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Highly conserved amino acids in Pax and Ets proteins are required for DNA binding and ternary complex assembly

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ABSTRACT

Combinatorial association of DNA-binding proteins on composite binding sites enhances their nucleotide sequence specificity and functional synergy. As a paradigm for these interactions, Pax-5 (BSAP) assembles ternary complexes with Ets proteins on the B cell-specific *mb-1* promoter through interactions between their respective DNA-binding domains. Pax-5 recruits Ets-1 to bind the promoter, but not the closely related Ets protein SAP1a. Here we show that, while several different mutations increase binding of SAP1a to an optimized Ets binding site, only conversion of Val68 to an acidic amino acid facilitates ternary complex assembly with Pax-5 on the *mb-1* promoter. This suggests that enhanced DNA binding by SAP1a is not sufficient for recruitment by Pax-5, but instead involves protein–protein interactions mediated by the acidic side chain. Recruitment of Ets proteins by Pax-5 requires Gln22 within the N-terminal β-hairpin motif of its paired domain. The β-hairpin also participates in recognition of a subset of Pax-5-binding sites. Thus, Pax-5 incorporates protein–protein interaction and DNA recognition functions in a single motif. The *Caenorhabditis elegans* Pax protein EGL-38 also binds specifically to the *mb-1* promoter and recruits murine Ets-1 or the *C.elegans* Ets protein T08H4.3, but not the related LIN-1 protein. Together, our results define specific amino acid requirements for Pax–Ets ternary complex assembly and show that the mechanism is conserved between evolutionarily related proteins of diverse animal species. Moreover, the data suggest that interactions between Pax and Ets proteins are an important mechanism that regulates fundamental biological processes in worms and humans.

INTRODUCTION

The regulation of tissue-specific transcription is a function of sequence-specific DNA-binding proteins, which bind short nucleotide sequences that may appear frequently in the context of a complex genome. To enhance discrimination of functionally important sequences *in vivo*, contacts between these proteins increase their avidity for binding DNA at adjacent or overlapping sites. These interactions also allow for functional synergy in the context of the composite regulatory sequences. Binding by proteins with different functional domains greatly increases the range of possible regulatory outcomes at a single composite element.

Pax-5 is a member of the Pax family, a group of proteins that share a highly conserved DNA-binding motif, the paired domain. The 128 amino acid paired domain is a bipartite DNA-binding structure including two helix–turn–helix motifs reminiscent of homeodomains separated by an extended polypeptide linker (1–3). At the N-terminus, a short region of antiparallel β-sheet, the β-hairpin, contacts the phosphate backbone of DNA and is followed by a β-turn that contacts the minor groove. The linker region contributes to DNA binding by contacting the minor groove. Nucleotide sequences recognized by paired domains are degenerate, which results in a greater range of potential binding sites but poses a problem for the identification of functional target sites *in vivo*. In addition to its paired domain, the C-terminal region of Pax-5 includes domains required for transcriptional activation, repression and protein–protein interactions that contribute to its context-dependent activities *in vivo* (4,5).

The Pax family includes nine members that are important regulators of tissue- and differentiation stage-specific transcription in mammals (reviewed in 6). Pax proteins are also important in lower organisms, where they control development of specific regions of the body plan. Essential roles of these factors in vertebrate development are evidenced by naturally occurring mutations resulting in developmental abnormalities and from studies of mice with targeted deletions of their *pax* genes. For example, the first congenital malformation to be linked to a transcription factor gene was the *undulated* mutation of mice, which results in anomalies of the vertebral

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column and sternum due to a missense substitution in the *pax-1* gene (7). Mutations in the *pax-3* genes of mice (*splotch*) and humans (Waardenburg syndrome type I) result in different phenotypes depending on whether the Pax-3 protein is deleted, truncated or altered by more subtle missense substitutions (8). Mutations in Pax-6 are expressed as the Small eye (*Sey*) mutation in mice or as aniridia or Peter's anomaly in humans (9). Mice with targeted deletions of both Pax-6 genes lack glucagon-producing α cells in the pancreas (10), while mice lacking Pax-4 do not develop insulin-producing β cells or somatostatin-producing δ cells (11). These and other results suggest that Pax proteins serve as 'master regulators' of cell fates *in vivo*. Perhaps the most striking demonstration of this function is the observation that Pax-6 and its *Drosophila* ortholog, Eyeless, are required for eye development in mice, humans and flies (12). Strikingly, ectopic expression of Pax-6 in *Drosophila* generates compound eyes from tissues as diverse as wings, legs and antennae, suggesting that it can direct cells of these tissues to adopt new fates (13).

Pax-5 and its orthologs may also direct cell fates during development. In the hematopoietic systems of vertebrates, Pax-5 specifies B lymphocyte lineage commitment over other cell fates during early cell differentiation (14,15). Pax-5 is required for the generation of normal antibody-producing B cells (16,17). In its absence, B cell progenitors do not progress past an early developmental stage but can become different types of hematopoietic cells under the influence of lineage-directing cytokines. These observations suggest that Pax-5 has two roles *in vivo*: activation of a program of lineage-specific genes and repression of 'promiscuous' gene expression, including receptors for cytokines that promote alternative cell fates. In *Caenorhabditis elegans*, the Pax-5-related (75.8% amino acid identity between their paired domains) EGL-38 protein specifies the fate of cells of the hindgut, hermaphrodite egg laying system and spicules of the male tail and is required for viability at hatching (18,19). In the absence of EGL-38, anterior uterine cells and rectal epithelial cells fail to become different from their neighbors, suggesting that EGL-38 is required for specific patterning events during *C.elegans* development.

As a model system for the control of tissue-specific transcription in lymphocytes, we have studied regulation of the early B lymphocyte-specific *mb-1* promoter by Pax-5. Similar to the *pax-5* gene, the *mb-1* gene is expressed at early stages of B cell differentiation, but is shut off in terminally differentiated plasma cells (20). Pax-5 binds the *mb-1* promoter by itself *in vitro*, but also assembles ternary complexes with Ets proto-oncogene proteins (21), a family of 45 known transcriptional regulators that bind the core sequence GGA through a conserved winged helix motif, the ETS domain (22). Similar to Pax proteins, Ets proteins are important regulators of tissue-specific transcription and cell differentiation. Binding sites for Pax-5 and Ets proteins are required for *mb-1* promoter function in transfected cells and data suggest that factors bound to these sites functionally synergize to increase transcription *in vivo* (21). In support of these data, *mb-1* gene transcription is greatly reduced in Pax-5-deficient mice (23).

Our previous studies identified the DNA-binding domains of Pax-5 and Ets-1 as sufficient for mediating ternary complex assembly, but did not reveal the identities of the amino acids that mediate protein–protein contacts (21,24). For this report, we determined specific amino acid requirements for DNA recognition and interactions between Pax and Ets proteins *in vitro*. Strikingly, a *C.elegans* Pax protein, EGL-38, recruits Ets proteins from mice or worms to bind DNA. Sequences required for Pax–Ets interactions are highly conserved between worms and humans, suggesting that Pax–Ets interactions are an evolutionarily conserved mechanism that regulates transcription in diverse animal species.

MATERIALS AND METHODS

Plasmids and *in vitro* mutagenesis

Mutagenesis of plasmid DNA sequences was performed as described (24). All plasmids were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA).

hSAP1a plasmids. Plasmids for generating synthetic RNA for *in vitro* translation of human SAP1a were made as follows. The wild-type SAP1a DNA-binding domain (DBD) plasmid (BSSAP1a DBD) was constructed by PCR amplification of pKOZ-hSAP1a (21) using primers 5′-ACCATGGACAGT-GCTATCACCCTGTGG (hSAP1a DBD sense) and 5′-CTAC-TACTATTAAATCTCTGGATAAGAGAC (hSAP1a DBD antisense). Fragments were ligated into the *Ecl*136II site of Bluescript KS+. The plasmid for expression of hSAP1a DBD with the V68D mutation was similarly prepared using the previously reported V68D template (21). For each of the other SAP1a mutants, mutated SAP1a sequences were prepared by PCR amplification using the BSSAP1a DBD as template, followed by joining of overlapping fragments to complete the mutated sequence for ligation into Bluescript KS+. Two fragments were generated for each mutation using the hSAP1a DBD sense primer and a mutated antisense primer or the Bluescript polylinker antisense primer 5′-GGTCGACGGTATC-GATAAGC and a mutated antisense primer. Mutated antisense and sense primers used were: Q37D, 5′-CTGCGTCCA-AAAGCTTAAACTGCCCAT and 5′-TTTGGACGCAGAA-GAGGTGGCTCGTCTC; V68E, 5′-TCTTCTCATAATAG-TATCTGAGGGCT and 5′-TATTATGAGAAGAATATCA-TCAAAAAAGT; V68K, 5′-TCTTCTTATAATAGTATCTG-AGGGCTCGG and 5′-TATTATAAGAAGAATATCATCA-AAAAAGT; V68Q, 5′-TCTTCTGATAATAGTATCTGAG-GGCTC and 5′-ACTATTATCAGAAGAATATCATCAAA-AAAGT; V68R, 5′-TCTTTCGATAATAGTATCTGAGGG-CTC and 5′-TATTATCGAAAGAATATCATCAAAAAAGT. Gel purified 5′- and 3′-fragments were combined and amplified using the hSAP1a DBD sense and Bluescript polylinker antisense primers. To make the SAP1a DBD K17E/P18Q/V68D mutant (3× Mut), a 5′-fragment was generated by amplifying the template pKOZ-hSAP1a (K17E/P18Q) with the hSAP1a DBD sense primer and 5′-TTCTTGTCATAATAGTATCT-GAGG (V68D antisense) and a 3′-fragment by amplifying pKOZ-hSAP1a (V68D) with the hSAP1a DBD antisense and 5′-TTATGACAAGAATATCATCAAAAAAGTG (V68D sense) primers.

Ets-1 plasmids. Plasmids were constructed in two steps. First, plasmids containing amino acids 333–420 were generated. Plasmid BSEts-1(333–420)wt was constructed by PCR

amplification of pSKc-Ets-1.6 (25) using primers 5′-ACCATGGGGCCGATCCAGCTGTGGCAG (Ets-1 DBD sense) and 5′-CTACTAGCTCTGCAGGTCGCACACAAA (Ets-1 DBD antisense) for ligation into the blunted (Klenow fragment) *Xho*I and *Ecl*136II sites of Bluescript KS+. Ets-1 mutants were prepared using BSEts-1(333–420)wt as template. For each mutation, the first primer of the following pairs was used together with the Ets-1 DBD sense primer and the second together with the Bluescript polylinker antisense primer to generate two fragments. Mutations and primers were: T346Q, 5′-CCTATCCTGGAGTAATTCCAGAAGAAA and 5′-TTACTCCAGGATAAGTCTTGTCAGTCC; K388T, 5′-TCAACGTTTCATAATTCATCTTAGGTT and 5′-ATGAAACGTTGAGCCGTGGCCTTCGCTA; R391K, 5′-GGCCTTTGCTCAGTTTCTCATAATTCATC and 5′-AGCAAAGGCCTTCGCTACTATTATGAC; R394K, 5′-ATAGTACTTAAGGCCACGGCTCAGTTT and 5′-GGCCTTAAGTACTATTATGACAAAAATATCA; Y395F, 5′-AGAAGCTTAGGCCACGGCTCAGTTT and 5′-CCTCTCCGCTTCTATTATGACAAAAATATCATCC; Y395N, 5′-TAGTTTCGAAGGCCACGGCTCAGTTT and 5′-CCTTCGAAACTATTATGACAAAAATATCATCC; Y395R, 5′-TACCTTCGAAGGCCACGGCTCAGTTT and 5′-CCTTCGAAGGTATTATGACAAAAATATCATCC; Y395V, 5′-TAGACGCGTAGGCCACGGCTCAGTTT and 5′-CCTACGCGTCTATTATGACAAAAATATCATCC; D398E, 5′-ATTTTTCTCATAATAGTAGCGAAGGCC and 5′-TATTATGAGAAAAATATCATCCACAAG; D398K, 5′-ATTTTTCTTATAATAGTAGCGAAGGCC (c-Ets-1 D398K, antisense) and 5′-TATTATAAGAAAAATATCATCCACAAG; D398Q, 5′-ATTTTCTGATAATAGTAGCGAAGGCC and 5′-TATTATCAGAAAAATATCATCCACAAG; D398R, 5′-ATTTTTTCGATAATAGTAGCGAAGGCC and 5′-TATTATCGAAAAAATATCATCCACAAG; D398V, 5′-ATTTTTGACATAATAGTAGCGAAGGCC and 5′-TATTATGTCAAAAATATCATCCACAAG; K399A, 5′-GTGGATGATATTAGCGTCATAATAGTAGCGAAGGCC and 5′-TACTATTATGACGCTAATATCATCCACAAGACGGCG. Amplified fragments were gel purified and combined in subsequent PCR reactions using the Ets-1 DBD sense and Bluescript polylinker antisense primers. Fragments were ligated into the blunted (Klenow fragment) *Xho*I/136II site of Bluescript KS+. To increase the coding sequences of each plasmid to include amino acids 333–440 of Ets-1, the *Acc*I fragment of pSKc-Ets-1.6 was inserted into each plasmid following digestion with *Acc*I.

Pax-5 plasmids. Plasmid pET-Pax-5(1–149) for expression of the human Pax-5 DBD was described previously (24). Pax-5 mutants were prepared using pET-Pax-5(1–149) as template. For each mutation, the first primer of the following pairs was used together with the Pax-5 sense primer 5′-TGGATTTAGAGAAAAATTATCCG and the second primer was used together with the Pax-5(1–149) antisense primer 5′-GGCGGCAAGCTTATTGGTTGGGTGGCTGCT to generate two fragments. Mutations and primers were: G19R,- 5′-AAGCTGATTCACACGTCCATGTCCTGTCCTGCTGGT and 5′-ACAGGACATGGACGTGTGAATCAGCTTGGGGGGGTT; N21A, 5′-CCCCCCAAGCTGAGCAACTCCTCCATGTCCTGTCCT and 5′-CATGGAGGAGTTGCTCAGCTTGGGGGGGTTTTTGTG; Q22A, 5′-CCCAAGAGCATTCACTCCTCCATGTCCTG and 5′-GTGAATGCTCTTGGGGGGGTTTTTGTGAA; G24R, 5′-CACAAAAACCCCACGAAGCTGATTCACTCCTCCATG and 5′-GTGAATCAGCTTCGTGGGGTTTTTGTGAATGGACGG; F27A, 5′-CCGTCCATTCACAGCAACCCCCCCAAGCTGATTCAC and 5′-CTTGGGGGGGTTGCTGTGAATGGACGGCCACTCCCG; N29A, 5′-GAGTGGCCGTCCAGCCACAAAAACCCCCCCAAGCTG and 5′-GGGGTTTTTGTGGCTGGACGGCCACTCCCGGATGTA (1–149); G30S, 5′-CGGGAGTGGCCGGGAATTCACAAAAACCCCCCCAAG and 5′-GTTTTTGTGAATTCCCGGCCACTCCCGGATGTAGTC; R31A, 5′-ATCCGGGAGTGGAGCTCCATTCACAAAAACCCCCCC and 5′-TTTGTGAATGGAGCTCCACTCCCGGATGTAGTCCGC. Amplified fragments were gel purified and combined in subsequent PCR reactions using Pax-5 sense and Pax-5(1–149) antisense primers described above. Resulting fragments were ligated into the blunted (Klenow fragment) *Nde*I site of pET-11a. All constructs were sequenced as described above.

EGL-38 plasmids. The plasmid pET-EGL-38wt for expression of EGL-38(22–156) was constructed by PCR amplification of PRD17/ex787(A) phage DNA (26) using primers 5′-TGCATCACCACCACCATCATGATCCCCTACTGTCAGATGGC (EGL-38 sense) and 5′-CTATGACTTATTTCGTACAATTCGATTAAT (EGL-38 DBD antisense) for ligation into the blunted (Klenow fragment) *Nde*I site of pET-11a. Plasmid pET-EGL-38(Q35A) was created using PCR mutagenesis as described above and pET-EGL-38wt as template. Two fragments were generated using primer pairs EGL-38 sense and 5′-TACACCGCCCAAAGCATTGACGCCCGTGTGGGAGCC and 5′-ACGGGCGTCAATGCTTTGGGCGGTGTATTTGTCAAC and EGL-38 DBD antisense. Amplified fragments were gel purified and combined in subsequent PCR reactions using the EGL-38 sense and EGL-38 DBD antisense primers described above. Resulting fragments were gel purified and cloned into the blunted (Klenow fragment) *Nde*I site of pET-11a. Both constructs were sequenced as described above.

Caenorhabditis elegans Ets proteins. Phages yk418d10 and yk505d9 were kindly provided by Dr Yuji Kohara (National Institute of Genetics, Mishima, Japan). Plasmids for *in vitro* expression of LIN-1 and T08H4.3 proteins from *C.elegans* were constructed using PCR. For expression of the LIN-1 DBD, BSLIN-1 was made by amplifying phage yk418d10 DNA (using primers 5′-ACCATGGACTCAATCATAACCCTGTGGC and 5′-CTAGGCGTCAGTAGTTACAAAGCGATA) for ligation into the blunted (Klenow fragment) *Ecl*136II and *Hin*dIII sites of pBluescript KS+. For expression of the T08H4.3 DBD, BST08H4.3 was made by PCR amplifying yk505d9 phage DNA using primers 5′-CTCACCATGGGCAGTGGACAGACTCAGCTCTGGC and 5′-CTACATCGCATGAGAATTAAAGTAATCCTG for ligation into the blunted (Klenow fragment) *Ecl*136II and *Xho*I sites of pBluescript KS+. Both constructs were sequenced as described above.

Production of recombinant proteins and electrophoretic mobility shift assays (EMSA)

For *in vitro* translation of recombinant proteins, plasmids for expression of the SAP1a, Ets-1, LIN-1 or T08H4.3 DBD were linearized using *Asp*718. Templates were transcribed using T7 RNA polymerase (Promega Life Sciences, Madison, WI) and

synthetic RNA transcripts were purified using QiaTip20 columns (Qiagen, Chatsworth, CA) essentially as previously described (27). For translation of SAP1a DBDs, 1 µg synthetic RNA was incubated in a 50 µl translation reaction with wheat-germ extract (Promega Life Sciences) for 2 h at 25°C. For translation of Ets-1, LIN-1 and T08H4.3 DBDs, 1 µg synthetic RNA was incubated in a 25 µl translation reaction with nuclease-treated rabbit reticulocyte lysate (Promega Life Sciences) as suggested by the manufacturer for 2 h at 30°C. All translations were initiated by addition of 40 µM unlabeled methionine and cysteine. For reference translation reactions, 1 µl Pro-mix *in vitro* cell labeling mix with L-[^{35}S]methionine and L-[^{35}S]cysteine (>1000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) was included for analysis of protein synthesis using 18% SDS–PAGE. Quantitation of labeled proteins was performed using a Molecular Dynamics STORM 860 PhosphorImager system (Amersham Pharmacia Biotech). For binding assays, unlabeled translation reactions were diluted to normalize protein concentrations relative to detection of ^{35}S-labeled proteins.

Pax-5(1–149) and EGL-38 proteins were overexpressed in *Escherichia coli* BL21(λDE3)pLysS (Novagen, Milwaukee, WI) harboring plasmid dnaY (28). Single colonies were picked from plates of freshly transformed bacteria and inoculated into 50 ml cultures of Luria broth supplemented with carbenicillin P (500 µg/ml), chloramphenicol (34 µg/ml) and kanamycin (50 µg/ml). Bacteria were cultured at 37°C to an $OD_{600\ nm}$ of 0.4 and induced by culturing for 2 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were harvested by centrifugation and resuspended in ice-cold buffer Z (25 mM HEPES pH 7.0, 100 mM KCl, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). Bacterial suspensions were sonicated for 2 × 10 s and centrifuged for 30 min in a Sorvall SS34 rotor at 15 000 r.p.m. to remove bacterial debris. Protein concentrations of supernatants were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Relative expression of specific proteins was determined by analysis of 5 µg total bacterial protein using 18% SDS–PAGE, staining with Coomassie Brilliant Blue and comparing concentrations using a Nucleovision Imaging Workstation (Nucleotech, San Carlos, CA).

Preparation of DNA probes, nucleotide sequences and conditions for EMSA were described previously (24). DNA binding assays were quantitated using a Molecular Dynamics STORM 860 PhosphorImager system (Amersham Pharmacia Biotech).

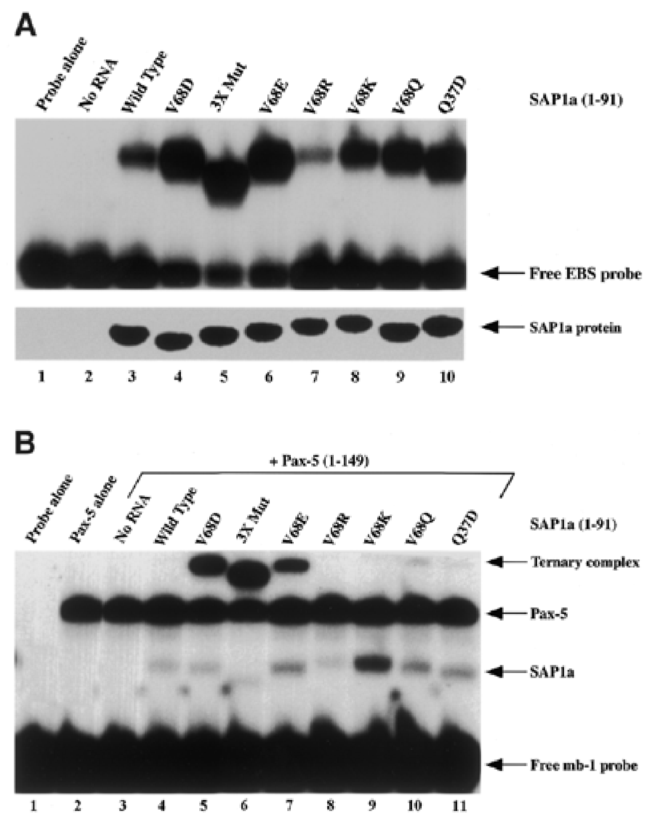

Figure 1. DNA binding by wild-type and mutated SAP1a ETS domain proteins in the absence or presence of Pax-5. (**A**) Binding of SAP1a(1–91) polypeptides to the optimized Ets-1 binding site (EBS) probe sequence (5′-CCGGAAG). (Top) EMSA of SAP1a binding to the EBS probe. Synthetic RNAs were translated in wheatgerm extracts *in vitro*. An aliquot of 1 µl of each programmed extract was incubated with ^{32}P-labeled EBS probe for EMSA as described (24). 3X Mut, K17E/P18Q/V68D. (Bottom) An aliquot of 5 µl of each ^{35}S-labeled translation reaction was fractionated by 18% SDS–PAGE, dried and quantitated on a Molecular Dynamics PhosphorImager. (**B**) Assembly of Pax-5–SAP1a ternary complexes. *In vitro* translated SAP1a ETS domain proteins were incubated with the Pax-5 paired domain (1–149) as shown for analysis of binding to ^{32}P-labeled mb-1 probe. No RNA, unprogrammed wheatgerm extract. Bands representing SAP1a alone, Pax-5 alone or Pax-5–SAP1a ternary complexes are indicated on the right.

RESULTS

ETS domain amino acids required for DNA binding and recruitment by Pax-5

We previously showed that recruitment of Ets proteins by Pax-5 is facilitated by a conserved aspartic acid residue near the DNA recognition α-helix (α3) of the ETS DNA-binding domain (21). Multiple Ets proteins that possess this residue at the equivalent position, including Ets-1, Elk-1, Net, Fli-1 and GABPα, are efficiently recruited by Pax-5 to bind the *mb-1* promoter *in vitro*. In contrast, SAP1a has a valine at the equivalent position (Val68) and is not recruited by Pax-5. When Val68 is mutated to aspartic acid, recruitment of SAP1a by Pax-5 to bind the *mb-1* promoter is increased nearly 100-fold. A survey of amino acid sequences of ETS domains showed that, in addition to aspartic acid or valine, the amino acid at this position can be glutamic acid (e.g. ER81, ERM and LIN-1), arginine (ER71 and Yan), lysine (PU.1, Spi-B, Ets-4, TEL and Elf-3/ESE-1/ESX/ERT/jen) or glutamine (PEA3, E74, NERF and Elf-1). To identify other potential Ets partners and further characterize requirements for their recruitment by Pax-5, we substituted aspartic acid, glutamic acid, arginine, lysine or glutamine for Val68 in the ETS domain of SAP1a (amino acids 1–91) and examined the abilities of the mutated proteins to bind DNA in the presence or absence of Pax-5 (amino acids 1–149).

We first determined whether amino acid substitutions affect Pax-5-independent DNA binding by SAP1a (Fig. 1A). Synthesis of recombinant SAP1a proteins in wheatgerm

extracts was confirmed by analysis using SDS–PAGE (Fig. 1A, bottom). For EMSA analysis, we examined binding of equivalent amounts of SAP1a proteins to a probe including an optimized binding site for Ets-1 (EBS probe, 5′-CCGGAA) that is also recognized by SAP1a. Binding products were fractionated on a non-denaturing polyacrylamide gel for visualization and quantitation of binding using a PhosphorImager. To test the effects of acidic amino acids in place of Val68, we mutated this residue to either aspartic acid or glutamic acid. Because we previously noted that SAP1a K17E/P18Q bound DNA more efficiently in the absence of Pax-5 relative to wild-type SAP1a (21), we tested these mutations together with the V68D mutation (SAP1a K17E/P18Q is not recruited by Pax-5). Mutations V68D (lane 4), K17E/P18Q/V68D (lane 5) and V68E (lane 6) each increased binding to the optimized EBS probe by 4- to 5-fold relative to wild-type SAP1a. These results confirm that Val68 participates in an autoregulatory mechanism that inhibits DNA binding by wild-type SAP1a, possibly through intramolecular interactions within the DBD (29,30). Mutation of Val68 to other amino acids produced mixed effects. Arginine inhibited binding to half that of wild-type (Fig. 1A, lane 7), while a lysine at the same position increased binding 2-fold (lane 8). Substitution of glutamine enhanced EBS binding to an intermediate level.

We next determined the abilities of mutated SAP1a DNA-binding domain polypeptides to be recruited by Pax-5 (Fig. 1B). Binding of the ternary complex sites of the *mb-1* promoter sequence (mb-1 probe) by Pax-5 alone is detected as a single band in the absence (lane 2) or presence of unprogrammed wheatgerm extract (lane 3). Addition of extract programmed with RNA for wild-type SAP1a (lane 4) results in weak binding by SAP1a itself, as detected by a faster migrating band, but does not result in detectable ternary complexes. In contrast, addition of *in vitro* translated SAP1a V68D results in a greater than 100-fold increase in assembly of ternary complexes with Pax-5 (lane 5). SAP1a K17E/P18Q/V68D was recruited 3-fold more efficiently by Pax-5 relative to SAP1a V68D (lane 6). Mutation of Val68 to glutamic acid also resulted in enhanced recruitment by Pax-5, but at half to one-third the level observed with aspartic acid at this position (lane 7). Of the other amino acids tested, only the V68Q mutation detectably increased recruitment of SAP1a (~3-fold relative to background). We conclude that an acidic amino acid at this position is an essential component of ETS domain interactions with Pax-5.

Recruitment of mutated SAP1a polypeptides suggests that an acidic amino acid in the region following the α3 helix participates in protein–protein interactions. However, mutations in V68D and V68E proteins each increased binding to the optimized EBS in the absence of Pax-5. To determine whether the increase in DNA binding is solely responsible for enhanced recruitment, we mutated Gln37 of SAP1a to aspartic acid. Similar to the V68D mutation, DNA binding of SAP1a is increased by the Q37D mutation, potentially by disrupting an intramolecular interaction involving these two amino acids (29). SAP1a Q37D protein bound the optimized EBS as efficiently as the V68D mutant protein (Fig. 1A, lane 10), but was not detectably recruited by Pax-5 (Fig. 1B, lane 11). These data show that recruitment by Pax-5 is not simply a function of enhancing the DNA-binding ability of the ETS domain, but involves a specific role for an acidic amino acid following the DNA recognition α-helix.

To further address requirements for ternary complex assembly we mutated amino acids in the context of the ETS domain of Ets-1 (amino acids 333–440), which is efficiently recruited by Pax-5(1–149). Recombinant Ets-1 polypeptides were synthesized at similar levels in reticulocyte lysates (Fig. 2A, bottom) for analysis using EMSA. Similar to SAP1a, we first examined binding of wild-type and mutated Ets-1 ETS domains to the EBS probe. Introduction of the T346Q mutation in Ets-1, which changes the residue to that in SAP1a, did not affect binding to the optimized EBS probe (lane 4). K388T, a mutation that changes the nucleotide sequence specificity of Ets-1 to that of the Ets proteins Elf-1 and E74 (binding to 5′-CCGGAT, but not 5′-CCGGAA, is reduced; 31), also did not affect binding to the EBS probe (lane 5). Mutation of arginine residue 391 or 394, which are highly conserved among Ets proteins, to lysine each completely abolished binding to the EBS probe (lanes 6 and 7). Mutation of Tyr395 to other amino acids had variable effects on EBS binding. Mutation to phenylalanine reduced binding to EBS by half, while mutation to asparagine (as found in the Ets proteins PU.1 and Spi-B) reduced binding by >80% (lanes 8 and 9). Mutation of Tyr395 to arginine or valine reduced binding to nearly background levels (9 and 2% of wild-type binding; lanes 10 and 11). Although Asp398 is not predicted to contact DNA, mutations at this position had different effects on DNA binding. Conversion of Asp398 to glutamic acid had no detectable effect on EBS probe binding (lane 12), while other substitutions, including lysine, glutamine, arginine and valine, reduced binding to the EBS by varying degrees (to 21, 43, 27 and 46%, respectively; lanes 13–16). Mutation of the adjacent amino acid Lys399 to alanine reduced EBS binding to one-third of wild-type.

To examine the effects of Ets-1 mutations on recruitment, binding of the mb-1 probe by mutated Ets-1 polypeptides was examined in the presence of Pax-5 paired domain (Fig. 2B). At the concentrations tested, none of the Ets-1 proteins bound the mb-1 probe in the absence of Pax-5 (data not shown). In contrast, the wild-type Ets-1 ETS domain was efficiently recruited by Pax-5 (lane 4). Ets-1 proteins with T346Q or K388T mutations were recruited similarly (lanes 5 and 6). In contrast, R391K and R394K mutant proteins, which did not bind the EBS, were not recruited (lanes 7 and 8), suggesting that these residues are essential for binding both sequences. While binding to the EBS by these proteins was similar, mutation of Asp398 to glutamic acid greatly reduced recruitment by Pax-5 (to 33% of levels with aspartic acid; lane 13). These data suggest that the longer side chain of glutamic acid is not as effective as aspartic acid for precise interactions with Pax-5. Mutation of the aspartic acid to other amino acids did not result in detectable ternary complexes. Interpreting the effects of other mutations, e.g. Y395A and K399A, is more difficult because mutations of these amino acids resulted in decreased binding both to the EBS and in ternary complexes with Pax-5 (lanes 8–11 and 17).

Paired domain amino acids required for DNA binding and recruitment of Ets-1

We previously showed that recruitment of Ets-1 to bind the *mb-1* promoter involves Gln22, an amino acid within the

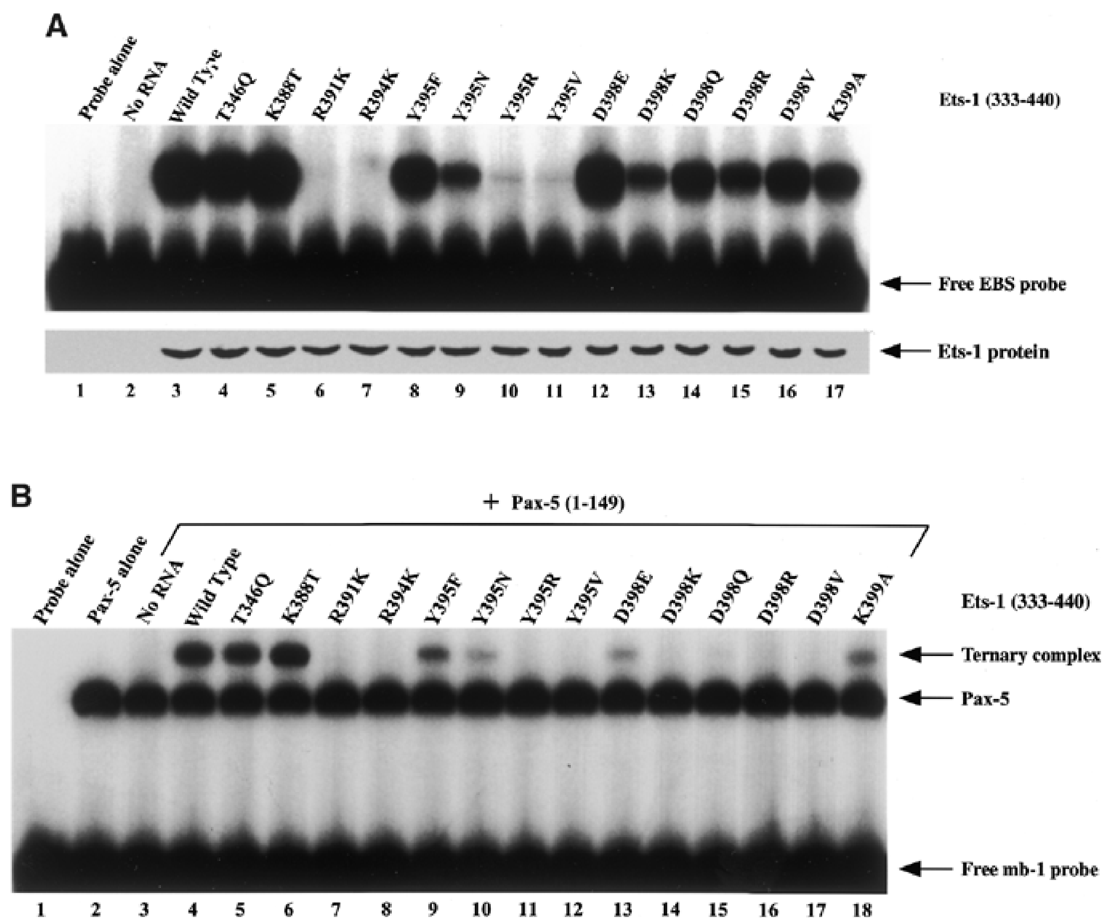

Figure 2. DNA binding by wild-type and mutated Ets-1 ETS domain proteins in the absence or presence of Pax-5. (**A**) Binding of Ets-1(333–440) polypeptides to the optimized EBS probe. (Top) EMSA of Ets-1 binding to the EBS probe. Synthetic RNAs were translated in reticulocyte lystates *in vitro*. An aliquot of 1 µl of each programmed extract was incubated with ^{32}P-labeled EBS probe for EMSA as described (24). (Bottom) An aliquot of 5 µl of each ^{35}S-labeled translation reaction was fractionated by 18% SDS–PAGE, dried and quantitated on a Molecular Dynamics PhosphorImager. (**B**) Assembly of Pax-5–Ets-1 ternary complexes. *In vitro* translated SAP1a ETS domain proteins were incubated with Pax-5 paired domain (1–149) as shown for analysis of binding to ^{32}P-labeled mb-1 probe. No RNA, unprogrammed reticulocyte lysate. Bands representing Pax-5 alone or Pax-5–Ets-1 ternary complexes are indicated on the right.

N-terminal β-hairpin motif of the Pax-5 paired domain (24). Mutation of Gln22 to alanine had little or no effect on binding to the mb-1 probe or to a high affinity site from the murine *CD19* promoter with a significantly different nucleotide sequence (CD19 probe, ACCCATGGTTGAGTGCC, versus mb-1 probe, GGCCAGTGGAGCCCATC; matching bases underlined), but decreased recruitment of Ets-1 to bind the mb-1 probe by 75%. To identify other amino acids involved in Pax-5 DNA binding and Ets recruitment, we mutated amino acids individually within the β-hairpin and adjacent β-turn sequences and examined binding to the mb-1 or CD19 probe. Mutations included two (G24R and G30S) identified previously in *pax* genes of mice with developmental abnormalities. Wild-type (Fig. 3, lane 2) or mutated Pax-5 (amino acids 1–149) was expressed in *E.coli* and equivalent amounts of each polypeptide were tested for binding to the mb-1 or CD19 probes using EMSA. Binding of Pax-5 Q22A was essentially the same as wild-type Pax-5, while mutations G19R and N29A reduced binding to either probe by approximately half (lanes 4, 6 and 9, relative to lane 3). In contrast, mutations N21A, G24R and F27A reduced binding to the two probes by very different degrees. The mutated proteins bound the CD19 probe one-third (N21A and F27A) to one-quarter (G24R) as well as wild-type Pax-5 (lanes 5, 7 and 8), but binding to the mb-1 probe was nearly undetectable (<1% of wild-type). Therefore, amino acids of the β-hairpin are important for binding some, but not all, nucleotide sequences recognized by Pax-5. The G24R mutant is of special interest because this substitution corresponds to a missense mutation in Pax-3 that results in the Splotch^{d} phenotype in mice (32). Selectively reduced binding to the mb-1 probe by Pax-5 R31A was also observed. Mutation of Gly30 to serine, which corresponds to a mutation first reported in the Pax-1 protein in *undulated* mice (7), completely blocked binding to either site. These data support previous observations that the conserved glycines in Paired or Pax-6 proteins are important for DNA recognition (2,3), but also reveal that amino acids of the β-hairpin (e.g. Asn21) participate in highly selective DNA recognition.

We next examined the abilities of mutated Pax-5 polypeptides to recruit Ets-1(333–440) to bind the mb-1 probe (Fig. 4).

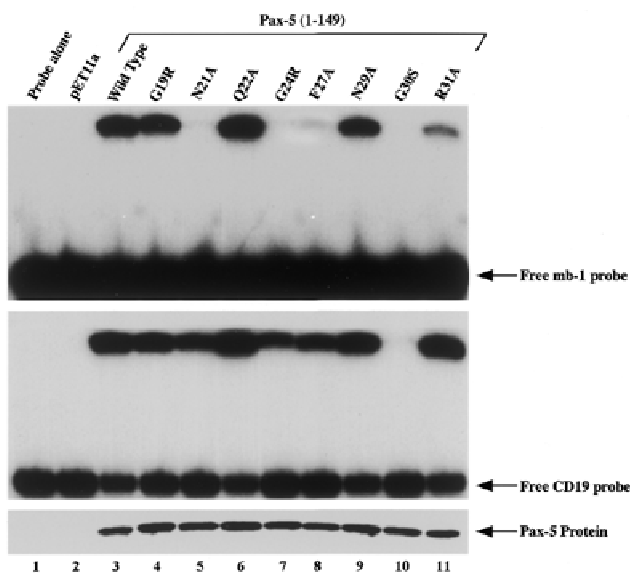

Figure 3. Relative binding of wild-type or mutated Pax-5 paired domain (1–149) polypeptides to mb-1 or CD19 probe DNAs. Proteins were synthesized in *E.coli* as described in Materials and Methods. (Top) EMSA showing binding of Pax-5 to the mb-1 probe. (Center) EMSA showing binding of Pax-5 to the CD19 probe. (Botttom) Relative expression of Pax-5 paired domain proteins in *E.coli* extracts was determined by analysis of 5 µg total bacterial protein using 18% SDS–PAGE, staining with Coomassie Brilliant Blue and comparing concentrations using a Nucleovision Imaging Workstation (Nucleotech, San Carlos, CA).

Relative binding of Ets-1 by itself to the EBS is shown in lane 3. When the same amount of Ets-1 was added to binding reactions with wild-type or mutated Pax-5, the ratio of ternary complex to Pax-5 binding by itself was similar for all mutants except one, Q22A, which showed reduced ability to recruit Ets-1 (reduced to one-fifth that of wild-type Pax-5). Although it is difficult to exclude dual roles for these amino acids in DNA binding and Ets recruitment, we conclude that of the amino acids tested, only Gln22 mediates interactions between Pax-5 and Ets-1.

Ternary complex assembly is an evolutionarily conserved property of Pax and Ets proteins

We previously noted that the glutamine and adjacent residues of the paired domain β-hairpin motif are perfectly conserved among Pax proteins, including those of sea urchins, jellyfish, nematodes and sponges (33–36). As shown in Figure 5A, 97 of the 128 amino acids of the Pax-5 paired domain are identical to those of the *C.elegans* protein EGL-38 (18 others are conservative replacements), which places both proteins in the Pax2/5/8 subfamily. EGL-38 can bind the CD19 probe, suggesting that DNA recognition properties are conserved between the *C.elegans* protein and mammalian Pax-5 (33). To test whether other functional properties of Pax-5, including recruitment of Ets proteins, are conserved in EGL-38, we tested the ability of its paired domain to bind the mb-1 probe and recruit murine Ets-1.

Recombinant EGL-38 paired domain (amino acids 22–156) was expressed in *E.coli* and tested for binding to the mb-1 probe in an EMSA (Fig. 5B). The specificity of EGL-38 DNA binding was demonstrated by incubating the protein with mb-1 probe DNA in the absence (lane 2) or presence of increasing amounts of excess unlabeled competitor oligonucleotides. Although EGL-38 binds the mb-1 probe with somewhat lower affinity relative to Pax-5 (data not shown), specific binding of EGL-38 is evidenced by competition with the wild-type *mb-1*

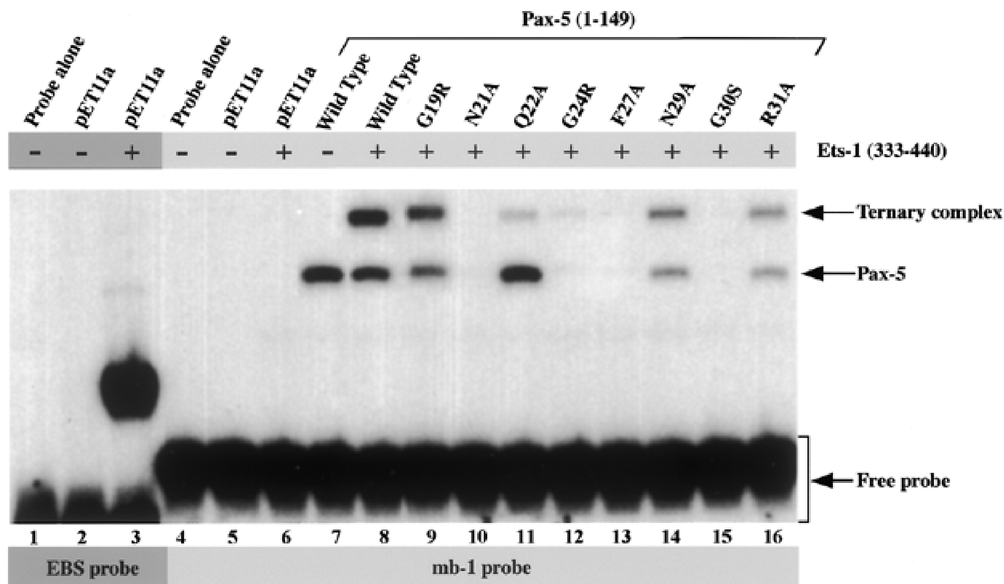

Figure 4. Recruitment of the recombinant Ets-1 ETS domain by wild-type or mutated Pax-5 paired domain proteins. Control bacterial lysate generated from *E.coli* containing empty expression vector (pET11a) or lysates containing bacterially expressed wild-type or mutant Pax-5(1–149) proteins were added to binding reactions as indicated above and analyzed using EMSA. Addition of *in vitro* translated wild-type Ets-1 ETS domain (333–440) is indicated by + or – above. Binding of Ets-1 alone to the EBS probe (lanes 1–3) or with Pax-5 to the mb-1 probe (lanes 4–16) is indicated below. Bands comprising Pax-5 alone or Pax-5–Ets-1 ternary complexes are indicated on the right.

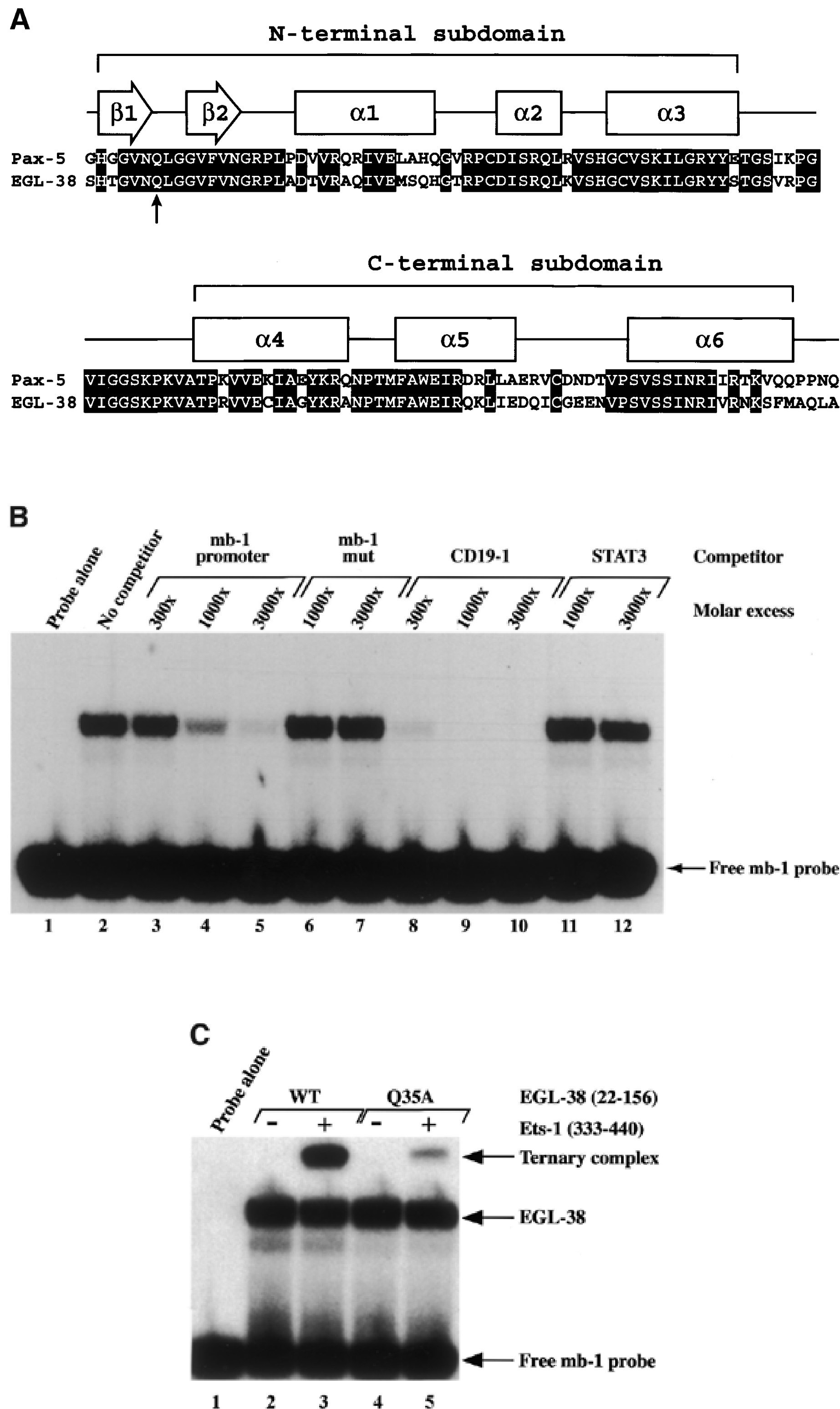

Figure 5. *Caenorhabditis elegans* Pax protein EGL-38 binds *mb-1* promoter DNA specifically and recruits murine Ets-1. (**A**) Alignment of amino acid sequences of Pax-5(16–149) versus EGL-38(29–162). The paired domain of Pax-5 is defined as amino acids 16–143 (1). The predicted secondary structure of human Pax-6 is indicated above for reference (3). Boxes, α-helical regions; arrows, β-sheet. Approximate boundaries of the N- and C-terminal subdomains are highlighted. The filled arrow indicates the position of alignment of Gln22 of Pax-5 and Gln35 of EGL-38. (**B**) Competitive EMSA with the EGL-38 paired domain (22–156). Increasing amounts of double-stranded oligonucleotide competitor DNAs were incubated as indicated above with bacterially expressed EGL-38 and the ^{32}P-labeled mb-1 probe prior to fractionation on a non-denaturing polyacrylamide gel. (**C**) EGL-38 recruits murine Ets-1 to bind the *mb-1* promoter. EMSA was performed using the mb-1 probe with EGL-38 and recombinant murine Ets-1(333–440) as indicated.

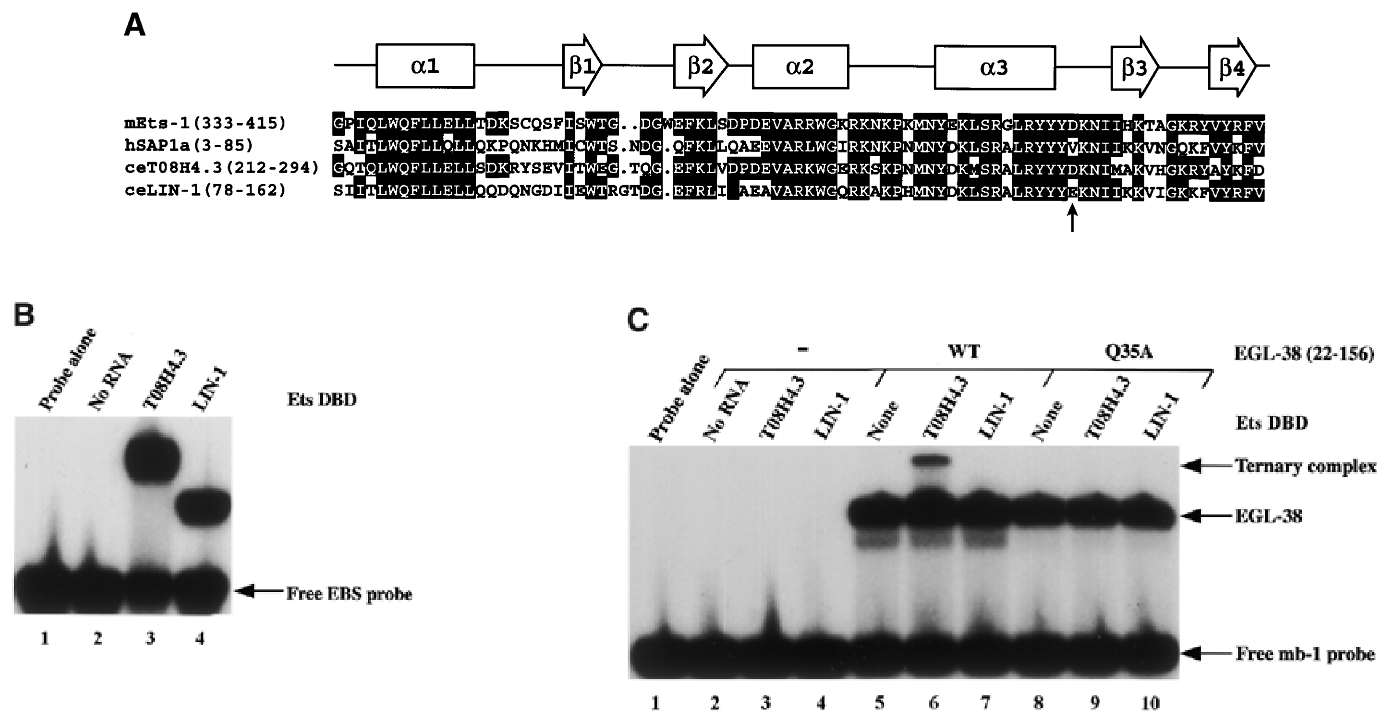

Figure 6. Recruitment of *C.elegans* Ets proteins by EGL-38. (**A**) Alignment of the amino acid sequences of the ETS domains of murine Ets-1, human SAP1a, *C.elegans* LIN-1 and *C.elegans* T08H4.3. Predicted secondary structure of murine Ets-1 is indicated above (22). Boxes, α-helical regions; arrows, β-sheet. The filled arrow indicates alignment of amino acids with Asp398 of Ets-1. (**B**) Binding of *C.elegans* Ets proteins to the EBS probe. EMSA was performed with EBS probe and Ets proteins synthesized in reticulocyte lysates. (**C**) Recruitment of the T08H4.3 ETS domain, but not that of LIN-1, by the EGL-38 paired domain to bind the mb-1 probe. Recombinant proteins were incubated with mb-1 probe as shown for analysis using EMSA. Bands including EGL-38 alone or ternary complexes with Ets proteins are indicated on the right.

promoter sequence (lanes 3 to 5), but not by competitor oligonucleotides with 3 nt substitutions (lanes 6 and 7) that disrupt binding by Pax-5 (21). Binding was inhibited more efficiently (~3-fold) by oligonucleotides comprising the *CD19* promoter site (lanes 8–10). Competition was not observed when STAT3 binding sites were added at 3000-fold molar excess (lanes 11 and 12). Together, these results show that DNA recognition by EGL-38 is very similar to that of Pax-5.

We next examined whether EGL-38 can recruit Ets proteins to bind the mb-1 probe *in vitro* (Fig. 5C). Recombinant EGL-38 bound the mb-1 probe by itself (lane 2) and addition of the ETS domain of murine Ets-1 resulted in slower migrating complexes (lane 3). Therefore, amino acids required for Ets recruitment are conserved in EGL-38. Because mutation of the homologous glutamine in Pax-5 results in greatly decreased (by 80%) recruitment of Ets-1 (24), we mutated Gln35 of EGL-38 to alanine. Like the wild-type protein, EGL-38 Q35A bound the mb-1 probe by itself (lane 4), but showed a greatly reduced ability to recruit Ets-1 (lane 5). We conclude that the ability to recruit Ets proteins is conserved between Pax-5 and EGL-38, suggesting that Pax–Ets interactions may be employed as a regulatory mechanism in *C.elegans*.

Our experiments suggest that EGL-38 has the capacity to interact functionally with Ets proteins *in vivo*, but genetic data are not available to suggest the identities of its Ets partners in worms, or of genes regulated by these proteins. To determine the potential of Ets proteins to interact with EGL-38 *in vivo*, we first examined the sequences of Ets proteins that are expressed in *C.elegans* (37). LIN-1 and T08H4.3 each have acidic amino acids following their α3 helices (Fig. 6A). Overall, the percent amino acid identity between the ETS domains of LIN-1 and Ets-1 and SAP1a is 57.8 and 71.1%, respectively. T08H4.3 shows 66.3% identity with Ets-1 and 56.6% with SAP1a. As a potential difference, LIN-1 and T08H4.3 feature glutamic acid or aspartic acid, respectively, following the α3 helix of their ETS domains (equivalent to Asp398 of Ets-1). To test whether these proteins can be recruited by EGL-38, we expressed their ETS domains in reticulocyte lysates *in vitro*. Similar levels of binding to the optimized EBS probe were detected in an EMSA by each of the recombinant ETS domains (Fig. 6B, lanes 3 and 4). Binding of either ETS domain to the mb-1 probe was not detected in the absence of paired domain proteins (Fig. 6C, lanes 3 and 4). However, the T08H4.3 protein was recruited by EGL-38 to bind the mb-1 probe (lane 6), although less efficiently than murine Ets-1. Recruitment of T08H4.3 by EGL-38 is similar to recruitment of Ets-1 by Pax-5, because mutation of the highly conserved glutamine of the β-hairpin motif (Q35A) blocked ternary complex assembly (lane 9). In contrast to recruitment of T08H4.3, ternary complexes comprising EGL-38 and LIN-1 were not detected (lane 7). This observation is somewhat surprising in the light of the recruitment of mutated SAP1a or Ets-1 with a glutamic acid at the key position. We conclude that Pax–Ets interactions involve the acidic amino acid and other residues that have diverged in LIN-1, which

weakens its ability to be recruited by EGL-38. Therefore, of the two Ets proteins, we conclude that only T08H4.3 is a candidate for functional interactions with EGL-38 on *mb-1* promoter-like sites in *C.elegans*, but interactions between EGL-38 (or other paired domain proteins) and LIN-1 cannot be ruled out in the context of other nucleotide sequences.

DISCUSSION

In this report, we have further characterized the roles of amino acids involved in Pax–Ets ternary complex assembly. Mutation of a valine following the DNA recognition α-helix of SAP1a to an acidic amino acid results in both increased binding to the optimized EBS probe and recruitment by Pax-5. Increased DNA binding by mutated SAP1a proteins does not solely account for their enhanced recruitment by Pax-5. Instead, a key acidic amino acid near the α3 helix is critical for recruitment of Ets-1. With the exception of substitutions for Asp398, mutation of amino acids in or near the DNA recognition α-helix have similar effects on Ets-1 binding to the EBS probe or to the mb-1 probe with Pax-5. Mutations in Pax-5 reveal a role for the β-hairpin and β-turn motifs for sequence-specific DNA binding. Of the mutations tested, only Gln22 is clearly involved in recruitment of Ets proteins. The evolutionary conservation of Pax–Ets ternary complex assembly is demonstrated by recruitment of murine Ets-1 or *C.elegans* T08H4.3 by EGL-38 to bind *mb-1* promoter DNA. Requirements for assembly of these complexes *in vitro* are similar to assembly of mammalian Pax-5–Ets-1 complexes, because a glutamine in the EGL-38 β-hairpin motif is required for ternary complex assembly. Paradoxically, although either aspartic acid or glutamic acid facilitated recruitment of SAP1a or Ets-1 by Pax-5, LIN-1, which has a glutamic acid following the α3 helix, is not recruited by EGL-38. These results suggest that LIN-1 lacks other amino acids required for recruitment of Ets proteins that are present in SAP1a and Ets-1. We conclude that assembly of Pax–Ets complexes is a highly conserved mechanism that exploits combinatorial association to bring together functionally diverse proteins *in vivo*.

Requirements for recruitment of Ets proteins

Assembly of Pax-5–Ets complexes is an informative model system for studying short-range interactions between DNA-binding proteins. Recently, we determined that the apparent equilibrium dissociation constant (K_d) for Pax-5 binding to the promoter with Ets-1 (0.056 nM) is significantly lower than without Ets-1 (0.51 nM) (D.Fitzsimmons and J.Hagman, manuscript in preparation). Other experiments determined an apparent K_d of 110.7 nM for Ets-1 binding the promoter by itself, indicating that Pax-5 enhances Ets-1 DNA binding by >1000-fold. The remarkable increase in binding is due, in part, to the ability of Pax-5 to facilitate Ets-1 binding to the suboptimal site in the *mb-1* promoter, 5′-CCGGAG. The last G of this sequence inhibits binding by Ets-1, but is necessary for binding by Pax-5. Mutation of this G residue to an A reduces Pax-5 binding to the promoter by >95%, but increases binding by Ets-1 in the absence of Pax-5 by >100-fold (21,24). Despite these changes in binding by the individual factors, the mutated site can assemble ternary complexes nearly as well as the wild-type promoter sequence. These data suggest that Pax-5 and Ets-1 bind the promoter in very close proximity to each other and ternary complex assembly likely involves contacts between multiple residues in each protein.

Asp398 of Ets-1 and equivalent acidic residues in other Ets proteins mediate recruitment of the ETS domain by Pax-5. Structural determinations suggest that Asp398 and structurally equivalent amino acids in other ETS domains are oriented away from the DNA for interactions with nearby proteins (30,38–40). In SAP1a, the valine at the same relative position of the ETS domain has also been implicated in protein–protein interactions with serum response factor on serum response elements. In addition, the valine contributes to DNA recognition by interacting with a neighboring tyrosine (Tyr65 in SAP1a, the equivalent of Tyr395 in Ets-1) in the recognition α-helix, thus altering the conformation of the tyrosine and other side chains that contact DNA (29,30). Interestingly, structural studies of the Elk-1 ETS domain show that Asp69 (the equivalent of Asp398) adopts alternative conformations that may influence the positioning of Tyr66 for interaction with DNA (40). Based on these observations, we propose that acidic side chains in ETS domains, such as Asp398 of Ets-1, are available to contact Pax-5 directly through ionic interactions with a basic side chain of the paired domain. Alternatively, the acidic side chain may mediate conformational changes in the ETS domain necessary for binding the suboptimal site of the *mb-1* promoter. Structural studies of Pax-5–Ets-1 interactions are necessary to confirm this hypothesis.

The Pax-5 β-hairpin is required for sequence recognition

Results from our laboratory and others suggest that DNA recognition by Pax-5 involves different sets of amino acids for binding to different nucleotide sequences. This conclusion is supported by the definition of the bipartite structure of the paired domain as the basis for its unusual DNA sequence specificity. Deletions in Pax-5 that remove much of the C-terminal subdomain decrease the ability of the protein to bind some, but not all, sequences bound by the full-length protein (1,24). This property has been used to define sets of degenerate sequences recognized by Pax-5: one set recognized by the complete paired domain and one set that does not require the C-terminal subdomain. DNA recognition is also mediated by other sequences, including the β-turn and linker (2,3).

We confirmed that amino acids of the paired domain β-turn, including Gly30 and Arg31, participate in selective DNA recognition. Surprisingly, the Pax-5 β-hairpin is also important for specific DNA recognition. Mutation of Asn21, Gly24 and Phe27 each have relatively small effects on binding to the CD19 probe, but decrease binding to the mb-1 probe to nearly background levels. In the related Pax-6 structure, the equivalents of Asn21 and Phe27 contact the phosphate backbone. These contacts may be more significant for binding to the mb-1 probe than the CD19 probe but, alternatively, folding of the β-hairpin may be unpredictably disrupted by these mutations. G24R, which corresponds to the Pax-3 protein Splotch^d mutation (32), may disrupt β-hairpin folding and other intramolecular interactions. Together, these 'altered specificity' mutants define roles of the β-hairpin motif for binding to select DNA sequences. Expression of Pax proteins with these mutations in cells may result in decreased expression of a subset of potential target genes *in vivo*.

Our studies confirmed that Gln22 mediates recruitment of Ets-1 by Pax-5 and identified dual roles for the β-hairpin in DNA recognition and protein–protein interactions. However, we did not identify other amino acids involved in ternary complex assembly. It is possible that other amino acids involved in DNA recognition by Pax-5 also have roles in recruiting Ets partners. Structural studies will be necessary to identify amino acids at the interface of the Pax-5–Ets-1 ternary complex.

Evolutionary conservation of Pax–Ets interactions

We previously showed (24) that the ability to recruit Ets-1 to bind the *mb-1* promoter is shared by representatives of three of the mammalian Pax subfamilies: Pax-2 and Pax-5 (Pax-2/5/8 subfamily), Pax-3 (Pax-3/7 subfamily) and, to a lesser degree, Pax-6 (Pax-4/6 subfamily). In the current study we have demonstrated that interactions between Pax and Ets proteins are conserved between humans, mice and nematodes. The DNA binding specificity of the *C.elegans* Pax protein EGL-38 overlaps significantly with that of Pax-5, as it binds specifically to *mb-1* promoter and *CD19* promoter sites *in vitro*. EGL-38 also has conserved amino acids required for protein–protein interactions because it efficiently recruits murine Ets-1 to bind *mb-1* promoter DNA. The ability of EGL-38 to recruit Ets proteins involves a mechanism similar to that employed by Pax-5, because mutation of the glutamine in the β-hairpin greatly diminishes recruitment of Ets-1. The glutamine and adjacent amino acids of the β-hairpin are perfectly conserved in Pax proteins of sponges and coelenterates, including jellyfish, coral and hydra (34,41,42). Indeed, sequences of the β-hairpin are as conserved as sequences of the α3 helix that interact with the major groove of DNA (43). Taken together, these observations suggest that functions of this motif in DNA binding and protein–protein interactions are conserved in Pax proteins of lower organisms as well.

Our data suggest a means of identifying Ets proteins as potential partners of Pax proteins *in vivo*. A survey of sequences of ETS domains for acidic amino acids following the α3 helix suggests that mammalian Ets-1, Ets-2, ERG, Fli-1, FEV, GABPα, Elk-1 and Net/SAP2, which all possess aspartic acid at the key position, and PEA3, ER81 and ERM, which feature a glutamic acid, are potential partners of Pax proteins (22,44). In *Drosophila*, Pointed and ELG both possess aspartic acid following α3. Using these criteria, we were able to predict the identity of a potential partner of EGL-38 in *C.elegans*. Although we detected similar binding of the *C.elegans* Ets proteins LIN-1 and T08H4.3 to the EBS probe, only T08H4.3 was recruited by EGL-38 to bind the mb-1 probe. Overall, the two ETS domains share a high percentage of identical residues with each other and with murine Ets-1. The key aspartic acid present in Ets-1 and other Ets proteins is present in T08H4.3, but is substituted by glutamic acid in LIN-1. Although glutamic acid at the key position can mediate recruitment by Pax-5, we found that aspartic acid mediates recruitment by Pax-5 more efficiently. We also conclude that other amino acids within the ETS domains of these proteins contribute to the recruitment mechanism. Differences in recruitment efficiency may also be due, in part, to subtle differences in the DNA-binding specificities of Ets proteins.

Conservation of the ability to interact *in vitro* suggests that functional interactions *in vivo* between Pax and Ets proteins are similarly conserved. T08H4.3 is a candidate partner of EGL-38. The biological functions of this Ets protein are currently unknown. LIN-1 would appear to be ruled out as a partner for EGL-38, but they may functionally interact on different nucleotide sequences. Another potential Ets partner in *C.elegans* is the Fli-1 homolog C42D8.4, but its expression throughout development in a discrete set of head neurons exclude it as a potential partner of the hindgut-expressed EGL-38 protein (37). Evidence for interactions between EGL-38 and Ets proteins *in vivo* has not yet been obtained and genetic screens are underway to further examine this possibility. As a candidate mutation that may involve these interactions, EGL-38 sy287 is a temperature-sensitive missense mutation of Gly37 to serine that may disrupt protein–protein interactions, DNA binding or both (19). Future studies will address the importance of interactions between EGL-38 and Ets proteins for nematode development and of the roles of related proteins in other developmental systems.

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