The Murine Polycomb-Group Gene eed and Its Human Orthologue: Functional Implications of Evolutionary Conservation

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INTRODUCTION

In the mouse, a considerable number of developmental control genes have been identified by mutational analysis (Reith and Bernstein, 1991; Copp, 1995; Meisler, 1996). One of these genes is encoded by the classical mouse gastrulation locus eed (embryonic ectoderm development), which disrupts anterior–posterior (A-P) patterning of the early primitive streak (Niswander et al., 1988, 1989; Faust et al., 1994; Holderer et al., 1995a). eed was isolated recently by positional cloning and encodes a 441-amino-acid protein with five WD motifs (Schumacher et al., 1996). The protein is 55% identical and 74% similar to Drosophila ESC (Extra Sex Combs). (Gutjahr et al., 1995; Sathe and Harte, 1995a; Simon et al., 1995), and this high degree of evolutionary conservation comprises 83% of the Eed sequence without a single gap or insertion (Schumacher et al., 1996). Remarkably, the amino terminus (residues 1–76) has evolved at a much faster rate than the large WD-motif-containing region and conservation is as low as 24% identity and 41% similarity.

eed is a member of the large Polycomb-group (PcG) of genes and as such is required for maintenance of transcriptional repression of homeotic genes (Struhl and Akam, 1985; Struhl and White, 1985; Gutjahr et al., 1995; Sathe and Harte, 1995a,b; Simon et al., 1995). Evidence is increasing that PcG function is executed by multimeric protein complexes modifying higher order chromatin structures, thereby repressing transcription of homeotic and other putative target genes (Paro and Harte, 1996). Accordingly, loss-of-function mutations in Drosophila PcG genes cause derepression of homeotic genes resulting in transformation of all body segments to an eighth abdominal segment identity (Simon, 1995). Functional analysis of murine homologues of Drosophila PcG genes indicates conservation of transcriptional regulation of homeotic genes across species (Schumacher and Magnuson, 1997; Gould, 1997; van Lohuizen, 1998). For example, conservation of eed function was evident by the finding of highly penetrant, dosage-sensitive posterior ho-
morphic transformations of the axial skeleton in the hypomorphic 17Rn519895B allele (Schumacher et al., 1996). However, eed is unique among murine PcG genes analyzed thus far by being essential during embryonic development (Faust et al., 1994; van der Lugt et al., 1994; Akasaka et al., 1996; Coré et al., 1997; Takihara et al., 1997).

Despite the high degree of conservation, both expression and functional differences are notable between eed and Drosophila esc. During early embryogenesis, esc is uniformly expressed at relatively high levels both maternally and zygotically (Gutjahr et al., 1995; Sathe and Harte, 1995a,b; Simon et al., 1995). After germ band extension, transcription becomes restricted to specific neuroblasts and certain regions in the brain at levels near detection limits (Gutjahr et al., 1995). Drosophila embryos lacking both the maternal and the zygotic esc gene product display transformation of all body segments into an eighth abdominal segment identity and show widespread derepression of homeotic genes (Struhl, 1981). Maternally provided esc alone is sufficient for homozygous mutant embryos to develop into viable adults, and lack of zygotic esc expression confines phenotypic alterations to extra sex combs in the adult fly (Slifer, 1942; Struhl, 1981). eed expression analysis indicated a large maternal mRNA pool in oocytes, and the minute amount of transcripts detected during preimplantation development probably represent remnants of the maternal eed pool (Schumacher et al., 1996). The apparent onset of embryonic eed expression occurs at E5.5 followed by constitutive expression throughout development and in the adult mouse (Schumacher et al., 1996). In agreement with these expression patterns, complex phenotypic alterations throughout embryonic development have been observed in an allelic series of point mutations at the eed locus. The developmental defects ranged from disruption of primitive streak function during gastrulation in null mutant embryos (Faust et al., 1994), to midgestation phenotypes in compound heterozygotes (A. Schumacher and T. Magnuson, unpublished results), and to homeotic transformations in newborns carrying a hypomorphic allele (Schumacher et al., 1996). Furthermore, phenotypic analysis of the 17Rn533545B null allele demonstrated an earlier role of eed during development than was predicted from esc function. esc is required to maintain A-P patterning of genetically determined segmental structures by transcriptional regulation of homeotic genes but does not implement polarity in the unsegmented egg (Struhl, 1981, 1983). In contrast, disruption of primitive streak polarity in homozygous null embryos reflects involvement of eed in pattern formation before segmentation occurs during somitogenesis (Faust et al., 1994).

These findings raise the question of whether the apparent increase in complexity of eed developmental pathways compared with Drosophila esc is based solely on different temporal and spatial expression pattern or whether protein sequence divergence could also be a contributing factor. Although functional differences residing in the highly conserved carboxy-terminal 83% of the Eed sequence cannot be excluded, the divergent amino terminus may account more readily for such properties. Questions regarding the structure and function of the Eed amino terminus have also been raised in recent experiments by Denisenko and Bomsztyk (1997). Based on in vitro translation, initiation of eed mRNA translation was suggested to begin at a putative GTG codon located 282 nucleotides upstream of the previously predicted start methionine (Schumacher et al., 1996).

Here an evolutionary strategy is employed to gain further insight into the potential importance and predicted length of the Eed amino terminus. Given the 80 to 100 million years of evolutionary separation between mouse and human (Novacek, 1992), absence of selective pressure should have resulted in protein sequence divergence of the human eed orthologue. However, stringent functional constraints were found to operate on all residues of Eed by virtue of 100% amino acid identity of the human orthologue. This sequence identity includes the amino terminus as defined by translation initiation at the most 5' ATG. Since it represents the region of greatest divergence between Eed and ESC, complete sequence conservation between divergent mammals is consistent with the hypothesis that some of the additional developmental function(s) of Eed in mammals may reside in this part of the protein. In contrast, significant sequence divergence between Eed and its human orthologue upstream of the most 5' ATG does not substantiate the findings by Denisenko and Bomsztyk (1997) of a putative amino-terminal extension implemented by initiation of eed mRNA translation at an upstream start site.

**MATERIALS AND METHODS**

**Library screening and sequence analysis.** The mouse 1.95-kb full-length eed cDNA was used as a probe to screen approximately 500,000 pfu of an oligo(dT)- and random-primed human adult brain cDNA library constructed in λ phages (Stratagene). Hybridization was performed at 65°C in Church buffer (0.5 M sodium phosphate, pH 7.5, 1 mM EDTA, 7% SDS, 1% BSA). The filters were washed to 0.1% SDS at 65°C. Autoradiograph signals appeared after 1 h exposure is shown here.

The mouse 1.95-kb full-length λ phage clone containing the putative eed cDNA was obtained from the human λ phage collection (Shinya et al., 1997). Northern blot hybridization. A 1.9-kb Eed cDNA probe was hybridized to a Multiple Tissue Northern (Clontech) with 2 μg of poly(A)+ RNA from adult human tissues. Hybridization was done at 65°C in Church buffer, and the blot was finally washed in 0.1× SSC, 0.1% SDS at 65°C. Autoradiograph signals appeared after 1 h exposure, and an 8-h exposure is shown here.
Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) was performed on unstained slides as described before (Sullivan et al., 1996). The 1.9-kb 19S phase probe was labeled with biotin-14–dATP by nick-translation (BioNick Labeling System 18247-015; Gibco BRL); the digoxigenin-labeled INT2 probe was obtained from ONCOR, Inc. The chromosomes were counterstained with DAPI. Twenty metaphases were analyzed for the presence of probes utilized on both the normal and the deleted chromosome. Digital images were captured using a confocal microscope and/or a Zeiss epifluorescence microscope equipped with a cooled CCD camera (Photometrics CH250) controlled by an Apple Macintosh computer. Gray scale source images were captured separately with DAPI, fluorescence, and rhodamine filter sets, merged, and pseudocolored using Gene J on software (Yale University).

WD motif alignment. The Munich Information Centre for Protein Sequences was searched for WD motifs. Cluster 00361 (release 12/97) provides a multiple sequence alignment of WD motifs of which a profile was constructed with PROFILESMAKE which in turn was aligned with the first WD motif of the β-subunit of the bovine G. protein using PROFILEGAP (Devereux et al., 1984; Gribkov et al., 1987). The second WD motif of Eed/EED was aligned against this profile under the same conditions. The bovine protein structure (code 1GOT) was retrieved from the Brookhaven Protein Data Bank.

RESULTS

cDNA Isolation and Sequence Analysis of EED

The human orthologue of eed, designated EED, was isolated by screening an oligo(dt)- and random-primed adult brain cDNA library using the mouse 1.95-kb full-length eed cDNA as a probe. Four overlapping phage clones were obtained containing cDNA inserts between 0.8 and 1.9 kb. The largest insert is deemed to be approximately full length based on Northern blot hybridization (see below). Conceptual translation of the 1.9-kb cDNA demonstrates a 441-amino-acid protein with a relative molecular mass of 50,000 (M, 50K) and a pl of 6.6. The predicted human protein is 100% identical to Eed (Fig. 1), and hence, all putative functional domains are conserved, including five WD motifs (also known as β-transducin repeat, WD-40 repeat, GH-WD repeat) (Neer and Smith, 1996), a bipartite nuclear targeting signal, a PEST sequence, and three GD-WD repeat) (Neer and Smith, 1996). Imporantly, 22% of the fourfold degenerate sites are divergent between eed and EED, indicating that protein identity across species is associated with significant departure at the nucleotide level (Fig. 1).

Nucleotide sequence conservation in the 344-bp 5′ untranslated region of EED is high and amounts to 84% compared with eed (Fig. 2a). Both the mouse and the human 5′ untranslated regions are entirely contained within the first exon, which in turn constitutes part of an approximately 1.7-kb CpG island (Schumacher et al., 1996; A. Schumacher and T. Magnuson, unpublished results). The G+C content in the 5′ untranslated region of eed and EED is 71 and 69%, respectively, and CpG and GpC dinucleotides are equally abundant (Fig. 2a). The ratio of observed over expected CpG (Gardiner-Garden and Frommer, 1987) is 1.01 in mouse and 0.98 in human. In addition to these DNA sequence criteria for CpG islands (Bird, 1986), functional analysis of the murine CpG island using methylation-sensitive restriction enzymes revealed that the rare-cutter sites are indeed unmethylated in genomic DNA (Holdener et al., 1995).

Conceptual translation of the 5′ untranslated region indicates that the reading frame is “open” in a 5′ direction in both eed and its human orthologue (Fig. 2b). To distinguish this region from an amino terminus beginning at the most 5′ methionine, it is herein referred to as the “putative amino-terminal extension.” Database searches and sequence analysis of the putative amino-terminal extension did not reveal any homology with known proteins or motifs. In both mouse and human, an extended amino terminus would contain 28% charged and 38% polar residues (Fig. 2b). The prevalence of hydrophobic residues would be slightly different between mouse (33%) and human (29%), yet enrichment for glycine and serine residues is notable in both species. Thus, the overall physicochemical properties of the putative mouse and human amino-terminal extensions appear similar. However, sequence alignment demonstrates significant amino acid divergence caused by nucleotide gaps and insertions. The 204 nucleotides immediately upstream of the predicted start methionine would constitute a human polypeptide with a single amino acid insertion and two conservative (A → S, S → N) and two neutral substitutions (both S → G) based on the BLOSUM62 substitution matrix (Fig. 2b). More 5′ sequences include a 2-bp deletion at position 142 and four single-basepair deletions at positions 23, 47, 50, and 58 of the human 5′ untranslated region (Fig. 2a). Likewise, the mouse 5′ untranslated region contains a single-basepair deletion at position 141. These deletions cause frameshifts and complete amino acid divergence in these regions of the predicted polypeptides (Fig. 2b). For example, as a result of the 2-bp deletion at position 142 of the human 5′ untranslated region, there are no in-frame GTG and CTG codons upstream of that deletion (Fig. 2a). A valine residue in the human N-terminal extension, which maps 4 residues upstream of the putative valine translation initiation site in the mouse, would be encoded by a GTA codon and can be excluded as a start site. In addition, both the GTG putative translation initiation codon and the weak flanking Kozak sequences are not conserved in EED (Fig. 2a).

Expression Analysis of EED

Northern blot analysis of poly(A)+ RNA comprising eight human adult tissues (pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart) reveals constitutive EED expression (Fig. 3). An approximately 2.2-kb band, consistent with a full-length cDNA when a poly(A) tail of 100 to 200 bp is taken into account, is detected in all lanes. In addition, an approximately 1.7-kb band is found in skeletal muscle, placenta, brain, and heart. Similar mRNA expression patterns in adult tissues have been described for eed.
FIG. 1. Nucleotide and predicted protein sequence alignment of eed and its human orthologue EED. The mouse nucleotide sequence is shown on top aligned with the human sequence below. Dots indicate identical nucleotides between mouse and human; in case of a mismatch, the human nucleotide is indicated. The predicted protein sequence, which is identical between mouse and human, is given at the bottom. The predicted eed/EED protein contains five tandemly repeated WD motifs (stippled background). Residues 22 to 52 encompass a potential PEST sequence (underlined). Residues 65 to 80 define a potential bipartite nuclear targeting signal (dashed underlined). Three potential asparagine-linked glycosylation sites at amino acid positions 157, 283, and 349 are double underlined. Two ENU-induced eed point mutation alleles (Schumacher et al., 1996) are indicated by arrows. The T₁₀₄₀C transition in the l7Rn533SB allele changed a leucine to a proline residue (L196P) in the second WD motif. The T₁₀₃₁A transversion in l7Rn51989SB allele substituted an asparagine residue for an isoleucine (I193N).
Under high-stringency conditions, there are no eed- and EED-related sequences in the mouse and human genome, respectively (A. Schumacher and T. Magnuson, unpublished results), suggesting that the smaller band is derived from alternatively spliced pre-mRNA. In fact, RT-PCR analysis of various mouse tissues demonstrated numerous possible eed mRNA splice variants (A. Schumacher and T. Magnuson, unpublished results).

Chromosomal Mapping of EED

The 1.9-kb EED cDNA was used to isolate a series of overlapping phage clones from a human genomic library (A. Schumacher and T. Magnuson, unpublished results). The 14.8-kb insert of λ9H-1.95 derived from the 5′ end of the transcription unit and was used to map EED to the long arm of chromosome 11 by FISH (Fig. 4). More refined localization was achieved by two-color mapping to metaphase chromosomes from a female with a deletion in 11q [del(11)(q14.2q22.3)] (S. Schwartz, unpublished results). As demonstrated in Fig. 4, wildtype chromosome 11 shows hybridization of both the λ9H-1.95 insert and the INT2 probe, whereas the deletion chromosome hybridized only with INT2. From these results it is predicted that EED is located in a conserved linkage group between Tyrosinase (TYR; 11q14.3) and the Olfactory marker protein (OMP; 11q13.4-q14.1) (see Discussion).
Predicted Locations of eed Point Mutations

The β-subunit of the bovine G protein provides a structural reference for the five WD motifs identified in Eed/EED. The eed null mutation L196P found in the 17Rn53354SB allele (Schumacher et al., 1996) aligns with residue 60 of the first WD motif of the β-subunit of the bovine G protein (Fig. 5a). This residue is internal and maps near the apex of the V-shaped β-propeller blade.
The hypomorphic mutation I193N found in the l7Rn51989SB allele (Schumacher et al., 1996) aligns with cognate residue 57 (Fig. 5a). This position also maps near the apex of the β-blade but on the surface (shown in blue in Fig. 5b). Alignment of the five WD motifs of Eed/EED against this profile is consistent with their folding into β-propeller blades (data not shown), similar to the seven blades of the β-subunit of the G protein (Sondek et al., 1996; Wall et al., 1995) and those proposed for ESC (Ng et al., 1997). Figure 5b illustrates that, if Eed/EED does assume such a β-propeller fold, then the null mutation probably disrupts the core of the propeller. In contrast, the predicted location of the hypomorphic mutation on the protein surface is unlikely to interfere with propeller packing.

**DISCUSSION**

Comparison of murine eed and its human orthologue EED demonstrated 100% identity of the predicted pro-
proteins across 80 to 100 million years of evolutionary separation of the two taxa (Novacek, 1992). Slow accumulation of mutations across the eed transcription unit attributable to the properties of large chromosomal domains such as DNA replication or nucleosome positioning (Boulilakis, 1992) could be excluded, since introns and flanking sequences are diverged (A. Schumacher and T. Magnuson, unpublished results). Further evidence for functional constraints operating at the level of the encoded protein could be inferred from conservation at fourfold degenerate sites, which is only 78%. In contrast, a recent database analysis of 27 identical mouse and human proteins revealed a lower divergence at these sites with conservation between 84 and 98% (Makalowski et al., 1996).

Generally, biological functions residing in the 5′ untranslated regions of genes comprise properties of transcriptional regulation of gene expression. In this light, high evolutionary conservation of the 5′ untranslated region between eed and EED region may be attributed to functional constraints imposed by a CpG island encompassing the 5′ end of the transcription unit. Ubiquitous expression and high GC content in the 5′ untranslated region of both eed (Schumacher et al., 1996) and EED (present study) are in line with presence of a 5′ CpG island in all constitutively expressed genes analyzed thus far (Larsen et al., 1992). Furthermore, retention of the CpG island in EED was expected, since loss of CpG islands between mouse and human seems to occur exclusively in genes with tissue-specific expression (Antequera and Bird, 1993). There is increasing evidence that 5′ CpG islands participate in regulation of gene expression by containing transcription factor binding sites and/or rendering access to such sites through formation of a promiscuous secondary structure (Bird, 1987). Whereas the former typically corresponds to short sequence elements, the latter is likely to comprise long stretches of DNA whose identification may be elucidated by cross-species sequence comparison. For example, a 165-bp GC-rich region with 100% identity between mouse Zfx and human ZFX has been implicated in formation of a functionally important secondary promoter structure (Luoh et al., 1995). Likewise, the highly conserved nucleotide stretches in the 5′ untranslated region of eed and EED, such as an identical 87-bp GC-rich polynucleotide, are probably concerned with promoter function.

Alternatively, eed sequence conservation in the 5′ untranslated region may point to a longer open reading frame and use of an upstream translation initiation signal. Support for this hypothesis comes from eed in vitro translation experiments suggesting utilization of a GTG trinucleotide as a translation start site located 282 nucleotides upstream of the most 5′ ATG (Denisenko and Bomsztyk, 1997). Interestingly, in yeast two-hybrid screens, this putative amino-terminal extension interacted with the heterogeneous nuclear ribonucleoprotein K protein (Denisenko and Bomsztyk, 1997). Conceivably such an interaction should be highly conserved across mammals on the basis of high sequence conservation of the protein domains involved. Conceptual translation of the human EED cDNA, however, revealed significant protein sequence divergence between the putative mouse and human amino-terminal extensions. Whereas the 68 amino acids immediately upstream of the most amino-terminal methionine are 93% identical and 96% similar, the other 26 residues are completely divergent due to a frameshift. Most importantly, however, both the GTG putative start codon and the flanking Kozak sequences are not conserved between mouse and human. Thus, from an evolutionary point of view, it is unlikely that the eed transcription unit encodes a larger protein than initially predicted. Rather, the in vitro translation results by Denisenko and Bomsztyk (1997) arose from the weak Kozak sequence surrounding the most 5′ ATG in eed such that translation initiation is low at that codon in the absence of appropriate regulatory factors in vitro. In addition, the fact that the reading frame is "open" upstream of the most 5′ ATG can be explained by the 5′ untranslated region being part of a CpG island which, by definition, is poor in adenine and thymine nucleotides and, hence, has a low potential for stop codons.

EED has been mapped to human chromosome 11q14.2–q22.3. It is likely that EED is in fact located in 11q14.3 and forms part of a conserved linkage group demarcated by TYR (11q14.3) and OMP (11q13.4–q14.1). This is consistent with findings in the mouse wherein Tyr maps about 1–2 cM proximal to eed (Holdener-Kenny et al., 1992) and about 5 cM proximal to Omp (Brown et al., 1992) on MMU7. To the best of our knowledge, no congenital disorder other than the recessive type I oculocutaneous albinism (OCA1) has been mapped to 11q14.3. Surprisingly, a recent study showed that in the vast majority of cases, OCA1 resulted from point mutations or frameshifts in TYR and only 1 of 250 patients displayed a deletion of the gene (Spritz, 1994). The striking absence of large chromosomal deletions in a homozygous state in clinical samples is most likely due to the proximity of TYR to EED since, similar to the mouse (Niswander et al., 1988, 1989), deletion of both EED alleles should cause early embryonic lethality. Deletion hemizygosity is unlikely to be of clinical significance since TYR is completely dominant, resulting in normal skin pigmentation. Furthermore, mice hemizygous for various large chromosomal deletions encompassing the tyr → Omp linkage group do not display any gross phenotype (Holdener-Kenny et al., 1992). With regard to somatic mutations, frequent loss of heterozygosity at loci in 11q14–q24 in lung cancer (Lizuka et al., 1995) and structural abnormalities of 11q14 in various types of lymphoproliferative disorders (Mitelman et al., 1994) point to the presence of a putative tumor suppressor gene(s) in this large chromosomal region. At present, however, there is no evidence to implicate EED in any of these processes.

Like other WD motif-containing proteins, including ESC (Ng et al., 1997), Eed/EED is likely to adopt a tori-
dal β-propeller structure. β-propeller folds are stabilized by contacts within and between the β-blades (Neer and Smith, 1996; Sondek et al., 1996; Wall et al., 1995), and the extensive evolutionary conservation of ESC is thought to be enforced by the structural requirements of these highly interactive β-blades (Ng et al., 1997). Compelling evidence for this hypothesis comes from the protein identity between Eed and EED, indicating that stringent functional constraints are operating on all residues. This implies that even small sequence alterations may jeopardize protein function, and indeed, missense mutations in null and hypomorphic eed alleles have been identified (Schumacher et al., 1996). Alignment of the five WD motifs of Eed/EED with those of the β-subunit of the G protein maps the L196P eed null mutation to the internal core of the inner end of the β-propeller blade. By virtue of its ability to cause sharp kinks in chain conformation (MacArthur and Thornton, 1991), a proline substitution in this location is prone to disrupt the cooperative nature of the β-propeller fold, which could completely abolish protein function, thereby matching the null phenotype. Experimental evidence for the importance of structural integrity of individual β-propeller blades for protein function has been obtained by deletion analysis of WD proteins from yeast and Drosophila (Williams and Trumbly, 1990; Hu et al., 1994; Ng et al., 1997). In contrast, the I193N eed hypomorphic mutation maps onto the surface of the β-propeller blade near the central cavity. Given the low steric restrictions of asparagine on the polypeptide backbone (Stites and Pranata, 1995), the relative phenotypic refractivity of the hypomorphic mutation is likely to result from a disrupted surface interaction due to alterations in regional iso electrical properties rather than compromised propeller packing.

Sequence comparison between Eed/EED and Drosophila ESC discloses that the amino terminus (residues 1–76) and the WD motif-containing region (residues 77–441) have evolved at different rates (Schumacher et al., 1996). Whereas the compact WD structure is highly conserved (55% identity and 74% similarity), the amino terminus is significantly more divergent, and conservation is as low as 24% identity and 41% similarity. Analysis of ESC homologues in distantly related insect species revealed that the amino terminus is also the region of greatest sequence divergence within those taxa (Sathe and Harte, 1995b; Ng et al., 1997). For example, the housefly (Musca domestica) homologue of Drosophila melanogaster ESC, which has an estimated divergence time of 100 million years, showed only 44% identity in the amino-terminal portion compared with 76% in the WD-motif-containing region (Ng et al., 1997). These data represent yet another line of evidence of stringent functional constraints operating across the entire length of the mammalian Eed/EED protein since the evolutionary distance between fruitfly and housefly matches that of mouse and human. Complete intolerance of the Eed/EED amino terminus toward sequence alterations raises the question of which tertiary structure this region is likely to adopt. As disclosed by crystallography, the amino terminus of the β-subunit of the G protein forms a distinct and solvent-accessible α-helical chain that girds the top of the toroidal body, thereby constituting the binding region with the γ-subunit (Wall et al., 1995; Lambright et al., 1996). Similarly, the Eed/EED amino terminus, which is also unlikely to fold into a β-propeller blade, may extend out of the plane of the toroidal β-propeller structure and form part of a protein–protein interface(s). Thus, it is tantalizing to speculate that Eed/EED acts in a modular fashion such that the compact WD structure at the carboxyl-terminal end implements A-P patterning of segmented axial structures and the amino terminus exerts other function(s) during development, for example, during gastrulation.

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Note added in proof. While this manuscript was under review, another paper describing the EED human sequence was published (Sewalt et al., Molec. Cell. Biol. 18: 3586–3595, 1998).

REFERENCES


