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SEQUENCE SPECIFIC DNA BINDING AND TRANSCRIPTIONAL REGULATION BY THE PROMYELOCYTIC LEUKEMIA ZINC FINGER PROTEIN

by

JIA-YUAN LI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

SEQUENCE SPECIFIC DNA BINDING AND TRANSCRIPTIONAL REGULATION BY THE PROMYELOCYTIC LEUKEMIA ZINC FINGER PROTEIN

by
Jia-Yuan Li

Advisor: Jonathan D. Licht, M.D.

The Promyelocytic Leukemia Zinc Finger gene (PLZF) is rearranged in the chromosomal translocation t(11;17)(q23;21) associated with a retinoic acid resistant form of acute promyelocytic leukemia. The translocation fuses the retinoic acid receptor α gene to PLZF producing reciprocal fusion proteins hypothesized to play a prominent role in leukemogenesis. PLZF encodes a 81 kD transcription factor with nine Kruppel like C2-H2 zinc fingers located in a single domain near the C-terminus of the protein. The last seven of PLZF's zinc fingers are retained in the t(11;17) fusion protein RARα-PLZF suggesting that RARα-PLZF plays a role in leukemogenesis by competing with wildtype PLZF for response elements. In this thesis, I identified several PLZF DNA binding sites which suggests that zinc finger proteins in general have the ability to recognize a spectrum of DNA sequences with different affinities. One binding site recognized specifically by the PLZF protein is the β-retinoic acid response element. The biological implications of such an interaction is that PLZF may regulate a subset of retinoic acid responsive genes and that restoration of the RARα pathway by all-trans retinoic acid may not compensate for transcriptional deregulation by RARα-PLZF, an aberrant transcription factor. Through PCR based binding site selection, I also identified a high affinity binding site for PLZF and showed that PLZF binds to this site through its most carboxyl seven zinc fingers. In cotransfection experiments, PLZF repressed

transcription through this high affinity binding site. Transfected RARα-PLZF resulted in significant loss of repression (compared to that of wildtype PLZF). To identify important functional domains within its effector region, various fragments of the PLZF were fused to the Gal4p DNA binding domain. Constructs encoding these chimeric proteins were cotransfected with a reporter plasmid containing five copies of Gal4p DNA binding site. These experiments demonstrate that transcription repression by PLZF is mediated by two separate domains residing between amino acids 1-100 and amino acids 200-300. The presence of two non-contiguous repression domains implies that PLZF may repress transcription by effecting more than one molecular target. There is also a transcription activation domain within the PLZF effector domain which resides between amino acids 100-200. The presence of both activation and repression domains within one transcription factor implies that PLZF may be bi-functional depending on the presence of other factors, post-translation modification or the promoter architecture of target genes.

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First of all, I would like to thank my thesis mentor, Jonathan Licht for his insights, enthusiasm and patience. I feel truly blessed to have the opportunity to work with Jon on this exciting project and to say that I have learned a lot from him is an understatement. I would also like to thank the members of my thesis advisory committee, Jim Bieker and Leslie Pick for years of discussion, advice and encouragement.

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To my family

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Chapter 1

INTRODUCTION

Acute promyelocytic leukemia: histology and associated syndromes. Acute promyelocytic leukemia (French-American-British [FAB] M3) represents 10% of all acute myeloid leukemias and is defined as the clonal expansion of malignant myeloid cells blocked at the promyelocyte stage of development (Clarkson *et al.*, 1991; Frankel *et al.*, 1993; Grignani *et al.*, 1994; Tallman and Rowe, 1994; Warrell *et al.*, 1993). The cytoplasm of these promyelocytes are filled with large azurophilic granules containing pro coagulants which activate the coagulation cascade, generate thrombin and deplete fibrinogen clotting factors and platelets (Reviewed by Holland *et al.*, 1993). Compared with other subtypes of acute myeloblastic leukemias, there is an excess of early mortality, usually related to intracranial hemorrhage (Reviewed by Holland *et al.*, 1993; Warrell *et al.*, 1993). Long-term prognosis, however, is favorable with a 5 year survival rate of 35% to 45% (Reviewed by Holland *et al.*, 1993; Warrell *et al.*, 1993). Clinical trials have shown that greater than 90% of APL patients can be induced to undergo complete remission with oral all-trans retinoic acid (ATRA) (Castaigne *et al.*, 1990; Chen *et al.*, 1991; Hwang *et al.*, 1993; Warrell *et al.*, 1991). Current evidence suggests that this is achieved through the differentiation of promyelocytes into granulocytes (Chen *et al.*, 1993; Elliott *et al.*, 1992). The remission, however, is short-lived and a high relapse rate occurs in APL patients treated with ATRA alone. ATRA along with conventional chemotherapy increased overall survival of APL patients (Kanamaru *et al.*, 1995; Wu *et al.*, 1993). On the molecular level, APL is consistently associated with chromosomal translocation t(15;17)(q22; q12) which is often the only detectable chromosomal anomaly in these patients (de The *et al.*, 1990; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Larson *et al.*, 1984; Rowley *et al.*, 1977; Rowley *et al.*, 1977). The breaks occur within the second intron of the retinoic acid receptor α

(RARα) gene and within two major sites of the PML gene (exons 4 and 7). This translocation generates two sets of fusion proteins PML-RARα and RARα-PML (Figure 1).

Translocation t(15;17)

Retinoic acid receptor α.

RARα gene is one of three retinoic acid receptor genes identified. RARα, β and γ are nuclear proteins that belong to the steroid/thyroid hormone receptor superfamily. They act as ligand dependent, DNA binding transcription factors which transactivate target genes by forming heterodimers with the RXR family of proteins (Reviewed by Chambon *et al.*, 1996). RARs can bind either 9-cis or all-trans retinoic acid as ligand whereas RXR proteins only bind to 9-cis retinoic acid (Mangelsdorf *et al.*, 1992; Repa *et al.*, 1993). In the absence of ligand binding, RAR-RXR heterodimers have been shown to mediate transcription repression by binding to corepressors (N-CoR and SMRT) (Chen *et al.*, 1996; Chen *et al.*, 1995; Horlein *et al.*, 1995; Kurokawa *et al.*, 1995). RAR proteins are divided into six regions (A-F) (Reviewed in Chambon, 1996). Each RAR gene encodes isoforms which are products of alternative splicing and differential promoter choice. Regions B through F are common in all isoforms of the same RAR subtype; the differences within the subtypes occur mainly within the 5' untranslated regions of the mRNA and the A domains. Mutagenesis experiments demonstrate that the E region is responsible for ligand binding and dimerization and the C region is responsible for DNA binding. Regions A and B contain transactivating functions. The functions of regions D and F are unknown (Reviewed by Chambon, 1996). Retinoic acid response elements (RAREs) have been characterized as having a core motif of (A/G)G(G/T)TCA(X)n(A/G)G(G/T)TCA (Durand *et al.*, 1992; Mader *et al.*, 1993). RAR-RXR dimers have the highest affinity for a spacing of 5 although binding to spacings of 1 or 2 have also been observed.

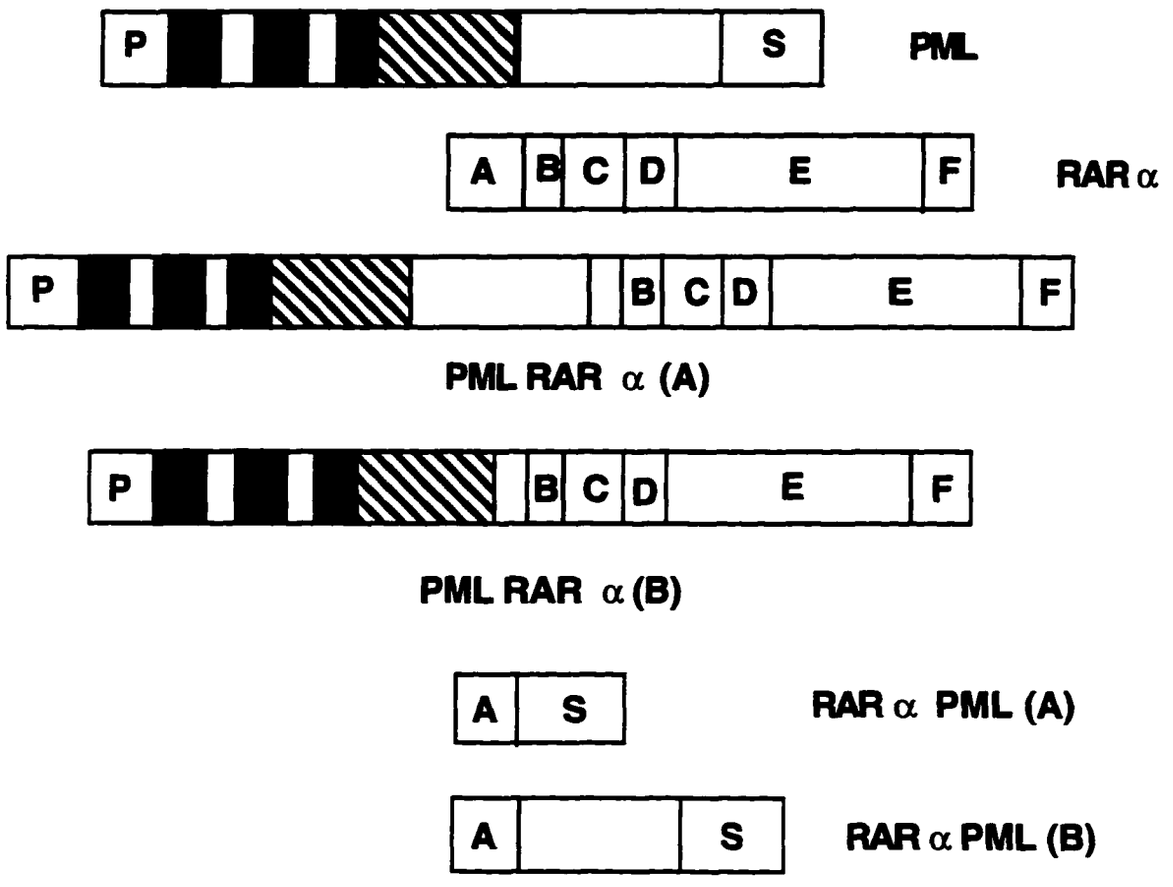


Fig. 1. Schematic representation of PML. RARα and t(15;17) fusion proteins PML RARα and RARα PML. On the PML protein: "P" denotes a proline rich region; Black box denotes the ring finger motif; Gray boxes denotes B-boxes B1 and B2; Hatched box denotes the coiled-coil motif; S denotes a serine proline rich region. The RARα proteins are divided into regions A-F. Regions A and B are transactivation domains. Region C is the DNA binding and dimerization domain. Region E is the ligand binding domain.

In addition, other variations of this core motif (palindromes, inverted palindromes, etc.) have also been identified as RAREs (Reviewed by Chambon, 1996).

RARα and Myeloid Differentiation.

Several lines of evidence strongly suggest that RARα plays a significant role in the differentiation of myeloid cells. In HL60 cells, retinoic acid can induce RARα mediated neutrophilic differentiation (Breitman and Collins, 1981; Collins *et al.*, 1990). Supporting this observation, it was demonstrated that a dominant negative form of RARα inhibited this differentiation (Tsai and Collins, 1993) and in the multipotential cell line FDCPmixA4, dominant negative RARα blocked GM-CSF induced neutrophilic differentiation at the promyelocyte stage (Tsai *et al.*, 1992).

PML

The partner of RARα in the reciprocal translocation t(15;17) is the PML gene which encodes phosphoproteins that belong to the RING finger class of zinc finger proteins. This class of proteins includes the recombinase activating RAG-1 protein (Rodgers *et al.*, 1996), TRAF6 (Ishida *et al.*, 1996), a TNF signal transduction molecule and the DNA repair protein, RAD18 (Lovering *et al.*, 1993) (Figure 2).

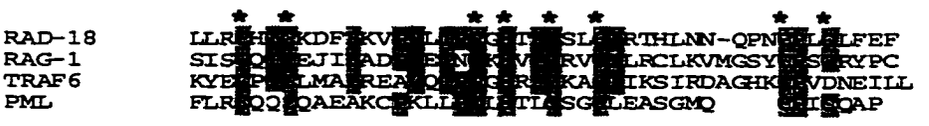


Fig. 2: Sequence alignment of the RING finger proteins. Stars indicate Zn^{2+} ligands. Residues that are conserved are shown in gray.

NMR and other biophysical studies revealed that RING finger motifs of different proteins are structurally diverse which implies that these RING fingers may be functionally diverse as well (Borden *et al.*, 1995). The function of the PML protein is thus far unclear although current evidence suggests it may function in several modes. PML protein has been shown to suppress anchorage independent growth and tumorigenicity of t(15;17) APL derived NB4 cells (Mu *et al.*, 1994). PML also suppressed transformation of early passage REF cells by H-ras and mutant p53 and transformation of NIH 3T3 cells by activated neu oncogene (Mu *et al.*, 1994). Another supporting evidence for PML's effect on cell growth comes from a study which showed that overexpression of PML protein in NB4 cells rendered these cells incapable of propagation in culture (Ahn *et al.*, 1995). On the molecular level, PML has been shown to associate or co-localize with the nuclear matrix and several proteins of known and unknown functions (Boddy *et al.*, 1996; Chang *et al.*, 1995; Dent *et al.*, 1996; Zuber *et al.*, 1995). The significance of its associations is still under investigation. PML is also induced by interferon which suggests that it may play a role in interferon's antiproliferative effects (Lavau *et al.*, 1995; Nason-Burchenal *et al.*, 1996; Stadler *et al.*, 1995). PML proteins are localized in subnuclear structures known as nuclear bodies or PODs (PML oncogenic domains) which were observed to disassemble during mitosis and failed to localize with centromeres, coiled bodies, nucleoli, spliceosomes or sites of DNA replication initiation (Daniel *et al.*, 1993; Dyck *et al.*, 1994; Kastner *et al.*, 1992; Koken *et al.*, 1994; Weis *et al.*, 1994). Moreover, mutations in the RING finger of PML prevent POD formation which suggests that an intact RING finger is necessary for the localization of PML proteins (Borden *et al.*, 1996). Recently, mice homozygous for PML mutation (at the RING finger B-Box region) have been generated. Although these mice appear to be normal, they show a high susceptibility to bacterial infections. Examination of their hematopoietic system revealed that in addition to having

altered immunological response, they have an abnormal proliferation of plasma cells with reduced numbers of granulocytes and monocytes (Pandolfi *et al.*, 1995).

PML-RARα acts as an aberrant RARα

Since the translocation involves the disruption of one allele of each gene, it has been hypothesized that the t(15;17) fusion proteins act as dominant negative mutants inhibiting the functions of the wildtype RARα and PML gene products. It has been shown that PML-RARα can bind to retinoic acid response elements either as homodimer or heterodimer with RXR (Pandolfi *et al.*, 1991; Perez *et al.*, 1993). Therefore, sequestration of RXR and competition for DNA binding sites may be ways in which PML-RARα contribute to leukemic phenotype. Compared with RARα-RXR, however, PML-RARα dimers have a different profile of affinities with the direct repeats suggesting that it may deregulate a set of target genes which includes but are not limited to the target genes of RARα (Perez *et al.*, 1993). Moreover, many studies have shown that PML-RARα acts as a ligand dependent aberrant RARα with significantly reduced transcription activation capabilities (Licht *et al.*, 1996; de Thé *et al.*, 1991; Kastner *et al.*, 1992). Cotransfected with RARα, PML-RARα significantly reduced transactivation in response to retinoic acid (Licht *et al.*, 1996; de Thé *et al.*, 1991; Kastner *et al.*, 1992).

The effect of retinoic acid on the subnuclear localization of PML antigens

PML-RARα may also disrupt the function of wildtype PML. Immunocytochemistry data show that there is a difference in the localization pattern of PML in the absence and in the presence of PML-RARα (Daniel *et al.*, 1993; Dyck *et al.*, 1994; Kastner *et al.*, 1992; Koken *et al.*, 1994; Weis *et al.*, 1994). As stated earlier, PML is localized in PODs which are approximately ten small round structures in the nucleus. (This number changes from cell type to cell type.) When anti-PML antibodies

were used to analyze PML antigen distribution within NB4 cells or PML-RARα transfected COS cells, a fine speckled pattern was seen in the nucleus (Weis *et al.*, 1994). Retinoic acid treatment of cells expressing PML-RARα revealed that the localization of PML antigens gradually changed to the localization pattern of wildtype PML (Daniel *et al.*, 1993; Dyck *et al.*, 1994; Kastner *et al.*, 1992; Weis *et al.*, 1994). In NB4 cells, RXR colocalized with PML-RARα and upon retinoic acid treatment, this colocalization was abolished (Weis *et al.*, 1994). The distribution of RXR resumed a weak diffuse pattern seen in non-APL cells (Weis *et al.*, 1994) supporting the hypothesis that sequestration of RXR by PML-RARα may be a critical factor in leukemogenesis (Weis *et al.*, 1994).

The effects of PML-RARα on cellular differentiation

There is much evidence to suggest that PML-RARα disrupts cellular differentiation and growth. Expression of the PML-RARα in the myeloid precursor cell line U937 has rendered these cells incapable of differentiation in response to signals such as vitamin D3 and transforming growth factor beta 1 (TGFβ–1) (Grignani *et al.*, 1996; Grignani *et al.*, 1993; Rousselot *et al.*, 1994). It was also shown that at low levels of retinoic acid, PML-RARα blocked differentiation of U937 cells but at high levels of retinoic acid, PML-RARα actually enhanced U937 response (Grignani *et al.*, 1996; Rousselot *et al.*, 1994).

Animal models of t(15;17) APL

To assess whether PML-RARα alone is sufficient to cause APL, four groups have attempted to generate animal models. Altabef *et al.* reported that infection of chick embryos with retroviruses encoding PML-RARα resulted in acute leukemia within two weeks of hatching. However, the cells transformed in these animals resemble multipotential hematopoietic precursors rather than promyelocytes (Altabef *et al.*,

1996). Furthermore, these cells do not differentiate in response to ATRA treatment (Altabef *et al.*, 1996). Three groups have generated transgenic mouse lines expressing the fusion protein PML-RARα. In the first transgenic mouse experiment, PML-RARα was placed under the control of the CD11b promoter which directs cell type specific expression in myelomonocytic cells (Early *et al.*, 1996). The mice generated with the use of this promoter displayed normal peripheral blood leukocyte and neutrophil counts and do not develop leukemia after two years of follow-up (Early *et al.*, 1996). However, these mice are more susceptible to sublethal doses of radiation and death was associated with leukopenia and granulocytopenia. *In vitro* clonal growth assays revealed a lower number of GM-CSF responsive myeloid precursor cells in the peripheral blood of transgenic mice versus control suggesting that the transgene had an effect on myeloid development (Early *et al.*, 1996). Transgenic mice were also generated with the use of the cathepsin G promoter, which targets the expression of the transgene to promyelocytes (Grisolano *et al.*, 1997). These mice have an expansion of myeloid cells at all stages of maturation in the bone marrow and a significant expansion of myeloid cells was also seen in the spleen (Grisolano *et al.*, 1997). *In vitro*, splenic promyelocytes expressing the transgene disappear after incubation with ATRA, probably by apoptosis (Grisolano *et al.*, 1997). A striking result of this experiment is that 30% of the PML-RARα expressing transgenic mice developed acute myeloid leukemia after a long latent period (Grisolano *et al.*, 1997). This strongly suggests that although PML-RARα plays a key role in leukemogenesis, another event is necessary for cellular transformation, in agreement with a multi-step progression of oncogenesis. Comparing the phenotypes of these transgenic mice with human APL patients, both groups exhibit thrombocytopenia and anemia. However the severe leukocytosis and organ infiltration found in the transgenic mice are absent in human patients (Grisolano *et al.*, 1997). The last transgenic mouse experiment is closest in the recapitulation of human APL. In this experiment, the expression of PML-RARα is driven by the *hMRP8* promoter which is

not down-regulated during granulocytic differentiation unlike the cathepsin G promoter. These PML-RARα expressing transgenic mice develop APL phenotype including anemia and thrombocytopenia (Brown *et al.*, 1997). Examination of the bone marrow revealed that the normal hematopoietic mix was completely replaced by leukemic cells of promyelocytic morphology (Brown *et al.*, 1997). Severe infiltration of leukemic cells in the kidneys, reproductive organs and meninges were observed in addition to the complete destruction of splenic and lymphatic architecture (Brown *et al.*, 1997). The most striking observation in this study was that the leukemic cells from these transgenic mice can be induced to differentiate by ATRA both *in vitro* and *in vivo* (Brown *et al.*, 1997). Mice treated with ATRA have been shown to undergo complete remission although relapse does occur (Brown *et al.*, 1997), mirroring the effects seen with human APL patients.

Translocation t(11;17)

Identification of t(11;17) APL and cloning of the PLZF gene

Variant translocations have been identified in cases of APL. One variant reciprocal translocation associated with APL was found by Chen *et al.* in an initial study of 32 APL patients (Chen *et al.*, 1993). This translocation t(11;17) fuses the RARα gene to a previously unidentified gene PLZF (promyelocytic leukemia zinc finger gene). Southern blot revealed that the breakpoint on chromosome 17 was within the second intron of RARα, approximately 2 Kb downstream of the exon encoding the A2 region of RARα. The breakpoint in PLZF gene occurs within the region that encodes the zinc finger domain, near the carboxyl terminus of the protein (Chen *et al.*, 1993). By RT-PCR analysis, it was shown that PLZF-RARα fusion transcripts are present in the bone marrow of the index patient and absent in the bone marrows of patients with t(15;17) APL, HL60 cells and NB4 cells (Chen *et al.*, 1993). Currently, six APL patients have

been identified with t(11;17). All six patients responded poorly to ATRA and chemotherapy treatments (Licht *et al.*, 1995).

The PLZF protein

The PLZF gene encodes a 673 amino acid transcription factor that possess nine C2-H2 Kruppel-like zinc fingers in the carboxyl region (Chen *et al.*, 1993) (Figure 3). In its N-terminal region, there is a domain of note, the evolutionarily conserved POZ/BTB (pox virus zinc finger)/BTB (broad-complex, tramtrack, bric a brac) domain. POZ/BTB domains (Figure 4) consist of a stretch of 120 amino acids that are generally located within the N-terminal region of some zinc finger proteins including tramtrack, the GAGA factor, ZF5 and BCL-6 (Bardwell and Treisman, 1994; Chang *et al.*, 1996; Numoto *et al.*, 1993; Seyfert *et al.*, 1996; Zollman *et al.*, 1994). Some POZ/BTB proteins are thought to mediate transcription repression although this is not the case for all POZ/BTB containing proteins (Chang *et al.*, 1996; Numoto *et al.*, 1993; Xiong *et al.*, 1993). Domain mapping experiments, including the ones presented in this thesis, demonstrate that the POZ/BTB domain overlaps a region responsible for transcription repression (Chang *et al.*, 1996; Li *et al.*, 1995; Numoto *et al.*, 1993; Seyfert *et al.*, 1996). The exact function of the POZ/BTB domain is unclear; however, it has been shown to be important for self dimerization of proteins including BCL-6 and PLZF (Bardwell and Treisman, 1994; Chang *et al.*, 1996; Dong *et al.*, 1996; Seyfert *et al.*, 1996) and the subnuclear localization pattern of PLZF and ZID (Bardwell and Treisman, 1994; Dong *et al.*, 1996). PLZF is nuclearly localized and is distributed in faint speckles, partially overlapping with PODs (Licht *et al.*, 1995). It is phosphorylated on serine and threonine residues and has a very specific temporal and spatial pattern of expression (Avantaggiato *et al.*, 1995; Cook *et al.*, 1995; Licht *et al.*, 1995).

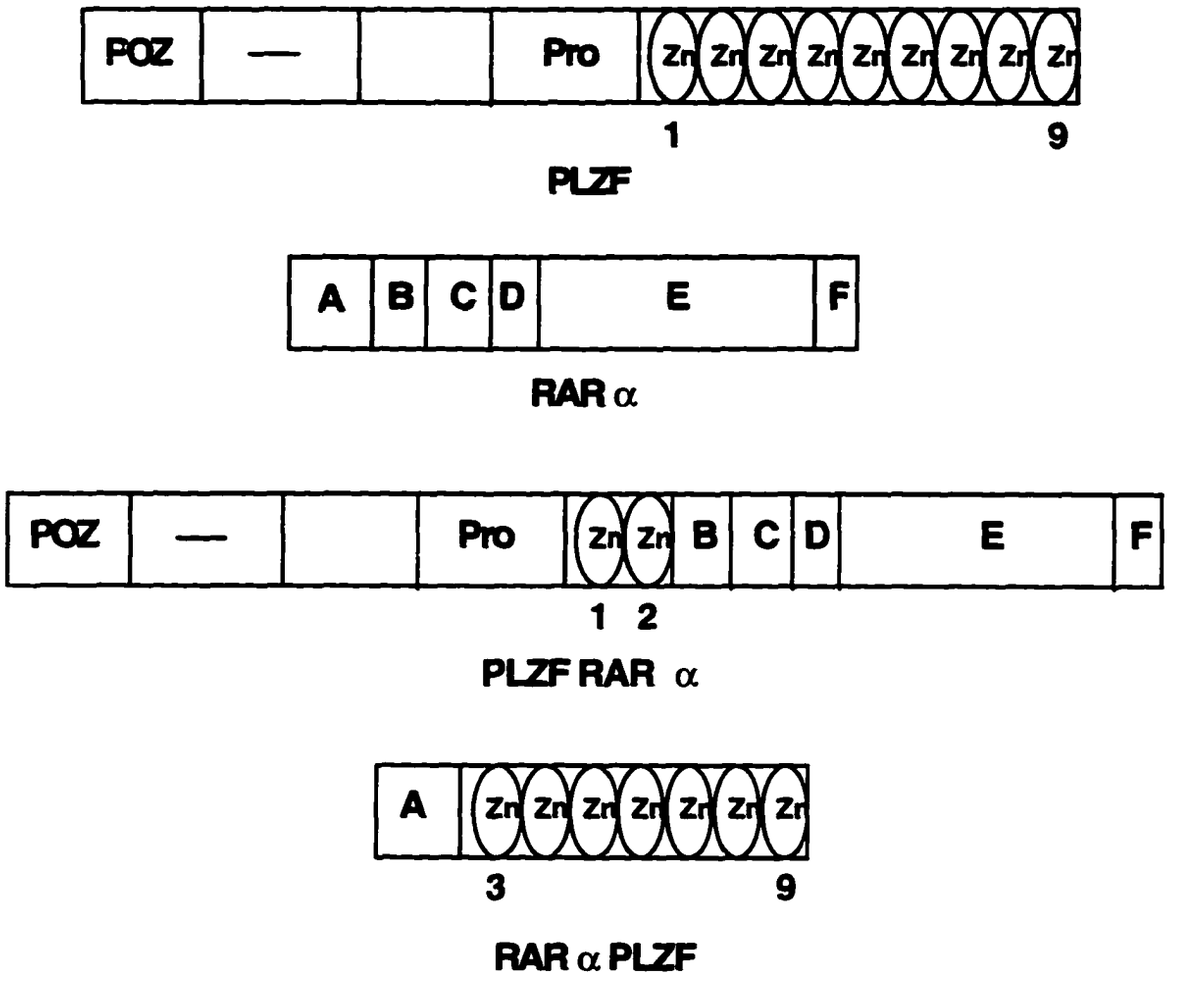


Fig. 3. Schematic representation of PML, RARα and t(11;17) fusion proteins PLZF RARα and RARα PLZF. POZ: PLZF POZ/BTB domain; Pro: Proline rich region; oval with Zn: Zinc finger motifs; "-": acidic region of PLZF; RARα is divided into regions A-F as described in text.

```
M D L T K M G M I Q L Q N P S H P T G L L C K A N Q M R L A      PLZF
M A S P-----A D S C I Q F T R H A S D V L L N L N R L R S R      BCL-6
M A D D E-----Q F S L C W N N F N T N L S A G F H E S L C R      E(Var)
M S L P M N S L Y S L T W G D Y G T S L V S A I Q L L R C H      93-3D
M K M A S---Q R F C L R W N N H Q S N L L S V F D Q L L H A      GAGA
----------Q Q F C L R W M N Y Q T N L T T I F D Q L L Q N      BAB

G T L C D V V I M V D S Q E F H A H R T V L A C T S K M F E      PLZF
D I L T D V V I V V S R E Q F R A H K T V L M A C S G L F Y      BCL-6
G D L V D V S L A A E G Q I V K A H R L V L S V C S P F F R      E(Var)
G D L V D C T L A A G G R S F P A H K I V L C A A S P F L L      93-3D
E T F T D V T L A V E G Q H L K A H K M V L S A C S P Y F N      GAGA
E C F V D V I L A C D G R S M K A H K M V L S A C S P Y F Q      BAB

I L F H R---------N---S Q H Y T L D F L S P K T F Q Q I L E      PLZF
S I F T D Q L K C N L S V I     L D P E I N P E F F C I L L D      BCL-6
K M F T Q M---P S N T H A I V F L N N V S H S A L K D L I Q      E(Var)
D L L K N T---P C K---H P V V M L A G V N A N D L E A L L E      93-3D
T L F V S H---P E K---H P I V I L K D V P Y S D M K S L L D      GAGA
T L L A E T---P C Q---H P I V I M R D V N W S D L K A I V E      BAB

Y A Y T A T L Q A K A E D L D D L L Y A A E I L E I E Y L E      PLZF
F M Y T S R L N L R E G N I M A V M A T A M Y L Q M E H V V      BCL-6
F M Y C G E V N V K Q D A L P A F I S T A E S L Q I K G L T      E(Var)
F V Y R G E V S V D H A Q L P S L L Q A A Q C L N I Q G L A      93-3D
F M Y R G E V S V D Q E R L T A F L R V A E S L R I K G L T      GAGA
F F Y R G E I N V S Q D Q I G P I L R I A E M L K V R G L A      BAB

E Q C L K M L E T I Q A S D D N D T E A T M A D G G A E E E      PLZF
D T C R K F I K A S E A E M V S A I K P P R E E F L N S R M      BCL-6
D N D P A P Q P P Q E S S P P P A A P H V Q Q Q Q I P A Q R      E(Var)
P Q T V T K D D Y T T H S I Q L Q H M I P Q H H D Q D Q L I      93-3D
E V N D D K P S P A A A A A G A G A T G S E S T A T T P Q L      GAGA
D V T                                                          BAB
```

Fig. 4: The POZ domains of six zinc finger proteins.

Similar or identical residues are written in gray.

PLZF expression in mammalian development.

The earliest expression of PLZF was detected in the anterior neuroepithelium (Avantaggiato *et al.*, 1995; Cook *et al.*, 1995). Subsequently, its expression spreads to the entire neuroectoderm until E 10.5. From E 8.5, strong PLZF staining can be seen in parts of the forebrain, midbrain and hindbrain (Avantaggiato *et al.*, 1995; Cook *et al.*, 1995). Its expression in the hindbrain is particularly striking because of its dynamic nature. As the hindbrain develops, it is subdivided into rhombomeres where PLZF levels become markedly restricted both temporally and spatially. Once evenly distributed in the hindbrain, PLZF becomes down-regulated in rhombomeres 3 and 5 by E 8.5 (Cook *et al.*, 1995). By E 10, PLZF expression was observed only in the rhombomeric boundaries (Cook *et al.*, 1995) suggesting that PLZF plays a significant role in defining hindbrain subdivisions. As development progress, PLZF was detected in the developing lung, kidney, liver and heart of the fetal mouse (Cook *et al.*, 1995). After parturition, PLZF levels increase dramatically in the kidney, liver and heart suggesting that PLZF may also play a role in metabolic adaptations required of newborns (Cook *et al.*, 1995). In the adult animal, a high level of PLZF was seen in the heart, with lower levels detected in the lung, muscle, brain and spleen (Cook *et al.*, 1995).

PLZF expression in the hematopoietic system

In the hematopoietic system, PLZF expression was detected in myeloid cell lines KG1, HL60, and NB4 but absent in cells of erythroid or lymphoid lineages (Chen *et al.*, 1993). PLZF mRNA levels decline as HL60 cells and NB4 cells are induced to differentiate by retinoic acid suggesting that the down regulation of PLZF plays a role in the maturation of these cells (Chen *et al.*, 1993). Supporting this conclusion, PLZF mRNA levels was shown to be high in early multipotential progenitor cell lines such as FDCPmixA4 and B6SUtA and lower in lineage-restricted myeloid cell lines such as 416B and J774.2 (Reid *et al.*, 1995). In addition, overexpression of PLZF in the non-

tumorigenic myeloid cell line 32DCL3G/GM resulted in severe growth suppression which was accompanied by the accumulation of these cells in G1 phase of the cell cycle and an increased incidence of apoptosis (Shaknovich *et al.*, 1997). PLZF overexpression also inhibited the differentiation of 32DCL3G/GM cells into granulocytes and monocytes (Shaknovich *et al.*, 1997). Combined, these data strongly suggest that PLZF is a growth inhibitor whose disruption in t(11;17) may play a role in oncogenesis.

PLZF-RARα acts as an aberrant RARα

Translocation t(11;17) results in two sets of fusion proteins RARα-PLZF and PLZF-RARα (Figure 3). PLZF-RARα encodes the entire N-terminal region of PLZF including the POZ/BTB domain and two of its nine zinc fingers fused to the B-F domains of RARα. Immunoflourescence studies performed with PLZF-RARα transfected CV-1 cells demonstrate that PLZF antigen distribution does not change with ATRA treatment suggesting that mislocalization of PLZF does not play a prominent role in t(11;17) APL (Licht *et al.*, 1996). PLZF-RARα has been shown to bind RAREs as homodimer and as heterodimer with RXR (Chen *et al.*, 1994; Dong *et al.*, 1996; Licht *et al.*, 1996). Transient transfection assays revealed that PLZF-RARα, like PML-RARα, act as an aberrant transcription factor, with significantly less ligand dependent transactivation capabilities than wildtype RARα. The loss in transactivation has been mapped to two regions of PLZF-RARα, the POZ/BTB domain and the first two zinc fingers of the PLZF moiety. When PLZF-RARα was cotransfected with wildtype RARα, it blocked ligand dependent activation through RAREs which indicates that it has a dominant negative effect on RARα transcription regulation (Chen *et al.*, 1994; Dong *et al.*, 1996; Licht *et al.*, 1995). These data suggest that PLZF-RARα may promote leukemogenesis by sequestration of RXR, competition with RARα /RXR dimers for DNA binding sites, or having aberrant effector functions. All experimental evidence gathered to date suggests that the effects of PML-RARα and PLZF-RARα on RARα transcription regulation are

similar and yet a fundamental difference exists between t(15;17) and t(11;17) APL: Patients with t(11;17) APL do not respond to ATRA treatment. Thus, combined, these data argue that the non-responsiveness of t(11;17) patients to ATRA may lie in the disruption of PLZF transcription regulation by RARα-PLZF. It is worthy to note that both RARα and RARα-PLZF are regulated by a promoter containing RAREs. Thus, retinoic acid treatment can also cause an elevated level of RARα-PLZF expression, exacerbating the deregulation of PLZF target genes.

RARα-PLZF possesses either the A1 or A2 domain of RARα fused to the most carboxyl seven zinc fingers of PLZF (Figure 3). Since the A1 and A2 domains of RARα replaces the entire effector domain of PLZF, these fusion proteins may act as novel transcription activators with the DNA binding specificity of PLZF. To date, no experimental data has been presented on the effect of RARα-PLZF on PLZF transcription regulation. This is mainly due to the fact that little is known about the PLZF DNA binding site. Simultaneous with the studies presented in this thesis, a DNA binding site was fortuitously discovered in a yeast two hybrid experiment (Sitterlin *et al.*, 1997). This study demonstrates that PLZF zinc fingers bound specifically to the Lex A operator. Furthermore, it has narrowed the DNA binding activity of PLZF to the last five zinc fingers which strongly suggests that RARα-PLZF can compete with PLZF for binding sites. Although the results of this study yields a potential system to study PLZF transcription regulation, it does not address transcription regulation by either PLZF or RARα-PLZF and it does not provide insights into the identity of PLZF target genes in mammalian cells.

DNA binding by zinc finger transcription factors

Zinc fingers are sequence specific DNA binding domains which were first identified in the Xenopus transcription factor TFIIIA (Miller *et al.*, 1988). NMR and

crystallography studies show that the C2-H2 zinc finger motif is composed of 28 to 30 amino acids structurally arranged in an antiparallel β-sheet followed by an α-helix (Lee *et al.*, 1989; Pavletich and Pabo, 1991). Two invariant cysteines located near the turn of the β-sheet and two invariant histidines in the α-helix chelate a single zinc ion (Lee *et al.*, 1989; Pavletich and Pabo, 1991). Two conserved hydrophobic residues, a phenylalanine and a leucine, located at the loop region of the zinc finger, are thought to stabilize the zinc finger structure (Figure 5) (Lee *et al.*, 1989; Pavletich and Pabo, 1991).

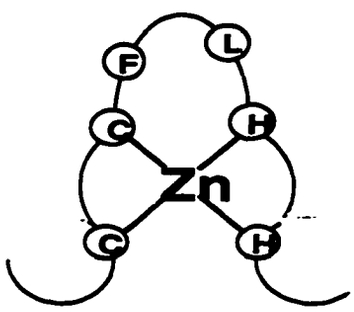


Fig. 5: Schematic representation of a zinc finger. The circles with C and H represent cysteines and histidines at invariant positions. They chelate a singe zinc ion (Zn). Conserved hydrophobic residues phenylalanine and leucine are depicted as circles with F and L.

X-ray crystallography studies of zif 268 revealed that each zinc finger recognizes discreet triplets of DNA with two bases in the triplet making direct contact with amino acids on the zinc finger (Pavletich and Pabo, 1991). Although this base triplet recognition theory was useful in predicting the DNA binding activity of some zinc finger proteins, zinc finger-DNA interactions may be more complex than once envisioned. X-ray crystallography data from tramtrack-DNA (Fairall *et al.*, 1993) complex demonstrates that DNA binding by zinc finger proteins may occur through

different structural arrangements compared with those of zif 268. Moreover, it has been demonstrated that zinc finger proteins may bind specifically to many DNA sequences. The Drosophila tramtrack protein which possess two zinc fingers bind to many sequences that have little in common other than a GGA (Fairall *et al.*, 1993; Fairall *et al.*, 1992). There are also many binding sites found for WT1 proteins that have little in common (Rauscher III *et al.*, 1990; Wang *et al.*, 1993). This may be due to many reasons. It has been shown that the WT1 proteins recognize different DNA sequences depending on which subset of zinc fingers is employed. In addition, not all zinc fingers on a given protein bind DNA bases. In TFIIIA and tramtrack, zinc fingers have been shown to contact phosphodiester backbones or may not contact DNA at all (Fairall *et al.*, 1993; McBryant *et al.*, 1996; Pavletich and Pabo, 1991). Crystallography studies of DNA binding by tramtrack and GLI also indicate that zinc finger contacts can be made on both strands of DNA (Fairall *et al.*, 1993; Pavletich and Pabo, 1993). There is now accumulating evidence which suggests that DNA architecture may play a large role in DNA binding by zinc finger proteins. Zinc finger 4, shown to be vital for high affinity DNA binding by TFIIIA, is thought to recognize specific DNA structure rather than sequence (McBryant *et al.*, 1996). In addition, DNA binding by zinc finger protein has been correlated with DNA deformation. Structural changes at an ATA sequence has been shown to occur when tramtrack zinc fingers bind DNA (Fairall *et al.*, 1993). DNA binding by SP1 has been shown to accompany unwinding of the DNA strands (Shi *et al.*, 1996). Although most proteins bind to DNA with two or more zinc fingers, the GAGA factor has been shown bind DNA with high affinity and specificity utilizing one zinc finger flanked by basic residues (Pedone *et al.*, 1996).

Transcription factors

Transcription by RNA polymerase II is regulated by a multitude of protein factors that positively or negatively affect gene expression. Basal levels of gene

transcription are maintained by general transcription factors which are required for RNA polymerase activity (Reviewed by Pugh and Tjian, 1992). These factors have been shown to assemble onto the promoter region in a sequential manner, with TFIID being the first to contact the TATA box or initiator element followed by TFIIA, B, F, E, H and J. Fractionation experiments have shown that some general transcription factors are multi-protein complexes. TFIID, for example is composed of the TATA binding protein (TBP) and TBP associated factors (TAFs) (Reviewed by Pugh and Tjian, 1992).

Basal transcription is upregulated by activators which have been shown to elevate gene expression in a DNA binding dependent manner (Reviewed by Ptashne and Gann, 1997). Activators are thought to stimulate gene expression by recruiting the transcriptional machinery to the initiation site. Evidence supporting this hypothesis comes from biochemical studies which demonstrate physical interactions between activators and general transcription factors. For example, the yeast Gal4p has been shown to associate with both TFIID and TFIIB (Lin *et al.*, 1991; Wu *et al.*, 1996). Moreover, experiments utilizing mutants of the Gal4p activation domain have demonstrated that the affinity of Gal4p for these general transcription factors is correlative with the degree of transcription activation (Wu *et al.*, 1996).

There are also evidence which suggests that the RNA polymerase holoenzyme may be a target of activators as well. Experiments performed in yeasts have also revealed that the introduction of a Lex A DNA binding domain into a component of the RNA polymerase holoenzyme results in transactivation which is dependent on the presence of Lex A binding sites (Barberis *et al.*, 1995). This experiment supports the hypothesis that activation can be achieved by the recruitment of RNA polymerase II to the site of transcription initiation. Activators can also induce post-translation modifications of holoenzyme components resulting in an increased level of transcription. Activators,

such as the HIV Tat protein and the adenovirus E1A protein, have been shown to enhance the elongation of transcripts, by promoting the phosphorylation of the CTD domain of RNA polymerase II by TFIIH, and CDK8 respectively (Gold *et al.*, 1996; Parada *et al.*, 1996).

Unlike activators, proteins that repress transcription act by inhibiting the assembly of the transcriptional machinery. The evenskipped repressor protein has been shown to physically block TFIID from assembling onto the TATA box; this repression can be abrogated by preincubating the TATA box with TFIID (Austin *et al.*, 1995). Similarly, the Drosophila Kruppel protein has been shown to associate with a component of TFIIE and this association is correlated with Kruppel's ability to repress transcription (Sauer *et al.*, 1995). Genetic experiments revealed that the NC2 protein repress transcription by interacting with components of the RNA polymerase II (Gadbois *et al.*, 1997).

Repressors can also function by competing with activators for DNA binding sites. An example is seen on the immunoglobulin heavy chain (IgH) enhancer which contains E-boxes that are the sites of contention between the E2A activator and the ZEB repressor (Genetta *et al.*, 1994). Transcriptional repression can also be the result of a specific interaction between a repressor and an activator. The Kruppel protein, for example, block transcription activation by SP1 but not that of Gal4p suggesting that it represses transcription through very specific protein-protein interactions (Licht *et al.*, 1993).

Recently, it has been increasingly apparent that nucleosomes play a large role in transcriptional regulation. They have the potential to block transcription through steric hindrance, although the position that they occupy on the promoter can be modified. Post translational modifications of the core histones such as acetylation can destabilize the

nucleosome structure and enhance transcription initiation. Acetylation of core histones is thought to maintain the promoter in an "open" conformation to facilitate transcription initiation. Conversely, histone deacetylation is thought to play a role in transcription repression. This is supported by many lines of investigation which show that some DNA binding transcriptional repressors act by recruiting deacetylases to nucleosome. For example, unliganded, DNA bound steroid hormone receptors are linked to a histone deacetylase by a N-CoR/SMRT mSin3A complex. These protein interactions ultimately results in transcription repression in the absence of steroid hormone (Alland *et al.*, 1997; Thorsten *et al.*, 1997).

Specific Aims

The involvement of PLZF in t(11;17) APL suggests that its disruption plays a key role in the genesis of this disease and in the non-responsiveness of t(11;17) APL patients to retinoic acid treatment. The establishment of a system to study PLZF's effect on transcription may provide insights into PLZF's biological function and the role of t(11;17) proteins on the expression of PLZF target genes. Therefore the goals of this thesis are:

1. To find the cognate DNA binding site(s) of PLZF and if possible establish a binding site consensus
2. Deduce the DNA binding activities of the t(11;17) fusion proteins.
3. Characterize PLZF's effect on transcription through its cognate DNA binding site(s).
4. Characterize the effect of t(11;17) fusion proteins on transcription through PLZF's cognate DNA binding site.
5. Map the functional domains of the PLZF effector region.

Chapter 2

MATERIALS AND METHODS

Plasmid Constructions

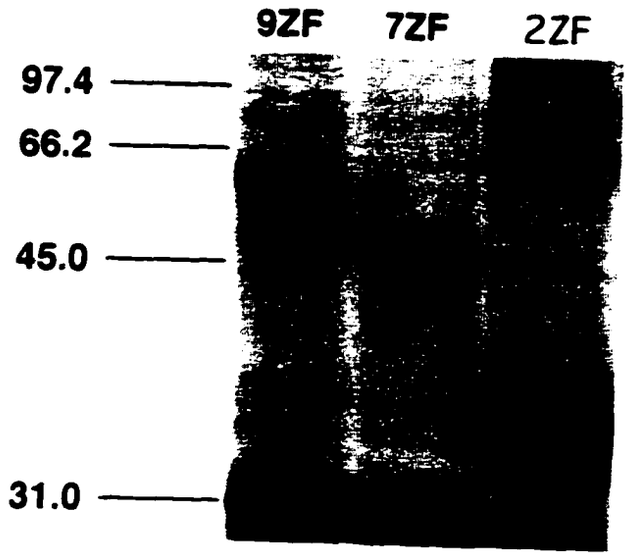
To produce purified protein for binding site selection, the nine zinc fingers of PLZF was expressed as a glutathione S-transferase fusion protein (GST-9ZF) (Figure 6). To construct GST-9ZF, the PLZF cDNA (Chen *et al.*, 1993) was digested with *Nco* I to release the sequences encoding the N-terminal effector domain and recircularized. Sequences encoding the nine zinc fingers of PLZF were then excised by digestion with *Eco*R 1 and inserted into *Eco*R 1 digested pGEX-3X (Pharmacia, Uppsala, Sweden). GST-7ZF, which includes PLZF zinc fingers 3-9 (Figure 6), was synthesized by digesting the PLZF cDNA with Sac I and *Eco*R 1 and inserting this fragment into pGEX-2T (Pharmacia, Uppsala, Sweden) digested with *Bam*H1 and *Eco*R 1 utilizing an adapter of the sequence:

5'GATCGTTAACGAGCT 3'
3'CAATTGC5'

GST-2ZF, which includes the most N-terminal two zinc fingers of PLZF (Figure 6), was constructed by amplification of a portion of the PLZF cDNA with the N terminal primer 5' GGATCCGGATCCCCGTGGGCATGAAGTCA 3' and the C-terminal primer: 5' GAATTCGAATTCAGAACTGCTGCTCTGGGT 3' The amplified fragment was digested with *Bam*H 1 and *Eco*R 1 and subcloned into pGEX-3X digested with *Bam*H 1 and *Eco*R 1. SG5-PLZF, RARα-PLZF and PLZF-RARα expression plasmids, described previously (Chen *et al.*, 1994; Licht *et al.*, 1996), contain the SV40 promoter/enhancer included in the SG5 plasmid (Green *et al.*, 1988). To map the PLZF functional domains, various segments of PLZF were fused to the DNA binding domain of Gal4p (amino acids 1-147). Gal4p-PLZF fusion constructs were made by amplification of segments of the PLZF coding sequence by PCR, digestion of these fragments with *Bam*H 1 and *Xba* I and insertion of these sequences

GST-

A



B

5′ GGGACAATTCAACTGCCATCTAGGC N20 ACACCGAGTCAGAAGGATCCTACG 3′
3′ CCCTGTTAAGTTGACGGTAGATCCG N20 TGTGGCTCAGTCTTCCTAGGATGC 5′

Oligomers used in the first set of binding site selection

5′ GGGACAATTCAACTGCCATCTAGGC N13 TAAA N13 ACACCGAGTCAGAAGGATCCTACG 3′
3′ CCCTGTTAAGTTGACGGTAGATCCG N13 TAAA N13 TGTGGCTCAGTCTTCCTAGGATGC 5′

Oligomers used in the second set of binding site selection

Fig 6. Proteins and oligomers used in the PLZF binding site selection. *Panel A*, SDS PAGE analysis of GST fusion proteins. GST-9ZF, GST-7ZF and GST-2ZF with expected sizes of 60kD, 57 kD, and 33 kD, respectively. *Panel B*, In the first set of binding site selection, the oligomers used were 70 base pairs in length flanked by regions of known sequences which are 25 base pairs in length.. The random sequence regions in these oligomers were 20 base pairs in length. In the second set of binding site selection, the oligomers used were 80 base pairs in length with 13 base pairs of random DNA sequence flanking a central TAAA. On both ends of these oligomers are regions of known DNA sequence of 25 base pairs in length.

into pGBX a plasmid encoding Gal4p amino acids 1-147, digested with *Bam*H 1 and *Xba* I.

Sequences encoding PLZF fragments beginning with amino acid 1 were amplified with the N-terminal primer:

5'CGCGGATCCGTATGGATCTGACAAAAATG3'.

Sequences encoding fragments of PLZF beginning with amino acid 99 were amplified with the N-terminal primer:

5'CGCGGATCCGTCTGGATGACCTGCTGTAT.

Sequences encoding fragments of PLZF beginning with amino acid 200 were amplified with the N-terminal primer:

5'CGCGGATCCGTAAGGCTGCAGTGGACAGTTTG3'.

Sequences encoding fragments of PLZF beginning with amino acids 300 were amplified with the N-terminal primer:

5' CGCGGATCCGTGAGGAGAGTCGCCTCGAGCAG3'.

Sequences encoding fragments of PLZF ending with amino acid 100 were amplified with the C-terminal primer:

5'GCTCTAGAGATCCAGGGCCTCCGCCTTG3'.

Sequences encoding fragments of PLZF ending with amino acid 200 were amplified with the C-terminal primer:

5'GCTCTAGAGCTTGGTGGGACTCATGGCTGA3'.

Sequences encoding fragments of PLZF ending with amino acid 300 were amplified with the C-terminal primer:

5' GCTCTAGAGCTCTCGCCCATAGTGTAG 3'.

Sequences encoding fragments of PLZF ending with amino acid 400 were amplified with the C-terminal primer:

5' GCTCTAGAGCCGGCTCTCTGACTT 3'.

Flag-9ZF encodes the nine zinc fingers of PLZF fused in-frame to the flag epitope. It was constructed by digesting the plasmid AZ1 with *Nco* I to release the sequence encoding the effector domain and the remaining plasmid was recircularized as previously described. The cDNA sequence encoding the nine zinc fingers was excised by digestion with *Bam*H 1 and *Eco*R1 and ligated to *Bam*H 1, *Eco*R 1 digested pBFT4 (a gift from Dr. Xin-Yuan Fu). The pBFT4 plasmid contains a sequence encoding the flag epitope upstream of the polylinker region of pBluescript (KS^+).

Reporter construct G_5 *tk*-CAT was described previously (Shi *et al.*, 1991).

Reporter 15(n) tk-CAT was constructed by ligating one (n=1), two (n=2), or three (n=3) copies of duplex 15A oligomer:

5' TCGACTTAAAGGAAGCCACCATGAAG 3'
3' GAATTTCCTTCGGTGGTACTTCAGCT 5'

in the *Sal* 1 site of the polylinker region of pBLCAT5 (Boshart *et al.*, 1992).

Reporter construct A(n) *tk*-CAT was constructed by ligating one (n=1), two (n=2) or three (n=3) copies of the duplex oligomer:

5' TCGATATTGAAGCTAAAGTTTGATCTG 3'
3' ATAACTTCGATTTCAAACTAGACAGCT 5'

in the *Sal* 1 site of the polylinker region of pBLCAT5 (Boshart *et al.*, 1992).

Binding Site Selection

Bacterial expression plasmids were transformed into the Dna J deficient E. coli K12 strain CAG748 (New England Biolab, Beverly, MA). To produce Glutathione *S*-transferase (GST) fusion proteins, exponentially growing cultures of transformed bacteria were induced with IPTG for 4 hours at 37°C, lysed by sonication with the fusion protein purified on glutathione agarose beads as described (Ausubel *et al.*, 1989; Smith and Johnson, 1988). GST protein coated agarose beads were stored at -20°C in a buffer containing 50 mM HEPES pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, 1 mM DTT and 50% glycerol (Fainsod *et al.*, 1991).

To prepare a pool of random oligomers for the first set of binding site selections, an oligonucleotide (500 ng) of the sequence:

5' GGGACAATTCAACTGCCATCTAGGC $(N)_{20}$ ACACCGAGTCAGAAGGATCCTACG 3'

was hybridized to primer RP2 (250 ng) of the sequence:

5'CGTAGGATCCTTCTGGACTCGGTGT 3'

in a total volume of 10 μl. Five μl of the annealed DNA was extended to make a duplex random oligonucleotide pool with the Klenow fragment of DNA polymerase I (New England Bioloabs) with a final concentration of 0.25 mM of each dNTP at 37°C in a volume of 50 μl. After an hour incubation, dNTPs were added to a final concentration of 0.25 mM (each) and the incubation in 37°C was continued for another 30 minutes. The duplex

oligomers were ethanol precipitated, resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA), phosphorylated with T4 kinase and γ-[^{32}P] ATP (6000 Ci/mM), and purified using a spin column (Clonetech, Palo Alto, CA).

PLZF binding sites were selected by incubating the random oligomers with 50µl of a 50% slurry of GST-9ZF coated agarose beads (approximately 3 µg protein) in siliconized ependorf tubes in a buffer containing 10 mM Tris-HCl pH7.5, 1 mM EDTA, 4 mM DTT, 1 mM $MgCl_2$, 50 mM NaCl and 5% glycerol (Buffer A). After one hour of incubation on ice, the beads were collected by centrifugation, the supernatant containing unbound DNA was removed, and the beads were washed twice with 1 ml of ice cold buffer A. The oligomers retained by the beads were released by addition of 20 µl of deionized water and boiling for five minutes. The resulting DNA was subjected to PCR amplification with primer RP2, described above and primer RP3 of the sequence:

5'GGGAGAATTCAACTGCCATCTAGGC3'

The reaction (50 µl) contained 0.25 mM dATP, dGTP and dTTP, 0.05 mM dCTP and 3µl of α-[^{32}P] dCTP (3000 Ci/mmol) to radiolabel the PCR products. Twenty cycles of PCR were performed, consisting of 1 minute in 95°C, 1 minute in 59°C and 1 minute at 72°C. Subsequently, 1 µl of mixture of 2.5 mM of each dNTP was added to the reaction followed by one cycle of 5 minutes in 95°C, 2 minutes in 59°C and 15 minutes in 72°C. The amplified DNA was purified by spin column and used in a subsequent round of binding site selection. The success of each selection round was monitored by the increasing percentage of radiolabeled probe oligomer retained of the GST-PLZF beads. After six rounds of selection, the selected DNA was reamplified using radiolabeled dCTP as above, purified and incubated with approximately 3 µg of GST-9ZF protein in a 20 µl reaction containing buffer A. The resulting DNA-protein complexes were resolved by

electrophoresis through a 6% (30:1 acrylamide:bisacrylamide) gel. The retarded DNA-protein complex was excised, crushed and soaked overnight in 300 μl of 0.5 M ammonium acetate, 1mM EDTA. The eluted DNA was ethanol precipitated, digested with *Bam*H 1 and *Eco*R 1, subcloned into the polylinker region of pBluescript SK+ (Strategene, La Jolla, CA) transformed into E. coli and sequenced. A total of 29 clones were sequenced in this set of selection.

In the second set of binding site selection, the random oligomers used had the sequence:

5' GGGACAATTCAACTGCCATCTAGGC $(N)_{13}$ TAAA $(N)_{13}$ ACACCGAGTCAGAAGGATCCTACG 3'

Duplex oligomers were created by primer extension with the RP2 primer and fours rounds of binding site selection was performed as described above. The selected DNA was digested with *Bam*H 1 and *Eco*R 1 subcloned into pBluescript (SK^+) and 42 individual clones were sequenced.

Protein Production

GST Fusion Proteins

GST-PLZF fusion proteins (GST-PLZF, GST-9ZF, GST-7ZF and GST-2ZF) were produced as described in the binding site selection section with modifications.

To purify GST fusion proteins for mobility shift assays, 250 μl agarose beads were incubated with 10 ml of crude bacterial extract for 1 hour in 4°C were washed three times in 15 mls of ice cold PBS. To elute the bound GST fusion proteins from agarose beads, 250 μl of protein bound beads were incubated at room temperature for 1 hour in 1 ml of elution buffer which contained 20 mM of reduced glutathione, 100 μl of

1M Tris pH 8.45 and 900 µl of water. At the end of the incubation, the beads were centrifuged and the supernatant was collected. The concentration of the protein was determined by using spectrophotometry at the wavelength of 280 λ. A reading of 1 unit of optical density is equivalent to 0.5 mg/ml protein (Current Protocols).

Some mobility shift assays were performed with crude bacterial cell extracts which were produced in the following way: Bacterial cells were transformed, grown and induced as previously described. Sonication was performed in 10 ml of ice cold PBS. The resulting extract was aliquoted, subjected to flash freezing in liquid nitrogen and stored in -70°C.

In vitro Translation (Reticulocyte Lysate System)

All *in vitro* translations were performed with either T3, T7 based vector using the TNT rabbit reticulocyte lysate system (Promega, Madison, WI). Radiolabeling of the protein was performed by adding ^{35}S-methionine to the translation reaction. The protein was visualized by SDS-PAGE and autoradiography.

Whole Cell Extract

CV-1 cells were transfected with protein expression plasmids using a standard calcium phosphate protocol described below. The cells, harvested 48 hours post transfection was washed twice in ice cold TBS (140 mM NaCl, 5 mM KCl, 25 mM Tris pH 7.4) . The cells were resuspended in 150 µl ice cold lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 2% NP-40) and incubated on ice for 30 minutes. Cells were centrifuged for 20 minutes at 4°C and the supernatant was collected. The extract was subjected to flash freezing and stored in -70°C.

Electrophoretic Mobility Shift Assay (EMSA)

Synthetic duplex oligomer 15A of the sequence:

5'TCGACTTAAAGGAAGCCACCATGAAG3'
3' GAATTTCCTTCGGTGGTACTTCAGCT 5'

or synthetic duplex oligomer probe A of the sequence:

5' TCGATATTGAAGCTAAAGTTTGATCTG 3'
3 ' ATAACTTCGATTTCAAACTAGACAGCT 5'

was labeled by filling in with the Klenow fragment and α-[^{32}P] dTTP (3000 Ci/mmol) and purified by spin column.

Synthetic duplex oligomer β-RARE of the sequence:

5'AGCTTGGGTAGGGTTCACCGAAAGTTCACTCGA3'
3'ACCCATCCCAAGTGGCTTTCAAGTGAGCTTCGA 5'

was labeled by filling in with the Klenow fragment and α-[^{32}P] dATP (3000 Ci/mmol) and purified by spin column.

Each binding reaction (20 μl) contained approximately 2 μg of GST, GST-9ZF, GST-7ZF, or GST-2ZF as measured by A_{280} (unless otherwise indicated in figure legends) and 26 fmol of labeled DNA (approximately 0.6ng) in a buffer of 20 mM ZnCl, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT. The binding reactions were incubated on ice for one hour. In competition electrophoretic mobility shift assays, unlabeled DNA competitors were pre-incubated with GST-9ZF or GST-7ZF

for 15 minutes on ice, followed by the addition of labeled duplex oligonucleotide and incubation on ice for 45 minutes. The resulting DNA-protein complexes were separated by electrophoresis at 200 volts though either 4% or 6% polyacrylamide gels (30:1acrylamide/bis-acrylamide) for 3.5 hours at room temperature. The competitors used in the EMSAs are as follows:

β-RARE 5'AGCTTGGGTAGGGTTCACCGAAAGTTCACTCGA3'
3'ACCCATCCCAAGTGGCTTTCAAGTGAGCTTCGA5'

AP1 site 5'TGAGCATGAGTCAGACAC3'
3'TCGTACTCAGTCTGTGGT5'

Gal4 operator 5'GCCTCCTGTCATGAGGCCTAG3'
3'GATCCGGAGGACAGTACTCCG5'

p53 DNA binding site: 5'CTAGGGACATGCCCGGGCATGTC3'
3'CCTGTACGGGCCCGTACAGGATC5'

WT1 DNA binding site: 5'TCGACGCGTGGGAGTAG3'
3'GCGCACCCTCATCAGCT5'

Base transversion mutants of binding site A are as listed in Fig. 4.

Transient Transfections

CV-1 cells

CV1 cells were grown in DMEM supplemented with 10% calf serum and penicillin/streptomycin in a 5% CO_2 environment. One day before transfection, $1x10^6$ cells were plated in 100 mm tissue culture dishes. Reporter and effector plasmids in the amounts indicated in the figure legends along with 1 μg of a growth hormone (GH) internal control reporter gene (Selden *et al.*, 1986) were co-transfected by calcium phosphate precipitation as described (Licht, 1994; Reddy, 1995); Ausubel, 1989 #673]. At 48 hours post transfection, the cell media was collected and assayed for GH (Nichols Institute, San Juan Capistrano, CA) and the transfected cells were harvested

and cell lysates were assayed for chloramphenicol acetyl-transferase (CAT) (Ausubel *et al.*, 1989). The percent conversion of acetylated chloramphenicol was quantified by analysis of chromtography plates on a Phosphoimager using Imagequant software (Molecular Dynamics, Sunnyvale, CA). CAT activity is defined as the percentage CAT conversion. Relative percent CAT conversion is calculated by assigning the value of 100% to the CAT activity obtained when SG5 was used as effector. When required, chromatographs were analyzed by densitometric analysis (NIH Image 1.56) of scanned images. Graphics were scanned on a Agfa Arcus II scanner (Germany) and assembled on a Power Macintosh 7100/66 computer (Apple, Cupertino, CA).

Hematopioetic Cells

U937, K562 and HL60 cells were transfected using standard DMRIEC protocol with modifications. Cells were grown in a 5% CO_2, 37°C environment in RPMI media supplemented with penicillin/streptomycin and 10% heat inactivated fetal calf serum. The day of the transfection, K562 and U937 cells were spun down and suspended to the density of 2 X 10^5 cells per 200 µl. HL60 cells were suspended in the same media at the density of 4 X10^6 cells per 200 µl.

Reporter plasmid (2 µg), effector plasmid (2 µg), and MTGH (0.2 µg) were added to eppendorf tubes and suspended in 1 ml of Optimem reduced serum media (Gibco/BRL). 4 µl of DMRIEC reagent (Gibco/BRL) was then added to each tube and the mixture was allowed to incubate at room temperature for 1 hour so that the DNA liposome complexes could form. At the end of the hour, the mixture was pipetted into wells of a 6-well plate along with 200 µl of cell suspension. The cells were incubated in this mixture for 12 hours.

After the incubation, 1 ml of RPMI (with 10% heat inactivated fetal calf serum and pennincillin/streptomycin) was added to each well and the cells were allowed to grow in this media for another day. The cells were then harvested and cell lysates were assayed for chloramphenicol acetyl-transferase (CAT) (Ausubel *et al.*, 1989). The percent conversion of acetylated chloramphenicol was quantified by analysis of chromtography plates on a Phosphoimager using Imagequant software (Molecular Dynamics, Sunnyvale, CA). CAT activity is defined as the percentage CAT conversion. Relative percent CAT conversion is calculated by assigning the value of 100% to the CAT activity obtained when SG5 was used as effector. Graphics were scanned on a Agfa Arcus II scanner (Germany) and assembled on a Power Macintosh 7100/66 computer (Apple, Cupertino, CA).

Production of antibodies against PLZF

GST and full length GST-PLZF was produced as previously described. To produce polyclonal anti-PLZF antisera, GST-PLZF was injected into rabbits by Dr. Thomas Moran of the Hybridoma and Cell Center Core facility of the Mount Sinai Medical Center. Anti-PLZF activity was further purified by removing anti-GST antibodies. This was performed by subjecting the antisera to a chromatography column containing 2 ml of swollen GST coated agarose beads. The flow-through was tested for anti-PLZF activity by radio-immuno assay performed by Dr. Moran and Western blot analysis performed by Rita Shaknovich.

To produce monoclonal antibodies against PLZF, GST-PLZF was injected into mice by Dr. Thomas Moran. Six distinct hybridoma cell lines were produced. Antibodies from 800 ml of supernatant from each hybridoma was purified by perfusion chromatography on a protein G column using standard methods. Purified IgG antibodies from the supernatants of all six cell lines were used by Rita Shaknovich in a Western Blot

Analysis to assess their activities against GST, GST-PLZF and GST-9ZF. All six reacted only to GST-PLZF. One particular hybridoma clone (2A9) was subjected to further characterization. GAL-PLZF deletion constructs were transfected into CV-1 cells and whole cell lysates were prepared as previously described. The resulting lysates were given to Rita Shaknovich for use in a dot blot analysis. Antibodies produced by clone 2A9 recognized amino acids 100-200 of the PLZF effector domain

Chapter 3

PLZF IS A SEQUENCE SPECIFIC DNA BINDING PROTEIN

INTRODUCTION

Most proteins that regulate transcription have domains that confer sequence specific high affinity DNA binding activity. Historically, transcription factors such as TFIIIA and SP1 have been purified based on their ability to tether cis-acting elements in promoters (Briggs *et al.*, 1986; Engelke *et al.*, 1980). With the characterization of many DNA binding protein motifs, a newly cloned cDNA can be predicted to encode a transcription factor based on deduced amino acid sequence alone. However, in order to confirm these initial predictions, the DNA binding site(s) of the putative transcription factor must be identified.

Currently, several methods are available for this purpose. Each method of binding site selection utilizes two main steps: 1. the separation of bound DNA from unbound DNA and 2. the amplification of selected DNA for subsequent rounds of enrichment. In a method called CASTing (Cyclic Amplification and Selection of Targets) (Figure 7), the protein of interest is immobilized on a solid matrix (such as an agarose bead). It is then incubated with radiolabeled oligomers consisting of random DNA sequences flanked by regions of known sequences. Bound DNA is separated from unbound DNA by a series of washes and then used as template in a polymerase chain reaction. The primers used in the amplification reaction are designed to anneal to the regions of known sequences on the oligomer. The selection and amplification procedures are repeated several times to enrich the pool of binding sites. The success of each round of site selection can be monitored by taking Cerenkov counts of the protein coated agarose beads at the end of the washes. At the end of the selection protocol, the selected DNA is

subcloned and sequenced. Using the CASTing method, the binding sites of transcription factors such as Ikaros and myogenin have been identified (Molnar *et al.*, 1994; Wright *et al.*, 1991).

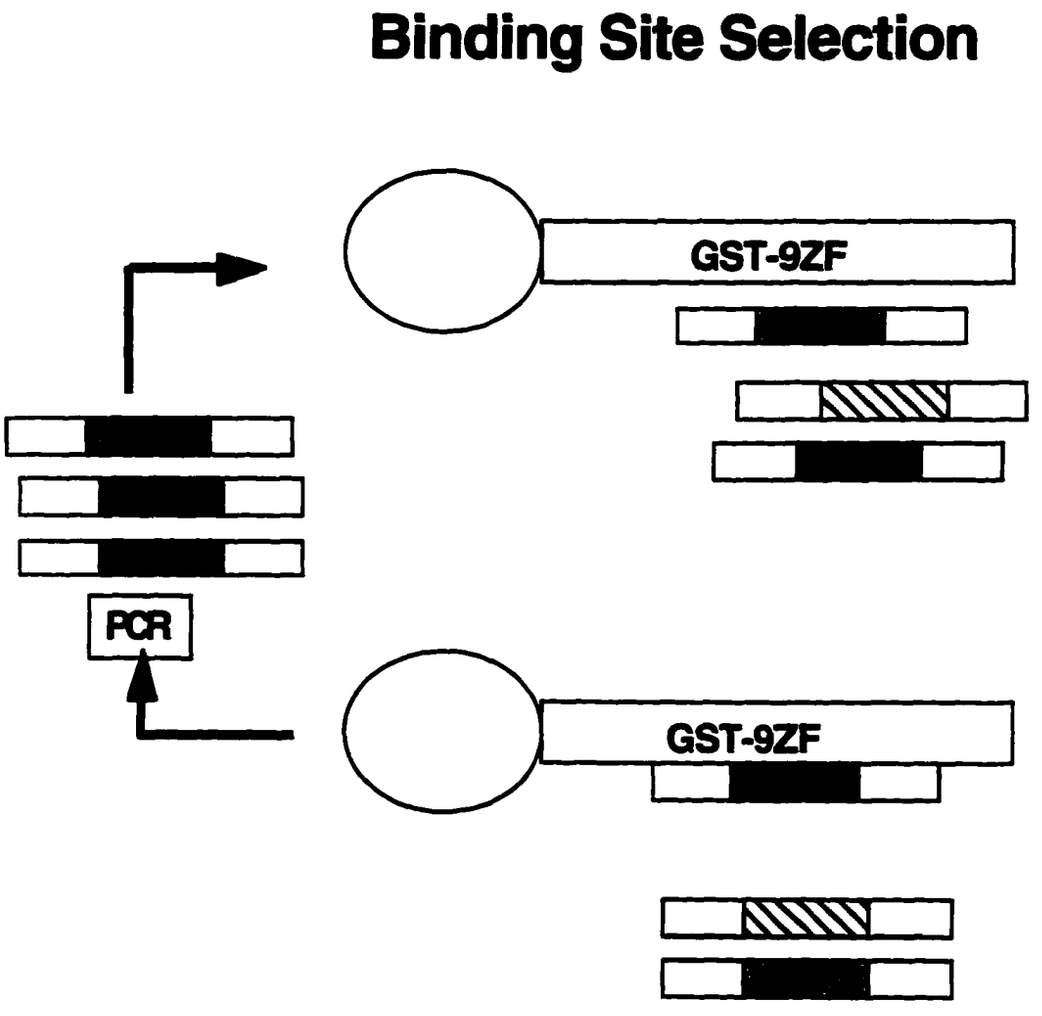


Fig. 7. The CASTing method of binding site selection. The DNA binding domain of PLZF (GST-9ZF) is expressed as a GST fusion protein and immobilized on glutathione coated agarose beads. It is then incubated with oligomers of random sequences. Unbound DNA is washed away and the DNA remaining on the beads is amplified by PCR and used another round of binding site enrichment.

RESULTS

Binding Site Selection

To identify the DNA binding site(s) of PLZF, its DNA binding domain was expressed as a glutathione S-transferase fusion protein (GST-9ZF) (Figure 6). GST-9ZF was immobilized on glutathione coated agarose beads and incubated with a pool of oligomers with 20 base pairs of random sequence (Figure 6). The success of binding site enrichment was monitored by measuring the radioactivity bound to the GST-9ZF coated agarose beads after the washes. Binding site selection was also performed with GST coated agarose beads as a control. At the end of six rounds of selection, approximately 6% of the input DNA (as measured by Cerenkov counts) bound to the GST-9ZF compared with only 2% bound to GST. The radiolabeled DNA was incubated with GST-9ZF in EMSA reactions (Figure 8). The DNA in the shifted band was eluted, amplified by PCR, and subcloned. Twenty-nine independent clones were sequenced (Table I).

Although a PLZF binding site consensus was not readily established from this result, it was observed that most of the selected sequences contained either TAA or TAAA. The most highly represented sequence, 15A, was found in 5 out of 29 clones which suggests that it may be a high affinity PLZF binding site. To test this hypothesis, competition EMSAs were performed using GST-9ZF, radiolabeled 15A, and unlabeled competitors (AP1 binding site, Gal4 operator and β-RARE) (Figure 9). A 10 fold molar excess of 15A completely abrogated the shifted complex in contrast to the 100 fold molar excess of AP1 and Gal4p sites needed to accomplish the same result. This suggests that PLZF binds to 15A with some specificity. However, only a 1 fold molar excess of β-

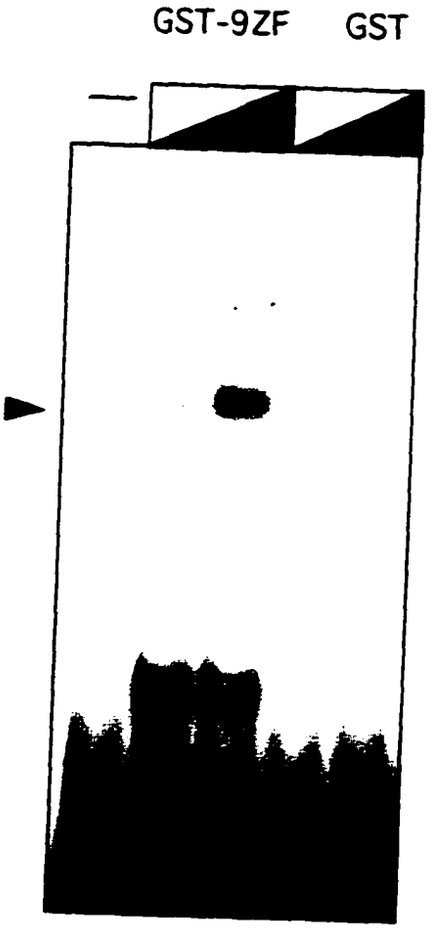


Fig. 8. EMSA performed after the sixth round of binding site selection. DNA amplified after the sixth round of selection was radiolabeled and incubated with either purified GST-9ZF or GST as indicated. GST-9ZF and GST were added in the amounts of 12 μg and 24 μg. The indicated bands were sliced out of the gel. The DNA in the slices were eluted, amplified, subcloned, and sequenced.

Table I

PLZF Binding Sites Selected After the First Set of Binding Site Selection

	Number of Clones
TAAAGGAAGCCACCATGAA	(5)
GCTAACTTCTTAAATAGACGA	(4)
TGGAGCGTCAAGGTGCAAAC	(2)
CAAATAAAGGCCCTCGTACT	(2)
TCGGGCTGAATCTAACCATG	(2)
ATAACTAATCGAAGGACCTG	(2)
GTATATAAACCTGTAGTAAC	(2)
TGGGTGCTTCTGTGTCCCT	(2)
ACAGTAGAATCGGTAATAAT	(1)
TGGAGACTGTGCAACAGAAT	(1)
GCACAGGTAATAGGGGTAAA	(1)
CTGTAAGGTTGGTATCCTCA	(1)
AGGGGTTTCGGTGTGGGCAA	(1)
CCATAAATTAGAACAACTAT	(1)
GTGTTGGGAATAATTTGTGTTA	(1)
TAACACAAATTATTCCCAAC	(1)

Table I. Sequences selected in the first set of binding site selection. Most of the sequences contained either TAA or TAAA (underlined). One of the sequences which contained TAAA was found in 5 out of 29 clones (Clone 15A, in *italic*).

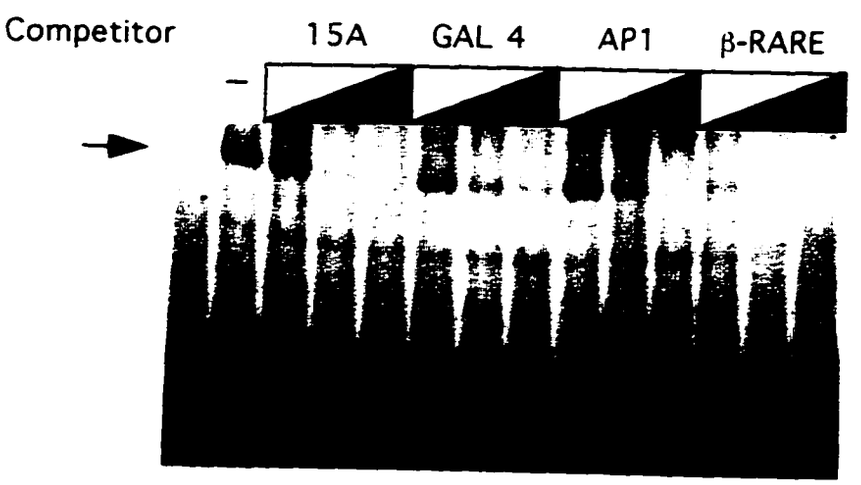


Fig. 9: Competition EMSA analysis of 15A. EMSAs were performed as described in "Materials and Methods" using GST-9ZF, radiolabeled 15A binding site and competitors as indicated. Each competitor was added in a molar excess of 1,10, 100 fold. The protein-DNA complex of interest is labeled with an arrow. Lower bands are thought to be composed of degradation products of GST-9ZF.

RARE was required to completely abrogate the shifted band suggesting that PLZF has a higher affinity for β-RARE than 15A.

The affinity of GST-9ZF to β-RARE was further investigated in another set of EMSAs. In these experiments GST-9ZF was incubated with radiolabeled β-RARE in the presence of non-labeled competitors (the AP1 binding site, the Gal4 operator, and β-RARE) (Figure 10). As little as 10 fold molar excess of β-RARE completely abrogated the shifted band. This is in contrast the to the 80 fold molar excess of Gal4 operator needed to achieve the same effect. Even at a molar excess of 80 fold, the AP1 binding site was not able to compete with the labeled β-RARE for GST-9ZF binding. In a second EMSA experiment, the interaction between GST-9ZF and β-RARE was tested using unlabeled 15A as competitor (Figure 11). As previously seen, a 10 fold molar excess of unlabeled β-RARE completely abolished the labeled complex whereas 80 fold molar excess of unlabeled 15A was needed to achieve the same effect. These results demonstrate that PLZF has a higher affinity for β-RARE than for the AP1 binding site, the Gal4 operator and 15A. Attempts to demonstrate transcriptional regulation by PLZF through either the 15A binding site or β-RARE were not successful (data not shown). Combined, these results suggest that PLZF's interaction with 15A is of relatively low affinity. Therefore, a new set of binding site selection was performed.

In the second selection experiment, the sequence TAAA, frequently found in the first set of selected DNA sequences, was embedded in the middle of the random sequence region of the oligomer (Figure 6). Four rounds of selection were performed. Again, the success of each round was monitored by taking measurements of the radioactivity remaining on the protein coated beads after the washes. By the end of the fourth round, 46% of the input DNA bound to the GST-9ZF in contrast to the 0.26% bound to the GST coated agarose beads (as estimated by Cerenkov counts). The selected DNA was subcloned

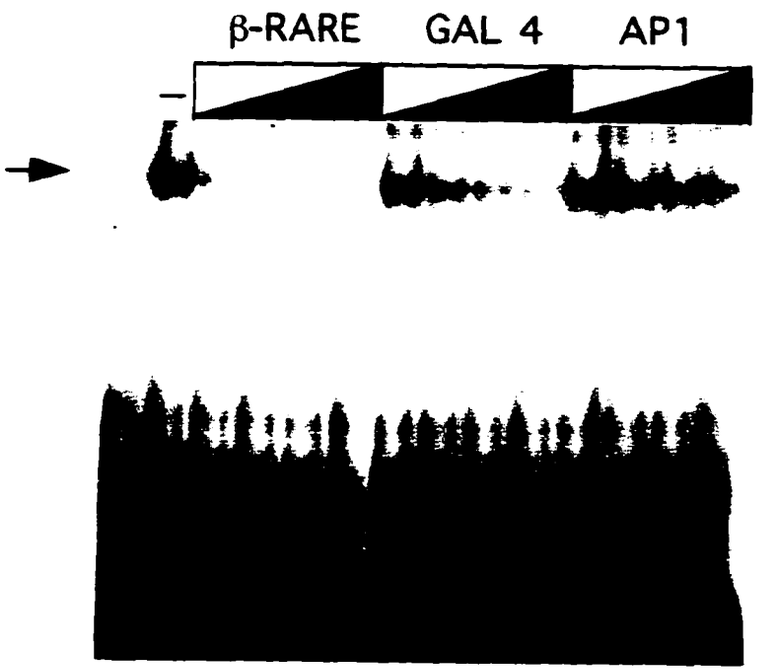


Fig. 10. Competition EMSA analysis of β-RARE. EMSAs were performed as described in "Materials and Methods" using GST-9ZF, radiolabeled β-RARE, and competitors as indicated. Each competitor was added in the molar excess of 10, 20, 40, 80 fold molar excess. "-" indicates the lane where no competitors were added.

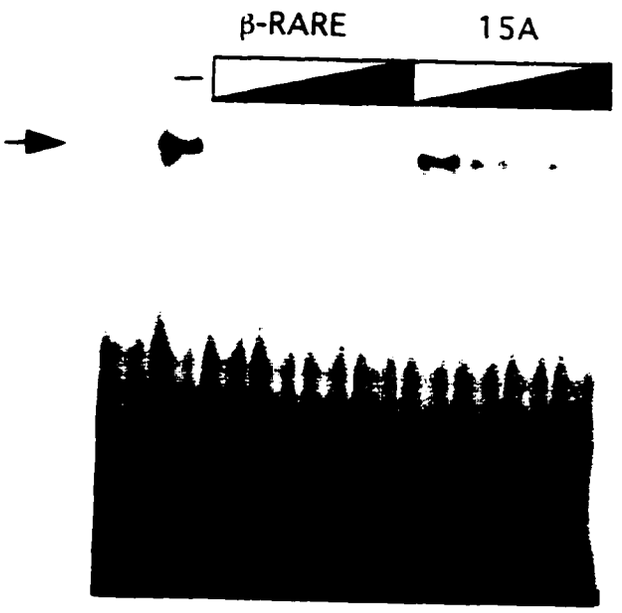


Fig. 11 Competition EMSA analysis of β-RARE. EMSAs were performed using GST-9ZF and radiolabeled β-RARE and competitors as indicated. Competitors were added in a molar excess of 10, 20, 40. 80 fold molar excess. "-" indicates the lane where no competitors were added.

and sequences for 42 individual clones were determined (Table II). Only 8 independent sequences were obtained. One of the selected binding sites (site A) was found in 19 out of 42 clones (Table II). Because binding site A was the most frequently selected sequence, its interaction with GST-9ZF was further investigated in a series of competition mobility shift assays. In figure 12, interactions of GST-9ZF with 15A and β-RARE are compared. As little as 10 fold molar excess of unlabeled site A completely abrogated the labeled A/GST-9ZF complex. In contrast, greater than 80 fold molar excess of 15A and greater than 10 fold molar excess of β-RARE are required to completely abrogate the shifted band.

To investigate the relative affinity of GST-9ZF for other sequences selected in the second binding site selection, competition EMSAs were performed using radiolabeled site A and unlabeled site A, site B (selected 7 out of 42 clones) and D (selected 2 out of 42 clones) as competitors (Table II) (Figure 13). As previously seen, 10 fold molar excess of unlabeled A completely abolished the shifted complex whereas 80 fold molar excess of binding sites B and D did not compete with the radiolabeled site A for GST-9ZF binding.

In a subsequent set of EMSA experiments, the affinity of GST-9ZF for binding site A was further tested using unlabeled site A, Gal4 operator, p53 and WT1 binding sites as competitors (Figure 14). As little as 10 fold molar excess of unlabeled A completely abrogated the labeled complex while the addition of up to 1000 fold molar excess of Gal4p, p53 and WT1 binding sites did not significantly compete with labeled site A for GST-9ZF binding.

To map residues of importance in more detail, six mutants of oligonucleotide A, with short stretches of base transversions were synthesized (Table III) and used as unlabeled competitors in EMSA experiments performed with radiolabeled binding site A

Table II

Sequences selected after the second set of binding site selection

	Number of Clones	
TTGTATTGAAGC**TAAA**GTTTGATCTGTTC	19	A
ACTAGTCGTCCGG**TAAA**TCATTAATAAAGT	7	B
AGTCATTCGGTTT**TAAA**GCTGTGCTAGTTC	6	C
CGGACTCATGTTG**TAAA**AGATTGCCGTGTG	2	D
GTGCAGGAA**TAAA**CATCATGTTACAC	2	
GTCTAGGTTAT**TAAA**ACCGAATCTGCAT	2	
GTCGAGACGTTG**TAAA**CATAGTAATCGCG	2	
GAGGCACTAGGTCGA**TAAA**CAAAGTTATGGG	2	

Table II. Sequences selected in the second set of binding site selection.

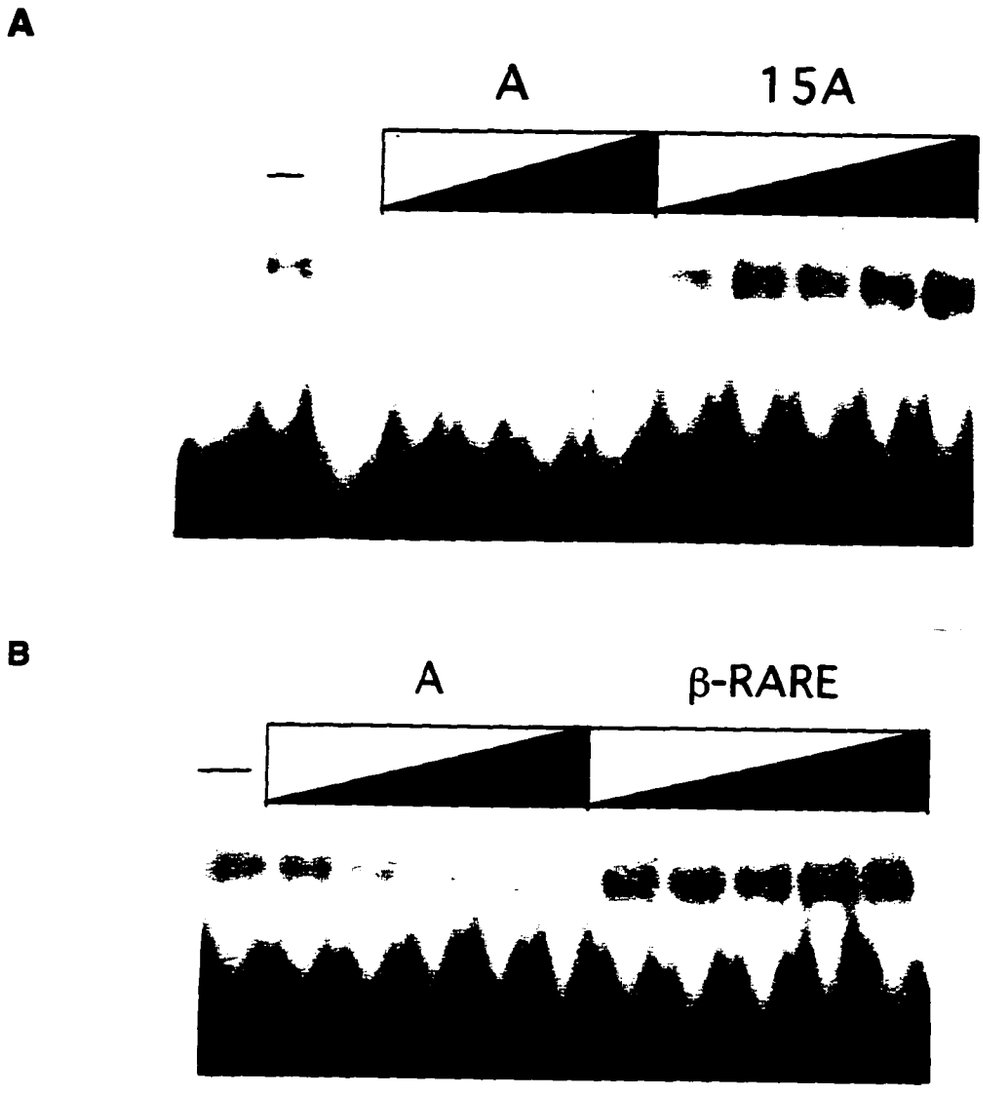
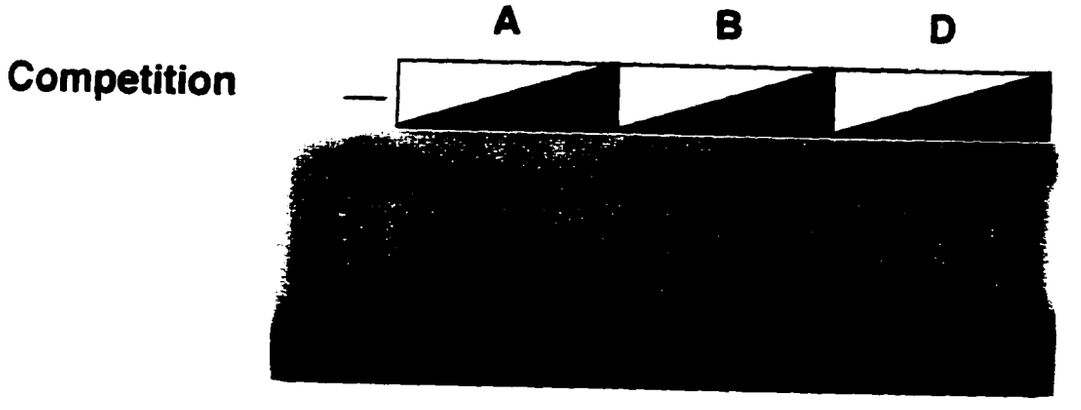


Fig. 12. EMSA comparing the affinity of GST-9ZF for binding site A, 15A and β-RARE. EMSAs were performed using purified GST-9ZF and radiolabeled binding site A. In *panel A*, unlabeled competitors, binding site A was added in a molar excess of 10, 20, 40, 80 fold. Competitor 15A was added in the molar excess of 1, 10, 20, 40, 80 fold. "-" indicates where no competitor was added. In *panel B,*. unlabeled competitors binding site A was added in a molar excess of 0, 1, 2, 4, 8, 10 fold. b - RARE was added in the molar excess of 1, 2, 4, 8, 10 fold.



A	TTGTATGAAGCTAAAGTTTGATCTGTTC	19 X
B	ACTAGTCGTCCGGTAAATCATTAATAAAGT	7 X
D	CGGACTCATGTTGTAAAAGATTGCCGTGTG	2 X

Fig. 13. A comparison of the affinity of GST-9ZF for binding sites A, B, and D. EMSAs were performed using radiolabeled A and competitors as indicated. Each competitor was added in a molar excess of 10, 20, 40, 80 fold. "-" indicates the lane where no competitor was added.

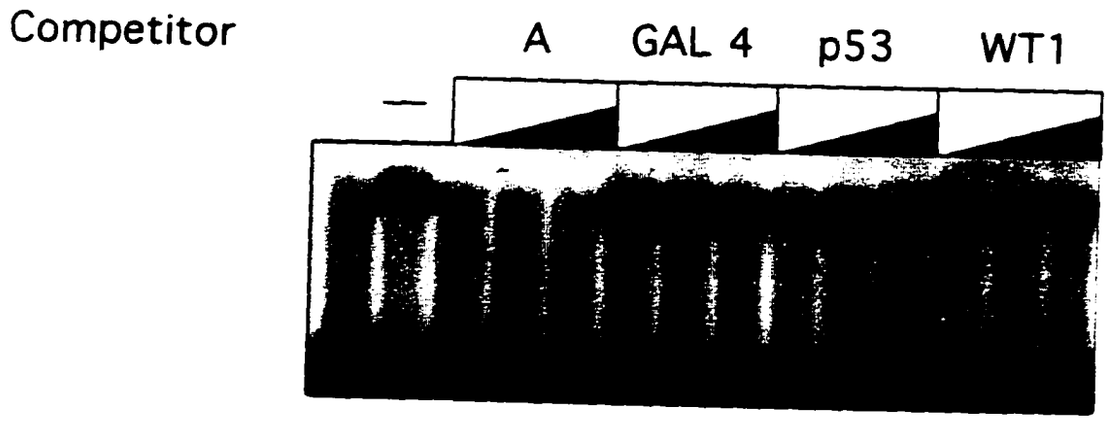


Fig. 14. GST-9ZF binds to site A in a sequence dependent manner. EMSAs were performed with GST-9ZF, radiolabeled site A and unlabeled competitors as indicated. Each competitor was added in a molar excess of 10, 100, 1000 fold molar excess. "-" indicates the lane in which no competitors were added.

Table III

TCGATTGTATTGAAGCTAAAGTTTGATCTGTTC	WT
TCGAGGTGCGGGAAGCTAAAGTTTGATCTGTTC	M 1
TCGATTGTATTTCCTATAAAGTTTGATCTGTTC	M 2
TCGATTGTATTGAAGCGCCCGTTTGATCTGTTC	M 3
TCGATTGTATTGAAGCTAAATGGGTCGCTGTTC	M 4
TCGATTGTATTGAAGCTAAAGTTTGATAGTGGA	M 5
TCGATTGTATTGAAGCGCCCTGGGGATCTGTTC	M 6

Table III Transversion mutants of binding site A. Changed areas are underlined.

(Figures 15, 16). The amount of radioactivity in the retarded complex was quantified and plotted as a function of the competitor concentration present in the binding reaction (Figure 17). The value of 100% was given to the counts per minute measured in the shifted complex in the absence of any unlabeled competitors. The effectiveness of each competition was measured by the amount of competitor required to displace 50% of the bound probe. As little as 10 fold molar excess of mutant M1 completely abrogated the complex (Figures 15, 17), suggesting that sequences mutated in this oligomer do not contribute significantly to the interaction with GST-9ZF. Both binding site A and M1 were very effective in competing with the labeled DNA for binding with GST-9ZF. M2 is approximately 4 fold less effective as a competitor compared to site A suggesting that the residues changed in this oligomer are involved in DNA binding by GST-9ZF, although not essential (Figures 15, 17). The binding curves for M4 and M5 overlap each other suggesting that residues changed in these oligomers contribute to PLZF DNA binding to the same extent (Figures15, 16, 17). Both are at least 10 fold less effective as competitor than M2 and 40 fold less effective than binding site A. Based on the first set of binding site selection, the sequence TAAA was hypothesized to be important for PLZF DNA binding. When TAAA was changed to GCCC in M3, the effectiveness of this oligomer was 60 fold less than that of binding site A (Figures15, 16, 17). Changing the TAAAGTTT in binding site A to GCCCTGGG (M6), resulted in a 200 fold decrease in its effectiveness as competitor compared to binding site A (Figures 15, 16, 17). Combined these results suggest that the core of the PLZF binding site is TAAAGTTTGATCGTTC and that the TAAA sequence is most important for DNA binding by PLZF.

Translocation t(11;17) produces two sets of chimeric proteins, PLZF-RARα and RARα-PLZF (Figure 3). PLZF-RARα retains the first two zinc fingers of PLZF whereas RARα-PLZF retains the last seven. To assess whether either or both chimeric proteins have the potential to bind to site A, sequences encoding the first two

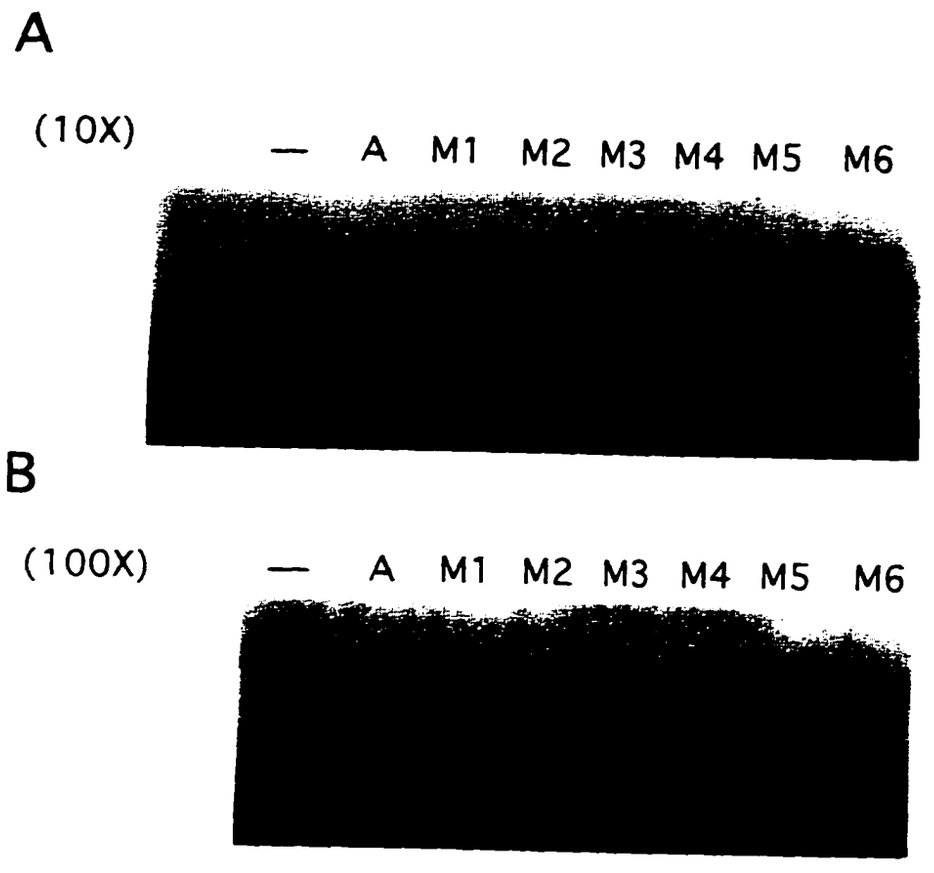


Fig.15. EMSA analysis using transversion mutants of binding site A as unlabeled competitor. GST-9ZF was incubated with radiolabeled site A. Competitors added are as indicated. "-Pr" indicates a lane where no protein was added. "-" indicates the lane where no competitors were added. *Panel A*, a 10 fold molar excess of competitors were added to each reaction. *B*, a 100 fold molar excess of competitors were added.

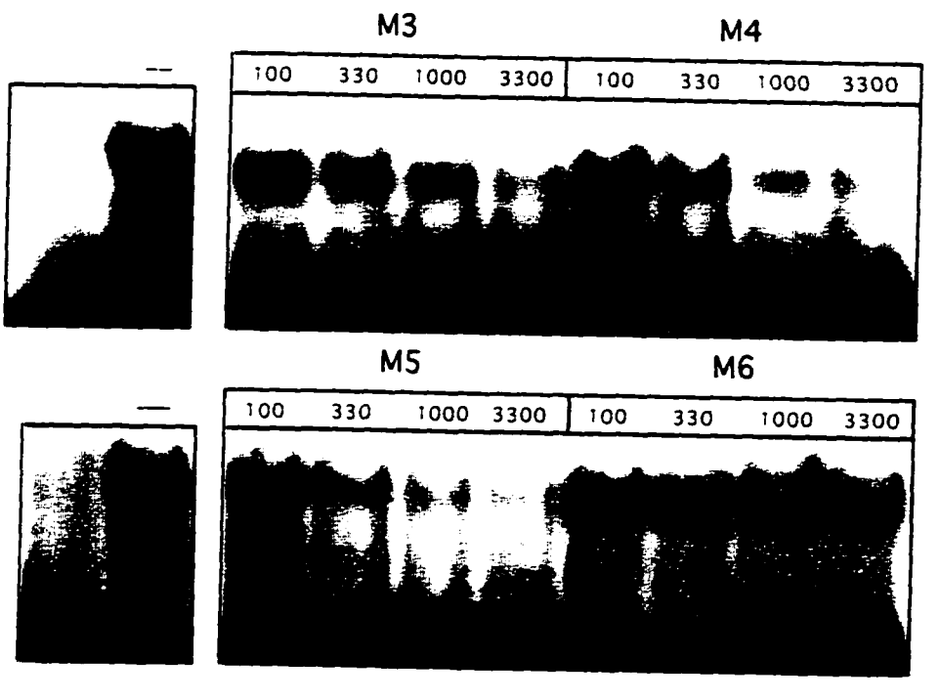


Fig. 16. EMSA analysis using transversion mutants of binding site A as unlabeled competitor. EMSA was performed with GST-9ZF, radiolabeled A, and unlabeled competitors as indicated. Each competitor was added in a molar excess of 100. 330. 1000, 3300 fold. "-Pr" indicates the lane where no protein was added. " - " indicates the lane in which no competitor was added.

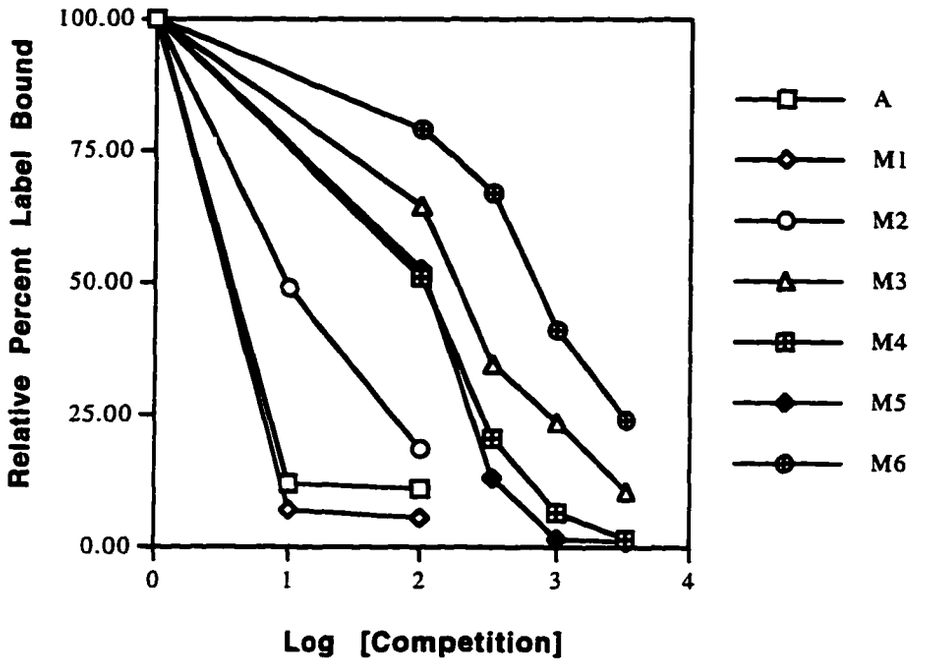


Fig. 17: A comparison of the relative affinity of GST-9ZF for binding site A and its transversion mutants. The protein bound radiolabeled binding site A in the mobility shift assays of figures 15 and 16 were quantitated using phosphorimager analysis. The relative percent bound when no competitors were addd was given the value of 100%.

and the last seven zinc fingers of PLZF were fused in-frame to the GST gene. The resulting fusion proteins (GST-2ZF and GST-7ZF respectively) were assayed for their ability to bind labeled site A by EMSA (Figure 6, 18). GST-7ZF but not GST-2ZF bound to site A (Figure 18). The affinity of GST-7ZF for binding site A was further tested by competition EMSA (Figure 19). As previously observed with GST-9ZF, 10 fold molar excess of unlabeled site A completely disrupted the GST-7ZF-site A complex whereas 1000 fold molar excess of Gal4p, p53 or WT1 binding sites did not.

Discussion

A major step in the characterization of PLZF is the identification of its DNA binding site. To accomplish this goal, we used the CASTing method in two sets of site selection. Although no binding site consensus was formulated from the results of the first set of selection, these experiments yielded 16 different sequences of which 11 possessed either a TAA or TAAA sequence. Subsequent EMSA analysis revealed that the interaction between GST-9ZF and the most highly selected site 15A was relatively non-specific. Although GST-9ZF favored this site over the binding sites of AP1 and Gal4p, it bound to the β-RARE with higher affinity. Transient transfection assays performed with a 15A regulated CAT reporter failed to demonstrate any transcriptional regulation by PLZF.

GST-9ZF bound to the β-RARE with approximately 10 fold higher affinity than it did to site 15A (Figures 9, 11). Whether the interaction between GST-9ZF and β-RARE is significant enough to propagate a biological response remains to be determined. Attempts to demonstrate a PLZF transcriptional effect through the β-RARE were inconclusive mainly due to the fact that it was very difficult to dissect the effects of PLZF from that of the endogenous RARs.

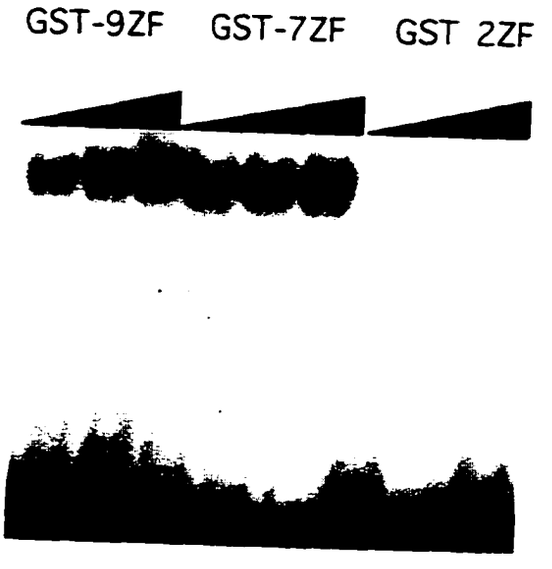


Fig 18. EMSA analysis performed with GST-9ZF, GST-7ZF and GST-2ZF and radiolabeled binding site A. Each protein was added in the amounts of 1, 2, and 3 μg per reaction.

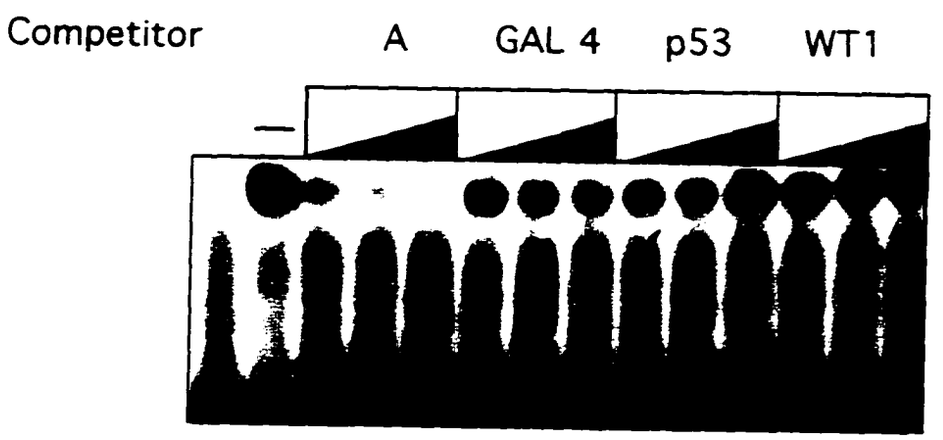


Figure 19. GST-7ZF binds site A with sequence specificity. EMSAs were performed using GST-7ZF radiolabeled A and competitors as indicated. Each competitor was added in a molar excess of 10, 100 and 1000 fold. "-" indicates the lane in which no competitors were added.

There remains a possibility that PLZF and RARα can effect a common set of target genes *in vivo*. If this is the case, a model for the molecular etiology of t(11;17) APL can be proposed (Figure 20). The importance of the disruption of the RARα pathway in leukemogenesis is apparent since both t(15;17) and t(11;17) translocations involve the RARα gene. In addition, transient transfection assays have demonstrated that both PML-RARα and PLZF-RARα transactivate through RAREs in response to pharmacological levels of ATRA (de Thé *et al.*, 1991; Kastner *et al.*, 1992) suggesting that both fusion proteins retain some of the characteristics of wildtype RARα. Thus, the unique non-responsiveness of t(11;17) APL patients to ATRA might be attributed to the fusion protein RARα-PLZF which contains the DNA binding activity of PLZF. Since RARα-PLZF is regulated by the RARα promoter, it escapes the down regulation that PLZF undergoes in response to retinoic acid induced granulocytic differentiation (Chen *et al.*, 1993). If RARα-PLZF recognizes and binds to the same sequence as wildtype RARα and PLZF-RARα, it may compete with both proteins for DNA binding sites, thwarting the transactivation of retinoic acid responsive genes. Data presented in chapter 5 will demonstrate that RARα-PLZF has a very weak transcriptional effect. Since the effects of RARα-PLZF may not be affected by the presence of ATRA, the disease phenotype persists despite ATRA treatment.

The results of the second set of binding site selection yielded site A as the most frequently selected PLZF binding site. To test the affinity of PLZF for site A, we performed EMSA studies which confirmed that GST-9ZF had a higher affinity to site A over all of the binding sites tested including the β-RARE. To identify important areas of protein contact on binding site A, six mutants of binding site A were synthesized (Table III) and used as unlabeled competitors in EMSA studies. These studies revealed that PLZF contacts only the 3' half of site A. Thus, the PLZF binding site has been narrowed to TAAAGTTTGATCGTTC. The importance of the TAAA in binding site A was underscored by

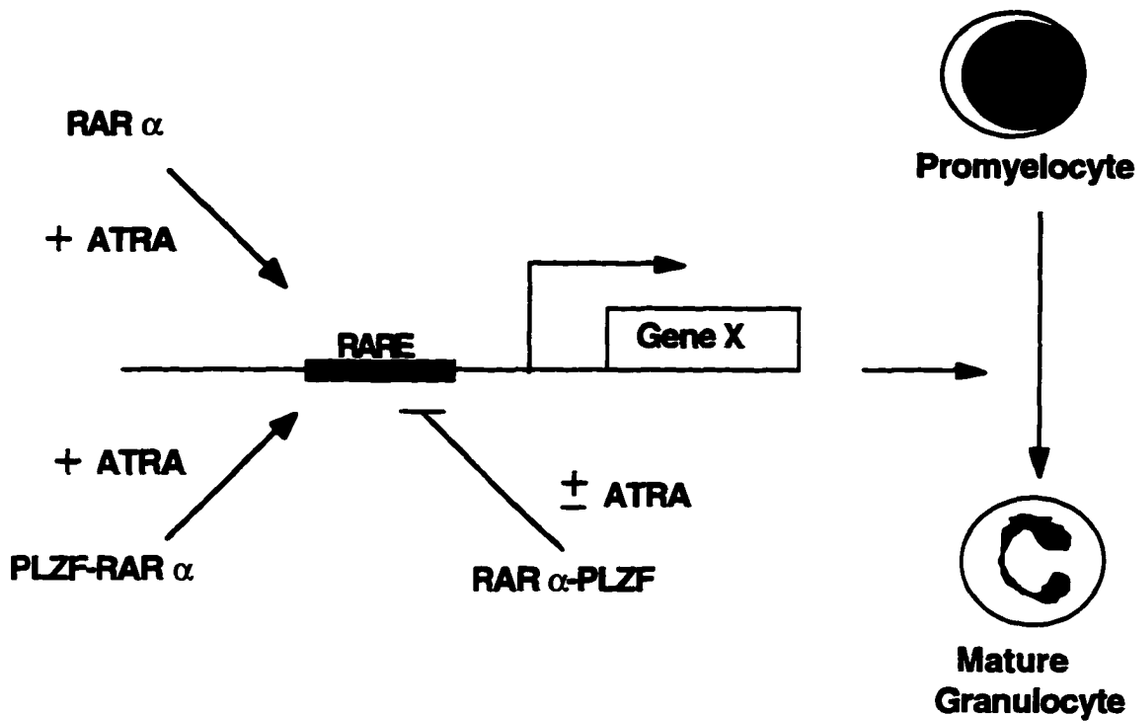


Fig. 20. Schematic model of t(11;17) APL leukemogenesis. Because RARα-PLZF retains most of the DNA binding domain of PLZF, it has the potential to tether RAREs and compete with RARα and PLZF-RARα for binding sites. Both the RARα and PLZF-RARα transactivate in response to ATRA. This is not the case for RARα-PLZF which has a weak effect on transcription in the presence or absence of ATRA. This competition for RAREs and may ultimately explain why t(11;17) APL patients fail to undergo remission with ATRA treatment.

the fact that changing this sequence to GCCC rendered the duplex oligonucleotide virtually incapable of competing with site A for binding with PLZF.

As in the first set of PLZF site selection, no apparent PLZF cognate consensus could be established from the results of the second set. There are, however, striking similarities between the three most highly represented sites of the second binding site selection (Table II). In site A, a selected TAAAGT is present as part of the engineered TAAA (Table II). In binding site B (selected 7 out of 42 times), a TAA and a TAAAGT were found outside of the engineered TAAA. PLZF binding site C (selected 6 out of 42 times) possesses a GTTC which is present in site A at exactly the same position relative to the engineered TAAA (Table II). When this GTTC was mutated in binding site A (M5), the affinity of PLZF for this site was severely diminished (Figures 16, 17) which suggests that GTTC may be a site of protein-DNA contact. A striking observation is that the GTTC is one helical turn away from the engineered TAAA. The presence of two sites of protein contact separated by one helical turn is reminiscent of DNA binding by TFIIIA (McBryant *et al.*, 1996).

It is now apparent that PLZF may bind to several DNA sequences with high affinity. Recently, Sitterlin *et al.* described another PLZF DNA binding site, the Lex A operator (Sitterlin *et al.*, 1997). Although it is evident that the Lex A operator is not a biological target of PLZF, their results clearly contributed to our understanding of DNA binding by PLZF. Dr. Helen Ball, a postdoctoral fellow in our laboratory, conducted studies which confirmed that the interaction between GST-9ZF and the Lex A operator is one of high affinity and sequence specificity. EMSAs performed with GST-9ZF and labeled binding site A results in the formation of a protein-DNA complex which migrates close to the unbound DNA. When the Lex A operator was labeled in EMSA, a band of lower mobility than that of site A was seen. The reason for this observation is unclear,

however, it strongly suggests that different structural arrangements are favored in the binding of these two sites. Recently, Dr. Ball also identified a PLZF binding site selected from a CpG island library (Figure 21) (Ball *et al.*, 1996). EMSA studies also revealed that PLZF bound to this site with high affinity and specificity and, like the Lex A operator, EMSA studies performed with this binding site produced a major band of low mobility. It also produced a minor band of similar mobility observed with site A (Ball *et al.*, 1996). Analysis of the DNA sequences revealed little similarity between binding site A, Lex A operator, and Dr. Ball's genomic PLZF binding site although a loose consensus can be formulated (Figure 21). All experiments performed to date suggest that PLZF has definite affinity for some DNA sequences over others. Whether a common thread exists between these sites or how sequence preference is made by PLZF is currently unknown.

Sitterlin *et al.* mapped the DNA binding activity of PLZF to the last five zinc fingers (Sitterlin *et al.*, 1997) which confirmed my observations that DNA binding to site A resides in the most carboxyl seven zinc fingers of PLZF (GST-7ZF) retained in t(11;17) fusion protein RARα-PLZF. These results strongly suggest that RARα-PLZF could act as a dominant negative mutant by competing with PLZF for DNA binding sites.

To assess whether binding site A represents a biologically relevant PLZF site, promoters of potential target genes were searched. A PLZF binding site in the promoter of cyclin A2 was found (Figure 21). This putative PLZF response element matches 12 out of 15 base pairs spanning the TAAA region of site A (Figure 21). Data accumulated by Dr. Patricia Yeyati, a postdoctoral fellow in our laboratory, suggests that PLZF binds to this site with a higher affinity than to binding site A suggesting that PLZF may indeed act through this site to regulate cyclin A2 transcription (Yeyati *et al.*, 1996). Cell cycle analysis of murine myeloid cells expressing PLZF showed an accumulation in the

G1 to S transition of the cell cycle and that this arrest is correlated with a decrease in cyclin A expression (Yeyati *et al.*, 1996).

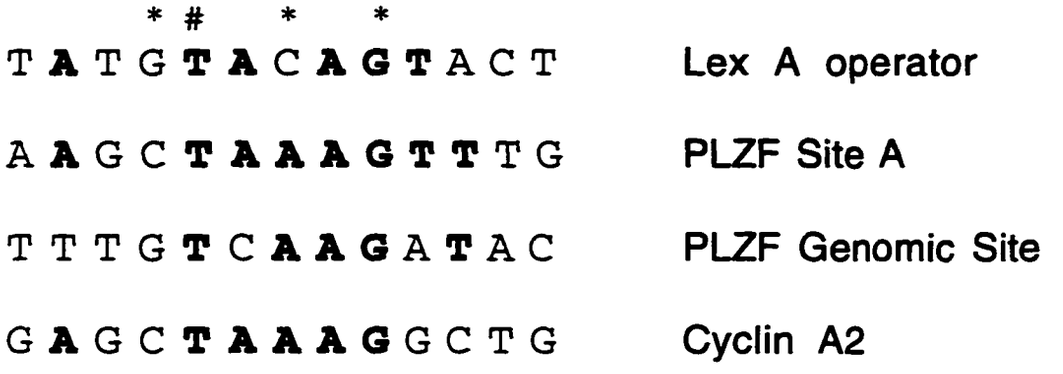


Figure 21. A comparison of PLZF binding sites. The PLZF A binding site was compared to sites found within the cyclin A2 promoter, a human genomic genomic DNA fragment and the Lex A operator. "*" denotes guanine/cytosine residues of importance as defined by methylation interference of the Lex A operator. "#" denotes a thymine residue of importance for PLZF's interaction with the human genomic DNA sequence. Bold letters indicate conserved residues. A lose consensus of A (T/G)(G/C)T(A/C)(A/C)AGT can be derived from this comparison

Chapter 4

MAPPING THE FUNCTIONAL DOMAINS OF PLZF

INTRODUCTION

Transcription factors are hypothesized to be modular in nature, composed of various functional domains. Most transcription factors contain a DNA binding domain and domains responsible for transcription activation and/or repression, however, many also contain regions responsible for ligand binding, co-factor interactions, and dimerization. The presence of a diverse group of effector domains in a given transcription factor may allow for a variety of functions depending on post translational modification, the presence of other factors or the architecture of cis-acting elements.

Little is known about the nature of domains that mediate transcription activation. Amino acid sequence analysis of activation domains revealed little similarity other than an abundance of acidic, glutamine or proline residues (Courey *et al.*, 1988; Lin *et al.*, 1991; Mermod *et al.*, 1989). Analysis of acidic activation domains revealed that they formed no definite structure in the absence of protein-protein interaction (Wu *et al.*, 1996). Experiments performed with various deletions of a Gal4p activation fragment revealed that the degree of transactivation is proportional to the length of the domain (Wu *et al.*, 1996), suggesting that Gal4p stimulates transcription by a general mechanism rather than one that entailed intricate structural arrangements. Furthermore, there is evidence which suggests that Gal4p activates transcription by interacting with TBP and TFIIB and that the strength of these interactions predicts the degree of transcriptional activation.

Like domains that mediate activation, much remains to be understood about the repression domains. The first repression domain to be characterized is that of the Drosophila Kruppel protein which has been described as alanine rich. Deletions of alanine residues in this domain, however, had little effect on repression by Kruppel (Licht *et al.*, 1993; Licht *et al.*, 1990). Analysis of its amino acid sequence predicted that this region forms an α–helix with a glutamine rich surface implying that a specific protein structure is important for repression function (Licht *et al.*, 1993). Some repression domains are conserved within families of transcription factors. The Kruppel associated (KRAB) box, for example, is estimated to be present in a third of all mammalian C2-H2 zinc finger proteins (Bellefroid *et al.*, 1991; Witzgall *et al.*, 1994). Site directed mutagenesis of conserved amino acids in the KRAB boxes resulted in a decrease in transcription repression suggesting that all KRAB box transcription factors utilize the same mode of repression (Witzgall *et al.*, 1994).

Although there are several notable exceptions, many activation and repression domains are conserved in function from yeast to man. Thus, it is thought that the underlying mechanisms of transcription regulation are conserved as well and that the specificity of action with respect to target genes seems to reside entirely in the interactions between the DNA binding domain of a transcription factor and the cis-acting elements present in a given promoter.

Results

To fully characterize the effector domain of PLZF, various segments of its N-terminal region were fused to the DNA binding domain of Gal4p. (Constructs encoding these chimeric proteins were co-transfected with a reporter (G_5 tk-CAT) containing 5 GAL4 operators (Figure 23). The entire effector domain of PLZF (amino acids 1-400) repressed the expression of the CAT reporter approximately 16 fold. This repression

function of PLZF was mapped to two regions in the effector domain. One of these regions is the POZ/BTB domain present in amino acids 1-100. The POZ/BTB domain of PLZF repressed transcription approximately 6 fold. When the POZ domain was absent from the effector domain of PLZF, the transcription repression was approximately 4 fold (Figure 23). Compare Gal4p 1-400 to Gal4p 100-400). Another more potent repression domain resides within amino acids 200-300. This domain repressed transcription approximately 13 fold. Unlike the POZ domain, this region does not have homology to any known protein motifs. Neither of these repression domains can repress transcription to the same extent as the entire effector domain. One region within the PLZF effector domain, noted for an abundance of acidic residues (amino acids 100-200), activated transcription approximately 3 fold. The effect of this domain, however, is weak since it can be masked by the presence of either the POZ domain or the second repression domain (Figure 23-Compare Gal4p 100-200 to Gal4p 100-300; or compare GaL4p 100-200 to Gal4p 1-200).

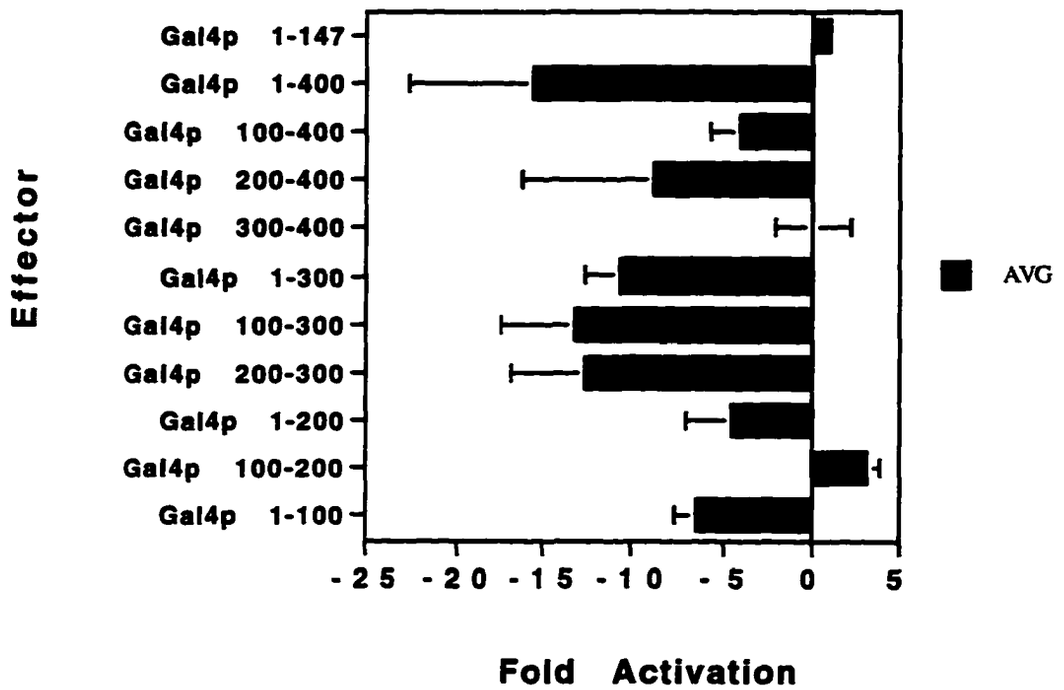


Fig 22. Mapping the functional domains of PLZF. The indicated amino acid stretches of PLZF were fused to the DNA binding domain of Gal4p. Constructs expressing these fusion proteins (1μg) were cotransfected in CV1 cells along with 2 μg G_5 *tk*-CAT as described in "Materials and Methods". The level of CAT expression when GAL 1-147 was used as effector was given the value of 1. This data represents the results of at least two separate experiments, each experiment represents a duplicate transfection of each effector.

Discussion

Domain mapping experiments are important because not only do they provide insight into the PLZF protein but they also contribute to the understanding of other homologous proteins. For example, the identification of zinc fingers as DNA binding motifs in TFIIIA made possible our prediction that PLZF will bind DNA in the absence of any biochemical data. This hypothesis was confirmed by the experiments presented in the previous chapter. In a similar vein, the dissection of the effector domain of PLZF may contribute to the understanding of not only PLZF but other transcription factors as well.

PLZF contains only one conserved protein motif in its effector region, the POZ/BTB domain. Although it has been described as a DNA binding inhibition domain in the ZID, tramtrack, GAGA and ZF5 proteins (Bardwell and Treisman, 1994; Kaplan *et al.*, 1997), data presented in this chapter argue that the POZ/BTB domain does not inhibit DNA binding by the zinc fingers of Gal4p, particularly since transfected Gal4p 1-400 and Gal4p 1-100 could clearly regulate transcription *in vivo*.

Dissection of the PLZF effector domain revealed that the POZ/BTB domain mediates transcription repression. Moreover, the removal of the POZ/BTB domain significantly diminished the activity of the full length PLZF effector domain from 16 fold to 4 fold repression. These observations concur with those made of the BCL6 POZ/BTB domain (Chang *et al.*, 1996; Seyfert *et al.*, 1996).

The POZ/BTB domain of BCL6 was also shown to repress activated transcription (Chang *et al.*, 1996; Seyfert *et al.*, 1996). Whether PLZF is also capable of repressing activated transcription remains to be determined. Experiments performed with the ZF5

protein indicate that its POZ/BTB domain can mediate transcriptional repression or activation depending on promoter context. Whether this is the case for the PLZF POZ/BTB domain also remains to be seen.

Like other zinc finger transcriptional repressors such as Drosophila Kruppel and the WT1 protein (Licht *et al.*, 1994; Sauer and Jackle, 1991; Wang *et al.*, 1993), PLZF contains both activation and repression domains. The PLZF activation domain is weak and its effect is masked when linked to the repression domains of PLZF in a Gal4p fusion construct. Nevertheless, the presence of an acidic activation domain within PLZF could potentially activate transcription. The presence of two repression domains within PLZF might allow PLZF to repress transcription through two distinct mechanisms.

Chapter 5

TRANSCRIPTIONAL REGULATION BY PLZF

INTRODUCTION

The data presented in chapters 3 and 4 demonstrate that PLZF binds to site A in a sequence specific manner. Moreover, this DNA binding activity resides in the most carboxyl seven zinc fingers of PLZF, retained in the fusion protein RARα-PLZF. In addition, the effector domain of PLZF when fused to a heterologous DNA binding was shown to mediate transcriptional repression. This effector domain is absent in the fusion protein RARα-PLZF. Combined these data suggest that PLZF acts as a transcription repressor by binding to site A and that this repression function is absent or altered in RARα-PLZF.

Results

To study the transcriptional effect of PLZF through its interaction with site A, the expression plasmids for PLZF, RARα-PLZF or the parental plasmid SG5 were co-transfected in CV-1 cells with a reporter construct containing three copies of site A placed upstream of a HSV thymidine kinase promoter (A_3*tk*-CAT). A representative experiment is shown in figure 23A. In this experiment, each transfection was performed in duplicate and the average of the results are plotted as relative percent CAT conversion. These results are representative of three independent experiments. Transfection of PLZF resulted in a 10 fold decrease of reporter activity which was not seen when RARα-PLZF was used as effector suggesting that RARα-PLZF may act as an aberrant PLZF, severely impaired in its ability to regulate PLZF target gene transcription. The result of this experiment is binding site dependent since transfection of PLZF with pBLCAT5 produced no such repression (Figure 23B). Another observation is that the addition of binding site A to the parental reporter plasmid pBLCAT5 (in A_3*tk*-

CAT) resulted in an increase in reporter expression which suggests that there may be an activator present in CV1 cells that binds to binding site A.

Results obtained from transient transfection assays using the hematopoietic cell line K562 produced the same results (Figure 24). Again, PLZF mediated transcription repression, this time 2.5 fold. No repression was seen when RARα-PLZF was transfected. As seen in CV-1 cells, the introduction of the A site into pBLCAT5 also stimulated transcription of the CAT reporter.

Discussion

Transfection experiments using A_3 *tk*-CAT revealed that PLZF is a transcriptional repressor and that this repression activity was severely diminished when RARα-PLZF was used as effector. RARα-PLZF expression is also under the regulation of the RARα promoter which suggests that in addition to its lack of repression function, RARα-PLZF may disrupt the temporal expression of PLZF target genes. This strongly suggests that a possible way in which RARα-PLZF can contribute to the t(11;17) APL phenotype is by deregulating the expression of PLZF target genes by competing with PLZF for binding sites in the promoter. The increase in CAT expression when the site A was introduced suggests that a potent transcriptional activator tethers the site A sequence. The fact that this activation through binding site A was seen in both CV-1 and K562 cells suggest that the activator is expressed in a wide variety of cells. The observation that PLZF is capable of mediating repression in the presence of this activator suggests that in addition to quenching the effects of transactivators, it may also compete with activators for binding sites.

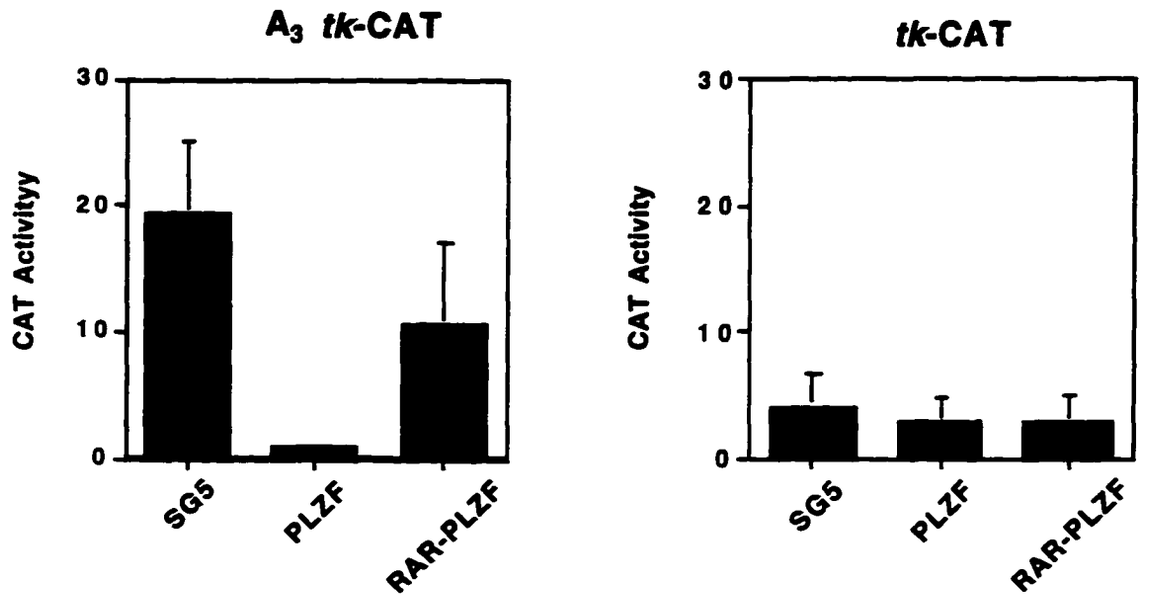


Fig. 23 Regulation of transcription by PLZF and RARα-PLZF. Constructs encoding PLZF, RARα-PLZF and the parental expression construct SG5 were cotransfected with either A_3*tk*-CAT or the parental reporter construct pBLCAT5 (as indicated). The results presented here represents those obtained from three independent experiments. These transfections were performed in the monkey kidney cell line CV-1.

K562

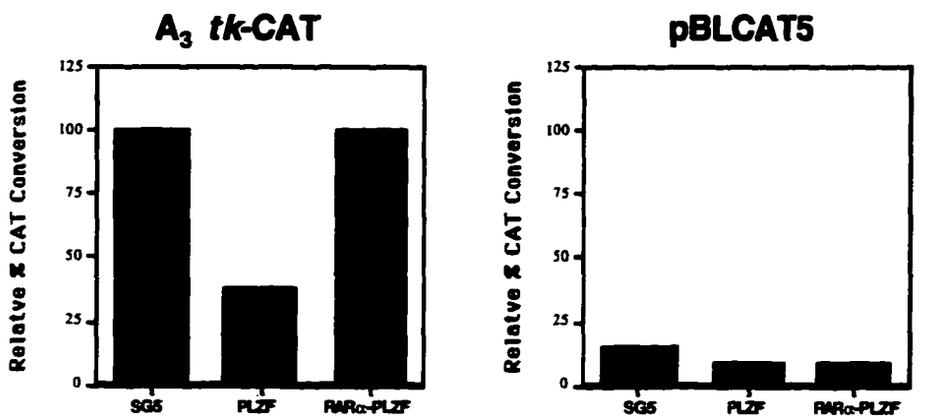


Fig. 24. Regulation of transcription by PLZF and RARa-PLZF. Constructs encoding PLZF, RARa-PLZF and the parental construct SG5 were cotransfected with either A_3*tk*-CAT or the parental reporter construct pBLCAT5 (as indicated). Relative percent conversion was calculated by assigning the value of 100% to the CAT activity obtained when SG5 was cotransfected with A_3*tk*-CAT. These transfections were performed in the human erythroleukemia cell line K562

Conclusion

When I started this body of work, the PLZF gene has just been cloned. In the initial study presented by Chen *et al.*, the PLZF gene was found to be involved in t(11;17) ATRA non-responsive APL and its disruption in t(11;17) led to the formation of fusion proteins PLZF-RARα and RARα-PLZF (Chen *et al.*, 1993). In the hematopoietic system, PLZF was shown to be expressed only in early CD34 + early progenitor cells and is down-regulated in granulocytic development (Chen *et al.*, 1993). Based on its predicted amino acid sequence alone, PLZF was predicted to encode a transcription factor.

Work presented in this thesis includes the identification of several DNA sequences as PLZF binding sites including the β-RARE and the artificially selected site A. Based on binding site A, a PLZF response element has been identified in the promoter of cyclin A2. Currently, several lines of evidence accumulated by Dr. Patricia Yeyati strongly suggest that cyclin A2 is indeed a biological target gene for PLZF.

Domain mapping experiments presented in this thesis revealed that the entire effector domain of PLZF mediates transcription repression and that this repression is partially mapped to the POZ/BTB domain. Data presented here also refutes the hypothesis that POZ/BTB domains act as a DNA binding inhibitory domain since Gal4p1-100, Gal4p1-400, and PLZF have been shown to mediate transcription repression in transient transfection assays. Domain mapping experiments also revealed that the effector domain of PLZF is comprised of two potent repression domains and one acidic activation domain which suggests that it may regulate transcription by more than one mechanism.

Transient transfection assays using a reporter under the regulation of PLZF site A demonstrates that PLZF repressed transcription through its binding site. Moreover, RARα-PLZF was not able to mediate the same level of repression. This strongly suggests that both leukemogenesis and non-responsiveness to ATRA treatment may be the result of competition for binding sites between wildtype PLZF and RARα-PLZF.

Based on the evidence produced in this thesis, a model for leukemogenesis in t(11;17) APL can be proposed. In this model, promyelocytes fail to differentiate because transcription regulation by RARα is disrupted by PLZF-RARα. This disruption, however, can be overcome because PLZF RARα can partially recover its transactivation function in the presence of pharmacological doses of ATRA. However, in t(11;17) APL, transcription regulation by PLZF is also disrupted, by the presence of RARα-PLZF. RARα-PLZF has the potential to compete with PLZF for cis-acting elements, one of which is present on the cyclin A2 promoter. However, the effects of RARα-PLZF on transcription regulation is not effected by the presence of ATRA which may explain why t(11;17) APL patients fail to respond to retinoic acid treatment. PLZF-RARα also has the potential to disrupt transcription regulation by PLZF because it contains the POZ/BTB domain. This domain may allow PLZF-RARα to act as a dominant negative mutant by sequestering co-factors or preventing dimerization by PLZF.

Model of t(11;17) APL

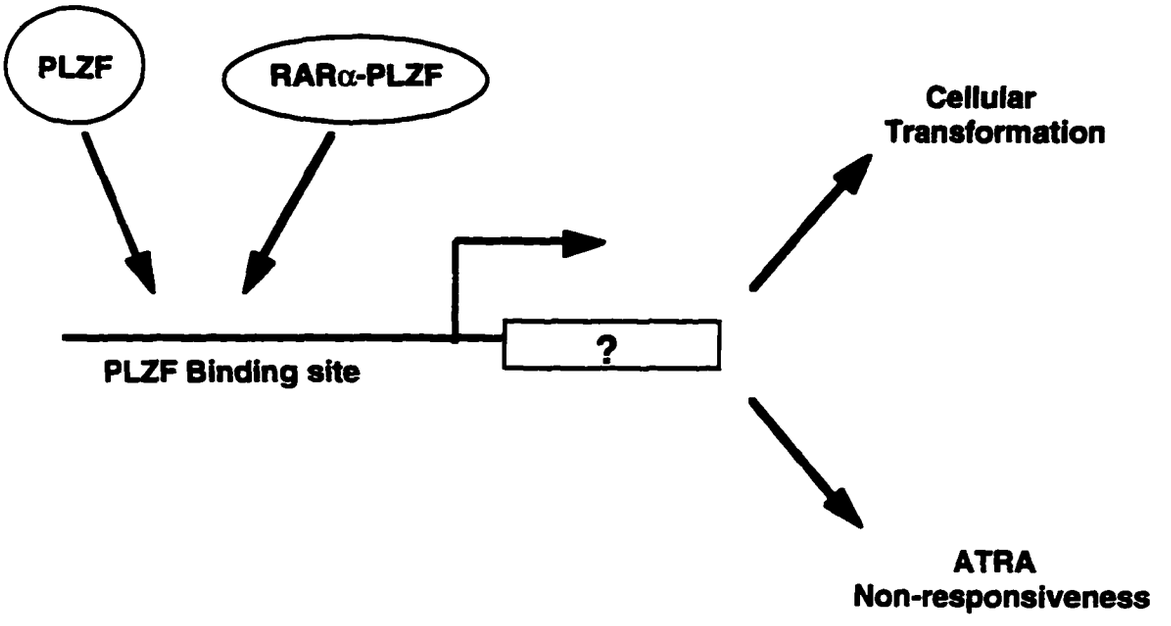


Fig. 25. A model of t(11;17) APL. RARα-PLZF may contribute to the APL leukemic phenotype by competing with PLZF for DNA binding sites and ultimately cause the deregulation of PLZF target genes. PLZF target genes may be involved in the progression of the cell cycle, cellular differentiation or apoptosis programs. Since, RARα-PLZF lacks the ligand binding domain of RARα, its function as a PLZF dominant negative mutant may not be affected by ATRA. This may contribute to the non-responsiveness of t(11;17) APL patients to ATRA therapy.

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