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Analysis of transcriptional modulation of the presenilin 1 gene promoter by ZNF237, a candidate binding partner of the Ets transcription factor ERM

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Abstract

DNA sequences required for the expression of the human presenilin 1 (PS1) gene have been identified between -118 and +178 flanking the major initiation site (+1) mapped in SK-N-SH cells. Several Ets sites are located both upstream as well as downstream from the +1 site, including an Ets motif present at -10 that controls 90% of transcription in SK-N-SH cells. However in SH-SY5Y cells transcription initiates further downstream and requires an alternative set of promoter elements including a +90 Ets motif. Ets2, ER81, ERM and Elk1 were identified by yeast one-hybrid selection in a human brain cDNA library using the -10 Ets motif as a bait. We have shown that ERM recognizes specifically Ets motifs on the PS1 promoter located at -10 as well as downstream at +90, +129 and +165 and activates PS1 transcription with promoter fragments whether or not they contain the -10 Ets site. We have now searched for ERM interacting proteins by yeast two-hybrid selection in a human brain cDNA library using the C-terminal 415 amino acid of ERM as a bait. One of the interacting proteins was ZNF237, a member of the MYM gene family. It is widely expressed in different tissues in eukaryotes under several forms derived by alternative splicing, including a large 382 amino acid form containing a single MYM domain, and 2 shorter forms of 208 and 213 amino acids respectively that do not. We show that both the 382 as well as the 208 amino acid forms are expressed in SK-N-SH cells but not in SH-SY5Y cells. Both forms interact with ERM and repress the transcription of PS1 in SH-SY5Y cells. The effect of both C-terminal and N-terminal deletions indicate that the N-terminal 120 amino acid region is required for interaction with ERM in yeast and furthermore single amino acid mutations show that residues 112 and 114 play an important role. The repression of transcription in SH-SY5Y cells also appears to require the N-terminal potion of ZNF237 and was affected by mutation of the amino acid 112. Data from electrophoretic mobility shift assays indicate that ERM and possibly ZNF237, interact with a fragment of the PS1 promoter.

INTRODUCTION

Presenilins (PS1 and PS2) are highly homologous multipass transmembrane proteins [11,19]. PS1 mutations have been linked to early onset familial Alzheimer's disease (AD) [28,32].

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Presenilins are required for the function of γ-secretase: a multiprotein complex that has also been implicated in the development of AD [5,10,14,31]. They may act as a catalyst or may be involved in the structure and metabolism of the complex itself. γ-secretase has been implicated in the development of AD because of its role in the cleavage of the amyloid precursor protein (APP) and the production of Aβ peptide which is central to the pathogenesis of AD [9]. Similarly the processing of the Notch receptor protein, which controls signaling and cell-cell communication has implicated the role of presenilin in development [16]. Presenilin and γ-secretase also appear to cleave a variety of Type 1 transmembrane proteins which all release intracellular fragments with the ability to interact with transcription coactivators [15,33]. They include CD44, a ubiquitous cell adhesion protein (24), and neuronal cadherin (N-cadherin) [20]. Hence it appears that presenilins may affect the expression of many genes through intramembrane proteolysis [33]. The control of the level of presenilins and its coordination to other components of the γ-secretase complex are likely to be tightly regulated, so we have studied the transcriptional control of the PS1 gene.

We have identified DNA sequences required for the expression of the human PS1 gene. A promoter region has been mapped in SK-N-SH cells and includes sequences from -118 to +178 flanking the major initiation site (+1). However we have shown that the promoter is utilized in alternative modes in SK-N-SH cells and its SH-SY5Y subclone [27]. The -10 Ets site controls 80% of transcription in SK-N-SH cells whereas by itself it plays only a minor role in SH-SY5Y cells. Conversely, the Ets element at +90, controls 70% of transcription in SH-SY5Y cells, whereas it affects transcription by less than 50% in SK-N-SH cells [27]. Nevertheless, in both cell types mutations at both the -10 and +90 Ets sites eliminate 90% of transcriptional activity indicating the crucial importance of these two Ets motifs [27]. In addition to controlling the level of gene expression Ets factors may direct the choice of the promoter elements in play as well, and therefore determine the selective combination of transcription factors involved and the regulatory pathways modulating transcription. We have identified several Ets factors that recognize specifically the -10 Ets motif using the yeast one-hybrid selection including avian erythroblastosis virus E26 oncogene homologue 2 (Ets2), Ets-like gene 1 (Elk1), Ets translocation variant 1 (ER81), and Ets related molecule (ERM) [25-27]. We chose to analyze further the role of ERM because little is known about its mechanism of action and particularly the transcription factors with which it interacts. ERM recognizes specifically Ets motifs located at -10 as well as downstream at +90, +129 and +165 on the PS1 promoter and it activates PS1 transcription with promoter fragments containing the Ets motif at -10 or not. In this report we have identified new ERM-proteins interactions by yeast two-hybrid selection.

RESULTS

Identification of proteins interacting with ERM by yeast two-hybrid screening of a human brain cDNA library

To help understand the function of ERM we sought to detect novel protein interactions. We chose to use as a bait the 984 bp region 3′ end of the coding sequence that corresponds to C-terminal of ERM. It includes the Ets domain, the central inhibitory DNA-binding domain (CIDD) and the C-terminal domain (Fig. 1) [8]. We sought to detect interactions specific to ERM and of the 3 domains, only the Ets domain is highly conserved among the 3 known PEA3 members. The N-terminal activation domain which includes an alpha-helix highly conserved among PEA3 members has been shown to act as a transcription activator in yeast [7] and we chose not to include it in the bait. We screened a total of $1.5x10^6$ transformants and we identified 6 clones conferring growth on 30 mM 3-AT 3 days after plating, and showing a reduced number of colonies at 60 mM 3-AT by 6 days. The expression of the *GAL1-HIS3* reporter in AH109 is leaky: a low level of expression occurs in the absence of GAL4 activation. 3-AT (an inhibitor of Histidine biogenesis) is used to quench the background of growth on His minus medium and the minimum level of 3-AT required varies depending on the bait. A minimum 3-AT level

of 5 mM 3-AT was suitable for screening of the library. Higher doses of 3-AT can be used to discriminate between the phenotypes of different positive clones. Resistance to higher levels of 3-AT generally should indicate tighter interaction. Two clones were identical and included the entire cDNA for the major 382 amino acid splice variant of ZNF237 except for the N-terminal 24 amino acids. 2 clones encoded the N-terminal portion of the chromodomain helicase-DNA-binding protein 3 (CHD3), and one clone included sequences from syntrophin alpha.

The ZNF237 gene is expressed as several forms derived by alternative splicing [30]. The major product encodes a 382 amino acid protein and corresponds to the mRNA identified in our two-hybrid selection. Alternative splicing also produces mRNAs encoding a 208 aa protein and a 213 amino acid protein found in lesser amounts. Figure 2 indicates the regions of homology between the 3 forms; it also outlines the homologies to ZNF198. ZNF198 includes tandem repeats of a novel putative zinc-binding motif MYM (or myeloproliferative and mental retardation motif) [29]. The 382 amino acid form of ZNF237 contains a single more diverged MYM motif which is absent in both of the shorter forms [30].

Expression of endogenous ZNF237 in SK-N-SH and SH-SY5Y neuroblastoma cells

The presence of mRNA encoding ZNF237 in SK-N-SH and SH-SY5Y neuroblastoma cells was examined by RT-PCR from total cellular RNA (Fig. 3A) with the primers ZNF237-1 (sense) and ZNF237-1130 (antisense) (Fig. 3B). In SK-N-SH cells a major product of about 1050 bp corresponded to the major 382 amino acid splice variant according to GenBank data (GI: 6010116), a less abundant transcript identified by a 750 bp fragment likely represented the 208 amino acid variant (GI: 6010118 and 13937379). The larger product close to 1.3 bp likely represents the largest mRNA from the ZNF237 family of proteins which encodes the 213 amino acid specie (GI: 6010118 or 7658706). The RT-PCR product corresponding to the shorter mRNA which also encodes the 213 amino acid form (GI: 6010120) is expected to be about 50 bp shorter than the 1050 bp major variant and is present in minor amounts. It appears as a slight shadow below the 1050 bp band in Figure 3A. The smaller bands below 400 bp were not constantly observed and their identity is unknown. No PCR product was observed with RNA from SH-SY5Y (Fig. 3A, lane 2). Therefore we have used SK-N-SH cells to obtain the entire cDNAs from ZNF237 for further analysis. Functional analysis itself including transfection assays was performed in SH-SY5Y cells because the SH-SY5Y cell line does not express ZNF237 where as SK-N-SH cells do. In addition, SH-SY5Y cell line is a neuronal cell line which expresses both PS1 and ERM. This report focuses on the role of the 382 and 208 amino acid species, the structure of their mRNAs is summarized in Figure 3B. Splicing results in the shorter mRNA as well as a frame shift producing a shorter protein of 208 amino acids where the last 16 residues are absent in the major form (Fig. 3B), the 208 amino acid protein being homologous to the major 382 amino acid form up to position 195.

ZNF237 inhibits PS1 transcription in transient transfection assays

cDNAs from the full length ZNF237 major form or the minor 208 amino acid splice product were inserted into the pC1 vector (Promega, Madison, WI) and transfected into SH-SY5Y cells. Cells were first transfected with increasing amounts of the pC1:ZNF237 constructs together with PS1CAT expression reporter. The total amount of vector pC1+pC1:ZNF237 remained constant at 3 μg. Generally, increasing amount of ZNF237 resulted in increasing inhibition of transcription of PS1 whether it was directed by either the -118/+178 promoter fragment (Fig. 4A) or the shorter +6/+178 fragment (Fig. 4B). With 1.2 μg only 40 to 60% of the promoter activity remained. Transcription of the +6/+178 fragment appeared to be more markedly affected with less than 20% promoter activity left with 3 μg of the plasmid encoding the 382 amino acid form. Hence the 382 and 208 forms both inhibit the transcription of PS1.

No significant difference was observed between the effects of the 2 forms with either template. We also examined the effects of ZNF237 and ERM in combination (Fig. 4C). The addition of ERM appears to block the inhibition of the transcription of PS1 by ZNF237 (Fig. 4C). The sequence of the PS1 promoter used in this experiment is shown in Fig. 4D.

The similar effect of both ZNF237 forms indicate that the MYM domain is not required for repression.

Delineation of the domain(s) required for PS1 repression in transfection assays

We examined the effects of increasing C-terminal deletions of ZNF237 on the transcription of the (-118/+178)PS1CAT reporter (Fig. 5A). Deletions to amino acid 190 or 162 showed similar level of repression, intermediate between the full length 208 amino acid form and the control with pC1 alone. Deletion to amino acid 141 resulted in further reduction of repressor activity, at the limit of statistical significance but consistently observed. Deletion to position 120 or further to residue 53 eliminated repression entirely. Similar results were obtained with the (+6/+178)PS1CAT reporter (data not shown).

Therefore, it appears that progressive C-terminal deletions of the 208 amino acid form have a somewhat gradual effect from the C-terminal to amino acid 120. Amino acids 162 to 208 appear to contribute to the repressor activity. Elimination of the repressor effect is only observed with large deletions to position 120 and beyond.

Identification of ZNF237 domains required for interaction with ERM in yeast

The effects of the mutations in ZNF237 were also tested in the yeast two-hybrid assay which should test more directly the interactions with ERM. Fragments with the same end points as the constructs tested in transfection assays (Fig. 5A) were inserted into the pACT2 vector and transformed into the AH109 strain pretransformed with the bait. Transformants were selected on Leu-Trp-SD medium and alteration in the ability of the truncated protein to bridge interactions between Gal4AD and Gal4BD was scored by the loss of resistance to increasing amounts of 3-AT (0, 5, 30, 60 mM) on Leu-Trp-His-SD medium. Deletion to amino acid 195 or 190 showed similar 3-AT resistance. As compared to the full length 208 amino acid their growth at 30mM after 3 days at 30°C was reduced by about 50%. Colonies appeared at 60 mM only after 5-6 days instead of 3-4 days for the wild type form (Fig. 5B). Further deletions to amino acids 162 , 141 or 120 showed growth similar to 190-195 amino acids at 30 mM but grew much slower at 60 mM with only a few small colonies after 6 days (Fig. 5B). The major effect was observed with deletion to amino acid 105: similar to amino acid 53, it totally eliminated 3-AT resistance as no growth was observed even at 5 mM 3-AT as with the bait alone. Hence it appears that the sequences which are crucial for interaction with ERM in yeast are located towards the N-terminus. The C-terminal border of the crucial domain is between position 120 and 105. However, the amino acids 190 to 208 which contain sequences unique to the 208 amino acid form appear to contribute to interaction with ERM (Fig. 5B, 60 mM 3-AT) and repression (Fig. 5A). Amino acids 162 to 190 which are common to 208 and 382 forms (Fig. 2A) also affect interaction with ERM (Fig. 5B, 60 mM 3-AT) and transcription activity (Fig. 5A). This is corroborated by the effects of N-terminal deletions in yeast two-hybrid assays (Fig. 6). Deletions of sequences from amino acid 109 to 120 eliminated interactions of ZNF237 with ERM as shown by the loss of resistance to 5 mM 3-AT.

Although they are not identical, the results from the transfection assays in neuroblastoma cells and from the yeast assays both indicate the presence of a crucial ERM binding domain N-terminal from residue 120. Both binding and transcription activity appear to be modulated by sequences in the C-terminal portion as well, including sequences located in the portion of the

gene 3′ from the alternative splice specific to the 208 amino acid form Yeast two-hybrid assays using the C-terminal domain of ERM only as a bait and transfecting pACT2:ZNF237 or any of its mutants failed to produce any growth after 8 days on 5 mM 3-AT (data not shown). This indicates that the region of ERM interacting with ZNF237 is located within the fragment including the CIDD and the Ets domains.

Identification of amino acids required for interaction of ZNF237 with ERM and the repression of transcription

The importance of the N-terminal 120 amino acid region was analyzed further by mutations in selective residues. First we mutated blocks of 3 residues. Mutations at positions 109-111 did not appear to affect ZNF237 interactions with ERM in yeast (Fig. 7A) as this mutant showed resistance to 3-AT similar to the wild type 208 amino acid form (FL). However mutations of the 3 other group of residues at 112-114, 115-117 and 117-120 all eliminated ZNF237-ERM interactions. More selective mutations of single amino acids indicated the importance of residues at position 112 and 114 (Fig. 7A). Furthermore, the arginine 112 also appears to play an important role in the repression of PS1 by ZNF237 also (Fig. 7B), as mutating it eliminated any detectable repression activity. It may be interesting to notice that mutating the amino acid 120 resulted in an increase in repression activity (Fig. 7B), although it did not appear to increase binding activity in yeast-2-hybrid assays (Fig. 7A).

Interactions of ZNF237 with ERM and the PS1 promoter in EMSA

We were not able to detect direct interactions of ERM and ZNF237 in coimmunoprecipitation assays using whole cell extracts from SH-SY5Y cells cotransfected with both expression vectors in the presence of Lipofectin. Coimmunoprecipitation of a mixture of both in vitro translated protein failed also. Next we attempted to visualize directly interactions between the two proteins in the presence of the PS1 promoter using an electrophoretic mobility shift assay (EMSA). The PS1 promoter probe including sequences -22/+6 and a mixture of both in vitro translated proteins were incubated in the presence of a goat polyclonal antibody raised against a 20 amino acid C-terminal peptide of ERM (sc-1955X, Santa Cruz Biotech). A high molecular weight DNA-protein complex was observed (Fig. 8C, band A) when in vitro translated ZNF237 was included (lane 3) instead of a control aliquot of in vitro translation reaction with the pCMV-Tag2 empty vector (lane 2) where mostly the complex B was observed. Complex B was absent in the absence of antibody (lane 1) and indeed is specific for anti-ERM antibody since addition of the ERM (C-20) P control peptide used to raise the antibody to the binding reaction eliminates complex formation (Fig. 8B, lane 2). In the absence of antibody (Fig. 8A), no complex A/B was observed with ERM (lane 2), ZNF237 (lane 5), or ERM and ZNF237 together (lane 3). The presence of antibody (and ERM) was required to observe complex formation, and presumably stabilizes the ERM-DNA complex (27). Hence the data indicates that ZNF237 interacts with the ERM-PS1 promoter complex.

DISCUSSION

ERM (Ets related molecule 5or ETV5-Ets translocation variant 5) is a member of the small PEA3 (polyoma enhancer A3) 15group within the Ets family of transcription factors including also PEA3 (or E1AF, ETV4) and ER81 (or ETV1) [8]. Their specific function is unknown, however they have been associated with various cellular processes, including cell proliferation, differentiation [3,4], and tumorigenesis [22,23]. To date most identified target genes of the PEA3 group have been proteases involved in the degradation of cell matrix [6,12]. More recently the *bax* gene from the Bcl-2 family of effectors of the apoptotic cascade was also shown to be activated by the PEA3 group including ERM [12]. It is interesting to note that presenilins also modulate proteolysis, and the metabolism of a number of proteins involved in

signaling and development [15,33]. The same pathways that control crucial steps in neuronal development may also be implicated in neurodegeneration and disorders including the early onset of Alzheimer's disease as well as cases of sporadic occurrence [15,33]. The regulation of the transcription of the presenilin genes likely plays a crucial role in development, and alterations in its control may also play a role in neurodegeneration processes. We have shown that both ER81 and ERM bind to the PS1 promoter and activate its transcription [26,27]. Little is known about the proteins interacting with the different domains of ERM [8]. Its α-helical acidic N-terminal domain functions as a transactivator and interacts with TAFII60 [7]. It functions independently as a transactivator in yeast [7] and was not included in our bait. TBP and TAFII40 also bind to ERM, however the interacting domain (s) have not been determined [7]. To better understand the function of ERM in the control of the PS1 gene we have selected interacting proteins by yeast two-hybrid selection. One of the partner proteins is ZNF237 which was first described as a member of the MYM (myeloproliferative and mental retardation motif) family of genes [30].

The MYM domain has been described as a putative zinc-binding motif likely involved in protein-protein interactions rather than DNA binding [29,30]. ZNF237 contains a single more diverged motif while multiple repeats are more common among the other members [30]. The MYM group is highly conserved in higher eukaryotes and its expression is ubiquitous [30] with no strict tissue specificity. However the ZNF237 gene gives rise to alternative transcripts corresponding to different protein forms which do show differential expression profiles in various tissues [30]. A number of proteins in this group are involved in chromosomal translocations associated with developmental disorders or malignancies. Hence it has been inferred that the fusion proteins themselves may lead to the abnormal phenotypes likely by oligomerization of the fusion protein leading to constitutive activation of the receptor [2,35]. Indeed, the fusion ZNF198-FGFR1 (fibroblast growth factor receptor 1) is observed in a stem cell myeloproliferative disorder [34] and it has been shown that the fusion protein by itself increases the resistance of Ba/F3 cells to apoptosis and their ability to progress through cell cycle in the absence of serum or cytokines characteristic of transformed cells [1,13]. The C-terminal proline rich region rather than the zinc finger appears to be required for oligomerization, downstream signaling activity and transformation [2,35].

However, the function of the intact MYM proteins is still unknown. ZNF198 itself appears to have the ability to interact with proteins associated with DNA repair, replication and chromosomal segregation [17] and this may enable it to affect these cellular pathways [13]. The acidic N-terminal domain of ZNF198 is largely homologous to ZNF237. However, to date the structure-function analysis of ZNF198 has not presented any analogy with functional areas in ZNF237. The region of ZNF198-FGFR1 required for protein-protein interactions appears to be contained in a proline rich region of ZNF198 outside of the region of homology between the two proteins. C-terminal deletion mapping of ZNF237 indicates that sequences essential for interaction with ERM in yeast are located in the N-terminal portion, before amino acid 120 (Fig. 5B) and hence are likely to contain the interaction interface. Indeed single amino acid mutations in this area eliminate interactions with ERM in yeast (residues 112 and 114), as well as the repression of transcription (residue 112). The effect of deletions in transfection assays in SH-SY5Y cells (Fig. 5A) is consistent with the interaction tests in yeast, however shorter deletions affect the repression activity to a much higher degree than in the yeast assay possibly due to alterations in required interactions with other transcription factors. The differential effect of the point mutation at residue 114 on transcription as compared to yeast binding assays could result from the fact that protein-protein interactions are not identical using the bait which is a fragment of ERM or the whole protein synthesized in eukaryotic cells. It could also result from different protein modifications or additional protein factors occurring selectively in the two systems. The mechanism of the repression observed in transfection assays has not been analyzed. ERM acts as an activator of PS1 [27]. ZNF237 may inhibit activation by ERM or

further, form an inhibitory complex with ERM that still binds to the promoter, hence having a dominant effect. ZNF237 may also interact with other members of the Ets family of transcription factors. Indeed it has also been found to interact with TEL (ETV6) [21]. Although no functional analysis has been described in that case TEL acts generally as a transcription repressor and it is widely expressed, hence ZNF237 may enable or enhance such a repressor activity in SH-SY5Y cells. The region of ERM interacting with ZNF237 in yeast two-hybrid has been narrowed to a region including the CIDD and the Ets domain (data not shown). This is consistent with TEL and ERM sharing the most significant homology in their Ets domain [18]. Understanding the function of the ZNF237 proteins in the modulation of the activity of Ets factors will require identification of the amino acid region of ERM involved in the interaction with ZNF237 and examination of the interactions with other Ets members including members of the small PEA3 group most related to ERM such as ER81 which we have already shown to activate PS1 transcription [26].

EXPERIMENTAL PROCEDURES

Yeast two-hybrid screening: construction of the Gal4$_{BD}$: bait gene fusion plasmid

The bait was constructed by inserting the 984 base pair 3′-terminal portion of ERM into the pGBKT7 vector (ClonTech, Palo Alto, CA) in frame with the *Gal4*$_{BD}$ (amino acids 1-147) to generate a fusion protein. ERM sequences were inserted between the *Eco*R I and *Bam*H1*sites* of the multiple cloning site (MCS) immidiatly downstream from the T7 promoter and a c-myc epitope tag located between *Gal4*$_{BD}$ and the MCS of the vector. The ERM insert was synthesized by PCR amplification with the sense primer

Mα-FW: 5′-GATCGAATTCTCCTCTGAGCTGTCGTCTTGTA-3′ and antisense

Mα-R: 5′-GATCGGATCCACTCCGCCACTCAGAAACTTAG-3′

where *Eco*R1 and *Bam*HI sites were added to ERM sequences to direct insertion. It included the central inhibitory DNA binding domain (CIDD), the Ets and C-terminal transactivation domains, but excluded the N-terminal α-helical acidic transactivation domain (Fig. 1) [8]. PCR conditions with Pfu DNA polymerase (Stratagene, La Jolla, CA) were for 30 seconds at 94°C, 30 seconds at 55°C and 90 seconds at 72°C. The ORF fusion junction was verified by DNA sequencing. The production of the fusion protein was also verified by Western analysis of *in vitro* translation products from T7 translated RNA using an antibody to the c-myc epitope tag (clone 9E10, #551101 BD Bioscience Pharmingen, San Diego, CA). Althought the ERM insert does not contain an ATG codon, translation can be initiated on the T7 produced RNA from an ATG start site present on the vector immidiatly upstream from the c-myc epitope and hence does not include *Gal4*$_{BD}$. In yeast, translation is initiated at the start codon of the *Gal4*$_{BD}$. In preliminary experiments the *Gal4*$_{BD}$:bait was tested for autoactivation. The plasmid was transformed into the AH109 reporter strain of *Sacccharomyces cerevisiae.* Plating on SD medium lacking tryptophane (Trp-SD) indicated the transformation efficiency. Autoactivation was monitored by the expression of the *GAL1-HIS3* reporter in AH109 and the growth of colonies on Trp-His-SD medium containing increasing 3-amino-1,2,4-triazole (3-AT) (Sigma, Saint Louis, MO) concentrations (from 0 to 5, 10, 30, and 60 mM). With the bait described above 5 mM 3-AT was sufficient to suppress any growth even after 7 to 10 days incubation at 30°C. Hence this concentration was chosen for the two-hybrid screening.

Yeast two-hybrid screening

The cDNA library (cat #HL4004AH in pACT2, ClonTech, Palo Alto, CA) was amplified and the plasmid DNA was used together with the bait in cotransformation of AH109 competent cells from ClonTech according to the manufacturer's instructions . The total number of transformants was counted by plating on SD medium lacking tryptophane or leucine. A total

of 1.5 x 10^6 independent transformants were screened. Transformants where the transcription of the *GAL1-HIS3* reporter was activated were selected on medium lacking tryptophane, leucine or histidine and containing 5 mM 3-AT. Clones appearing after 4-6 days were analyzed further. Yeast plasmid DNA was extracted and used to transform *E.coli* DH5α. In a round of secondary screening, in order to verify that phenotypic 3-AT resistance was indeed attributable to only one plasmid, the bacterial plasmid DNA was in turn retransformed in yeast. The resulting 3-AT resistant clones were analyzed by DNA sequencing.

Cloning of ZNF237 and its mutants

The entire cDNAs for both ZNF237 alternative splice products of 387 amino acids and 208 amino acids were obtained by PCR amplification using:

ZNF237-1: 5′-GATCACGCGTCGTGAGGCTTTGGCAGAAAT-3′ and

ZNF237-2: 5′-GATCGTCGACGTTATACATCCAGAACACGTGC-3′

as forward and reverse primers respectively with a template cDNA obtained by reverse transcription of total cellular RNA from SK-N-SH neuronal cells. PCR with Pfu DNA polymerase was according to the manufacturer's recommendations at 94°C, 60°C and 72 °C for 30, 30 and 90 seconds, respectively. The primers included restriction sites for *Mlu*1 and *Sal*1 in order to direct insertion into the corresponding sites of the pC1 vector (Promega, Madison,WI) downstream from the cytomegalovirus (CMV I) promoter. PCR products were separated on 1.4% agarose gels and the 1.2 and 0.85 kb fragments corresponding respectively to the 382 and 208 amino acid forms were eluted, digested with *Mlu*1 and *Sal*1 and inserted into pC1. All constructs were sequenced on both strands to determine their identity.

C-terminal deletions from the 208 amino acid form were derived by PCR from the cDNA clone above using the same sense primer ZNF237-1. The antisense primers to the deletion end points including also a UGA stop codon were:

ZNF-1130 5′-GATCGTCGACTCACCAGTTGTTTTCACAGGGAGAC-3′ (full length 208 form)

ZNF-740 5′-GATCGTCGACTCAAGGACTATGATGAGTTGC-3′

ZNF-693 5′-GATCGTCGACTCATGCCACATTCATTCTAC-3′

ZNF-639 5′-GATCGTCGACTCATCTTGAAAGACTGGAAGTGG-3′

ZNF-575 5′-GATCGTCGACTCACCATTCGATAAAACAGGAAGAC-3′

ZNF-512 5′-GATCGTCGACTCAATCAATAACAATTGTCTC-3′

ZNF-480 5′-GATCGTCGACTCAATCTCTTGAAGAAGGAG-3′

ZNF-300 5′-GATCGTCGACTCAATCTTCCACTGGTGAGTTC-3′

Constructs into pC1 of N-terminal deletions from the 208 amino acid form were derived by PCR from the full length cDNA clone using a set of complementary primers including a fusion between the first 4 amino acids of ZNF237 and the end point of the 3′-deletions at position 28, 68, 101, 109 and 120 respectively. Both sense and the complementary antisense primer for each deletion were:

Z3P-28F:5′-GCATGGAAAAATGTCTCATGGACATAGG-3′

Z3P-28R: 5′-CCTATGTCCATGAGACATTTTTCCATGC-3′

Z3P-68F: 5′-GCATGGAAAAATGTCAACCTCCTTCAATTTC-3′

Z3P-68R: 5′-GAAATTGAAGGAGGTTGACATTTTTCCATGC-3′

Z3P-101F: 5′-GCATGGAAAAATGTCCTTCTTCAAGAG-3′

Z3P-101R: 5′-CTCTTGAAGAAGGACATTTTTCCATGC-3′

Z3P-109F: 5′-GCATGGAAAAATGTCAGAAGGGAAATATAAG-3′

Z3P-109R: 5′-CTTATATTTCCCTTCTGACATTTTTCCATGC-3′

Z3P-120F: 5′-GCATGGAAAAATGTGATGATGAAGAGGAC-3′

Z3P-120R: 5′-GTCCTCTTCATCATCACATTTTTCCATGC-3′

For each deletion, the 5′ portion of the insert was generated using the ZNF237-1 primer containing an *Mlu*I site and the antisense mutant primer. The 3′ portion of the deletion was generated using the sense mutant primer and the ZNF1130 antisense primer containing a *Sal*I site. The entire insert was then synthesized by mixing aliquots of the first 2 fragments in a PCR reaction with the ZNF237-1 and ZNF1130 primers.

pC1 constructs including selective amino acid mutations were synthesized according to a similar protocol with the following sense and antisense primers for each mutation.

For the triple mutant at amino acid 109-111, 112-114, 115-117, 118-120, the following primers were used:

ZNF-10911S: 5′-GAGATTTGGCATCTGCGGCGGTAAATATAAGTGAG-3′

ZNF-10911A: 5′-CTCACTTATATTTACCGCCGCAGATGCCAAATCTC-3′

ZNF-11214S: 5′-CATCTCAGAAGGGACATGTAGGTGAGACAAATGTTATTG-3′

ZNF-11214A: 5′-CAATAACATTTGTCTCACCTACATGTCCCTTCTGAGATG-3′

ZNF-11517S: 5′-
GAAGGGAAATATAAGTGGGGCAGCTGTTATTGATGATGAAGA-3′

ZNF-11517A: 5′-
TCTTCATCATCAATAACAGCTGCCCCACTTATATTTCCCTTC-3′

ZNF-11820S: 5′-AGTGAGACAATTGGTGGTGGTGATGAAGAGGACG-3′

ZNF-11820A: 5′-CGTCCTCTTCATCACCACCACCAATTGTCTCACT-3′

For the single mutants at amino acids 112, 114, 116, 120, the following primers were used:

ZNF-112S: 5′-CATCTCAGAAGGGACATATAAGT GAGACAAATGTTATTG-3′

ZNF-112A: 5′-CAATAACATTTGTCTC ACT TATATGTCCCTTCTGAGATG-3′

ZNF-114S: 5′-CATCTCAGAAGGGAAATATAGGTGAGACAAATGTTATTG-3′

ZNF-114A: 5′-CAATAACATTTGTCTCACCTATATTTCCCTTCTGAGATG-3′

ZNF-116S: 5′-
GAAGGGAAATATAAGTGAGGCAATTGTTATTGATGATGAAGA-3′

ZNF-116A: 5′-TCTTCATCATCAATAACAATTGCCTC ACTTATATTTCCCTTC-3′

ZNF-120S: 5′-AGTGAGACAATTGTTATTGGTGATGAAGAGGACG-3′

ZNF-120A: 5′-CGTCCTCTTCATCACCAATAACAATTGTCTCACT-3′

Transfection assays in SH-SY5Y neuroblastoma cells

Transfection by calcium phosphate precipitation and glycerol shock was described previously [27]. Cells were seeded at a density of $10^4/cm^2$, 1 day before transfection. Promoter activity was determined by chloramphenicol acetyl transferase (CAT) assay in different samples and was standardized using the amount of protein present in the cellular extracts as an internal control as described previously [27]. Each experiment was repeated three times, with at the minimum triplicate tests of each construct and treatment.

Protein-protein interaction assays

The same set of C-terminal deletions from the ZNF237 cDNAs used in transfection was also tested for their ability to interact with the bait using the yeast two-hybrid assay. Forward and reverse primers were designed to incorporate *Eco*R1 and *Xho*1 sites respectively in order to direct insertion into the corresponding sites of the pACT2 polylinker and to generate a $Gal4_{AD}$:ZNF237 fusion protein with in frame insertion of ZNF237 starting at amino number 4 (underlined below). The common sense primer was

5′-GATCGAATTCGCTGTTCAGTGGGAGGATTAG-3′.

Antisense primers were

ZNF-1130 5′-GATCCTCGAGTCACCAGTTGTTTTCACAGGGAGAC-3′

ZNF-740 5′-GATCCTCGAGTCAAGGACTATGATGAGTTGC-3′

ZNF-693 5′-GATCCTCGAGTCATGCCACATTCATTCTAC-3′

ZNF-639 5′-GATCCTCGAGTCATCTTGAAAGACTGGAAGTGG-3′

ZNF-575 5′-GATCCTCGAGTCACCATTCGATAAAACAGGAAGAC-3′

ZNF-512 5′-GATCCTCGAGTCAATCAATAACAATTGTCTC-3′

ZNF-480 5′-GATCCTCGAGTCAATCTCTTGAAGAAGGAG-3′

ZNF-300 5′-GATCCTCGAGTCAATCTTCCACTGGTGAGTTC-3′

N-terminal deletions with the same end-points as the constructs in pC1 described above were generated using the set of sense primers including an *Eco*R1 site:

Z3A-28: 5′-GATCGAATTCTCATGGACATAGGGGATTCATTTGG-3′

Z3A-68: 5′-GATCGAATTCAACCTCCTTCAATTTCTGCTC-3′

Z3A-101: 5′-GATCGAATTCCTTCTTCAAGAGATTTGGCATCTCAG-3′

Z3A-109: 5′-GATCGAATTCAGAAGGGAAATATAAGTGAG-3′

Z3A-120: 5′-GATCGAATTCTCGATGATGAAGAGGACGTAG-3′

and the common antisense primer ZNF1130 above including an *Xho* I site.

Amino acid mutants were constructed with the method described above for the pC1 inserts and with the same mutant primers. The 5′ forward primer was the same used in the construction of the C-terminal deletions. The 3′ reverse primer was ZNF1130 also described above. Constructs were verified by DNA sequencing.

The pACT2:ZNF237 constructs were transformed into AH109 pretransformed with the $Gal4_{BD}$:ERM bait, and the double transformants were selected on Trp-Leu - His-SD medium. The resistance to 3-AT was then tested by resuspending individual colonies into 1 ml H2O, and plating patches with 4 μl aliquots on medium containing increasing amounts of 3AT.

Analysis of protein-protein interactions by EMSAs

To assay the interactions of ERM and ZNF237, the proteins were synthesized from pCI-based vectors with an *in vitro* transcription-translation kit as recommended by the manufacturer (Promega). In EMSAs,aliquots (1.5 μl) from *in vitro* translation reactions were added to 20-μl DNA binding mixtures including 12 mM Hepes, pH 7.5, 50mM NaCl, 1 μM $ZnCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 10-50pg of [^{32} P]-labeled DNA probe including the PS1 sequences (-22, +6), and 1 μg of poly[d(I·C)]. The goat polyclonal antibody sc-1955X (SantaCruz Biotechnology, Santa Cruz, CA) (2 μl) raised against a 20 amino acid C-terminalpeptide of the human ETV5/ERM was also included in the binding reaction in order to stabilize the complexes observed. Control reactions included corresponding to the ERM (C-20)P control peptide (sc-1955-P). Reactionswere incubated at 24°C for 30 min and analyzed by electrophoresison native 4.5% polyacrylamide gels containing 0.1% NP40 at 4°C as described previously (27).

ACKNOWLEDEGMENTS

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Glossary

Abbreviations:

Ets, Avian erythroblastosis virus E26 oncogene homologue; ERM, (Ets related molecule 5 or ETV5-Ets translocation variant 5); PEA3 (or E1AF, ETV4), Polyoma enhancer A3; ER81 (ETV1), Ets related protein 81 or Ets translocation variant 1; MYM, (myeloproliferative and mental retardation motif).

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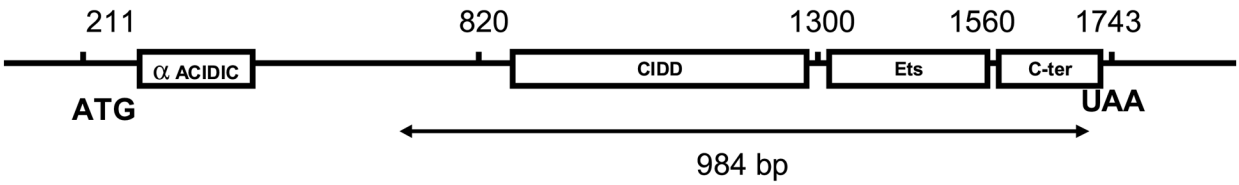


Figure 1.

ERM protein domains included in the yeast two-hybrid bait. The linear sequence of the cDNA for ERM is annotated according to GI: 33873571. The translation starts at nucleotide 211 and stops at position 1743. The domains conserved within the PEA3 family are boxed, including the N-terminal alpha-helical acidic domain contained within the first 72 amino acids, the Ets domain, the central inhibitory DNA-binding domain (CIDD) and the C-terminal domain. The fragment of cDNA included in the bait is indicated by arrows.

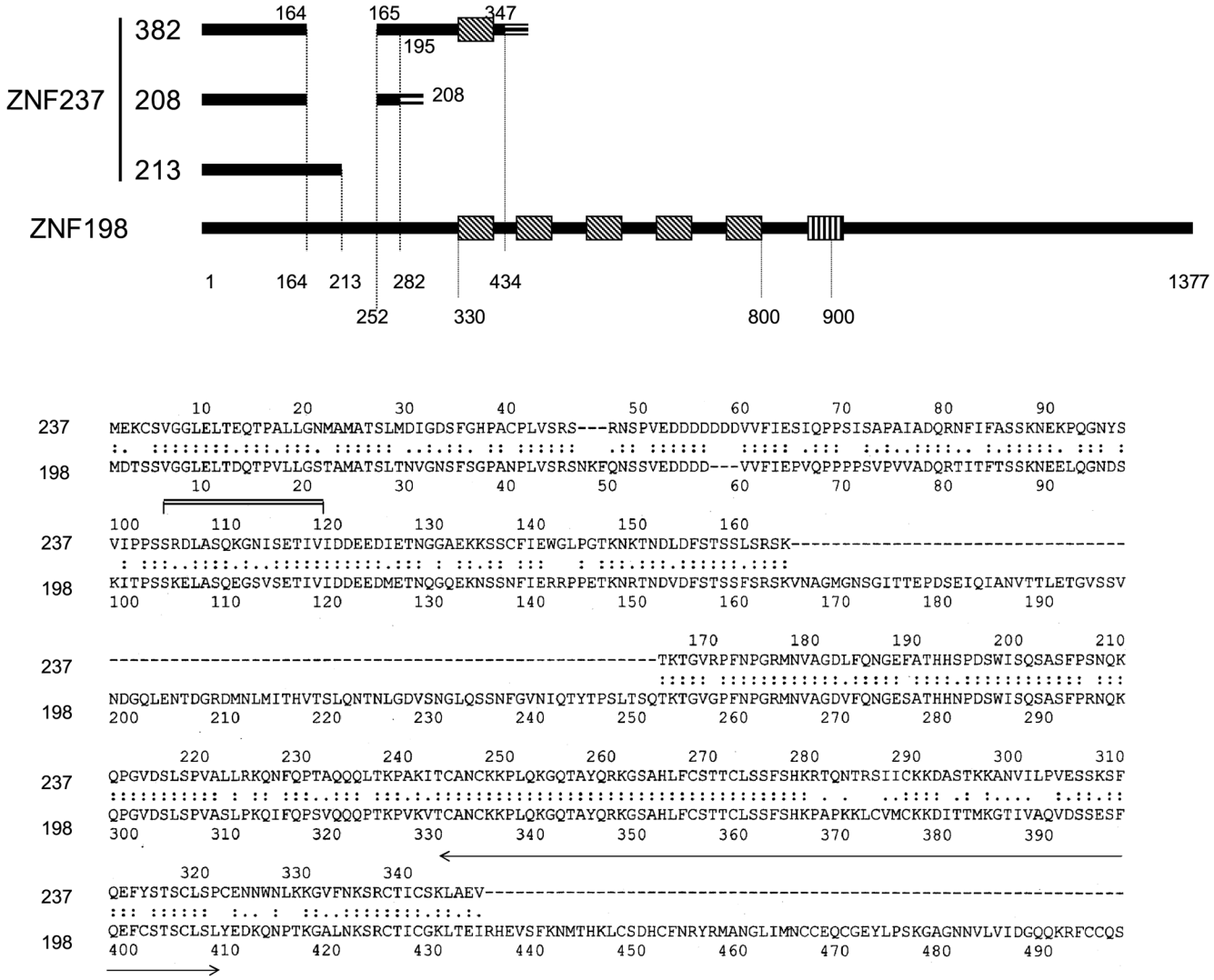
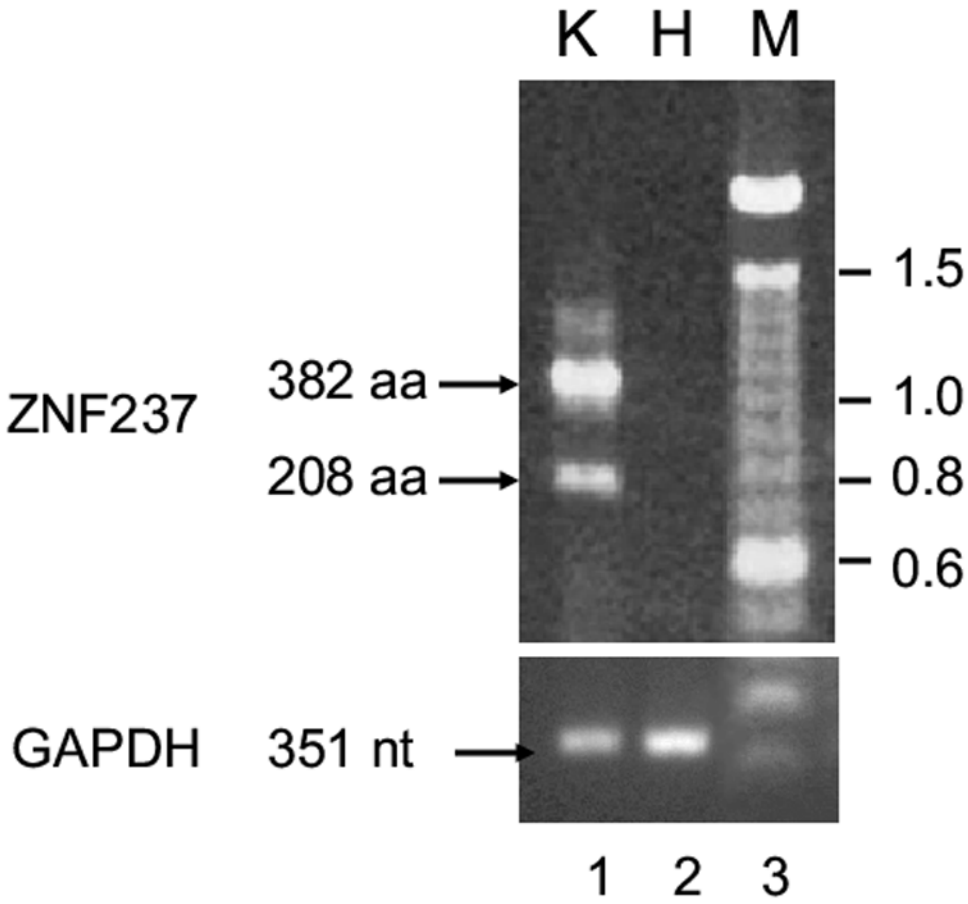
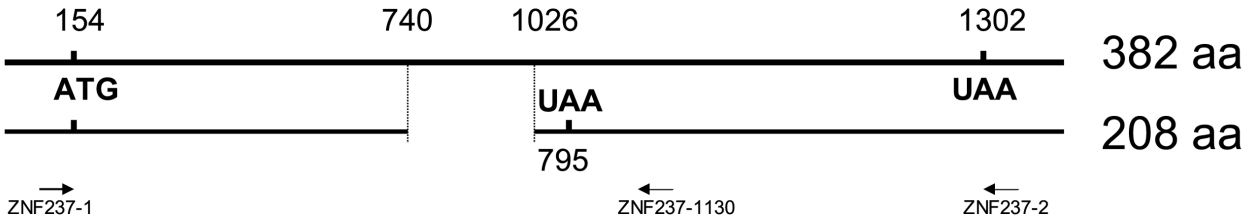


Figure 2.

Various ZNF237 proteins and their homologies. A. Homologous regions between ZNF237 and ZNF198. The 382 amino acid major ZNF 237 (GI: 6010116) form is aligned with the 2 minor 208 (GI: 6010118) and 213 (GI: 6010120) amino acid forms derived by alternative splicing as well as with ZNF198 (GI: 22137330) in order to display homologous segments. Black lines show homologous areas. Double lines mark heterologous regions: amino acid 195 to 208 of ZNF237 (208 aa form) are absent in ZNF237 (382aa form) or ZNF198. The triple line at amino acid 347 in ZNF237 (382 aa form) marks a region of divergence with ZNF198. The MYM motifs are indicated by boxes striped in diagonal, the proline rich region of ZNF198 is shown by a box with vertical stripes. The amino acid positions on the respective proteins are indicated by numbers. B. Amino acid sequences alignment of ZNF237 and ZNF198 . The N-terminal amino acids 1 to 347 of ZNF237 were aligned with the homologous region in the N-terminal of ZNF198. The area including an MYM domain is underlined with arrows. The protein region where mutations eliminate binding to ERM by yeast-two hybrid assays is indicated by brackets. Dotted lines indicate residues in ZNF198 that are absent in ZNF 237. Dots represent amino acids which are conserved between ZNF237 and ZNF198.





MEKCSVGGLELTEQTPALLGNMAMATSLMDIGDSFGHPACPLVSRSRNSPVE**D**DDDDDDVVFIESIQPPSISA

PAIADQRNFIFASSKNEKPQGNYSVIPPSSR**D**LASQKGNISETIVI**D**DEEDIETNGGAEKKSSCFIE**W**GLPGTKNK

TNDLDFSTSSLS**R**SKTKTGVRPFNPGRMNVAGDLFQNGEF**A**THHS **PDSWISQSASFPSNQKQPGVDSLSPVA**
PEMHLQRRLMSFFQ

LLRKQNFQPTAQQQLTKPAKITCANCKKPLQKGQTAYQRKGSAHLFCSTTCLSSFSHKRTQNTRSIICK

KDASTKKANVILPVESSKSFQEFYSTSCLSPCENNWNLKKGVFNKSRCTICSKLAEVWIFIPKLLFRLTVI

ILTFKCYYVLFHLHNARVLDV

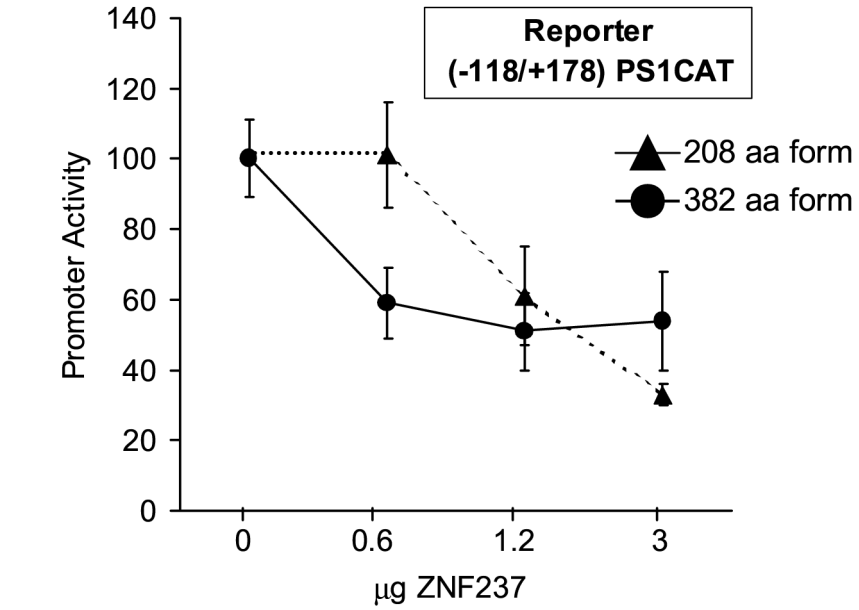
Figure 3.

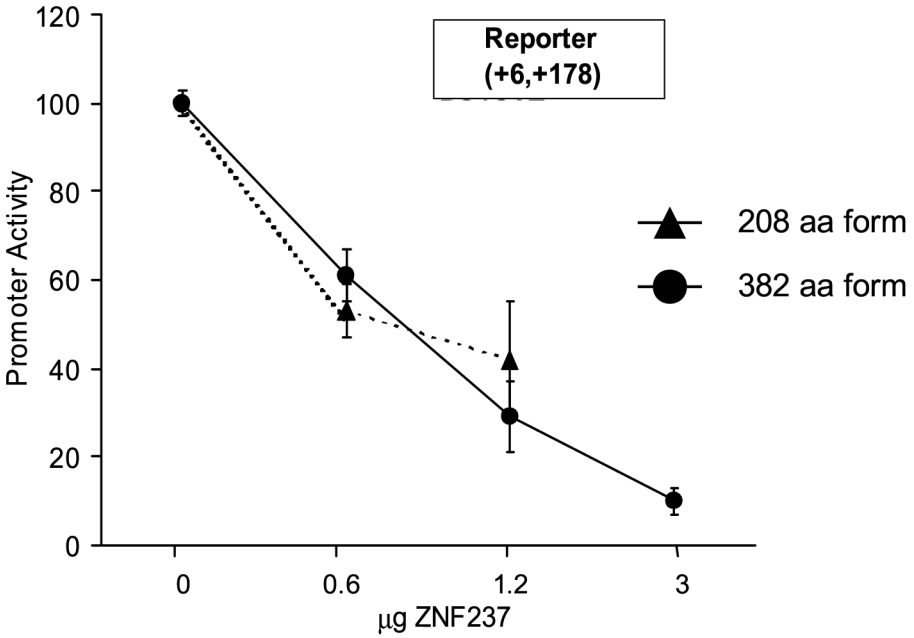
RNA and protein structure of ZNF237 major 382 amino acid form and 208 amino acid minor form. A. RT-PCR with total cellular RNA from SK-N-SH cells (lane 1) or SH-SY5Y (lane 2) with primers ZNF237-1 and ZNF237-1130. Molecular weight markers were run alongside (lane 3) and fragment size is indicated in kb on the right. Arrows show the fragments corresponding the major ZNF237 specie of 382 amino acids and the minor 208 amino acid form. Control PCR reactions to the human GAPDH gene were performed with each of the cDNAs. The expected 351 nt GAPDH product is indicated by an arrow. Primers used for the GAPDH gene were: sense

5′-AGCAATGCCTCCTGCACCACCAAC-3′

and antisense 5′-CTGCTTCACCACCTTCTTGATG-3′.

B. Top: the homologous regions of the cDNAs from the 382 and 208 amino acid forms are aligned. The numbers indicate the nucleotide positions according to GI: 6010116. The start (ATG) and stop (UAA) of translation are indicated. The position of primers used to identify and clone the cDNAs by RT-PCR is indicated by arrows. Bottom: the amino acid sequence of both proteins is summarized: amino acid 1 to 195 are common to both 382 and 208 species. The divergent C-terminal regions downstream from 195 are indicated in bold. The numbers near the amino acid sequence indicate the position of the underlined residues.





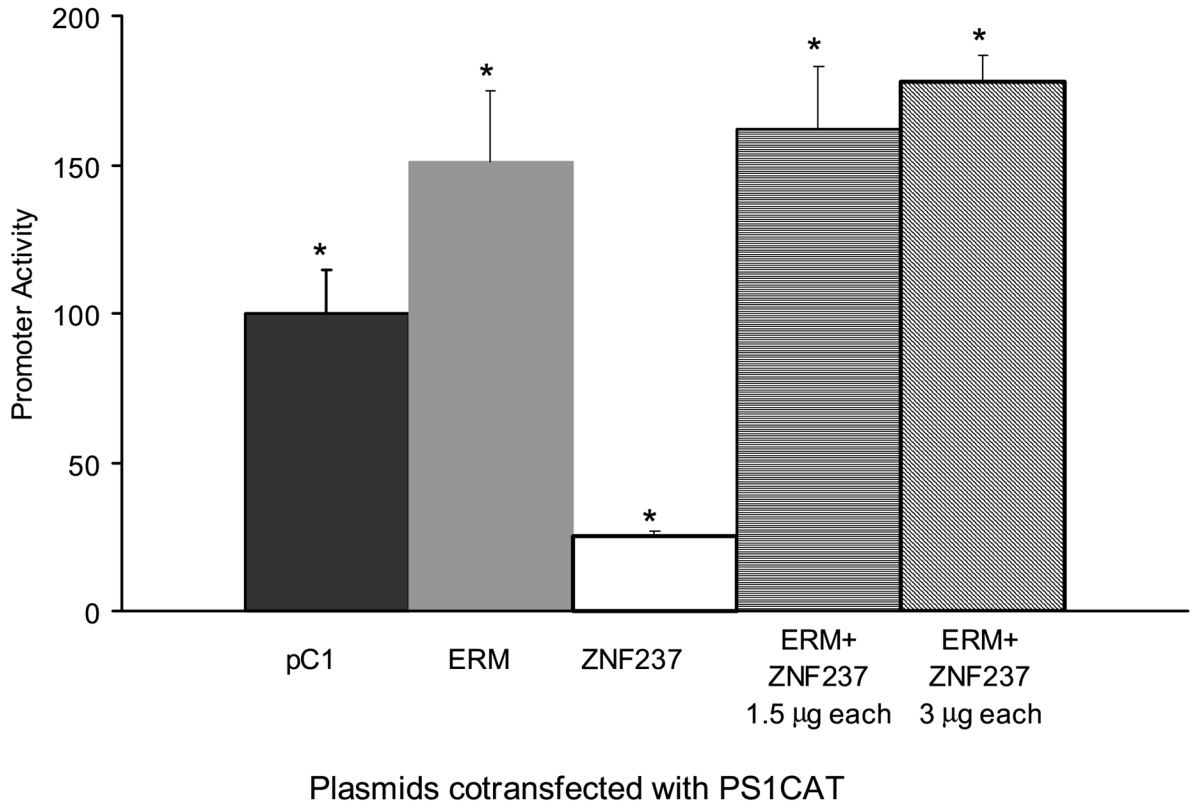


Figure 4.

Inhibition of PS1 transcription by ZNF237. SH-SY5Y cells were transfected with 6 µg of (-118,+178) PS1CAT (A) or (+6,+178) PS1CAT (B) reporter together with increasing amounts of ZNF237 major 382 (circles) or 208 (triangles) amino acid forms. The total amount of (ZNF237+pC1) was kept constant at 3 µg. Promoter activity in different samples was standardized using the amount of protein present in the cellular extracts as an internal control. Each experiment was repeated three times, with at the minimum triplicate tests of each

construct combination. (C) (-118,+176) PS1 CAT (5 μg) was cotransfected with expression vectors for ERM or ZNF237 (208 aa form) (3 μg), or a mixture of ERM and ZNF237 (1.5 μg each or 3 μg) with a minimum triplicate test of each construct combination. The symbol (*) indicates differences significant with $p<0.05$ bt t-test/ANOVA between the pC1 control as compared to each combination of plasmid (s) tested. (D) DNA sequence of the PS1 promoter region used in transfection experiments. The homologies to the Ets motif are underlined. The major transcription initiation site is indicated at +1.

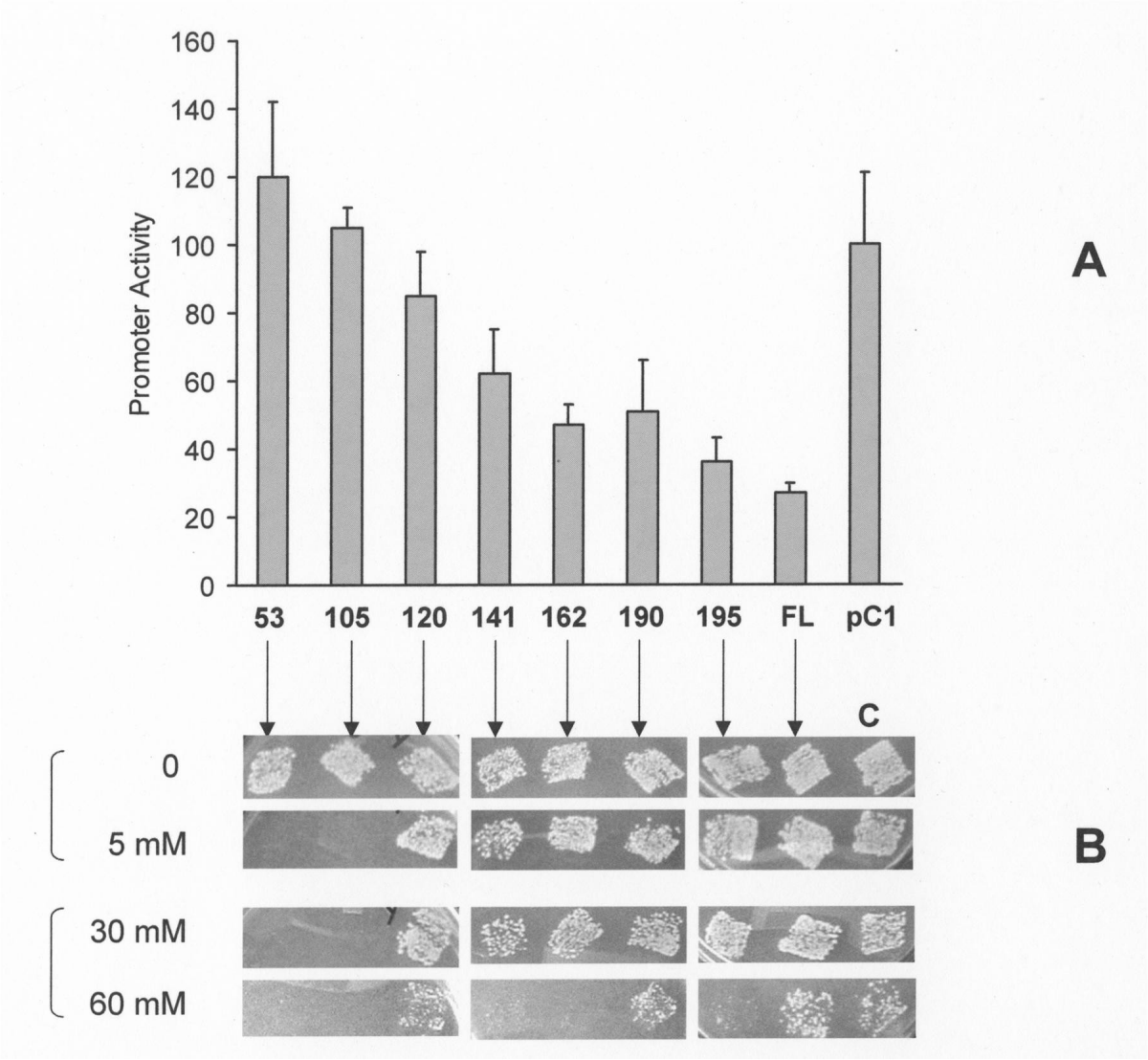


Figure 5.

Effect of C-terminal deletions on the inhibition of transcription by ZNF237 and on the binding to ERM. A. Effect of C-terminal deletions on the inhibition of transcription by ZNF237. SH-SY5Y cells were transfected with 6 μg of the (-118, +178)PS1CAT reporter together with 5 μg of ZNF237 full length or each C-terminal deletion with the indicated end-point (position 53, 105, 120, 141, 162, 190, 195, or the full length 208 amino acid form) or the empty pC1 vector. Promoter activity in different samples was standardized using the amount of protein present in the cellular extracts as an internal control. Each experiment was repeated three times, with a minimum of n=4 for each data point. B. Identification of ZNF237 domains interacting with ERM by yeast two-hybrid assay. The same C-terminal deletions of ZNF237 were introduced into pACT2 and transformed into AH109 pretransformed with the $Gal4_{BD}$-ERM fusion bait. The ability of the mutants to promote growth on SD medium lacking Tryptophane, Leucine or Histidine and including increasing amounts of 3-AT (indicated alongside on the left) was compared after 4 days (0 and 5 mM 3-AT) and after 5 days (30 and 60 mM 3-AT). Patches from the series C were transformants with the original cDNA from

ZNF237 obtained when screening the library that included most of the 382 amino acid form except for the first 24 amino acids.

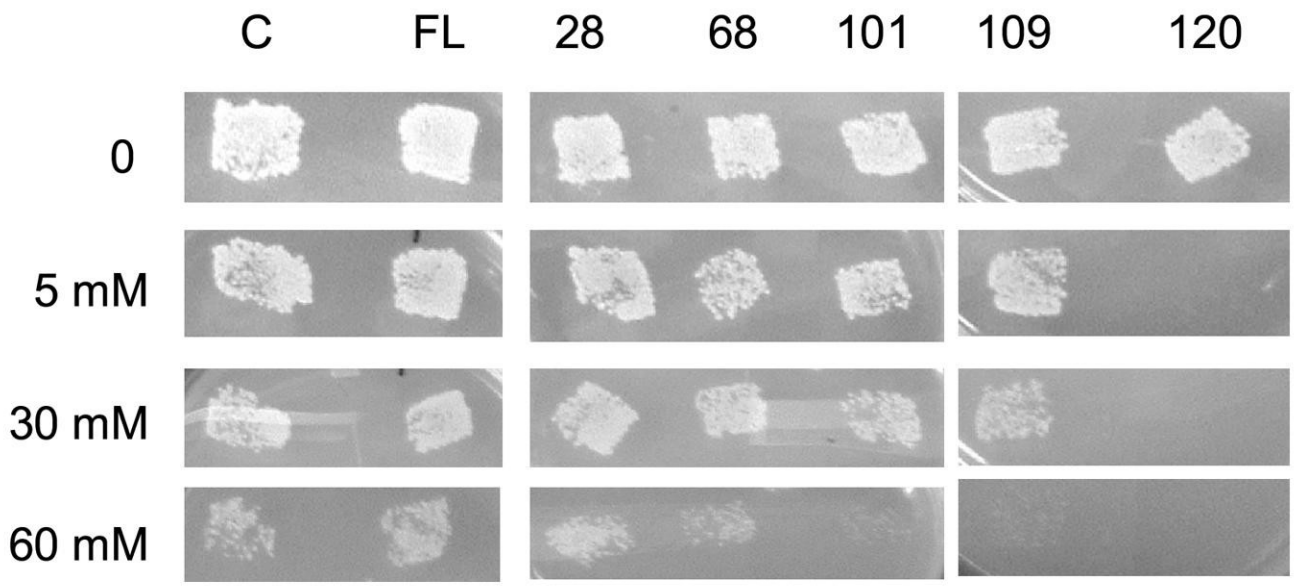
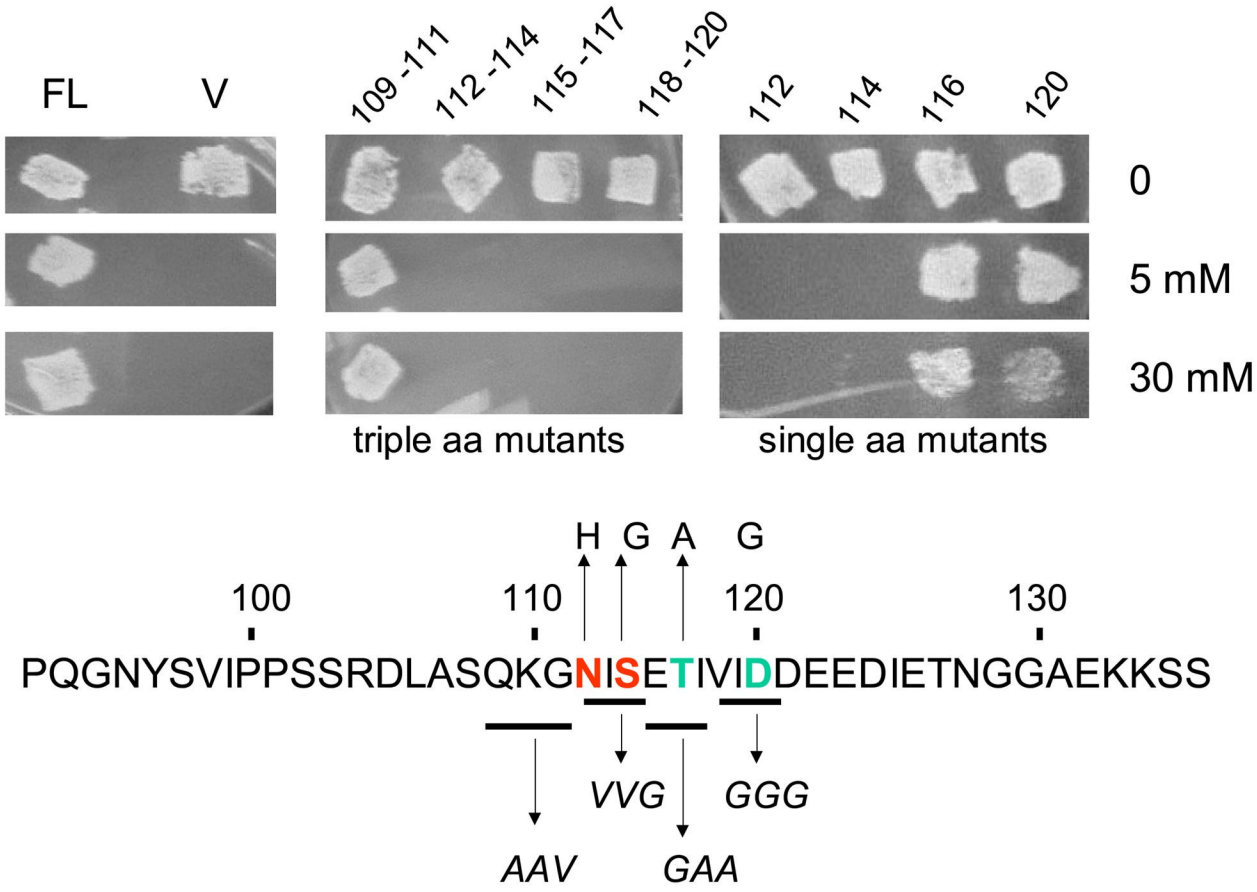


Figure 6.

Effect of N-terminal deletions on the interaction of ZNF237 with ERM: yeast two-hybrid assay. The N-terminal deletions of ZNF237 to amino acids 28, 68, 101, 109 and 120 were introduced into pACT2 and transformed into AH109 pretransformed with the $Gal4_{BD}$-ERM fusion bait. The ability of the mutants to promote growth on SD medium lacking Tryptophane, Leucine or Histidine and including increasing amounts of 3-AT (indicated alongside on the left) was compared after 4 days (0 and 5 mM 3-AT) and after 5 days (30 and 60 mM 3-AT). Patches from the series C were transformants with the original cDNA from ZNF237 obtained when screening the library that included most of the 382 amino acid form except for the first 24 amino acids. FL indicates the intact 208 amino acid form fused to the pACT2 polylinker and $Gal4_{AD}$ at amino acid 4.



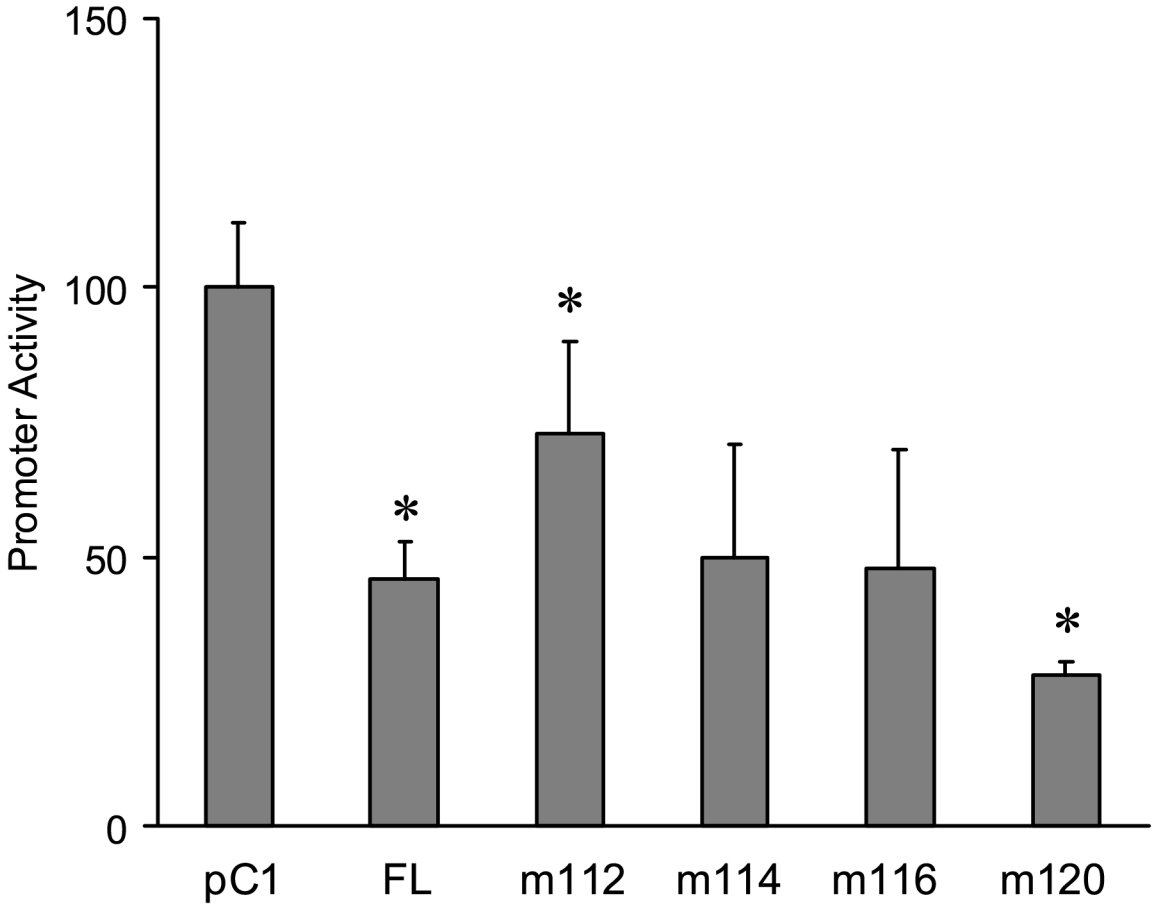


Figure 7.

Effect of selective amino acid mutations in ZNF237 on its binding to ERM and the inhibition of transcription of PS1. A. Effect of amino acid mutations of ZNF237 on its interaction with ERM in yeast two-hybrid assays. Either triple mutants or single amino acid mutants of the 208 amino acid form of ZNF237 were introduced into pACT2 and transformed into AH109 pretransformed with the $Gal4_{BD}$-ERM fusion bait. The ability of the mutants to promote growth on SD medium lacking Tryptophane, Leucine or Histidine and including 0, 5 and 30 mM 3-AT (indicated alongside on the right) was compared after 5 days to the wild type (FL) and the empty pACT2 (V). The amino acid sequence of the corresponding area of ZNF237 is indicated together with the amino acids mutated and the residues that were substituted. B. Effect of single amino acid mutations on the inhibition of PS1 transcription by ZNF237. SH-SY5Y cells were transfected with 5 μg of the (-118, +178)PS1CAT reporter together with 6 μg of ZNF237 wild type 208 amino acid form or each of the mutants at single amino acid position 112, 114, 116 and 120 respectively. Promoter activity was standardized using the amount of protein present in the cellular extracts. Activity in the presence of the empty pC1 control vector was considered 100%. Each experiment was repeated three times, with a minimum of n=4 for each data point. The symbol (*) indicates a significant difference with $p<0.05$ by t-test/ANOVA between the full length protein and m112 as well as between the full length protein and m120.

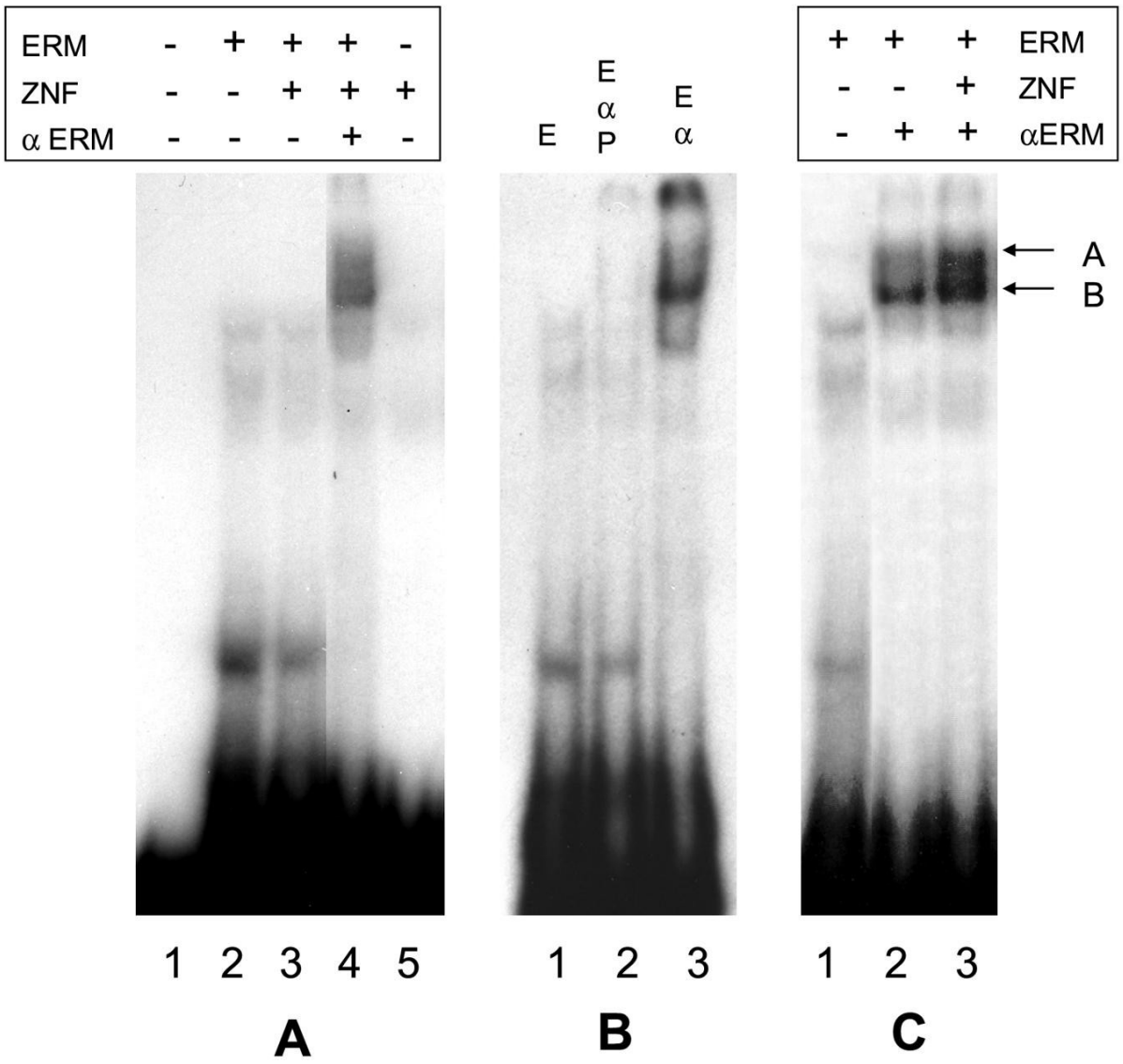


Figure 8.

ZNF237 interaction with the PS1 promoter. A. In vitro translated ZNF237 208 amino acid form and full length ERM were incubated together with a labeled probe containing PS1 promoter sequences from -22 to +6 and in the presence of the goat polyclonal anti-ERM antibody sc-1955X (αERM). Added components are marked (+). B. ERM (E) was incubated with the PS1 probe (lane 1), with added anti-ERM antibody (lane 3), or with both the antibody and the control peptide used to raise the antibody (P) (lane 2). C. ERM was incubated with the PS1 promoter (lane 1), together with anti-ERM antibody and ZNF237 (lane 3), or a control aliquot of an in vitro translation reaction with the empty pCMV-Tag 2 vector as a template instead of ZNF237 (lane 2). Arrows indicate the position of specific DNA-protein complexes.