows various non-B cells. HAFTL1, PD31 and 70Z/3 represent pre-B cells of increasing maturity, S194 is a plasma cell line. The non-B cells are as follows: BW5147 is a T cell line, WBM, is a mast cell line, NIH/3T3 is a fibroblast cell line and HeLa is a cervix carcinoma cell line. With the exception of HeLa cells (human), all other cell lines tested are of murine origin. (**D**) Modified EMSA with extracts (3 µg) from abl1.1 and the human Namalwa B cell line.

Discussion

Here we show that physiological concentrations of either Oct1 or Oct2 are insufficient to activate minimal octamer-containing promoters in non-B cells. In contrast, the same promoter elements are highly active in B cells, irrespective of the presence of the Oct2 protein. No other octamer-binding protein could be detected in these B cells. This suggests that the Oct1 protein is responsible for the transcriptional activity of this reporter in the absence of Oct2.

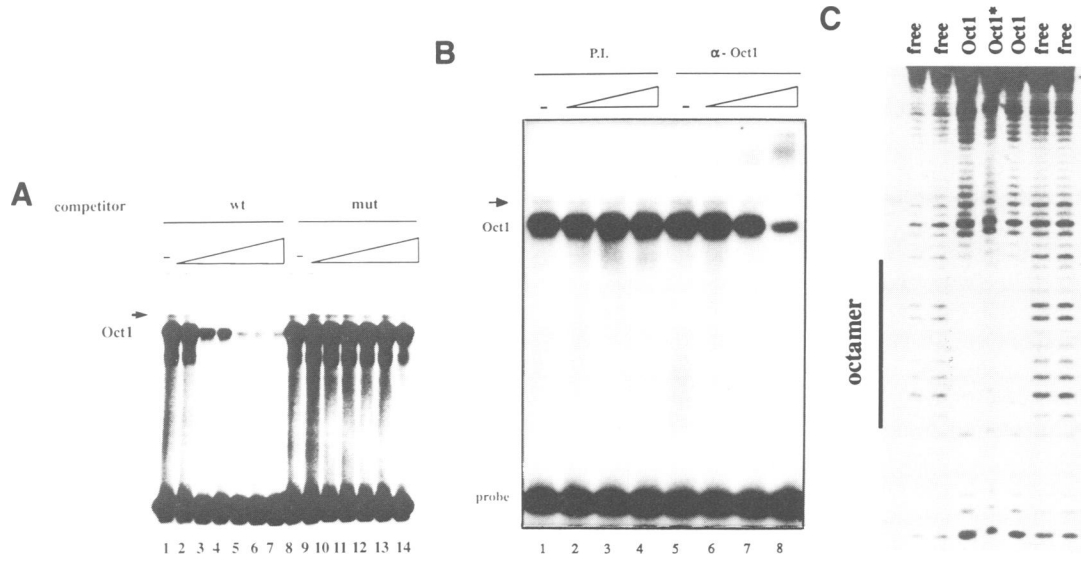

Fig. 6. Characterization of the retarded B-cell-specific complex. (**A**) Competition experiment with increasing amounts of unlabelled competitor DNA with either wild type (lanes 2–7) or point mutant versions of the octamer motif (lanes 9–14). Competitor DNA was present in 10-, 50-, 100-, 500-, 1000- and 2000-fold excess over the probe DNA in the various reactions. (**B**) EMSA in the presence of increasing amounts (1 μl each of a 1:100, 1:30 and 1:10 dilution) of either rabbit pre-immune serum (lanes 2–4) or identical dilutions of a specific antiserum raised against the N-terminus of Oct1 (lanes 6–8). (**C**) In-gel chemical footprinting of the free probe, the Oct1 complex or the retarded complex (Oct1*) with Cu-orthophenanthroline. The location of the octamer motif in the probe is highlighted.

Therefore, the ubiquitous Oct1 protein is much more active in B cells than in non-B cells and can confer a cell type-specific expression pattern. To our knowledge, this is the first example where cell type specificity of a given promoter element is not regulated at the level of a corresponding cell type-specific DNA-binding transcription factor.

When the octamer motif is present in a promoter proximal position, both Oct1 and Oct2 can function in concert with a B cell-specific activity. However, when the octamer motif is in a remote enhancer position, only Oct2, together with either the same or a distinct factor, can confer transcriptional activity. The most likely candidate responsible for mediating the octamer promoter activity is OCA-B (Luo *et al.*, 1992). This factor has been identified in a human B cell line and was shown to potentiate the activity of Oct1 and Oct2 *in vitro*. Although OCA-B did function with both Oct1 and Oct2, in EMSA experiments it showed preferential interaction with the Oct1 protein. We detected a retarded B cell-specific Oct1-containing complex which could contain the murine homologue of OCA-B.

However, several other explanations that could account for the B cell-specific activity of octamer promoters cannot yet be ruled out. The Oct1 and Oct2 proteins might be specifically modified in B cells. It has been shown that phosphorylation of octamer-binding proteins correlates with their activity in HeLa cells (Tanaka and Herr, 1990). Furthermore, cell cycle regulated phosphorylation of the Oct1 protein has been shown to be responsible for the cell cycle-dependent DNA-binding activity of the Oct1 protein on the histone H2B promoter (Boseman Roberts *et al.*, 1991; Segil *et al.*, 1991). We have addressed the question of whether the B cell-specific retarded complex detectable with the modified EMSA conditions might contain a hyper-phosphorylated form of Oct1. However, neither calf intestinal alkaline phosphatase nor potato acidic phosphatase affected this complex (data not shown). Alternatively, B cells might express a differentially spliced version of the Oct1 protein. Three lines of evidence argue against this latter hypothesis. First, in EMSA experiments using standard conditions with extracts from the Oct2-deficient B cell lines, only the Oct1 complex is detectable. Secondly, in Western immunoblots no difference can be detected between B cells and non-B cells with the Oct1-specific antibody (data not shown). Finally, overexpression of the normal Oct1 protein in B cells did not repress the octamer-dependent promoter (data not shown). This shows that the 'normal' Oct1 protein is functional in B cells.

By overexpression of Oct2 in normal B cells we had previously demonstrated that Oct2, but not Oct1, is capable of activating the octamer motif when present in a remote enhancer type position. This result could be confirmed in the Oct2-deficient cell lines. In these cells the enhancer element does not show any octamer-dependent activity. In fact, the enhancer element containing the wild-type octamer motif was reproducibly slightly less active than the construct bearing the mutant octamer motif. This indicates that Oct1 binding to this enhancer interferes with the function of another positively-acting transcription factor. Cotransfected Oct2 specifically stimulates the wild-type octamer-containing reporter. The results from the stable transfectants suggest that physiological levels of Oct2 suffice for this enhancer activation. In contrast to the parental abl1.1 cell line, activity of the reporter construct bearing the intact octamer motif in the enhancer position is not reduced in the stable transfectant. This is most likely due to the fact that the oestrogen receptor hormone-binding domain does not completely inactivate the Oct2 transactivation function. Importantly, hormone induction fully reconstitutes the activity of the octamer motif in the enhancer.

It is presently unclear whether the B cell-restricted activity that confers this Oct2-mediated enhancer function is identical to the promoter stimulating activity. Two findings argue against this possibility, however. First, at the promoter Oct1 and Oct2 can function equally well, whereas at the enhancer

only Oct2 can functionally interact with this activity. Secondly, we have identified at least one cell line (EL4 T) in which the octamer promoter is highly active, whereas the enhancer is inactive in these cells. This suggests that in these cells the promoter activating factor(s) are present, but the activity required for enhancer activation is missing, despite the presence of the Oct2 protein (A.Annweiler, S.Zwilling and T.Wirth, manuscript in preparation).

Recently, the mutation of both copies of the *Oct2* gene in a mature B cell line was reported (Feldhaus *et al.*, 1993). In this report, selectable markers had been inserted into the first coding exon of Oct2, just downstream of the AUG. Due to the presence of another in-frame AUG in the second exon, Oct2 production was not completely abolished but rather a truncated Oct2 protein that lacked the first 12 amino acids was produced. The level of Oct2 protein in B cells was reduced by a factor of 20 and this reduction led to Oct2 levels comparable with those present in normal pre-B cell lines. Therefore, these cells are Oct2-low rather than Oct2-deficient. Nevertheless, some of the conclusions reached with these cells resemble our results. Whereas no effect was seen on a simple promoter bearing a single octamer motif upstream of the TATA-box, a 10-fold reduction of activity was observed for a reporter bearing multimerized octamer motifs at a distance of 127 bp upstream of the cap site. The authors concluded that Oct1 in B cells can efficiently activate singular octamer motifs, whereas Oct2 is required for regulatory elements bearing multimerized octamer motifs. The reporter construct with the multimerized octamer motifs used by Feldhaus and colleagues (1993) most likely represents an intermediate between constructs bearing octamer motifs in promoter versus enhancer positions. Furthermore, the reason they observed only a partial effect with this reporter might be due to the fact that their cells still had residual Oct2 protein.

Octamer transcription factors seem to represent a novel class of transcriptional activators that strongly depend on cell type-specific cofactors to perform their activities. Several such interactions have been observed for various octamer-binding proteins. The activity of the TAATGARAT motif in the promoters of HSV immediate early genes requires a complex between the Oct1 protein, a host cell cofactor and the viral VP16 transactivator (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Stern *et al.*, 1989; Wilson *et al.*, 1993). Both Oct2 and Oct4 can efficiently stimulate activities from remote enhancer octamer motifs. However, both depend on cell type-specific cofactors. Oct4 has to interact with an E1A-like activity present in undifferentiated EC cells (Schöler *et al.*, 1991), while Oct2 interacts with an unknown B cell-specific factor. Finally, promoter octamer activation by Oct1 and Oct2 also requires the presence of a B cell-specific cofactor as shown in this report. This latter requirement can be partially overcome by highly overexpressing Oct1 or Oct2 ectopically in non-B cells. It is at present unclear whether the transactivation achieved by Oct1/Oct2 overexpression is mechanistically similar to the B cell-specific activation observed at much lower physiological protein concentrations. The interaction of sequence-specific DNA-binding proteins with cell type-specific cofactors might represent a novel strategy to achieve tissue-specific gene expression. By restricting the expression of the appropriate cofactor, the activity of the DNA-binding protein can be regulated. It will be interesting to see whether this regulating principle is unique to the octamer transcription factors or is also utilized by other transactivators.

Finally, these results bring up the question: how are the regulatory regions of Oct2-dependent genes organized? Clearly, Oct2 is an important regulator in B cells, as B cell development is impaired at a late stage in *oct2*-deficient mice (Corcoran *et al.*, 1993). Our results demonstrate that Oct2 is essential for octamer-dependent enhancer activity in B cells. However, the enhancer elements of affected genes need to have an organization with a lower functional redundancy than the Ig heavy chain enhancer. Although the octamer motif has been shown to contribute to the B cell-specific activity of this enhancer, this contribution is only seen with truncated versions of the enhancer or in the context of additional mutations (Lenardo *et al.*, 1987), and mutation of the octamer motif in the enhancer did not affect the activity of this element in transgenic mice (Jenuwein and Grosschedl, 1991; Annweiler *et al.*, 1992a). In addition, it is conceivable that not all octamer-containing promoter elements will be activated by Oct1 in B cells and there might be a subset of promoters that critically depend on Oct2. The identification of Oct2-regulated genes in B cells will allow us to distinguish between these possibilities.

Materials and methods

Plasmid constructions, DNA transfections and reporter assays

Expression vectors for stable transfections, containing the various Oct2 isoforms under the control of a retroviral LTR and a tk-neo cassette for selection, the octamer-dependent enhancer constructs (Annweiler *et al.*, 1992b) and the reporter constructs with the octamer motif in a promoter proximal position (Annweiler *et al.*, 1993), have all been described previously. The constructs containing the synthetic enhancer upstream of the chicken lysozyme promoter (EDCL and EdCL) were generated by inserting the hexameric enhancers as a *Xho*I fragment into a unique *Sal*I site at −579 relative to the transcriptional start site (Annweiler *et al.*, 1992b). For the generation of Ed.TATA and Ed.TATA, the respective enhancer fragments were inserted at position −38 of the truncated HSV-tk promoter, which contains only a functional TATA element. In all cases the orientation of the enhancer element with respect to the start site of transcription was identical. The Oct2.6−ER fusion was generated by creating a *Bgl*II site at the translational stop codon of an RSV expression vector of the Oct2.6 isoform (Annweiler *et al.*, 1992b). The oestrogen hormone-binding domain of clone HE14 (Kumar *et al.*, 1986) was then introduced as a *Bam*HI−*Eco*RI fragment.

Stable transfections were performed using either calcium phosphate [for NIH/3T3 cells (Wirth *et al.*, 1991)] or electroporation (Bio-Rad gene pulser, 960 μF, 250 mV for BW5147, abl1.1 and Cos1 cells). Stable transfectants were selected in medium containing 0.5 mg/ml G418 for NIH/3T3 and Cos1 cells, and 1.5 mg/ml for BW5147 cells. The stable transfectant of the abl1.1 cell line was obtained by cotransfection of the pSV_2−His expression vector and selection in the presence of 0.5 mg/ml L-histidinol. Transient transfections for PD31, abl1.1, abl1.2 and BW5147 cells were performed by electroporation using the same conditions as above. S194 and X63.Ag8 cells were transfected by the DEAE-Dextran method (Wirth *et al.*, 1987) and Cos1 and NIH/3T3 cells by calcium phosphate as above. Between 5 (DEAE-Dextran) and 20 μg of reporter plasmid were used, unless indicated otherwise. When Oct2 expression vectors were included in the transfection, only half as much reporter was used and identical amounts of expression vectors were included. Conditions for luciferase assays were as described (Annweiler *et al.*, 1992b). Transient transfections routinely contained a β-galactosidase expression vector CMV-*lacZ* for normalization of transfection efficiencies. Due to high endogenous galactosidase activities in the abl pre-B cell lines, transfections into these cells were, in addition, repeated several times with a minimum of two independent DNA preparations. The determination of the number of cells that took up DNA in the transient cotransfection experiment of NIH/3T3 cells (Figure 1D) was performed as follows. Cells on one dish, each of a triplicate transfection experiment, were fixed with 2% paraformaldehyde−0.2% glutaraldehyde, washed three times in PBS and then stained in X-gal buffer [1 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$] and

several hundred cells were counted on each dish. Stable Oct–ER transfectants of abl1.1 cells were grown in phenol-red free medium (Gibco BRL) and oestrogen was added to a final concentration of 1 μM where indicated.

Nuclear extracts, conditions for EMSA and Western immunoblots

Generation of nuclear extracts (Dignam *et al.*, 1983) and standard EMSA conditions (Wirth and Baltimore, 1988) were as described. The modified EMSA conditions were adapted from a published protocol (Luo *et al.*, 1992). In brief, nuclear extracts were incubated in 20 μl binding buffer (12.5 mM HEPES pH 7.9, 31.25 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 8% glycerol, 0.1 mg/ml BSA, 0.25 mM PMSF) with 50 000 c.p.m. labelled probe and 2 μg poly dI:dC for 30 min at room temperature. Complexes were separated on pre-run (1 h at 120 V) 4% polyacrylamide gels (19:1 crosslinked) containing 5% glycerol and 0.5 × TBE. For competition experiments, the unlabelled competitor DNA was included in the binding reaction. For supershift experiments, antibodies were preincubated with the nuclear extract for 30 min at room temperature. Probe was added together with poly dI:dC and, after a further incubation for 30 min at room temperature, samples were loaded onto pre-run gels. In-gel footprinting with Cu-orthophenanthroline was performed as described (Kuwabara and Sigman, 1987) and products were analysed on 15% denaturing polyacrylamide gels. For Western immunoblots, 100 μg nuclear extracts were immunoprecipitated with 1 μg affinity-purified Oct2-specific antibody coupled to 10 mg protein A Sepharose (Pharmacia) as described (Harlow and Lane, 1988). Precipitated proteins were released by boiling in SDS–sample buffer, separated on a 10% SDS–polyacrylamide gel, transferred to PVDF membrane and reacted with the affinity-purified Oct2-specific antibodies and alkaline phosphatase-labelled secondary antibodies. The blots were developed using the CSPD system (Tropix).

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