The EMBO Journal vol.13 no.24 pp.5967–5976, 1994

Transformation by homeobox genes can be mediated by selective transcriptional repression

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Communicated by W.Schaffner

Altered transcription is a recurrent theme in the field of cancer biology. But despite the central role of transcription in transformation, little is known about the mechanism by which dominant nuclear oncogenes induce malignancies. Homeobox family proteins are prominent examples of transcriptional regulators which control development and can function as oncogenes. Here we explore the molecular basis for transformation by this class of regulators using Oct-2 and Oct-1. We show that the DNA binding POU domains of these proteins are selective and sequence-specific transcriptional repressors that produce malignant lymphomas when they are expressed in T cells of transgenic mice. Mutagenesis experiments identified a specific set of promoters, those containing octamer regulatory elements, as the targets for transformation by selective inhibition of gene expression.

Key words: octamer binding transcription factor/POU domain/transcriptional regulation/transformation/transgenic mice

Introduction

A number of different genetic and epigenetic changes have been associated with malignant transformation. The genetic changes include alterations in genes that encode several categories of cellular and developmental regulators (for reviews see Bishop, 1991; Hunter, 1991). Prominent among these oncogenes are nuclear proteins that are normally involved in transcriptional regulation (reviewed in Cleary, 1991; Lewin, 1991; Rabbitts, 1991). The mutations that convert transcriptional regulators to oncogenes include truncations, translocations, point mutations and insertions that up-regulate expression. These pleiotrophic changes have led a number of investigators to postulate that dominant nuclear oncogenes can transform cells by either induction or inhibition of transcription of a specific set of growth-regulating target genes. Three mechanisms have been proposed to account for the transforming activity of the dominant transcription factor oncogenes. The simplest model suggests that high-level expression of an oncogenic transcription factor increases the production of growth-promoting proteins. A second possibility is that the overexpressed oncoprotein will activate transcription from a set of inappropriate promoters, perhaps by binding to low-affinity target sequences. The third model suggests that instead of increasing transcription, the deregulated oncogenic variant interferes with transcription of essential tumor-suppressing genes. The absence of clearly defined target genes has made it difficult to distinguish between these three hypotheses.

One approach to this problem has been to define the structural features of transcription factor oncogenes that are required for malignant transformation. The difficulty with this approach has been that many of the nuclear oncogenes belong to complex families of proteins that need to be paired with additional factors to bind to DNA. These protein–protein interactions frequently determine both the DNA binding specificity and the transcriptional regulatory activity of the oncogenes (Cleary, 1991; Lewin, 1991; Rabbitts, 1991). For example, c-Myc, a well-studied nuclear oncoprotein, has only low affinity for DNA and must interact with other basic–helix–loop–helix–leucine zipper proteins like Max/Myn to produce a heterodimer that binds to DNA (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991). The partners for Myc, Max/Myn, are in turn able to bind to either Myc, or other members of the family such as Mad, or Mxi1 (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Myc–Max is believed to stimulate transcription (Amati *et al.*, 1992; Kretzner *et al.*, 1992), but Max–Mad and Max–Mxi1 are thought to be transcriptionally inactive (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Thus, high-level expression of c-Myc could have both positive and negative effects on transcription by altering the normal stoichiometry of Myc–Max, Max–Mad and Max–Mxi1 complexes. Such combinatorial promiscuity makes analysis of the role of transcription in transformation a formidable task.

Homeobox proteins are sequence-specific transcriptional regulators that contain a conserved 60 amino acid DNA binding domain (reviewed in Scott *et al.*, 1989). Recently, a number of different homeobox family proteins have been linked with lymphoid malignancies. These include HOX 2.4 in myelomonocytic leukemia (Blatt *et al.*, 1988; Kongsuwan *et al.*, 1989), PBX-1 in acute childhood leukemia (Kamps *et al.*, 1990; Nourse *et al.*, 1990) and HOX-11 in human T cell acute lymphoblastic leukemia (Hatano *et al.*, 1991; Kennedy *et al.*, 1991; Lu *et al.*, 1991). In addition, several members of the PAX subfamily of homeoproteins have been shown to transform fibroblasts in tissue culture (Maulbecker and Gruss, 1993). Involvement of homeobox genes in malignant transformation is of particular interest because many of the members of this large family of proteins play an essential role in regulating cellular growth and differentiation (Scott *et al.*, 1989; McGinnis and Krumlauf, 1992). In vertebrates, the

regulatory functions of homeobox genes have been well implicated in pattern formation, as well as organogenesis of the central nervous system (McGinnis and Krumlauf, 1992) and the hematopoietic system (Rabbitts, 1991). These diverse functions appear to be mediated by transcriptional activation or repression of a number of different cellular genes whose regulatory elements contain a group of related homeobox binding sites (reviewed in Levine and Hoey, 1988; Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993).

The mutations that convert homeodomain proteins to oncogenes are similar to those that have been described for other nuclear oncogenes, and include translocations and insertions that up-regulate expression (Cleary, 1991; Lewin, 1991; Rabbitts, 1991). But, in contrast to many classes of nuclear oncoproteins, members of the homeofamily can bind to DNA autonomously as monomers, and have well-defined effects on transcription of specific promoters (Levine and Hoey, 1988; Scott *et al.*, 1989; Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993). We have taken advantage of the elegant work that defined the DNA binding and transcriptional activating domains of the homeobox family proteins to study the role of transcription in malignant transformation. Here we show that selective repressors of gene expression derived from the DNA binding domains of homeofamily proteins function as oncogenes *in vivo*. The mechanism that results in transformation requires repressor binding to a well-defined set of DNA target sequences.

Results

Selective transcriptional repression by the Oct-2 POU domain

We chose Oct-2 (Clerc *et al.*, 1988; Ko *et al.*, 1988; Müller *et al.*, 1988; Scheidereit *et al.*, 1988; Staudt *et al.*, 1988), a well-characterized, lymphoid-specific member of the POU subfamily of homeodomain proteins (Herr *et al.*, 1988), for our studies because of the propensity of homeodomain family members to produce lymphoid malignancies. Oct-2 has also been used extensively as a prototype for biochemical and structural studies, and it binds to a well-defined octamer (consensus ATGCAAAT) element present in the promoters of a number of different genes (for a review see Schöler, 1991; Verrijzer and Van der Vliet, 1993). The Oct-2 protein has a modular structure that includes a central DNA binding POU domain (Herr *et al.*, 1988) which is flanked by two transcriptional activating domains (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). The POU domain is a bipartite structure which consists of an N-terminal POU-specific (POU_S) and a C-terminal POU-homeo (POU_{HD}) subdomain, both of which are necessary for high-affinity sequence-specific DNA binding (Sturm and Herr, 1988; Ingraham *et al.*, 1990).

Previous studies have shown that removal of the transcriptional activating domains of Oct-2 produced a truncated protein that can bind to DNA with the same affinity and specificity as the full-length protein (Clerc *et al.*, 1988; LeBowitz *et al.*, 1988). However, this truncated form of Oct-2 had little or no transcriptional activation activity in a transfection experiment; in fact, the truncated protein has been shown to interfere with the function of the full-length protein (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). The competition experiment carried out by Gerster *et al.* (1990) demonstrated that when increasing amounts of the truncated Oct-2 POU domain protein were co-expressed with full-length Oct-2, there was inhibition of transcription induced by the intact protein. In addition, a natural alternative splicing form of murine Oct-2, which contains only the POU domain, has also been found to negatively modulate octamer-dependent transcription *in vivo* (Stoykova *et al.*, 1992). These studies suggested that the isolated POU domain might be used as a competitive transcriptional repressor.

To characterize further the mechanism of transcriptional repression by the truncated Oct-2, we used *in vitro* transcription assays and recombinant proteins (Figure 1A). Oct-1 and Oct-2 purified from HeLa cells (Pierani *et al.*, 1990; Luo *et al.*, 1992) were used as positive controls. DNA binding activity was determined by electrophoretic mobility shift assays (EMSA) with an IgH octamer sequence probe (Figure 1B). Transcriptional activation was measured in nuclear extracts prepared from the Namalwa B cell line that had been depleted of endogenous Oct-1 and Oct-2 (Luo *et al.*, 1992). The depleted Namalwa nuclear extracts showed low levels of octamer-dependent transcriptional activity but had normal levels of transcription from octamer-independent promoters (Luo *et al.*, 1992). IgH and H2B promoters were used to measure the octamer-dependent transcriptional activity because they represent two different classes of octamer-regulated promoters (Luo *et al.*, 1992). We found that the Oct-2 proteins prepared in *Escherichia coli* and HeLa cells had comparable levels of octamer binding and transcriptional activating activities (Figure 1B and C). In contrast to full-length Oct-2, the POU domain fragment (N154C376; Gerster *et al.*, 1990; herein referred to as POU-2) retained DNA binding activity but did not stimulate transcription of any of the promoters tested (Figure 1B and C). The results of our *in vitro* transcriptional analyses were in agreement with previous transfection experiments, showing that the POU domain by itself is sufficient to bind DNA (Clerc *et al.*, 1988; LeBowitz *et al.*, 1988) but lacks transcription activating activity (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990).

To determine how the truncated Oct-2 protein could repress transcription of octamer-containing promoters, we performed factor competition experiments with uncompromised Namalwa nuclear extracts. In contrast to the depleted extracts, these nuclear extracts were competent to activate transcription from both the IgH and H2B promoters. The addition of POU-2 protein produced a 10-fold inhibition of IgH transcription and a 2-fold inhibition of H2B transcription, but did not alter control 2×Sp1 transcription (Figure 1D, see quantitation). Inhibition was specific for POU-2, since full-length Oct-2 had no significant effect (Figure 1D). Furthermore, the inhibitory effect of POU-2 on H2B and IgH promoters could be largely prevented by the simultaneous addition of both the truncated and full-length proteins to the same reaction (Figure 1D, Oct-2 + POU-2). Although the degree of inhibition differed for H2B and IgH promoters, the octamer sequence was identical in the two promoters and repression was independent of the concentration of DNA template (data not shown). Thus, the difference between the two

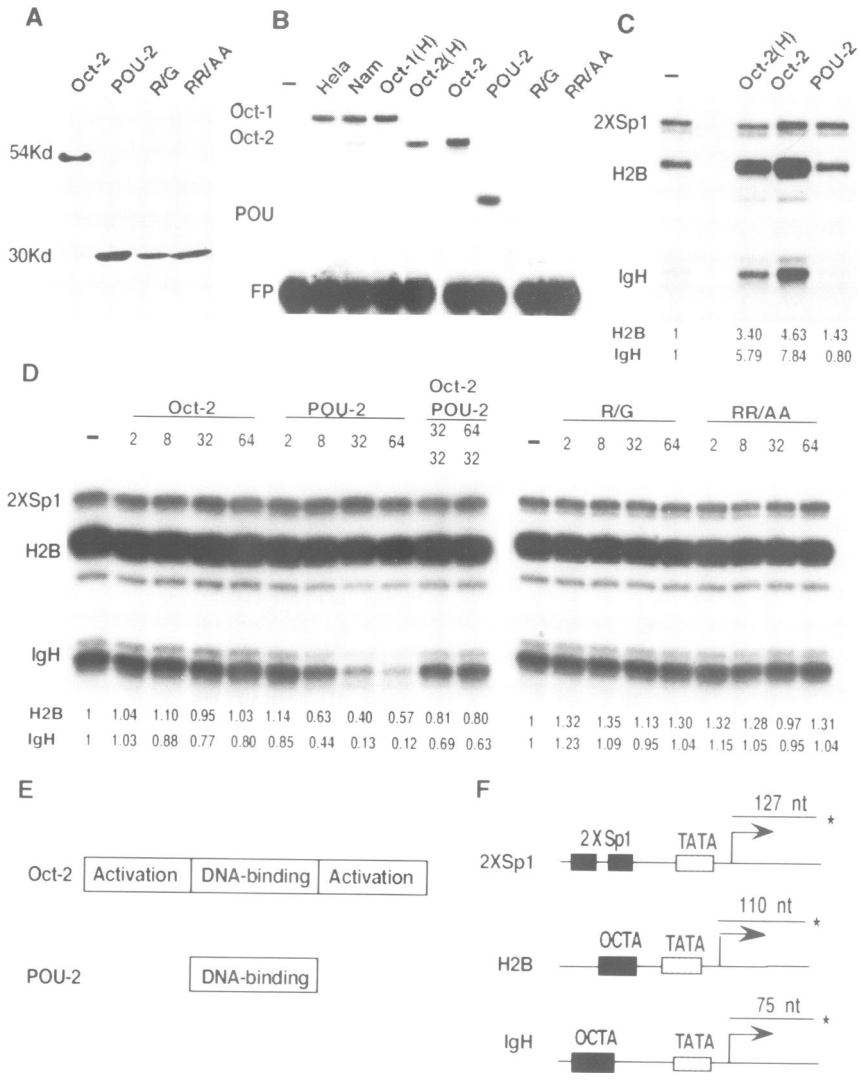

Fig. 1. Selective inhibition of octamer-dependent transcription by POU-2. (**A**) SDS–PAGE analysis of the purified bacterial recombinant proteins. Molecular weight is indicated in kDa on the left. Oct-2, full-length Oct-2 recombinant protein; POU-2, Oct-2 POU domain; R/G and RR/AA, the Arg198 to Gly single amino acid substitution and the Arg332/333 to Ala double amino acid substitution mutants of POU-2, respectively (see Materials and methods). (**B**) Octamer binding EMSA. 0.1 μl of nuclear extracts or 0.1 ng of purified proteins were used for the reaction. The specific complexes are indicated on the left. Hela, HeLa nuclear extract; Nam, Namalwa nuclear extracts; –, no nuclear extracts or protein added; FP, free probe. (**C**) Promoter stimulation assays in Oct-1 and Oct-2 immunodepleted Namalwa nuclear extracts. 20 ng of each of the purified proteins were added to a 25 μl *in vitro* transcription reaction with 12 μl of nuclear extracts. Transcription products were detected by primer extension, and the signals are indicated on the left. The relative transcriptional activities of H2B and IgH promoters are shown at the bottom. (**D**) Promoter repression assays in uncompromised Namalwa nuclear extracts. The amount of purified protein indicated at the top (in ng) was added to a 25 μl reaction containing 10 μl of the nuclear extract. (**E**) Schematic illustration of the full-length and truncated Oct-2 proteins. (**F**) Diagrams of the promoter templates used in the transcription assays. The *cis*-regulatory elements, the expected primer extension products and their sizes (in nucleotides) are illustrated.

promoters was not simply due to limiting concentrations of DNA or transcription factors, and may reflect the intrinsic properties of the two promoters (see Discussion). These experiments suggested that the Oct-2 POU domain was a potent and sequence-specific transcriptional repressor, but the level of repression varied with individual promoters.

T cell lymphoma in Oct-2 POU domain transgenic mice

It has generally been observed that oncogenic variants, including homeobox family proteins, all retain an intact DNA binding structure (Cleary, 1991; Lewin, 1991; Rabbitts, 1991). To test the idea that transformation could be mediated by an isolated DNA binding domain, we used the lck promoter (Chaffin *et al.*, 1990; Garvin *et al.*, 1990) to direct expression of POU-2 to the thymus (Figure 2A). We selected the thymus as a target organ because homeodomain proteins have been associated with human T cell malignancies (Hatano *et al.*, 1991; Kennedy *et al.*, 1991; Lu *et al.*, 1991) and thymic lymphomas in transgenic mice (Hatano *et al.*, 1992; Dedera *et al.*, 1993). Several founder mice were produced and two lines (TG.P2B and TG.P2C) were chosen for further characterization based on the steady state levels of transgenic POU-2 mRNA as measured by RNase protection. High levels of the POU-2

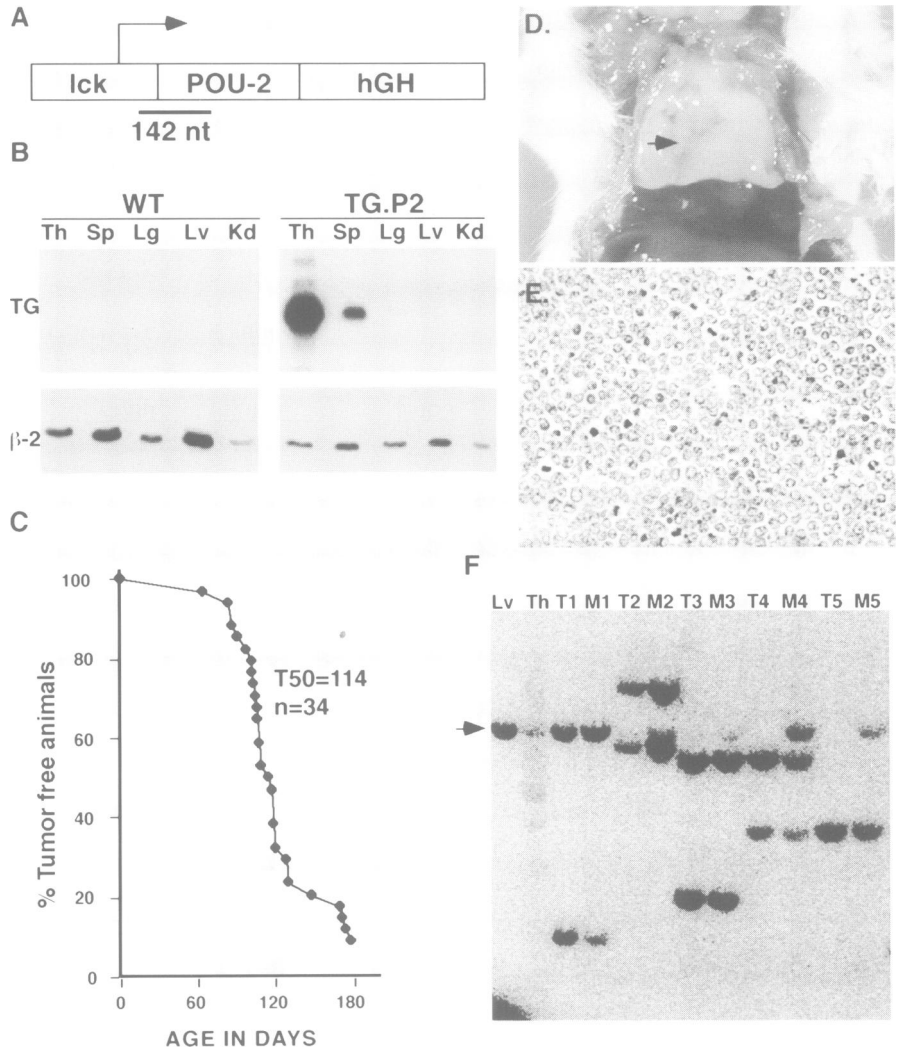

Fig. 2. Expression of POU-2 in transgenic mice produced clonal T cell lymphomas. (**A**) The POU-2 transgene construct (lck Pr, proximal promoter of the murine p56/lck gene; POU-2, human cDNA sequence encoding the truncated Oct-2 POU domain; hGH, human growth hormone genomic sequence). The RNase protection probe used in (B) and the size of the expected protected fragment (in nucleotides) are illustrated. (**B**) Expression of POU-2 mRNA was determined by RNase protection with 10 μg of total RNA from the designated tissues. WT, wild-type littermates; TG.P2, POU-2 transgenic line B; Th, thymus; Sp, spleen; Lg, lung; Lv, liver; Kd, kidney; TG and β2, the expected RNase protection signal of the POU-2 transgene and β2-microglobulin. (**C**) Kinetics of tumor occurrence in TG.P2B mice (F_5 generation). T50, 50% mortality indicated in days; *n*, number of mice scored. (**D**) Thymic tumor (indicated by the arrow) photographed *in situ*. (**E**) Histological section of thymic lymphoma stained with hematoxylin and eosin (×150). (**F**) Southern blot analysis of TCR gene rearrangements. Genomic DNA extracted from thymic tumors [T(1–5)] and spleen metastases [M(1–5)] from five representative animals was analyzed. DNA from liver (Lv) and thymus (Th) of wild-type mice was included as control. The blot was hybridized with a Jβ2 fragment probe. Arrow indicates the position of the germline band.

mRNA were found in the thymus and lower levels were found in spleen, whereas transgenic mRNA was hardly detectable in non-lymphoid organs (Figure 2B).

Expression of POU-2 was associated with rapid onset of thymic tumors in both TG.P2B and TG.P2C mice. Some 50% of the animals were affected by 4 months and 95% had succumbed before 1 year (Figure 2C). Histological examination of the tumors revealed lymphoblastic lymphomas that originated in the thymus (Figure 2D and E). The lymphomas were highly invasive and frequently produced systemic metastases to lymph nodes, spleen and visceral organs, including lung, liver and kidney (data not shown). All of the tumors were of T cell origin, as determined by analysis of lineage-specific cell surface markers. The majority of the lymphomas were of thymic intermediate cells that were positive for CD4 and CD8 and expressed intermediate levels of TCR (Table I). As in other transgenic tumor models the lymphomas were clonal (Stewart *et al.*, 1984; Adams *et al.*, 1985; Schmidt *et al.*, 1988). Analysis of TCR gene rearrangements by Southern hybridization with a Jβ2 probe revealed one or two distinct rearranged bands in both the primary tumor and the distant metastases (Figure 2F). The tumors were

Table I. Surface phenotype of TG.P2 T cell lymphomas

$CD4^+CD8^-$ $\alpha\beta TCR^-$	$CD4^+CD8^+$ $\alpha\beta TCR^{low}$	$CD4^+CD8^+$ $\alpha\beta TCR^{med}$	$CD4^+CD8^+$ $\alpha\beta TCR^{high}$
2 (6.3%)	10 (31.3%)	13 (40.7%)	7 (21.9%)

Number of tumors in any given category is indicated and the percentage is shown in parentheses.

readily transplanted between histocompatible mice, and tumor cells could be grown in tissue culture in the absence of exogenous growth factors. We concluded that POU-2 is a dominant oncogene that produces stochastically occurring clonal neoplasms whose characteristics are similar to those produced by traditional nuclear oncogenes in transgenic mice.

Oct-1 POU domain produces T cell lymphomas

Oct-2 is part of a family of proteins that bind to octamer sequences. Many of these proteins can activate transcription of octamer-containing promoters (reviewed in Schöler, 1991). Despite the similarities, members of this family appear to interact with different sets of co-factors and regulate different promoters. For example, Oct-1 is closely related to Oct-2, with 87% amino acid identity between Oct-1 and Oct-2 POU domains (Herr *et al.*, 1988), and the two proteins bind to DNA with similar specificities and affinities (LeBowitz *et al.*, 1988; Pierani *et al.*, 1990). However, only Oct-1 can interact efficiently with VP16 (Gerster and Roeder, 1988; O'Hare and Goding, 1988; O'Hare *et al.*, 1988; Kristie *et al.*, 1989; Stern *et al.*, 1989). The specificity of this interaction is largely determined by a single amino acid difference between Oct-1 and Oct-2 at position 22 in helix 1 of the homeo subdomain of Oct-1 (Lai *et al.*, 1992; Pomerantz *et al.*, 1992; Cleary *et al.*, 1993).

To investigate whether the transforming activity of POU-2 was due to a unique feature of Oct-2, we established lines of transgenic mice that carried the Oct-1 POU domain (referred to as POU-1) driven by the lck promoter. Seven independent lines were established, and all seven showed tumor development in their F_1 offspring with similar kinetics. When the transgenic protein levels were measured, there were 4- to 5-fold differences among the individual lines, but no clear correlation between the level of expression and the tumor occurrence was observed. Four lines with high levels of protein expression were further bred and characterized (TG.P1A, D, G and E; Figure 3A and B). Expression of either POU-2 or POU-1 resulted in the appearance of a single transgene-specific octamer binding protein in thymic tissue extracts (Figure 3B). We noted that despite high-level expression of the transgenes, there were no demonstrable feedback effects on the level of endogenous Oct-1 (Figure 3B). As with POU-2, all four transgenic lines that expressed POU-1 developed rapid onset thymic lymphomas (Figure 3C). Further, the lymphomas were histologically and antigenically identical to those that arose in TG.P2 animals (data not shown). These results suggested that the oncogenic activity of the POU domain was due to a structural feature which is shared by octamer family transcription factors.

DNA binding is required for transformation

Members of the POU subfamily of homeoproteins are known to possess two types of DNA binding activity. The homeo subdomain (POU_{HD}) is responsible for low-affinity binding to AT-rich sequences and the POU-specific subdomain (POU_S) confers high-affinity sequence-specific DNA binding (Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993). To determine whether transformation was dependent on interaction of the POU domain with the

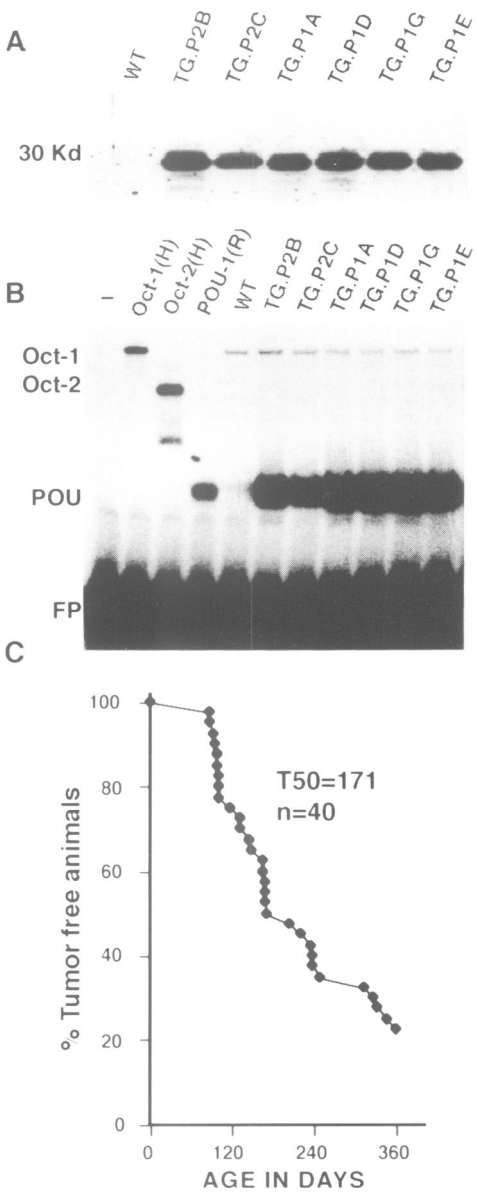

Fig. 3. T cell lymphomas in POU-1 transgenic mice. (**A**) Western blot analysis of transgene expression in four independent TG.P1 lines (A, D, G and E), two lines of TG.P2 (B and C) and a wild-type littermate. 5 μg of thymic tissue nuclear extracts were analyzed, and the transgenic proteins were detected by rabbit anti-POU domain antiserum. (**B**) Octamer binding activities in the tissue nuclear extracts. 0.2 μl of nuclear extracts (1.0–1.2 μg) were used for the reactions. Oct-1(H), Oct-2(H), POU-1(R) and POU-1 protein synthesized in reticulocyte lysates were included as controls. The protein–DNA complexes are indicated on the left. Transgenic lines were as in (A). (**C**) Kinetics of tumor occurrence in TG.P1A mice (F_1 and F_2 generations).

octamer sequence, we generated POU-2 mutants with altered DNA binding activities. Point mutations were introduced in positions predicted to be in the DNA contact surfaces of the POU domain (Sturm and Herr, 1988; Ingraham *et al.*, 1990), and the importance of these amino acids has been confirmed recently by solution and crystal structural analyses (Assa-Munt *et al.*, 1993; Dekker *et al.*, 1993; Klemm *et al.*, 1994). Mutant proteins produced in *E.coli* were screened for DNA binding by EMSA with the IgH octamer probe. Two mutations were selected for

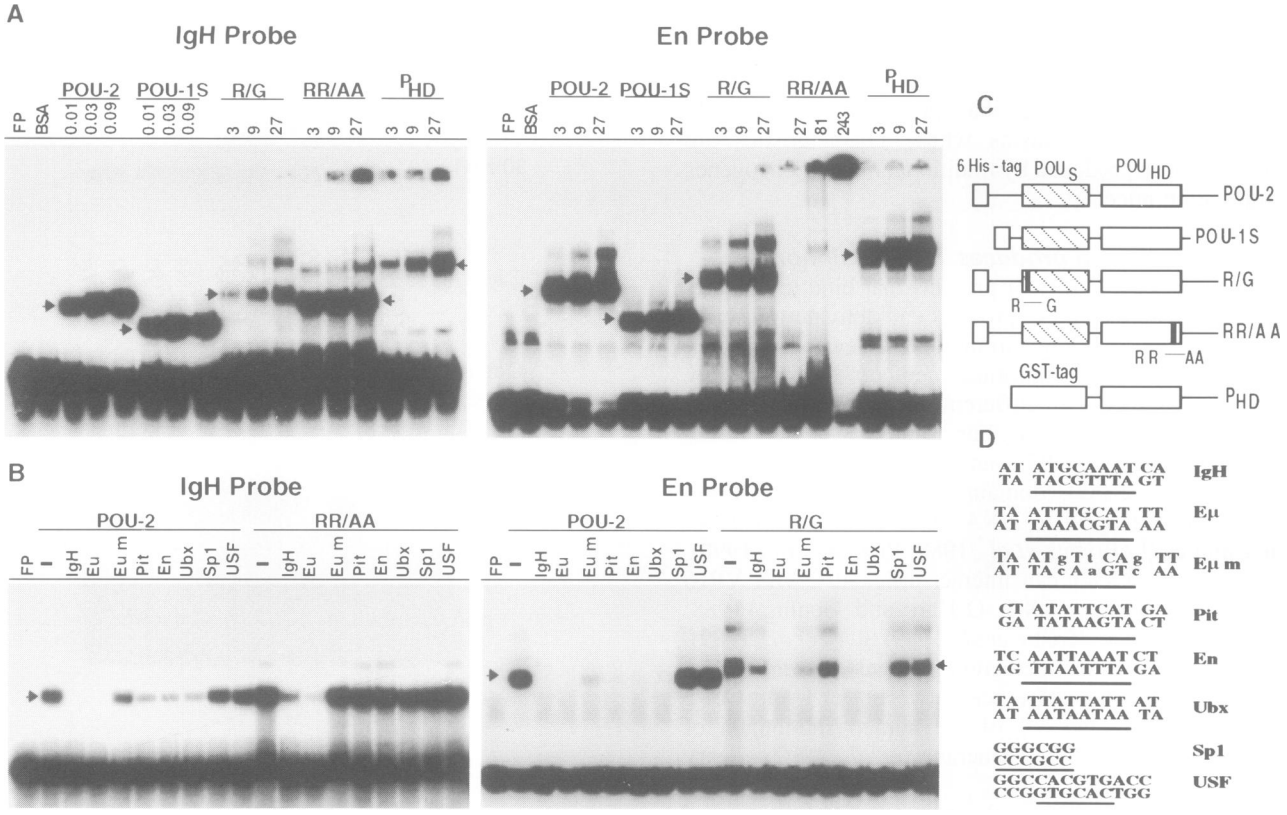

Fig. 4. DNA binding activities of mutant POU-2 proteins. (**A**) Binding activity titration. Amounts of purified proteins added to the reactions are indicated (in ng). The authentic monomer complexes are marked by arrows. Note that the RR/AA mutant tended to form non-specific aggregates which were retained on the top of the gel; slower migration of R/G protein is because the mutation in POU_S disrupts DNA bending activity (Verrijzer *et al.*, 1991). (**B**) Competition for binding to IgH and En probes with oligonucleotide competitors. 0.01 ng of the purified protein and a 400-fold excess of competitors (2000 fmol) were used for the POU-2 + IgH probe reactions (high-affinity binding), whereas 3 ng of protein and a 1000-fold excess of competitors (5000 fmol) were used for RR/AA + IgH, POU-2 + En and R/G + En reactions (low-affinity binding). Note, Ubx competitor is a tetramer; thus it had a higher avidity. (**C**) Diagrammatic illustration of the recombinant proteins. (**D**) Core sequence of the DNA probes and competitors: IgH, octamer binding site of the IgH promoter (H^-O^+; Poellinger *et al.*, 1989); Eμ, octamer binding site of the immunoglobulin μ enhancer (Schreiber *et al.*, 1988); Eμ m, the mutated octamer binding site of immunoglobulin μ enhancer (base substitutions indicated in lower case letters; Schreiber *et al.*, 1988); Pit, Pit-1 binding site of the prolactin promoter (Ingraham *et al.*, 1990); En, the engrailed homeobox binding site (NP-1; Desplan *et al.*, 1988); Ubx, the optimal ultrabithorax homeobox binding site (A1; Ekker *et al.*, 1991); Sp1, the consensus Sp1 binding site (Kadonaga *et al.*, 1987); USF, the USF binding site of the adenovirus major late promoter (Gregor *et al.*, 1990).

further study. Arg198 to Gly (referred to as R/G) is a single amino acid substitution in the first helix unit of the POU_S subdomain, and Arg332 and 333 to Ala (referred to as RR/AA) is a double substitution in the third helix of the POU_{HD} subdomain. The R/G mutation resulted in a 3000-fold decrease in octamer-specific DNA binding, whereas RR/AA caused only a 100- to 300-fold drop (Figure 4A, binding to IgH probe). The two mutants were characterized further by binding and competition studies with a set of octamer-related elements and AT-rich homeobox binding sites (Figure 4D). In contrast to the results for consensus octamer binding, low-affinity binding to AT-rich and octamer-related sequences was not significantly altered by the R/G mutation (Figure 4A and B, binding to En probe). Thus, this specific mutation in the POU_S subdomain selectively diminished high-affinity octamer-specific binding and left AT-rich sequence binding intact. Conversely, the RR/AA mutation in the POU_{HD} subdomain reduced both the octamer-specific and the low-affinity AT-rich sequence binding activities.

Transgenic mice that carried POU-2 with either the R/G or RR/AA mutation (TG.R/G and TG.RR/AA) did not develop lymphomas. A total of 13 independent founders and 91 F_1-F_3 mice from five separate lines have been observed for a period of up to 1 year without detecting a single thymic cancer. The absence of malignancies in the mice that carried TG.R/G and TG.RR/AA was not due to reduced levels of protein expression, as measured by Western blotting and EMSA on thymocyte nuclear extracts (Figure 5A and B). As expected from the results of DNA binding studies with *E.coli* proteins, the RR/AA mutant displayed a small residual amount of affinity for octamer sequences, and octamer binding to R/G was undetectable (Figure 5B). Thus, high-level expression of R/G and RR/AA in transgenic mice was not sufficient to produce lymphoma.

The transcriptional effects of the DNA binding mutations were measured in *in vitro* transcription assays. Neither R/G nor RR/AA had any inhibitory effects on transcription from H2B, IgH or the control promoters in uncompromised Namalwa nuclear extracts (Figure 1D). We concluded that octamer-specific DNA binding was required for transcriptional repression and for malignant transformation by the POU oncoproteins *in vivo*.

Discussion

Transgenic paradigms have established that a number of different transcriptional regulators can produce

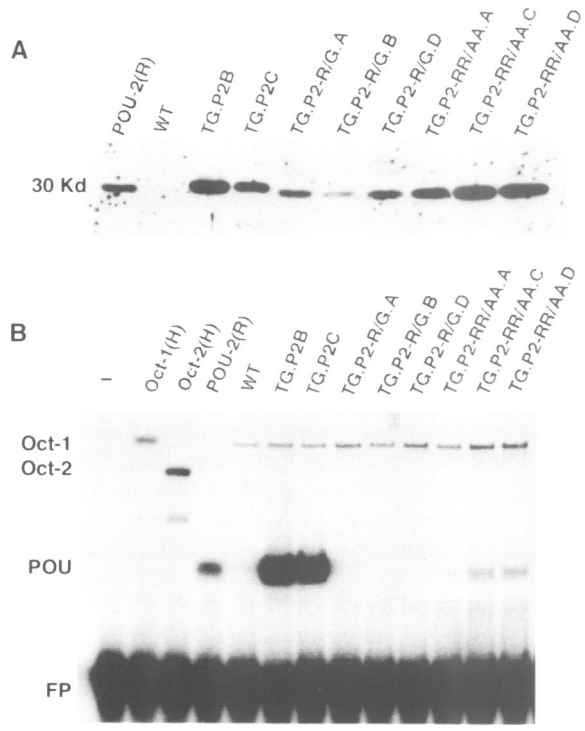

Fig. 5. Expression of mutant POU-2 proteins in transgenic mice. Three independent lines that carried TG.P2-R/G (A, B and D) and three lines of TG.P2.RR/AA (A, C and D) were compared with two lines of TG.P2 (B and C) and a wild-type littermate control. (**A**) Western blot analysis of transgene expression. (**B**) EMSA analysis of octamer binding activities in thymic tissue nuclear extracts.

malignancies *in vivo* when their expression is deregulated (Adams and Cory, 1991). The nuclear oncogenes are a diverse group of proteins that have been linked to a number of different cellular and developmental functions. Several of the homeoproteins are notable examples of developmental regulators that can induce transformation (Cleary, 1991; Rabbitts, 1991). Here we have used Oct-2 and Oct-1 to study the requirements for transformation by homeodomain-containing transcriptional regulators. Our experiments lend support to the idea that specific transcriptional repression is one of the mechanisms by which this group of genes can produce malignancies *in vivo* (Lewin, 1991).

The homeobox genes resemble other families of transcriptional regulators in that DNA binding and transcriptional activating functions are encoded by modular domains. Oct-2 and Oct-1 are members of the homeofamily by virtue of their highly conserved DNA binding domains (Herr *et al.*, 1988; Rosenfeld, 1991). The POU domain determines the DNA binding activity of the Oct proteins, and the DNA binding affinity and specificity of the POU domain is indistinguishable from that of the intact protein (Clerc *et al.*, 1988; LeBowitz *et al.*, 1988). However, the DNA binding domain has a little or no activity to stimulate octamer-dependent transcription (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). In fact, it has been shown that the isolated POU domain can interfere with the full-length protein and function as a dominant negative repressor both *in vivo* (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990; Stoykova *et al.*, 1992) and *in vitro* (this work). Transcriptional inhibition by the POU domain could be due to specific DNA binding and direct physical interference with promoter activity, or alternatively to a template-independent mechanism that involves protein–protein interactions in solution. Our *in vitro* experiments were most consistent with the idea that the inhibitory effects were due to specific DNA binding. First, inhibition required the presence of the octamer element in the target promoter. Second, point mutations in the POU-2 that lowered its affinity for the octamer sequence abolished the inhibitory activity. Finally, repression could be alleviated by a high concentration of the full-length protein.

Transcriptional inhibition was not equivalent in all octamer-containing promoters, as shown by a more profound effect (10-fold) on the IgH promoter than on the H2B promoter (2-fold). The difference is consistent with the possibility that octamer transcription factors interact with at least two classes of co-factors to activate transcription from different promoters. One group of co-factors, exemplified by VP16 (Gerster and Roeder, 1988; O'Hare and Goding, 1988; O'Hare *et al.*, 1988; Kristie *et al.*, 1989; Stern *et al.*, 1989) and PTF (Murphy *et al.*, 1992), contact both DNA and the POU domain. Factors in this group might be expected to be less sensitive to inhibition by truncated Oct-2 since they could continue to bind to DNA in the presence of the POU domain and thus achieve partial activation (Murphy *et al.*, 1992; Pomerantz *et al.*, 1992; Cleary *et al.*, 1993). The second class of octamer co-factors, exemplified by OCA-B, may be tethered to specific promoters mainly via a protein–protein interaction with octamer binding factors (Luo *et al.*, 1992). Promoters that rely on this second class of co-factors might be more sensitive to inhibition by truncated Oct-2, especially if the protein–protein interactions require protein segments outside the POU domain.

Interestingly, a short alternative splicing form of Oct-2, (designated MiniOct-2) which is closely related to POU-2, has been found as a natural product in the murine central nervous system (Hatzopoulos *et al.*, 1990). Like POU-2, MiniOct-2 is a repressor of octamer-dependent transcription in transfection experiments (Stoykova *et al.*, 1992). But unlike Oct-2 which is expressed in lymphocytes, MiniOct-2 is expressed only in the olfactory bulb which is one of the few regions in the central nervous system where growth and differentiation of sensory neurons occurs throughout adult life (Hatzopoulos *et al.*, 1990; Stoykova *et al.*, 1992). Based on the pattern of MiniOct-2 expression it was proposed that the protein plays an important role in self-renewal and continuous proliferation of olfactory neurons (Hatzopoulos *et al.*, 1990; Stoykova *et al.*, 1992). Our observation that POU-2 can induce deregulated growth and transformation in thymocytes is consistent with this idea.

To investigate the *in vivo* effects of the POU domain oncoprotein, we analyzed mRNA levels of several octamer-containing genes, including IL-2, H2B and p56/lck. We found no discernible changes in steady state mRNA levels in transgenic and control thymocytes, and in number of different tumor cell lines (X.-F.Qin, unpublished observations). One possible interpretation of these preliminary results is that *in vivo*, as *in vitro* (Figure 1D), not all the octamer-regulated genes are equally sensitive to inhibition by the POU domain. Alternatively, steady

state mRNA levels measured by Northern blot analysis may not directly reflect the rates of gene transcription. A more systematic approach is now being undertaken to identify the downstream targets of the POU oncogenes.

DNA binding is a common theme that ties together the transcription factor oncogenes (Lewin, 1991). Although this large group of proteins has a number of different DNA binding motifs, all of them contain a combination of sequence-specific and general DNA recognition features (Pabo and Sauer, 1992). Thus, transformation by overexpression of these oncoproteins could be the result of binding to inappropriate low-affinity target sites or to canonical *cis*-regulatory elements. We have taken advantage of the well-defined bipartite structure of the POU domain to examine the contribution of these two types of DNA binding activity to transformation. We found that high-affinity binding was essential for transformation, whereas low-affinity interactions with AT-rich sequences were not sufficient. We conclude that a specific set of promoters, those that contain octamer sequences, are the critical targets for transformation by POU domain oncoproteins.

An interesting question is whether full-length Oct-1 and Oct-2 can also induce tumors. To try to answer this question we made transgenic mice that carry lck-driven Oct-2. None of 51 F_1 and F_2 offspring of six independent lines developed tumors during a 1.5 year period of observation (X.-F.Qin, unpublished observations). However, the levels of the full-length Oct-2 transgene expressed in these animals were 10- to 100-fold lower than those of the truncated proteins in TG.P1 and TG.P2 animals. The lack of high-level expression of the full-length protein in mice appeared to be caused by both mRNA and protein instability, consistent with results obtained in tissue culture studies (Gerster *et al.*, 1990; Müller-Immerglück *et al.*, 1990). Although the data suggest that the intact activator lacks transforming activity, we cannot rule out the possibility that higher levels of intact Oct-2 expression would also lead to malignancies.

Decreased expression of growth inhibitory genes is thought to be an important part of the pathway that leads to malignant transformation (Marshall, 1991; Weinberg, 1991; Vogelstein and Kinzler, 1993). This could occur by one of two genetic mechanisms: either loss of specific activators, or aberrant expression of dominant repressors. The first mechanism, loss of specific transcriptional activators, is now well established. Elegant experiments with p53, a tumor suppressor gene associated with a large number of human malignancies, have established a direct link between decreased transcription and loss of growth control (El-Deiry *et al.*, 1993). The p53 protein functions as a sequence-specific transcriptional regulator that binds to a 20 bp *cis*-regulatory element and activates gene expression (Kern *et al.*, 1991). In the absence of p53, expression of genes that depend on p53 for transcription is believed to decrease. The group of genes that contain the p53-responsive sites includes WAF1 (also known as p21, Cip1; Harper *et al.*, 1993; Xiong *et al.*, 1993), which encodes a cyclin-dependent kinase (Cdk) inhibitor (Harper *et al.*, 1993; Xiong *et al.*, 1993). WAF1 controls cell cycle progression by modulating the activity of the Cdk kinases that are necessary for the G_1 to S phase transition (Harper *et al.*, 1993; Xiong *et al.*, 1993). It has been proposed that loss-of-function mutations in p53 deregulate cell growth and cause malignant transformation by decreasing WAF1 expression (El-Deiry *et al.*, 1993). The second genetic mechanism that may underlie tumorigenesis is down-regulation of growth inhibitory genes by transcriptional repression. This mechanism was originally proposed to explain transformation by v-ErbA (Damm *et al.*, 1989; Zenke *et al.*, 1990). However, recent experiments have shown that v-ErbA can either inhibit or activate transcription depending upon the promoter tested (Saatcioglu *et al.*, 1993), and transformation may be due to indirect effects mediated through interference with AP-1 and RARs (Desbois *et al.*, 1991; Sharif and Privalsky, 1991). Dominant negative repression has also been suggested as a mechanism for transformation by v-Rel (Ballard *et al.*, 1990), and chimeric oncoproteins like PML-RARα (de The *et al.*, 1991; Kakizuka *et al.*, 1991) could also be invoked in the case of PBX-1 oncogenic conversion (Kamps *et al.*, 1990; Nourse *et al.*, 1990). t(1;19) translocation results in the fusion of homeobox gene PBX-1 with E2A and produces a chimeric protein composed of the PBX-1 DNA binding domain and a transcriptional activation motif derived from E2A (Kamps *et al.*, 1990; Nourse *et al.*, 1990). This fusion protein can activate transcription from an artificial promoter containing PBX-1 binding sites (LeBrun and Cleary, 1994; Lu *et al.*, 1994); however, the role of the E2A activation motif in transformation has not been determined. PBX-1 is not normally expressed in the pre-B cells that are the targets for transformation by this oncogene (Kamps *et al.*, 1991; Monica *et al.*, 1991). In these cells the oncogenic fusion protein could compete with the endogenous regulators (Monica *et al.*, 1991) either to activate or to inhibit gene expression. Similarly, experiments with the other candidate oncogenes have failed to establish a direct connection between DNA binding, transcriptional activation or inhibition, and transformation. The repression model predicts that the DNA binding domains found in transcription factor oncogenes should be essential for transformation, and that the transcriptional activating domains may not be necessary. Our finding that only the DNA binding domain of Oct-2 and Oct-1 was required to produce lymphomas provides direct evidence to support the existence of such a mechanism.

The prevalence of tumor suppressor genes in human malignancies (Marshall, 1991; Weinberg, 1991; Vogelstein and Kinzler, 1993) and the correlation between inhibition of transcription and transformation by POU-2, makes it attractive to speculate that transformation by dominant oncogenes like POU-2 is due to down-regulation of tumor suppressor genes. The availability of transgenic mice that carry POU-2 oncogenes with well-defined DNA binding properties should facilitate the testing of this hypothesis.

Materials and methods

Cloning

Full-length and truncated Oct-2 (Oct-2 and POU-2) constructs were derived from the human Oct-2 cDNA clones 00 and N154C376 (Gerster *et al.*, 1990). The R/G (Arg198 to Gly) and RR/AA (Arg332/333 to Ala) mutants (numbering as in Scheidereit *et al.*, 1988) were made by site-directed mutagenesis (Kunkel, 1985) in N154C376. The POU-1 cDNA used for the transgene was generated by swapping nucleotides 530–1014 of N154C376 with the corresponding 889–1349 sequence of

pBS-Oct-1 (numbering as in Sturm *et al.*, 1988) encoding the POU domain. POU-1S is a short version of Oct-1 POU domain construct used to produce bacterial recombinant protein (Murphy *et al.*, 1992). The 6His fusion constructs were generated by in-frame insertion of the corresponding cDNA sequences between *Bam*HI and *Eco*RI of the 6His-pET11 expression vector (Hoffmann and Roeder, 1991). The lck-POU-2, lck-POU-1, lck-R/G and lck-RR/AA transgene constructs were generated by cloning of the corresponding cDNA sequences as *Bam*HI fragments into the lck-hGH expression vector (Chaffin *et al.*, 1990; Garvin *et al.*, 1990).

Recombinant proteins

E.coli BL21(DE3)/pLysS (Studier *et al.*, 1990) was used to produce 6His-tagged recombinant proteins (Hoffmann and Roeder, 1991). Bacterial culture and lysis were as described (Murphy *et al.*, 1992). Protein extracts were denatured in BC buffer/100 mM KCl [BC buffer: 20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 0.025% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride] containing 6 M guanidium hydrochloride on ice for 30 min. After centrifugation at 20 000 r.p.m. for 30 min, the supernatant was adjusted to 2 mM imidazole and loaded onto a Ni^{2+}-NTA column (Qiagen). The column was washed with 10 volumes of BC buffer/100 mM KCl containing 8 M urea followed by a salt-step wash with BC buffer containing 1000, 800, 500 and 300 mM KCl (three column volumes/step for 30 min) to allow renaturation of the bound protein. The column was then washed with BC buffer/300 mM KCl containing 20 mM imidazole, and the protein was eluted by BC buffer/200 mM KCl containing 200 mM imidazole. The eluate was dialyzed against BC buffer/100 mM KCl and applied to a heparin (Bio-Rad) column (Murphy *et al.*, 1992). The heparin column was step-washed with BC buffer containing 100, 200 and 300 mM KCl. All of the recombinant proteins were eluted with 300 mM KCl and were homogeneous as judged by Coomassie blue-stained SDS–polyacrylamide gels. P_{HD} was produced as a GST fusion protein by cloning of the *Sfi*I–*Eco*RI fragment (amino acid sequence N262C367, numbering as in Scheidereit *et al.*, 1988) of Oct-2 cDNA into the *Bam*HI–*Eco*RI site of pGEX-2T (Pharmacia). The fusion protein was purified by two cycles of glutathione affinity chromatography (Smith and Johnson, 1988). Recombinant Oct-1(H) and Oct-2(H) proteins from HeLa cells were prepared as described (Pierani *et al.*, 1990; Luo *et al.*, 1992). *In vitro* translation was carried out according to the manufacturer's protocol (Promega).

Nuclear extracts

HeLa and Namalwa cell nuclear extracts and immunodepletion of Oct-1 and Oct-2 were as described (Pierani *et al.*, 1990; Luo *et al.*, 1992). Thymic tissue nuclear extracts (5–6 μg/μl) were prepared from single-cell suspensions of thymocytes with a small-scale protocol according to Gerster *et al.* (1990).

In vitro transcription assays

In vitro transcription assays were performed essentially according to Luo *et al.* (1992). The reactions were carried out with three promoter templates (250 ng of H2B, 250 ng of IgH and 100 ng of 2×Sp1) and incubated at 30°C for 60 min. The transcription products were detected by primer extension with template-specific oligonucleotide primers (Luo *et al.*, 1992). The primers were labeled with [γ-^{32}P]ATP and had the same specific activity. Quantitation was performed on a PhosphorImager (Molecular Dynamics).

EMSA analysis

The IgH promoter sequence (H^-O^+) (Poellinger *et al.*, 1989) was used as the octamer binding site probe, and the En sequence (NP1; Desplan *et al.*, 1988) as the AT-rich homeobox binding site probe. DNA binding reactions were carried out in 10 μl with 5 fmol of the ^{32}P-labeled probe ($1.0–1.4\times10^4$ c.p.m./fmol) at room temperature for 30 min. The buffer conditions were as described (Luo *et al.*, 1992), except for the quantitative experiments of Figure 4. Poly(dI–dC):poly(dI–dC) was reduced to 1 μg/ml to measure the low-affinity binding activities. Protein–DNA complexes were resolved by electrophoresis on 6% 30:1 acrylamide: bisacrylamide gels with 0.25× TBE buffer at room temperature. Quantitation was performed on a PhosphorImager (Molecular Dynamics).

Transgenic mice

*Not*I was used to release the transgene fragments from vector DNA and the minigenes were microinjected into the pronuclei of FVB/N (Taconic Laboratory Animals and Services) mouse oocytes (Palmiter and Brinster, 1986). Animals carrying the transgenes were identified by transgene-specific PCR. The transgenic lines were established by heterozygous cross with FVB/N mice.

RNA and DNA analyses

RNase protection experiments were as described (Melton *et al.*, 1984). The transgene-specific probe overspans the lck promoter region that can differentiate the transgene signals from endogenous ones. RNase protection for β2-microglobulin was included as a control for the amount of mRNA present in each sample (Nussenzweig *et al.*, 1987). Genomic DNA was digested with *Hin*dIII, resolved on 1% agarose gels, transferred to a GeneScreen filter (NEN) and hybridized with a ^{32}P-labeled Jβ2 fragment probe (Fenton *et al.*, 1988; $8–9\times10^8$ c.p.m./μg) according to the manufacturer's protocol (NEN).

Western blot analysis

5 μg of nuclear extracts (5–6 μg/μl) were resolved on 10% SDS–polyacrylamide gels, electrotransferred onto a nitrocellulose membrane (Amersham) and probed with a 1:2000 dilution of rabbit anti-POU domain antiserum (Gerster *et al.*, 1990). The bound antibodies were detected by an HRPase-conjugated donkey anti-rabbit ECL system (Amersham).

FACS analysis

Tumor cells were stained with PE-conjugated anti-CD4 (Pharmingen), R613-conjugated anti-CD8 (Gibco-BRL) and FITC-conjugated anti-αβTCR (Pharmingen), and analyzed with a FACScan and Lysis II program (Becton Dickinson).

Digital image processing

Images were obtained either by phosphorimaging (Molecular Dynamics) or digital scanning of films, and processed with PhotoShop (Adobe) and PageMaker (Aldus) software.

Acknowledgements

We would like to thank Drs H.Hanafusa, C.Desplan, N.Heintz, N.Segil, L.Dailey, S.Jun and D.Wilson for critical reading of this manuscript, Dr T.Gerster for the Oct-2 cDNA clones, Dr W.Herr for the Oct-1 cDNA clone pBS-Oct-1 and Dr R.Perlmutter for the lck-hGH vector used in this study. We are also grateful to Drs J.-B.Yoon, C.-M.Chiang, H.Ge, E.Sinn and other members of Roeder's laboratory for reagents and helpful discussions, and Dr K.Sokol for assistance with pathology. X.-F.Q. was supported by a graduate student fellowship from the Rockefeller University and J.W. was supported in part by a National Research Service Award (grant) GM07982. Research was supported by grants to R.G.R. from NIH and the Pew Charitable Trusts, and to M.C.N. from HHMI.

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Received on August 24, 1994; revised on October 7, 1994