Using altered specificity Oct-1 and Oct-2 mutants to analyze the regulation of immunoglobulin gene transcription

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Oct-1 and Oct-2 represent the prototypical example of related transcription factors with identical DNA recognition properties. They bind functionally critical octamer elements found in diverse regulatory sequences. It has not been possible to determine directly if these factors are functionally redundant or selective when interacting with different regulatory sequences in the appropriate cell type. An equivalent pair of altered DNA-binding specificity mutants of Oct-1 and Oct-2 are used to examine their function from varied regulatory contexts in B cells. These factors function as redundant activators of immunoglobulin (Ig) gene promoters (V_K and V_H) and a histone H2B promoter. The structural basis of redundancy resides in the highly conserved DNA-binding POU domain, because this domain of either protein can activate transcription from both Ig and H2B promoters. We find that the nature of a distal enhancer dictates the relative potency of Oct-1 versus Oct-2 bound to a promoter. Oct-1 preferentially stimulates transcription from a V_H or V_K promoter in combination with enhancers from the IgH or Igk locus, respectively. In this context, the more potent action of Oct-1 is dependent on a region external to the POU domain. These results suggest that Oct-1 may be the critical regulator of Ig gene transcription during B cell development and provide an explanation for selective Ig isotype expression defects in Oct-2 and OCA-B null mice.

Keywords: altered specificity mutant/expression screening/immunoglobulin gene transcription/octamer-binding protein/POU domain

Introduction

Higher eukaryotic promoters and enhancers are generally complex in structure, consisting of multiple sequence motifs that serve as binding sites for various transcription factors (Tjian and Maniatis, 1994). Often, a binding site is recognized by different members of a family of regulatory proteins that have identical or overlapping DNA-binding specificities. For example, octamer-binding sites are recognized by Oct-1, Oct-2, Oct-3 or Oct-6 (Scholer, 1991), Sp1-binding sites by Sp1, Sp2 or Sp3 (Kingsley and

Winoto, 1992), and the GATA motif by GATA-1, GATA-2, GATA-3, GATA-4 or GATA-6 (Simon, 1995). When two or more members of a regulatory protein family occur in the same cell type, it becomes difficult to determine which member(s) controls transcription of different target genes *in vivo*.

The regulatory activity of a transcription factor is influenced both by the context of the binding site in the promoter or enhancer and by cell type-specific factors and co-activators (Roberts and Green, 1995). Therefore, it is important to study the activity of the factor in the context of the appropriate cell type. A common approach used to distinguish the regulatory activities of related transcription factors involves overexpression of individual family members in the appropriate cell type. However, analyzing the effects of a particular transcription factor on its target regulatory region in this manner is confounded by the presence of its endogenous counterpart and related family members. An alternative approach involves ectopic expression of individual family members. This approach has significant limitations since the cell types used may not express the other relevant cell type-specific transcription factors, and thus their contribution would go unaccounted for. For a complex promoter or enhancer, it is exceedingly difficult to reconstitute the appropriate combination and levels of other relevant regulatory proteins in a heterologous cell type. Targeted disruption of genes encoding individual family members represents a powerful means of analyzing their function. However, gene targeting of transcription factors may cause developmental defects resulting in loss of the relevant cell type(s). Even in cases where the appropriate cell type is present, the effects on target genes may be indirect. Also, gene targeting may fail to reveal the regulatory role of a particular family member due to partial or complete redundancies.

In this study, we describe a novel and general approach to analyze individually the regulatory activities of different members of a family of transcription factors in an appropriate cell type using native promoters or enhancers. The approach involves mutating a regulatory site and then rescuing the mutation using altered specificity mutant transcription factors (Figure 1). The first step involves a screen for severe binding site mutations that impair recognition by wild-type family members and consequently inactivate the regulatory region *in vivo*. The second step entails isolation of an altered specificity mutant family member that can bind to the mutant site. A related set of altered specificity family members is then generated by engineering the relevant mutation(s) in each protein. Finally, each altered specificity family member can then be expressed individually *in vivo* to determine its ability to rescue the function of the mutant regulatory region. This approach allows regulatory functions of individual related transcription factors to be assayed in

Fig. 1. Altered specificity mutants as tools for analyzing functions of related transcription factors. The left panel depicts an octamer element-dependent promoter in a B cell. It is recognized by two different transcription factors, Oct-1 and Oct-2, which have identical DNA-binding properties. A mutation in this octamer element severely impairs the promoter. Right panels depict how altered specificity mutants (asm) Oct-1 or Oct-2, which can bind the mutant octamer sequence, are used to measure their relative abilities to restore function in the presence of their wild-type counterparts.

the correct cell type(s) without the results being obscured by the activities of endogenous wild-type counterparts.

Immunoglobulin (Ig) gene promoters represent the prototypical examples of regulatory elements whose activities appear to be controlled by two related transcription factors with identical DNA-binding specificities. These promoters contain one conserved element, the octamer ATGCAAAAT, which is necessary for their activity in B cells (Bergman *et al.*, 1984; Falkner and Zachau, 1984; Mason *et al.*, 1985). Mutation of this element in the promoter of a rearranged Ig heavy chain transgene reduces expression 20- to 30-fold in lymphoid tissues (Jenuwein and Grosschedl, 1991). Furthermore, the octamer element activates heterologous promoters preferentially in B cells (Dreyfus *et al.*, 1987; Wirth *et al.*, 1987). Thus, the octamer element is both necessary and sufficient for the preferential activity of Ig promoters in B cells. Interestingly, the same motif is an important element of the promoters of ubiquitously expressed genes such as the U2 and U6 snRNA genes and the histone H2B gene (LaBella *et al.*, 1988; Murphy *et al.*, 1989). The ability of the octamer element to promote ubiquitous as well as B cell-specific gene expression was suggested initially to reside in its interaction with two distinct transcription factors, Oct-2 and Oct-1 (Singh *et al.*, 1986; Staudt *et al.*, 1986). While they contain related DNA-binding domains with identical recognition specificities, they are structurally quite distinct (reviewed in Herr and Cleary, 1995). Oct-2 and Oct-1 are members of the POU family of homeodomain proteins. POU proteins contain a 150–160 amino acid bipartite DNA-binding domain which consists of a conserved N-terminal POU-specific domain and a C-terminal POU homeodomain. The ATGC half of the octamer element is recognized by the POU-specific domain, whereas the AAAT half is recognized by the POU homeodomain. The *oct-1* gene is expressed in a variety of cell types, whereas *oct-2* expression is restricted to cells of the lymphoid and central nervous systems. Their differing distributions led to the proposal that Oct-1 regulates transcription of U snRNA and histone H2B

genes, while Oct-2 specifically activates Ig gene transcription (Schaffner, 1989).

Several studies involving ectopic expression of Oct-2 led to differing conclusions concerning its ability specifically to regulate the activity of Ig gene promoters in B cells (Junker *et al.*, 1990; Tanaka and Herr, 1990; Kemler *et al.*, 1991). Targeted disruption of the *oct-2* gene in mice and in a somatic B cell line have shown that Oct-2 is not essential for transcription of Ig genes (Corcoran *et al.*, 1993; Feldhaus *et al.*, 1993). However, the gene targeting studies may have failed to reveal Oct-2's role in Ig gene transcription due to partial or complete redundancy with Oct-1. We use altered specificity mutants of Oct-1 and Oct-2 to demonstrate that the two factors are functionally redundant in regulating Ig gene promoters in B cells. The structural basis of redundancy resides in the highly conserved POU domain, since this domain of either protein is sufficient to activate transcription. Interestingly, the potency of these activators from an Ig promoter is influenced by the type of remote enhancer. Oct-1 preferentially stimulates transcription from Ig gene promoters in combination with Ig gene enhancers. These results suggest that Oct-1 may be the critical regulator of Ig gene transcription during B cell development.

Results

Using expression screening for the isolation of altered specificity mutants

A widely used method for cloning transcription factors involves screening a bacteriophage λ gt11 cDNA expression library with a radiolabeled binding site probe (Singh, 1993). Since the screen serves as a simple but powerful assay for DNA binding by a large number of distinct recombinant proteins, it seemed possible to use the same approach to isolate altered specificity mutants of transcription factors. In this extension of the original method, the following steps are involved: (i) identification of a mutant binding site that severely reduces recognition by the wild-type transcription factor; (ii) generation of a λ gt11

Fig. 2. (A) Identification of severe mutations in the octamer site. All single base substitutions in the AAAT subsite of the octamer element were made, with the exception of A5T. Each mutant site was radiolabeled and used in a gel-shift assay to measure binding of Oct-2 isoforms and Oct-1 with a WEHI-231 B cell nuclear extract. OCTA denotes the wild-type site. P indicates the lane containing free probe. The positions of protein–DNA complexes formed by Oct-1, Oct-2B and Oct-2A are indicated on the right. Non-specific complexes are indicated with an asterisk (*). Six mutations that reduce the affinity of both proteins >50-fold are depicted below the sequence. (B) Structure of the Oct-1 POU domain bound to the octamer. Structural representation of the complex was generated using coordinates provided by Klemm *et al.* (1994) and Insight II software (Biosym Technologies). POU_S and POU_{HD} refer to the POU-specific and POU homeodomain respectively. The following residues in the complex are highlighted: Adenine 7 of the octamer (A7) makes specific hydrogen bonds with Asn51 (N51) of the POU_{HD}; N51 along with Val47 (V47) and Cys50 (C50) of the POU_{HD} were selected for mutagenesis; Asp41 (D41), in the turn between helix 2 and 3 of the POU_S domain, is the site of glycine substitution in the Oct-2 altered specificity mutant. (C) Isolation of an altered specificity Oct-2 mutant by expression screening with an A7G mutant probe. The figure shows the results of screening filters generated from platings of the wild-type phage (λOct-2) and an altered specificity (λOct-2 V47R D41G) recombinant phage. OCTA and A7G denote the wild-type and mutant probes respectively. Each probe contains three copies of the relevant sequence. (D) Results of screening filters generated from platings of the wild-type (λOct-2) and λOct-2 V47R N51R recombinant phage.

library expressing mutant forms of the transcription factor; (iii) screening the mutant expression library with the binding site probe; and (iv) characterization of the binding properties of the mutant protein in solution. We demonstrate the utility of this approach with two distinct altered specificity Oct-2 mutants.

In order to identify severe mutations in the octamer site, we performed a systematic single base substitution mutagenesis of the AAAT subsite. All possible single base substitutions in the AAAT subsite (with the exception of A5T) were engineered and tested for their ability to be recognized by Oct-2 and Oct-1 (Figure 2A). The A5T mutation had been shown previously to reduce affinity only 2-fold (Verrijzer *et al.*, 1992). The relative affinity of Oct-2 and Oct-1 for these mutant sites versus the wild-type site were also measured by competition analyses using gel-shift assays (data not shown). Six single base mutations were found to reduce the affinity of both proteins

>50-fold (A5G, A5C, A6C, A7T, A7G and A7C). None of the mutations at position 8 were as severe. Any of the six severe mutants were suitable for screening a mutant Oct-2 protein library for a corresponding altered specificity protein. We selected a mutation at A7, because this base makes specific and highly conserved hydrogen bond contacts with the amino acid N51 in the POU homeodomain recognition helix. Of the three possible substitutions of A7, A7G was used as it was the most severe mutation.

To isolate an altered specificity mutant Oct-2 that can recognize the A7G site with higher affinity, three residues in the POU homeodomain recognition helix (Val47, Cys50 and Asn51) were chosen for randomization (Figure 2B). We mutated codons for Val47 and Asn51 with the view that in classical homeodomain proteins, amino acids at these positions are in close proximity to the mutated base (Kissinger *et al.*, 1990). Furthermore, in the case of

classical homeodomains, position 9 (corresponding to Cys50) was shown to be a critical determinant of binding specificity (Hanes and Brent, 1989; Treisman *et al.*, 1989). A library of all possible single amino acid substitutions at these three positions was generated using a λ gt11 phage that encoded a β -gal-Oct-2 POU domain fusion protein (see Materials and methods).

Screening of 30 000 clones of the Oct-2 mutant library with the A7G probe resulted in the isolation of two identical recombinant phage. The protein encoded by the mutant phage contained two amino acid substitutions: Val47 to arginine (V47R) in the POU homeodomain and Asp41 to glycine (D41G) in the POU-specific domain (numbering follows that used in Klemm *et al.*, 1994). The mutant phage is designated λ Oct-2 V47R D41G, while λ Oct-2 denotes phage encoding wild-type Oct-2. In filter screening assays, Oct-2 V47R D41G has altered specificity; it binds the A7G site with increased affinity over the wild-type protein and retains high affinity recognition for the wild-type site (Figure 2C). Although the D41G mutation was probably generated by the infidelity of *Taq* polymerase during the PCR-based mutagenesis, it proved to be important for the binding properties of the mutant protein (see below).

As this phase of the study was being completed, Pomerantz and Sharp (1994) reported the isolation of an altered specificity mutant Oct-1 POU domain by screening a plasmid-based expression library. Coincidentally, the Oct-1 mutant POU domain was also isolated in a screen using a mutant A7G octamer. This mutant protein contained two amino acid substitutions, V47R and N51R, both in the POU homeodomain. Note that one of the mutations is shared with the V47R D41G mutant Oct-2 protein described above, and the other is different. The reason that the V47R N51R mutation was not isolated in our screen is because our library was not constructed to contain double mutations in the POU homeodomain. To demonstrate that our method can readily detect different altered specificity mutants, we constructed and screened an Oct-2 mutant phage carrying the V47R N51R mutations (Figure 2D). It can be seen that both types of Oct-2 mutant phage (V47R D41G and V47R N51R) give signals comparable with the A7G probe.

Binding properties of Oct-2 altered specificity mutants

To permit a direct comparison of the two types of altered specificity mutants that can recognize the A7G site with increased affinity as compared with wild-type, and to test if the D41G mutation was playing a role in alteration of specificity, the single and double mutations were engineered in the context of a full-length Oct-2 cDNA. It should be noted that the binding properties of the V47R N51R mutant protein, as reported by Pomerantz and Sharp (1994), were analyzed using a bacterially expressed protein A-Oct-1 POU domain fusion and not a native protein. Radiolabeled full-length Oct-2 proteins were produced by *in vitro* transcription and translation. The proteins were then tested for their ability to bind a wild-type octamer and the A7G mutant site by gel-shift assays (Figure 3A). Under these conditions, wild-type Oct-2 protein bound to the octamer with >100-fold higher affinity ($K_d = 2.5$ nM) than to the A7G mutant site. The V47R mutant protein

Fig. 3. (A) Comparison of DNA-binding properties of altered specificity mutants of Oct-2. Oct-2 protein–DNA complexes resulting from gel-shift assays, using the indicated proteins and the OCTA or A7G probes, are shown. Binding assays employed equivalent amounts of each *in vitro* translated protein. P indicates the lanes containing free OCTA or A7G probes. F denotes the position of free probe. (B) Altered specificity Oct-2 proteins rescue a mutant Ig promoter *in vivo*. The wild-type (V κ) or A7G mutant (V κ *) Ig promoter constructs (5 μ g each) were transiently transfected into A20.1 B cells along with a reference plasmid (2 μ g). Transfections in lanes 3–5 also included the indicated Oct-2 expression constructs (20 μ g each). Transfections in lanes 1 and 2 included an equivalent amount of the parent expression vector. Total RNA was assayed by RNase protection for accurately initiated transcripts (AIT) from the V κ promoters. Protection fragments from the reference plasmid transcripts are indicated by REF. Lane P contains the riboprobe and lane t represents a control hybridization with tRNA. Lane M contains molecular weight markers generated with an *Msp*I digest of pBR322 DNA.

exhibited 1.5-fold reduced affinity for the wild-type octamer, but 2.5-fold increased affinity for the A7G site, as compared with the wild-type protein. All relative affinity and dissociation constant (K_d) measurements are based on gel-shift assays employing equal amounts of radiolabeled proteins (see Materials and methods). Importantly, the V47R D41G double mutant protein bound the octamer with 2-fold reduced affinity and the A7G site with 4-fold higher affinity ($K_d = 90$ nM) compared with wild-type Oct-2. Thus the D41G mutation is important for the DNA-binding properties of the V47R D41G Oct-2 protein. This mutant protein has a selectivity ratio (OCTA/A7G) of 13. In contrast, the wild-type protein has a selectivity ratio of >100. The V47R N51R double mutant exhibited a 3-fold higher affinity for the A7G site ($K_d = 30$ nM) than the V47R D41G double mutant. Also, it bound less well to the octamer element than V47R D41G and displayed a selectivity ratio of 1. We also assessed the consequence of combining the three mutations, V47R, N51R and D41G, to see if this would further increase affinity for the A7G site. The triple mutant V47R N51R D41G did not have higher affinity for A7G than V47R N51R (Figure 3A). We examined the specificity of these proteins by measuring affinity for other severe octamer mutations. The V47R D41G and V47R N51R Oct-2 mutant proteins did not bind the A6C and A5C octamer mutant sites with higher affinity (data not shown). Further-

Fig. 4. Oct-2 and Oct-1 are redundant activators of an Ig promoter in B cells. (A) The POU domain V47R N51R (RR) mutation alters the DNA-binding properties of Oct-1 and Oct-2 equivalently. Protein-DNA complexes resulting from gel-shift assays using the indicated *in vitro* translated proteins and the OCTA, A7G or λB probes are shown. P indicates reactions in which each of the probes was incubated with unprogrammed reticulocyte lysate. Protein-DNA complexes observed in these reactions (lanes 1, 6 and 11) were non-specific. (B) Both Oct-2RR and Oct-1RR can restore activity of the mutant Ig promoter (Vκ*) in B cells. Transient transfections and RNase protection analysis using the indicated reporter (5 μg each) and expression constructs (10 μg each) were performed with MPC11 B cells as described for Figure 3B. (C) Oct-1RR- and Oct-2RR-mediated rescue of a mutant histone H2B promoter (H2B*) in MPC11 B cells. The mutant H2B promoter contains an A7G mutation in its conserved octamer element. (D) Western blot showing the levels of HA-tagged octamer proteins expressed in MPC11 B cells transiently transfected with the indicated expression vectors (10 μg each). Immunoreactive proteins in whole cell lysates were detected using the 12CA5 anti-hemagglutinin antibody. The positions of HA-Oct-1 and HA-Oct-2 proteins are indicated. IVT HA-Oct-1 and IVT HA-Oct-2 are *in vitro* translated proteins used as markers.

more, these mutant proteins do not have a general increase in non-specific binding affinity for DNA, because they do not recognize unrelated sites (see below; Figure 4A).

Altered specificity Oct-2 proteins rescue an A7G mutant Ig promoter *in vivo*

For functional analysis, we used a β-globin reporter construct driven by an Ig promoter (see Materials and methods). The promoter was from the kappa light chain gene of the myeloma, MOPC41 (Bergman *et al.*, 1984). This promoter (Vκ) contains an octamer element 45 bp upstream of the TATA box, and is preferentially active in B cells. The reporter construct contains an SV40 enhancer downstream of the β-globin gene, since the enhancerless construct has very weak activity. The A7G mutation was introduced into this Vκ promoter (Vκ*) and assayed by transient transfection into the B cell line A20.1. Accurately initiated transcripts (AIT) from wild-type or mutant Vκ promoters were quantitated using ribonuclease protection

assays after normalizing to transcripts from a reference construct (REF). All measurements of promoter activity represent an average of at least four independent experiments (see Materials and methods). The mutant promoter showed >10-fold reduced activity in B cells (Figure 3B, lanes 1 and 2). Expression constructs encoding wild-type Oct-2, Oct-2 V47R D41G and Oct-2 V47R N51R were used to examine the ability of each protein to rescue the function of the mutant promoter. Expression of Oct-2 V47R N51R resulted in a 4.5-fold activation of the mutant promoter (lane 4) and Oct-2 V47R D41G in 2-fold activation (lane 5). The control construct expressing wild-type Oct-2 did not activate the Vκ* promoter (lane 3). Furthermore, the altered specificity Oct-2 proteins did not stimulate transcription non-specifically, as evidenced by uniform levels of transcripts from the co-introduced reference construct. Stronger activation by Oct-2 V47R N51R compared with Oct-2 V47R D41G is probably due to its higher affinity for the A7G site. The substantial and

specific restoration of mutant promoter activity by Oct-2 V47R N51R provides the first direct demonstration of Oct-2's ability to regulate the activity of an Ig gene promoter in B cells. Furthermore, this approach permits an unambiguous assay of exogenously introduced Oct-2 in B cells without the results being obscured by the activities of endogenous Oct-2 and Oct-1.

Oct-2 and Oct-1 are redundant activators of immunoglobulin promoters in B cells

Given the above results, the POU domain V47R N51R (RR) mutation could be used to compare the ability of Oct-2 and Oct-1 to rescue the activity of various octamer-dependent promoters (Figure 1). The RR mutation was engineered into hemagglutinin (HA) epitope-tagged full-length Oct-2 and Oct-1 cDNAs. Epitope tagging was done to permit an accurate comparison of the relative levels of expression of the two activators in the transfected cells. As expected, the RR mutation altered the binding properties of Oct-1 in an identical manner to Oct-2 (Figure 4A). Both Oct-1RR and Oct-2RR mutant proteins have selectivity ratios (OCTA/A7G) of 1 and bind the A7G site with 12-fold higher affinity than their wild-type counterparts. Furthermore, neither of the two proteins bind detectably to a non-specific DNA probe (λ B).

The wild-type and altered specificity mutant versions of both proteins were tested for their ability to activate the $V\kappa^*$ Ig promoter in B cells (Figure 4B). Transient transfections were performed in the B cell line MPC11, because its higher transfectability permitted detection of transiently expressed proteins by Western blotting. Both Oct-1RR and Oct-2RR altered specificity mutants substantially restored activity of the mutant $V\kappa^*$ promoter, 12- and 15-fold respectively (Figure 4B, lanes 4 and 6). In contrast, expression of wild-type Oct-1 or Oct-2 showed a very low degree of activation, probably mediated by residual DNA binding (lanes 3 and 5). Similar results were obtained in the B cell line A20.1 (data not shown).

Immunoglobulin heavy chain promoters contain an octamer element in the opposite orientation to that of light chain promoters. To assess the ability of Oct-1 and Oct-2 to function from this context, we also made the A7G mutation in a V_H promoter and tested for rescue of promoter activity with the altered specificity mutants. Both Oct-1RR and Oct-2RR were able to restore significant activity to the A7G mutant V_H promoter (see below, Figure 7A). These experiments provide the first demonstration that Oct-2 and Oct-1 are functionally redundant in regulating Ig gene promoters in B cells.

Both Oct-2 and Oct-1 can regulate the activity of a histone H2B promoter in B cells

We next wanted to test if Oct-1 and Oct-2 could similarly regulate the activity of an octamer-dependent, but non-cell type-specific promoter. The histone H2B promoter is ubiquitously active and has been shown previously to contain a functionally critical octamer element (Fletcher *et al.*, 1987). For this analysis, we made use of a β -globin reporter construct driven by a histone H2B promoter in place of the Ig promoter. The octamer element in this promoter is 8 bp upstream of the TATA box (Wang *et al.*, 1996). The wild-type construct (H2B) or a mutant version containing the A7G mutation in the octamer element

(H2B*) were transfected into MPC11 B cells along with expression plasmids encoding the Oct-1 or Oct-2 altered specificity proteins. The A7G mutation caused a significant reduction in accurately initiated transcripts from the H2B promoter (6-fold, Figure 4C, lanes 1 and 2). Interestingly, both Oct-1RR and Oct-2RR rescued the activity of the mutant H2B promoter, 4- and 5-fold respectively (Figure 4C, lanes 4 and 6). Control transfections using wild-type Oct-1 and Oct-2 expression vectors exhibited a low degree of activation (2-fold, Figure 4C, lanes 3 and 5). Thus, while Oct-2 is expressed in a cell type-restricted manner, it, like Oct-1, is capable of activating a housekeeping gene promoter in B cells.

Figure 4D shows the levels of epitope-tagged Oct-1 and Oct-2 proteins in the transiently transfected B cells. Under conditions using equivalent amounts of expression vectors (10 μ g), the Oct-1 and Oct-1RR proteins were expressed at 5-fold higher levels than Oct-2 and Oct-2RR. It should be noted that the RR mutation does not affect the stability of either Oct-1 or Oct-2. We subsequently determined, by titrating various amounts of expression vectors, that 3 μ g of Oct-1/Oct-1RR expression vectors and 10 μ g of Oct-2/Oct-2RR expression vectors result in equivalent levels of expression of the four proteins (data not shown). Under these conditions, Oct-2RR activates Ig and H2B promoters 2-fold better than Oct-1RR (see below, Figure 6).

Efficient activation of Ig promoters in non-B cells requires OCA-B

A B cell-specific co-activator designated OCA-B/Bob1/OBF-1 has been described as a key regulator of Ig gene transcription (Gstaiger *et al.*, 1995; Luo *et al.*, 1995; Strubin *et al.*, 1995). OCA-B activates Ig promoters via protein-protein interactions with the POU domains of promoter-bound Oct-1 or Oct-2. To compare the relative potencies of Oct-1 and Oct-2 in activating Ig promoters in the absence or presence of OCA-B, we performed the experiments described below.

Wild-type and mutant $V\kappa$ - β -globin reporter constructs were transiently transfected into HeLa cells along with the indicated expression constructs (Figure 5A). These cells express endogenous Oct-1 but not Oct-2 or OCA-B. As has been reported by others, the MOPC41 $V\kappa$ promoter has detectable activity in non-B cells (Kemler *et al.*, 1991). This activity is octamer and Oct-1 dependent, as it is reduced significantly by the A7G mutation (Figure 5A, lanes 1 and 2). Expression of Oct-1RR and Oct-2RR activated the mutant Ig promoter comparably in the absence of OCA-B (4-fold, lanes 4 and 6). OCA-B did not activate the $V\kappa^*$ by itself (lane 3). However, co-expression of OCA-B with Oct-1RR or Oct-2RR further stimulated transcription (10-fold above the basal level, lanes 5 and 7). Neither wild-type Oct-1 nor Oct-2 significantly activated $V\kappa^*$ (data not shown). Western blot analysis of the transiently expressed proteins showed that Oct-1 and Oct-1RR were at 5-fold higher levels than Oct-2 and Oct-2RR (Figure 5B). Under conditions of equivalent expression of Oct-1RR and Oct-2RR (reduced amounts of Oct-1RR expression plasmid), Oct-2RR activates the Ig promoter 3-fold better than Oct-1RR (data not shown). These results are similar to those obtained in the transfections of MPC11 B cells (Figure 6). Furthermore, they

Fig. 5. (A) Oct-2RR and Oct-1RR function with OCA-B to rescue the activity of a mutant Ig V_k promoter optimally in non-B cells. Transient transfections and RNase protection analysis using the indicated reporter and expression constructs (15 μ g of Oct-1/2, 4 μ g of OCA-B) were performed with HeLa cells as described in Materials and Methods. AIT denotes accurately initiated transcripts from the V_k promoter and REF denotes transcripts from the reference plasmid. Lanes M, P and t denote molecular weight markers, free riboprobe and the control tRNA hybridization respectively. (B) Western blot showing the levels of HA-tagged octamer proteins expressed in transiently transfected HeLa cells as described in (A). The positions of HA-Oct-1 and HA-Oct-2 proteins are indicated. The vector control lane contains lysate from cells transfected with the vector only, and shows cross-reactive bands from the cell line.

demonstrate that the RR mutation in the POU domain does not perturb the interaction with OCA-B. The ability of the RR POU domain to interact with OCA-B has been verified by performing gel-shift assays (data not shown). Therefore, by expressing the mutant octamer-binding proteins and wild-type OCA-B, we have reconstituted the components regulating Ig promoter activity without being hampered by endogenous Oct-1.

POU domain-mediated rescue of immunoglobulin and histone promoters

Despite the fact that Oct-1 and Oct-2 have related DNA-binding domains, overall these two proteins are structurally quite different. Intriguingly, we have demonstrated that they function as redundant activators of Ig and histone H2B promoters. The structural basis of this redundancy may reside in the two proteins utilizing distinct types of activation domains. Alternatively, the redundancy may be the result of the transcriptional activation properties of the conserved POU domain. To examine this issue, we compared the ability of the altered specificity full-length proteins with that of their POU domain counterparts to activate the V_k^* and H2B* promoters. Both altered specificity POU domains were able to restore activity (12- to 15-fold) of the V_k^* promoter significantly in MPC11 B cells (Figure 6A, lanes 5 and 7). This level of restoration is similar to that seen with the corresponding full-length proteins (lanes 2 and 3). Importantly wild-type POU domains did not activate the mutant promoter (lanes 4 and 6). It should be noted that the POU domain constructs were HA-tagged like their wild-type counterparts, and were expressed comparably with each other but at higher levels than the full-length proteins (data not shown). Similar results were obtained with the histone H2B promoter. However, in this case, the overall level of activation

Fig. 6. (A) Altered specificity POU domains can rescue the activity of a mutant V_k promoter. Transient transfections and RNase protection analyses were performed in MPC11 B cells using the indicated expression and reporter constructs as detailed in Figure 4B. All expression plasmids were used at 10 μ g, with the exception of Oct-1RR, 3 μ g. Both the full-length and POU domain proteins were tagged with the HA epitope to enable detection. (B) Rescue of mutant histone H2B promoter activity by altered specificity POU domains. Analyses were performed as described in (A).

with the altered specificity derivatives was somewhat lower (5- to 8-fold above basal levels, Figure 6B). Therefore, the basis of functional redundancy of these two related activators resides in their conserved DNA-binding POU domain.

Ig enhancers dictate the relative potency of Oct-1 versus Oct-2 from Ig gene promoters

In the experiments described thus far, we have used a downstream SV40 enhancer in conjunction with both cell type-specific and ubiquitously active promoters. This was necessary because the activities of enhancerless wild-type promoter constructs were too weak to be detected reliably by RNase protection assays. The SV40 enhancer was used because it has been shown to act in conjunction with a variety of heterologous promoters and in numerous cell types. We next used the altered specificity mutants to examine the activities of Oct-1 and Oct-2 from Ig promoters in concert with their cognate enhancers from the Ig loci. The heavy chain intron enhancer (IE_H) is active throughout various stages of B cell development and has also been shown to be important for V(D)J recombination (Serwe and Sablitzky, 1993; Zou *et al.*, 1993). The Ig heavy chain locus contains an additional potent enhancer downstream of the C α constant gene segments (3'E_H). This enhancer preferentially functions in activated B cells and plasmacytes (Pettersson *et al.*, 1990; Lieberson *et al.*, 1995). A series of β -globin reporter constructs were generated in which the wild-type (V_H) or A7G mutant (V_H*) promoters were linked to either the heavy chain intronic or 3'-enhancers, or the SV40 enhancer. Each of these enhancers was positioned downstream of the β -globin reporter gene. The mutant promoter constructs were co-transfected with expression vectors encoding the altered specificity Oct-1 or Oct-2 proteins. In these transfections, the amounts of the two expression vectors were adjusted so as to express equivalent amounts of the two altered specificity derivatives (3 μ g of Oct-1RR and 10 μ g of Oct-2RR expression plasmid). Results of these transient transfection analyses are shown in Figure 7A. An A7G mutation in the octamer element drastically reduced the activity of reporter gene expression when the V_H promoter was coupled to any of the three enhancers (Figure 7A, lanes 1 and 2, 5 and 6, 9 and 10). As seen previously with the V κ * and H2B* promoters, both Oct-1RR and Oct-2RR could activate transcription from the V_H* promoter in conjunction with three distinct enhancers. However, when expressed at equivalent levels, Oct-1RR was a 2- to 3-fold more potent activator than Oct-2RR when functioning from a V_H* promoter coupled to either of the heavy chain enhancers. In contrast, Oct-2RR was a stronger activator than Oct-1RR from a V_H* promoter coupled to the SV40 enhancer (2-fold). Similar results were obtained using the V κ promoter in conjunction with cognate enhancers (intronic or 3') from the kappa locus (Figure 7B). Thus, the enhancer to which an Ig promoter is coupled significantly influences the relative potency of promoter-bound Oct-1 versus Oct-2. To determine if the POU domain was sufficient to permit the more potent action of Oct-1 in these contexts, the activities of Oct-1RR POU and Oct-2RR POU were assayed using the V κ *-3'E κ reporter construct. Unlike the full-length proteins, both altered specificity POU domains stimulated transcription equivalently (Figure 7C). Therefore, the more potent activity of Oct-1 from Ig promoters in conjunction with Ig enhancers is dependent on a region external to the POU domain. To our knowledge, this is the first demonstration of enhancers dictating the relative potencies

of related activators functioning from the same site at a promoter.

Discussion

Regulation of Ig gene transcription by Oct-1 and Oct-2 in B lymphocytes

The identical binding properties but differing distributions of Oct-1 and Oct-2 led to the initial idea that Oct-1, being ubiquitously expressed, regulates the expression of octamer-dependent housekeeping genes, whereas Oct-2 regulates transcription of Ig genes through functionally critical octamer elements in their promoters (Schaffner, 1989). This model was disproved by gene targeting of the *oct-2* locus in mice and somatic B cells. Ig transcription was shown to occur at wild-type levels in *oct-2* $-/-$ pre-B and B cells (Corcoran *et al.*, 1993; Feldhaus *et al.*, 1993). However, gene targeting experiments were unable to distinguish between redundancy and selectivity of these activators in regulating Ig genes. Results from the genetic studies, along with the discovery of the B cell-specific co-activator OCA-B, led to a revised view of the role of these activators. According to this model, OCA-B promotes B cell-specific transcription of Ig genes by interacting with either Oct-1 or Oct-2 (Luo *et al.*, 1995).

We wanted to address whether Oct-1 and Oct-2 are redundant or selective activators of Ig genes in B cells. Using equivalent Oct-1 and Oct-2 altered specificity mutants, we show that either factor can specifically promote transcription from Ig gene promoters in B cells. This represents the first direct demonstration of functional redundancy of these two transcriptional activators with respect to Ig promoters in the physiologically relevant context. Interestingly, Oct-1 is shown to be the more potent activator from an Ig promoter in conjunction with cognate Ig gene enhancers in B cells. This result provides molecular evidence that Oct-1 may be the critical regulator of Ig gene transcription during B cell development. Consistent with this view, developing B lineage cells express high levels of Oct-1, but significantly lower levels of Oct-2 (Miller *et al.*, 1991). The levels of OCA-B during early B cell development have not been reported. Furthermore, OCA-B-deficient mice show no impairment of Ig gene expression during B cell development (Kim *et al.*, 1996; Schubart *et al.*, 1996). Therefore, the basal levels of Ig gene transcription seen during the antigen-independent phase of B cell development may be regulated predominantly by Oct-1. Activation and terminal differentiation of B lymphocytes induced by antigen encounter result in a large increase in transcription of Ig genes. Such cells contain high levels of both Oct-2 and OCA-B in addition to Oct-1. The induced state of Ig gene transcription may be dependent on the overall concentration of Oct-1 and Oct-2, as well as the co-activator OCA-B. The former could effect increased occupancy of Ig promoters and the latter increased transcriptional efficiency. In this regard, *oct-2* null mice, as well as *scid* mice reconstituted with *oct-2* $-/-$ fetal liver progenitors, do have significantly reduced serum Ig levels (Corcoran *et al.*, 1993; Corcoran and Karvelas, 1994). Whereas this reduction has been attributed solely to a B cell proliferative defect upon antigen or lipopolysaccharide-mediated activation, it may be due additionally to the inability to transcribe Ig genes

Fig. 7. Relative potency of Oct-1 versus Oct-2 at an Ig promoter is influenced by distal enhancers. (A) The relative potency of Oct-1RR versus Oct-2RR examined on a V_H^* promoter with three different downstream enhancers. The reporter constructs contain a wild-type Ig promoter (V_H) or an octamer mutant (A7G) counterpart (V_H^*). Each of these promoters is coupled to one of three enhancers: Ig heavy chain intron (IE_H), 3' heavy chain ($3'E_H$) or SV40 (E_{SV}). (B) Relative potency of Oct-1RR versus Oct-2RR from a V_K^* promoter in combination with either kappa light chain intron enhancer (IE_K), 3' kappa enhancer ($3'E_K$) or SV40 enhancer (E_{SV}). (C) Relative potency of Oct-1RR POU versus Oct-2RR POU from a V_K^* promoter in conjunction with the 3' kappa chain enhancer ($3'E_K$). Transient transfections and RNase protection analyses were performed in MPC11 B cells as described in Figure 4B. The following amounts of expression vectors were used: Oct-1RR (3 μ g), Oct-2RR (10 μ g), Oct-1RR POU (3 μ g) and Oct-2RR POU (3 μ g).

optimally due to a reduction in the total level of octamer binding activators.

It has been proposed that Oct-1 regulates transcription of the housekeeping genes encoding histone H2B and U2 and U6 snRNAs (Fletcher *et al.*, 1987). Using altered specificity mutants, we directly compared the ability of Oct-1 and Oct-2 to regulate the activity of a histone H2B promoter in B cells. We demonstrate that both activators can regulate the activity of this non-cell-type-specific promoter. Although Oct-2 is clearly dispensable for H2B transcription, since it is not expressed in many cell types, its ability to function from this and other housekeeping gene promoters may be important during B cell activation. Antigen-driven activation of B lymphocytes results in extensive proliferation and high level expression of Ig genes. Therefore, there is a simultaneous demand for high level transcription of housekeeping genes, such as histone H2B and snRNAs, as well as Ig genes. It is possible that this demand represented the selection pressure to evolve the two related transcription factors, Oct-1 and Oct-2.

Transcriptional regulation by the POU domain

We show that the conserved DNA-binding POU domain of Oct-1 and Oct-2 can significantly promote transcription from Ig and histone H2B promoters. In the case of the Ig promoters, this activity may be explained by the ability of the POU domain to recruit the co-activator OCA-B (Luo *et al.*, 1995). Alternatively, the POU domain may have an activation potential that is not dependent on co-activators. In fact, Zwilling *et al.* (1994) have demonstrated that the POU domains of Oct-1 and Oct-2 physically

interact with TATA box-binding protein. This interaction may facilitate pre-initiation complex formation directly. In this regard, the Oct-1 POU domain stimulates snRNA gene transcription by direct interactions with SNAP_C (Mittal *et al.*, 1996). We show that the POU domain also activates transcription from the histone H2B promoter. The activity of this promoter is not dependent on OCA-B (Gstaiger *et al.*, 1995; Luo *et al.*, 1995). Thus it is likely that the POU domain stimulates transcription by promoting formation of pre-initiation complexes through specific interactions with general transcription factors. This intrinsic activity can be augmented through interactions with co-activators and linkage with activation domains.

Activator potency at a promoter is influenced by distal enhancers

Although Oct-1 and Oct-2 are functionally redundant as activators of Ig and H2B promoters in B cells, their relative potencies are different. In conjunction with a non-Ig enhancer (SV40), Oct-2 is more potent relative to Oct-1 from these promoters. Interestingly, this relative potency is reversed when Ig enhancers from the heavy or light chain loci are used with their cognate Ig V_H or V_K promoters. The more potent activity of Oct-1 in such a context depends on an activation region external to the POU domain. These observations represent a unique demonstration that the relative potency of two related activators binding to the same site in the promoter can be significantly influenced by the nature of the distal enhancer. Thus, differential action of a family of related factors binding to the same regulatory site provides another

mechanism for diversifying and specifying unique patterns of gene expression. Furthermore, this represents a powerful evolutionary strategy for extending the utility of the interaction between a conserved DNA-binding domain and its cognate site differentially to regulate gene activity.

The *oct-2* $-/-$ mice have a defect in expression of specific Ig isotypes (namely IgM, IgG1, IgG3 and IgG2B, but not IgA) (Corcoran and Karvelas, 1994). Similarly, Kim *et al.* (1996) have demonstrated that OCA-B $-/-$ B cells have a severe defect in expression of secondary Ig isotypes (all IgG subtypes, IgA and IgE) despite a normal ability to undergo switch recombination. They have suggested that closer apposition of the Ig V_H promoter to the more distal 3'-enhancer, brought about by switching, may increase the dependency of the promoter on OCA-B. Our observation that the potency of Oct-1 and Oct-2 at a V_H promoter can be influenced significantly by the nature of the distal enhancer provides evidence for this hypothesis. It also suggests the possibility that different switched IgH genes differ in their dependence on Oct-2 and/or OCA-B due to the relative configuration of downstream transcriptional enhancers.

DNA recognition of the A7G octamer by Oct-2/Oct-1 altered specificity mutants

To achieve increased affinity for the A7G mutant octamer site, at least two amino acid substitutions in the POU domain are necessary. In the case of the V47R N51R altered specificity mutant, both substitutions lie in the recognition helix of the POU homeodomain. However, in the V47R D41G mutant, one substitution lies in the POU-specific domain. Amino acid D41 is located in the unique extended turn between helices 2 and 3 of the POU-specific domain's helix-turn-helix motif. This residue has not previously been considered to be important for the binding of the Oct-1 POU domain to the octamer element in the crystallographic structure (Klemm *et al.*, 1994). Examination of the crystal structure reveals that this residue is only 4.4 Å away from the negatively charged phosphate residue between G4 and T5 of the octamer (Figure 2B). The charge repulsion due to this proximity is removed by substitution of the negatively charged aspartic acid residue with a neutral glycine. This may result in an increased affinity for weaker binding sites where the energetic contribution of this interaction becomes relatively important in combination with a favorable change at Val47.

Isolation of altered specificity mutants by expression screening

Given that a widely used method for the cloning of transcription factors involves screening a λ gt11 cDNA expression library with a radiolabeled binding site, it was appealing to modify this approach so as to extend it to the isolation of altered DNA-binding specificity mutants (Singh, 1993). The simplicity and power of this method made it an attractive choice in relation to other strategies for the isolation of altered specificity mutants (Strubin and Struhl, 1992; Hill *et al.*, 1993; Rebar and Pabo, 1994). We demonstrate the utility of this approach by successfully screening two distinct altered specificity Oct-2 mutants (D41G V47R and V47R N51R, Figure 2C and D). The V47R N51R was isolated initially using a plasmid-based expression screening strategy in the context of an Oct-1

POU domain fusion protein (Pomerantz and Sharp, 1994). Expression screening for altered specificity mutants provides several advantages over genetic screens in *Escherichia coli* or yeast. First of all it directly assays DNA binding, rather than transcriptional activation or repression. Secondly, it is much simpler to set up. This is especially true for transcription factors that have been isolated originally by screening λ gt11 cDNA expression libraries, such as Oct-1/Oct-2/Pit-1 (POU family), Ets-1/PU.1 (Ets family), E12/HTF-4/TEF-1 (bHLH family), IRF-1/ICSBP/Pip (IRF family), MBP-1/WZF-1 (Zn finger family) and EBP/CREB (leucine zipper family) (reviewed in Singh, 1993).

The general utility and power of the expression screening approach can be exploited more fully in cases where no high resolution structure nor detailed model of the protein-DNA complex exists, and therefore there is no information to suggest which amino acids make base-specific contacts. In combination with a codon-based mutagenesis strategy (Glaser *et al.*, 1992; Cormack and Struhl, 1993), one could readily employ expression screening to scan single and double amino acid substitutions across a large protein segment in tracts of 20 amino acids. All single amino acid substitutions across a 20 amino acid segment can be screened in a library of ~3000 clones (Cormack and Struhl, 1993). Approximately 30% of such a library would also contain double amino acid substitutions. Expression screening conveniently permits analysis of 10⁷ clones. Thus, the approach promises to be a useful method for isolating altered specificity mutants for analysis of transcription factor function as well as in identifying amino acids involved in DNA recognition.

A paradigm for using altered specificity mutants to analyze related transcription factors

Higher eukaryotes express a large number of related transcription factors with overlapping expression patterns. As elaborated in the Introduction, this makes it difficult to dissect the transcriptional regulatory activities of different co-expressed members of such families. This study demonstrates that altered specificity mutant proteins represent powerful tools to examine directly the issues of redundancy versus promoter selectivity among members of a family of related transcription factors. Furthermore, these mutant proteins can be used to explore the molecular basis of these differences *in vivo*. Altered specificity mutant transcription factors have been used previously to establish functionally important interactions with target sequences *in vivo* (Schier and Gehring, 1992; Hill *et al.*, 1993; Klein and Struhl, 1994). However, our study represents the first example in which such mutants are used to distinguish between regulatory activities of related factors.

Materials and methods

Engineering a λ gt11 phage library of Oct-2 DNA-binding domain mutants

A 1.1 kb partial Oct-2 cDNA from λ gt11 3-1 (Clerc *et al.*, 1988) was subcloned into pBS_T to generate pKSOct-2(1.1). The λ gt11 3-1 phage is denoted as λ Oct-2 in the text. The cDNA insert encodes the POU domain and a C-terminal segment of Oct-2A. Codons encoding Val47, Cys50 and Asn51 of the POU homeodomain were randomized individually using sequence overlap extension PCR. Representative clones were sequenced to verify the efficacy and absence of biases in the mutagenesis.

Mutant cDNAs were religated into λ gt11 phage arms and packaged using a Gigapack Gold kit (Stratagene). Recombinant phage resulting from mutagenesis of individual codons were pooled for expression screening. The λ Oct-2 V47R N51R mutant phage was constructed as follows: an *EagI*-*PstI* fragment of pKSOct-2(1.1) was replaced by the corresponding fragment from pHA-Oct-2RR(L); the 1.1 kb *EcoRI* fragment of the resulting construct was ligated into λ gt11 arms and subsequently packaged to generate the phage.

Expression screening

Screening of λ Oct-2 phage and its mutant derivatives was performed using either a wild-type octamer trimer or an A7G mutant octamer trimer. Each probe consisted of a directly repeated array. The sequence of the monomeric wild-type oligonucleotide is given below (see Gel-shift assays). Screening was performed as described by Singh (1993). The A7G mutant probe was used to screen 30 000 recombinants in the λ Oct-2 mutant library. Anti-Oct-2 antibodies were used to screen the recombinant plaques and verify that the mutant clones were producing the same level of Oct-2 protein as the wild-type phage.

Oct-2 and Oct-1 expression constructs

Full-length Oct-2 cDNA from pOEVI+ (Muller *et al.*, 1988) was subcloned into pGEM4Z for *in vitro* expression. This plasmid was used to generate the mutants V47R, D41G, V47R D41G, V47R N51R and D41G V47R N51R. These wild-type and mutant Oct-2 cDNAs were subcloned into pcDNA3 for *in vivo* expression. For expressing HA epitope-tagged proteins, Oct-2 or Oct-1 cDNAs were subcloned into pcDNA3-HA (Brass *et al.*, 1996). This vector introduces the hemagglutinin nonapeptide (12CA5 epitope) at the N-terminus. pHA-Oct-2(L) and pHA-Oct-2RR(L) contain wild-type and Oct-2 mutant (V47R N51R) cDNAs respectively. pHA-Oct-2(S) and pHA-Oct-2RR(S) have a truncated 3'-untranslated region (3'-UTR; 60 nucleotides). They delete a 460 bp *XbaI*-*BstEII* fragment from the 3'-UTR of the Oct-2 cDNA. pHA-Oct-1 was generated by subcloning the Oct-1 cDNA insert from pCMV-Oct-1 into pcDNA3-HA (Voss *et al.*, 1991). In this construct, PCR was used to introduce coding sequence for 21 N-terminal amino acids missing in pCMV-Oct-1 (Das and Herr, 1993). The POU domains of Oct-1 or Oct-2 were isolated by PCR using the following primers: ATGAGGCGGCCGCGAGGAGCCAGTGACCT, GTGGTCTAGATCATGGTTGATTCTTTTTC for Oct-1 and TGGTGGCGGCCGCGAGGAGCCAGTGATCTG, CCCGCTAGATCAGGGGTTGATGCGTTTCTC for Oct-2. The PCR fragments were then subcloned into pcDNA3-HA. Control experiments with *in vitro* translated proteins showed that the tagged Oct-2 and Oct-1 derivatives were equally reactive with the anti-HA antibody.

Reporter constructs and other plasmids

The V κ promoter construct is a derivative of Target-1 that was generated by replacing the minimal β -globin promoter (-43 to -10) with the MOPC41 V κ promoter (-100 to +25) as described by Feldhaus *et al.* (1993). This construct contains an SV40 enhancer (E_{SV}) downstream of the β -globin gene. The V $_H$ and H2B promoter constructs were generated by substituting the V κ promoter with, respectively, a V $_H$ promoter *SalI*-*XbaI* fragment (-154 to +57) and a H2B promoter (-102 to +68) obtained by PCR from the clone mm616-1.4 with the following primers: TCGCTGGATCCGGCATTTC and ACGCTGACTCTGCTGTAATC (Grosschedl and Baltimore, 1985; Wang *et al.*, 1996). The V κ^* , V $_H^*$ and H2B* reporter constructs contain an A7G mutation in the octamer element of the corresponding promoters. Accurately initiated transcripts from the wild-type and mutant kappa promoters were assayed using a riboprobe synthesized using pSKV κ 41 (Feldhaus *et al.*, 1993). The templates used to synthesize the V $_H$ and H2B riboprobes were generated by replacing the V κ promoter in pSKV κ 41 with the V $_H$ and H2B promoters respectively. The V $_H$ -IE $_H$ and V $_H$ -3'E $_H$ constructs were made by replacing the SV40 enhancer in the V $_H$ promoter construct with an *XbaI* intron-enhancer and a *StuI* 3'-enhancer heavy chain fragment, respectively (Dariavach *et al.*, 1991). The V κ -IE κ and V κ -3'E κ constructs were made by replacing the SV40 enhancer in the V κ construct, with the intron (*AluI* fragment) and 3' kappa (*Sac*-*XbaI* fragment) enhancers, respectively. Other plasmids used in this study have been described previously: pCATCH, pCATCH-NLS-Bob1 (Gstaiger *et al.*, 1995), Target-1 and OVEC-Ref (Muller *et al.*, 1988).

In vitro transcription and translation

In vitro translations were performed using 1 μ g of the indicated plasmid in 50 μ l of TNT-coupled transcription/translation reaction (Promega) as per the manufacturer's instructions. [35 S]methionine-labeled proteins

were resolved in a 10% SDS-polyacrylamide gel, dried and quantitated on a PhosphorImager system (Molecular Dynamics).

Gel-shift assays

Binding of Oct-2 and Oct-1 proteins was analyzed using a gel-shift assay with the following probe or its mutant derivatives: 5'-GAT-CCTTAATAATTTGCATACCTCA-3' 3'-GAATTATTAACGTATGGAGTCTAG-5'.

The octamer sequence and flanking base pairs are derived from the MOPC41 V κ promoter. To test for non-specific binding, control λ B sequence derived from an Ig lambda enhancer was used (Brass *et al.*, 1996). Complementary oligonucleotides were annealed and end labeled with [α - 32 P]dATP and the Klenow fragment. Nuclear extracts were prepared as described by Singh *et al.* (1986). Nuclear extract protein (20 μ g) or *in vitro* translated protein (10–30 fmol) was incubated with 4 or 1 μ g of poly(dIdC), respectively, and 5×10^5 c.p.m. (6–20 fmol) of the octamer probe in 20 μ l of binding buffer for 30 min at 25°C. The resulting complexes were resolved on a 6% native polyacrylamide gel using 1 $\times$ TGE buffer (Staudt *et al.*, 1988). Quantitation of protein-DNA complexes was performed using a PhosphorImager system (Molecular Dynamics). Relative affinity measurements represent an average of at least five independent binding experiments. Dissociation constants were determined using the following relationship, $K_d = ([P]_t - [D]_b) [D]_f / [D]_b$ where $[P]_t$ is total protein, $[D]_b$ is bound DNA and $[D]_f$ is free DNA.

Western analysis

Protein samples were resolved in a 10% SDS-polyacrylamide gel and transferred to a Immobilon P membrane (Millipore). Blots were incubated with 1:3000 dilution of rabbit anti-Oct-2 antiserum and 1:5000 dilution of donkey anti-rabbit horseradish peroxidase (HRP) conjugate. The anti-HA (12CA5) mouse monoclonal antibody was used at 2 μ g/ml with a rabbit anti-mouse Ig HRP secondary conjugate at 1:2500. Immuno-reactive proteins were detected using the ECL system (Amersham).

Transient transfection analysis

All transfections were performed at least four times.

MPC11 cells. Seventeen μ g of total DNA were transfected per 5×10^6 cells using a calcium phosphate protocol. This DNA included 3 or 10 μ g of Oct-1 expression construct and 10 μ g of the Oct-2 expression constructs pHA-Oct-2(S)/pHA-Oct-2RR(S), 5 μ g of the reporter construct and 2 μ g of the reference plasmid OVEC-Ref. The total amount of transfected DNA was kept constant in each transfection by including a compensating amount of the parent cytomegalovirus (CMV)-based expression vector pcDNA3. Total RNA was isolated 40–42 h after transfection.

A20.1 cells. Thirty μ g of total DNA were transfected per 10^7 cells using a DEAE-dextran-based protocol as described previously (Feldhaus *et al.*, 1993). This DNA included 20 μ g of expression vector, 5 μ g of reporter, 2 μ g of reference construct OVEC-Ref and 3 μ g of pBst carrier DNA. pHA-Oct-2(L) and pHA-Oct-2RR(L) were used for expressing Oct-2 and its derivatives in this cell line.

HeLa cells. Twenty-one μ g of total DNA were transfected per 10^6 cells using a calcium phosphate protocol. This DNA included 1, 5 or 15 μ g of Oct-2/1 expression construct, 4 μ g of pBst and 1 μ g each of the reporter construct and the reference plasmid OVEC-Ref. The total amount of transfected DNA was kept constant in each transfection by including a compensating amount of the parent CMV-based expression vector pcDNA3. In transfection experiments involving OCA-B, 4 μ g of pCATCH-NLS-Bob1 was used in place of pBst and pCATCH used as the parent empty expression vector. pHA-Oct-2(L) and pHA-Oct-2RR(L) were used for expressing Oct-2 and its derivatives in this cell line. Total RNA was isolated 40–42 h after transfection.

RNA isolation and analysis

Total cellular RNA was isolated from transiently transfected cells using RNazol B (Tel-Test). RNase protection analysis was performed using 10 μ g of total RNA with the pSKV κ 41 riboprobe, essentially as described previously (Miller *et al.*, 1991). Accurately initiated transcripts from the V κ promoter constructs generate a 240 nucleotide protection product. Reference transcripts from OVEC-Ref produce a 158 nucleotide protection product.

Acknowledgements

We would like to thank W.Schaffner and M.Gstaiger for providing the plasmids pCATCH and pCATCH-NLS-Bob1, W.Marzluff for the clone

mm616-1.4, R.Grosschedl for the V_H promoter construct and S.Pettersson for the Ig enhancer constructs. We also thank L.Staudt for the λ3-1 Oct-2 phage. The rabbit anti-Oct-2 antiserum was a gift of P.Sharp. We thank J.Klemm and C.Pabo for making coordinates of Oct-1 available before publication, and N.Assa-Munt and P.Wright for the NMR coordinates of Oct-1. We thank S.Sihag and S.Hsu for technical assistance and A.Brass and J.Walsh for their critical comments. This work was supported by the Howard Hughes Medical Institute. P.C.S. was supported in part by an NIH training grant in Biotechnology (GM08369).

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Received on July 16, 1997; revised on August 26, 1997