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*rep*E—the *Dictyostelium* homolog of the human xeroderma pigmentosum group E gene is developmentally regulated and contains a leucine zipper motif

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ABSTRACT

We have cloned and characterized the *Dictyostelium discoideum rep*E gene, a homolog of the human xeroderma pigmentosum (XP) group E gene which encodes a UV-damaged DNA binding protein. The *rep*E gene maps to chromosome 4 and it is the first gene identified in *Dictyostelium* that is homologous to those involved in nucleotide excision repair and their related XP diseases in humans. The predicted protein encodes a leucine zipper motif. The *rep*E gene is not expressed by mitotically dividing cells, and *rep*E mRNA is first detected during the aggregation phase of development when the cells have ceased dividing and replicating genomic DNA. The mRNA level plateaus by the time the developing cells have entered multicellular aggregates and remains at the same steady-state level for the remainder of development. In addition, we have demonstrated that the level of mRNA is very low in developing cells. These observations suggest that *rep*E may play a regulatory role in development. The data indicate that potential developmental roles for XP-related genes can be profitably studied in this system.

INTRODUCTION

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease that is marked by extreme sensitivity to UV light and a high predisposition to skin cancer in light-exposed areas of the body (1). Human XP cells are defective in nucleotide excision repair (NER). They are divided into eight complementation groups (A–G and a variant group) which differ from each other in the degree of their repair deficiency. The genes which correspond to the various XP groups represent different genes whose products are involved in the repair process (2). These XP-related genes (named *ERCC*, for excision repair cross-complementing) were identified by their ability to cross-complement a series of UV-sensitive rodent cell lines which were generated by mutagenesis and have been shown to comprise at least 11 complementation groups (2).

Many XP genes have been shown to share considerable homology with the yeast *RAD* genes and with *Drosophila* homologs of the XP genes (3). These homologies with the yeast *RAD* genes provided the initial clues for the repair activity of these genes and imply that the repair mechanism is highly conserved across these species. However, it is clear that significant differences in the repair mechanisms exist between species and each gene has unique additional functions in different organisms.

Of all the identified XP complementation groups, XPE is the least understood with respect to its role within the repair pathway. The NER defect in some XPE cell lines has been correlated with the absence of activity of the UV-DDB (UV-damaged DNA binding) protein. The cognate monkey and human genes have been cloned and sequenced (4–6). A yeast homolog has not been identified and no correlation to an existing *ERCC* gene has been made. The XPE protein has not been identified as part of the repairosome holoenzyme (7). However, recent work in which mammalian NER was reconstituted from purified protein components showed that XPE/UV-DDB plays an accessory role in NER (8).

One perplexing problem about XP patients is that even though their genetic defects are each in single repair genes, they exhibit a wide range of phenotypes, such as predisposition to cancer, stunted growth and neurological and developmental abnormalities (1). In yeast, some of the XP homologs are actually essential for viability (9,10). The underlying reason for this pleiotropy is that these repair genes are involved in multiple processes in addition to NER (11). Such processes involve transcription (7,12–18), DNA replication (19–22) and mitotic (23) and meiotic (24) recombination.

The involvement of NER gene products in transcription and other general processes in the cell begins to explain the wide range of phenotypes that are associated with XP. However, it does not explain why phenotypes which are associated with defects in such fundamental processes are not more severe and global. This consideration raises the possibility that these genes may be affecting specific developmental decisions. Identifying the nature of these affected processes remains one of the major challenges

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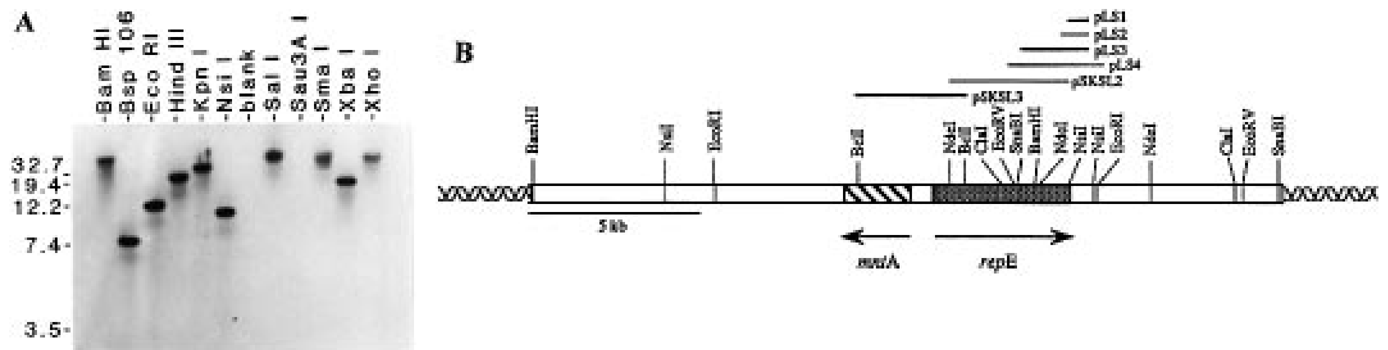

Figure 1. *rep*E exists as a single copy in the genome. (**A**) Southern analysis of genomic DNA from strain Ax3. Aliquots of 10 µg DNA per lane were digested with the various restriction enzymes and separated on a 0.8% agarose gel in TEA buffer. The gel was blotted onto nylon membrane and probed with a PCR-amplified fragment of the *rep*E gene (position 3555–3964 on the genomic sequence, Fig. 3). (**B**) Aliquots of 10 µg Ax3 DNA were digested with a combination of enzymes and separated on 0.8% gels. Each gel was blotted onto two filters, using the sandwich blotting technique and probed with two distinct fragments of the *rep*E clone pLS3: a 0.6 kb *Eco*RV–*Nde*I fragment and a 1 kb *Nde*I–*Nsi*I fragment. These fragments allowed the unambiguous alignments of fragments that resulted from double cuts. Sizes of the fragments were extrapolated from λ molecular weight markers.

of this field (25). Clearly these problems will benefit from studies on model developmental systems.

In this paper we report the identification and isolation of *rep*E, the *Dictyostelium* homolog of the primate *UV-DDB* gene. *rep*E is the first XP- or NER-related gene identified in this developing system. We demonstrate that expression of the *rep*E gene is developmentally regulated. The gene is not expressed in vegetatively growing cells, but *rep*E mRNA accumulates in the early stages of development. The protein has a leucine zipper motif, suggesting a role in gene regulation.

MATERIALS AND METHODS

Library screening

A cDNA library in λZap and a genomic library in pBLUESCRIPT were gifts from Herbert Ennis (Columbia University). The *rep*E gene was serendipitously identified as part of our studies on the discoidin genes of *Dictyostelium*. Two positive overlapping cDNA clones (see Results and Fig. 1B) were identified in an antibody screen of >75 000 phage from the λZap expression library while we were attempting to clone the discoidin II gene, using a specific polyclonal antibody as a probe. The sequence of these clones did not match any of the peptide sequences we had obtained for the discoidin II protein, but did reveal their homology to the primate *UV-DDB/XPE* gene. We have never explained this aberrant antibody reactivity. The antibody reacts with a single band on Western blots and it is possible that the cross-reactivity is at the level of secondary structure. The two cloned cDNA fragments were used to re-screen both the λZap and genomic libraries using standard procedures (26).

Cloning genomic DNA fragments

Genomic DNA cut with *Nde*I or *Bcl*I was size fractionated on a sucrose gradient. The DNA fragments from part of each fraction were separated by electrophoresis and blotted (27). The blots were probed with a 5′ fragment of the *rep*E cDNA clone, to identify the fractions containing the rest of the gene. The DNA from the peak fractions was cloned into the compatible vectors [pT7–7 (28) for *Nde*I and pUC19 for *Bcl*I] and the clones were screened by hybridization. All DNA manipulations were performed according to standard procedures (26). Sequencing was performed using a Sequenase kit from USB and [α-^{32}P]dATP. Routinely, plasmid DNA was purified using a 'Wizard prep' kit (Promega). The *rep*E gene was sequenced in its entirety on both strands.

Strains, developmental time course and RNA preparation

Strains Ax3 and DH1 were used throughout these studies. Ax3 is a derivative of the wild-type strain NC4, which is capable of growth on axenic medium. DH1 is a derivative of Ax3 with a deletion in the *pyr5-6* (UMP synthase) gene, so that it can be used in homologous recombination experiments with selection for uracil prototropy (a gift from R.Insall). For this work, the cells were grown on SM agar plates in association with *Klebsiella aerogenes* as a bacterial food source. When the *Dictyostelium* cultures reached mid log phase ($2–3 \times 10^8$ cells/100 mm plate) they were harvested and the remainder of the bacteria were washed away by differential centrifugation. The cells were plated on buffer-soaked black paper filters for development (10^8 cells/40 mm filter) (29). Development under these conditions is completely synchronous. When the cells reached the desired developmental stage, they were harvested by vortexing the cells off the filter. The cells were then pelleted and rapidly frozen. Total RNA was isolated at each time point as described (30) and used for Northern analyses or reverse transcription–PCR.

Reverse transcription–PCR

First strand cDNA synthesis was performed using a Life Technology (Gibco BRL) Superscript Preamplification system. For each time point, 2 µg of total RNA sample was treated with DNase I in a final volume of 20 µl 50 mM KCl, 20 mM $MgCl_2$. Aliquots of 2.2 µl of the DNase I-treated samples were used for the reverse transcription (RT) reaction, following the instructions of the manufacturer and using an oligonucleotide covering position 3473–3488 (3′→5′, oligo 6) as a primer. Aliquots of 2 µl of each sample were then amplified, using oligo 6 and an oligonucleotide covering position 2711–2726 (5′→3′, oligo 11) as primers. PCR was performed using a Coy thermocycler for 25 cycles under the following conditions: 1 min denaturation at 94°C, 2 min annealing at 40°C and 1 min 45 s elongation at 72°C. Each reaction contained 2 µl 20 µM primers, 10 µl 2.5 mM dNTP

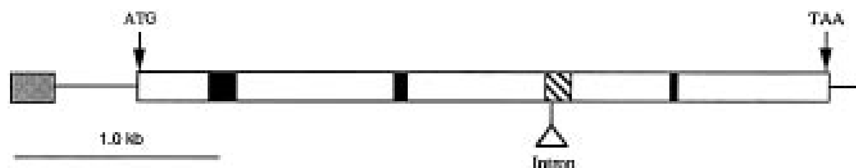

Figure 2. Schematic presentation of the sequence of the *rep*E gene. The 585 bp intergenic region is shown as a single line upstream of the *rep*E ATG initiation codon. The three black boxes are the poly(Asp) regions and the hatched box is the region encoding the leucine zipper. The position of the 92 bp intron is indicated. The stippled box at the left is the mannosyl transferase gene *mnt*A, which runs in the opposite orientation to *rep*E. The entire sequence is available through GenBank, accession no. U50042.

and 1 µl *Taq* polymerase (AmpliTaq; Perkin Elmer) in buffer containing 20 mM Tris–HCl, pH 8.4, 50 mM NaCl and 1.5 mM $MgCl_2$. Results were analyzed on 1.2% agarose gels in TBE.

Intron detection

The entire cloned genomic sequence was examined for introns using a reverse transcription-coupled PCR method as described (31). Briefly, cDNA was made from mRNA by reverse transcription. The cDNA was then amplified using overlapping sets of oligonucleotide primers which covered the entire length of the cloned sequence. A similar series of amplifications was performed on the genomic clone and the size of the equivalent PCR products were compared on agarose gels.

RESULTS

Cloning the *Dictyostelium repE* gene

Two overlapping cDNA clones (Fig. 1B, clones pLS1 and pLS2 of 824 and 845 bp, respectively) were identified in an antibody screen of a λZap cDNA library (see Materials and Methods). The sequence of these clones revealed homology to the primate *UV-DDB/XPE* gene. Keeping with standard *Dictyostelium* gene nomenclature (32), we have named the gene *rep*E (repair E). A subsequent screen of the cDNA library and a genomic library yielded an additional 1.6 kb cDNA clone (pLS3) and a genomic clone (pLS4) that covers 1.78 kb of the coding sequence and >1 kb of sequence 3′ to the *rep*E gene. Further attempts to clone the 5′-end of the *rep*E gene from either of these existing libraries were unsuccessful. Therefore, we established that the *rep*E gene appears as a single copy in the genome (Fig. 1A) and cloned the rest of the gene from a genomic DNA preparation.

We constructed a restriction map of the region surrounding the *rep*E gene using the pLS3 *rep*E clone as a probe (Fig. 1B). A 2.3 kb *Nde*I fragment which extends 1.6 kb from the 5′-end of the previously cloned genomic sequence seemed to be long enough to cover the rest of the gene, based on homology to the primate *UV-DDB* gene. A genomic *Nde*I digest was fractionated on a sucrose gradient and the appropriate size fraction was used to construct a mini-library, from which the 2.3 kb fragment was cloned. Sequence analysis of this fragment (clone pSKSL2) revealed that it was still missing the 5′-end of the gene due to the presence of an intron. We therefore repeated this procedure, cloning an overlapping *Bcl*I fragment (clone pSKSL3). This clone contained the 5′-end of the *rep*E coding sequence, 585 bp of 5′ upstream region and most of the neighboring upstream gene.

Characterization of the *repE* gene

The genomic sequence of the *rep*E gene is 3512 bp long (Fig. 2). There is a single 92 bp intron at position 2568 which was revealed by comparing the genomic and cDNA sequences. The absence of additional introns within the genomic sequence, for which we did not have corresponding cDNA clones, was established by comparing PCR products of the genomic segment to PCR products of RT cDNA from the same region (31).

We have sequenced 585 bp of the 5′ upstream region. This short untranslated region is shared with the neighboring gene, which runs in the opposite direction from *rep*E. This gene is the *Dictyostelium* homolog of the yeast *ALG1* gene, which encodes the mannosyl transferase which catalyzes the formation of dolichol pyrophosphate-GlcNAc$_2$Man in the pathway of *N*-glycosylation (33). We have named this gene *mnt*A.

There are a number of TAATA sequences between –70 and –39 bp upstream of the *rep*E ATG, but the exact position of the TATA box has not been established. In addition, the upstream region contains many features that are characteristic of *Dictyostelium* genes: There are three consecutive stop codons in-frame just upstream of the ATG; two G+C-rich regions and several stretches of poly(dT).

The location of the *rep*E gene within the *Dictyostelium* genome was determined by hybridization to the *Dictyostelium* YAC library (kindly performed by A.Kuspa and W.Loomis; 34,35). It maps towards the end of chromosome 4 between the *tag*B gene, which encodes a multidrug resistance transporter/serine protease (36), and *psd*A, which encodes the 3′,5′-cyclic nucleotide phosphodiesterase (37).

The two *rep*E exons comprise 3420 bp of open reading frame which encodes a predicted protein of 1140 amino acids with a molecular weight of 127.6 kDa. This is identical to the reported molecular weight of 127 kDa for the UV-DDB/XPE protein. Even though the codon usage is typical of *Dictyostelium* (38), it is noteworthy to mention that in most cases where there are two adjacent identical amino acids they are usually encoded by two different codons. The significance of this is not known.

At positions 110–135, 403–423 and 850–857 there are three poly(Asp) regions. We do not know whether these regions separate distinct functional domains. No similar sequences are found in the primate genes (see Discussion).

Homology to XPE-related genes

The predicted protein sequence of *rep*E shares a 40% identity and over 60% similarity with the primate *UV-DDB* gene (Fig. 3). A search of the NCBI non-redundant database, using the BLAST program (39) has not revealed any homology between the

Figure 3. Homology between *rep*E and the primate *UV-DDB* gene. Sequence homology between *rep*E and *UV-DDB* (GenBank accession no. pir/s37614) was established with the Align program of DNASTAR.

sequence of RepE or the primate UV-DDB protein and the previously identified proteins of the NER complex (such as the products of the yeast *RAD* genes or members of the *ERCC* family of genes). As such, the *rep*E and *UV-DDB*/*XPE* genes encode a novel, highly conserved family of proteins. A *Drosophila* homolog of primate UV-DDB (dm127) has recently been cloned. It shares 60% homology with the primate UV-DDB protein and about 40% homology with the RepE protein (M.Takao and M.Protic, personal communication).

Leucine zipper motif

The translated protein sequence of *rep*E contains a region which encodes a leucine zipper motif and an adjacent basic region, for a putative DNA binding site (double line in Fig. 4). This region exhibits a very high degree of sequence conservation between the RepE, the UV-DDB and the *Drosophila* dm127 proteins (64% identity and 82% similarity), as is shown in Figure 4. Moreover, there is a limited sequence homology to the bacterial UvrB protein, which is involved in the recognition step of the NER pathway in prokaryotes. This homology further highlights the functional importance of this sequence in these proteins.

Developmental pattern of expression of *rep*E mRNA

The RepE protein is expressed in the cells at very low levels. A number of attempts to identify the mRNA by conventional Northern analysis, as well as by RNase protection experiments, were unsuccessful. Thus, we prepared *rep*E-specific cDNA from equal amounts of mRNA from time points taken throughout the entire developmental sequence. The cDNAs were amplified using synthetic oligonucleotides as primers and analyzed by electrophoresis (Fig. 5). We were able to show that *rep*E mRNA accumulates in a developmentally controlled manner. It begins to accumulate at 3 h of development, during the onset of aggregation, peaks at 5–7 h of development, when the cells are forming discrete multicellular structures, and stays at a relatively constant level throughout the remainder of development. There is no measurable *rep*E mRNA during vegetative growth on bacteria.

DISCUSSION

The genes encoding the proteins involved in NER play a pivotal role in repairing DNA damaged by UV and chemical agents (3). Indeed, mutations in these genes result in the pathological conditions of XP,

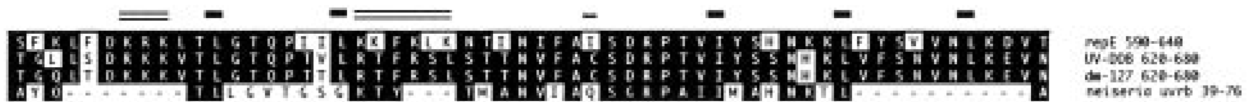

Figure 4. Sequence homology within the leucine zipper region. Comparison of the sequences representing the putative leucine zipper motif of RepE, UV-DDB, *Drosophila* dm127 and UvrB (GenBank accession nos: dm127, D82865; UvrB, U01743) was established using the Megalign program of DNASTAR. Similar sequences (2 units distance) are boxed and shaded. Leu/Ile residues, as well as the basic region, are indicated.

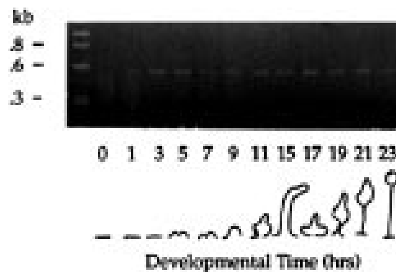

Figure 5. Developmental pattern of *rep*E expression. An aliquot of 2 µg total RNA purified from cells harvested from sequential developmental stages was reverse transcribed for first strand cDNA synthesis and the cDNA was then amplified by PCR as described in Materials and Methods. Samples of 15 µl of each PCR reaction were analyzed on 1.2% agarose gels in TBE buffer and the gels were stained with ethidium bromide and photographed.

Cockayne's syndrome (CS) and trichothiodystropy (TTD). Some of the NER proteins play dual roles as part of the basal transcription complex TFIIH, while others are involved in other fundamental processes in the cell. These interactions begin to shed some light on why individuals with these diseases often have associated developmental and neurological abnormalities. However, it does not explain why these pleiotropic effects are not more severe. It appears that these genes affect specific developmental decisions and that model systems are needed for the study of the role of these gene products in development.

This work presents the identification and characterization of the first XP-related gene in the cellular slime mold *Dictyostelium discoideum*. The organism is ideal for the study of the role of XP- and NER-related genes during development. *Dictyostelium* is a haploid single celled amoebae which grows by mitotic division, feeding on either bacteria or axenic medium. Following exhaustion or removal of the food source, cell division and genomic DNA replication cease and starvation signals the beginning of differentiation. The amoebae aggregate to form cohesive multicellular assemblies each containing 10^5 cells. These aggregates undergo a complex program of development, resulting in spatially localized spores resting on top of a cellular stalk (see illustration in Fig. 5). The synchronized development allows the correlation of biochemical and cellular events to the overall program of morphogenesis.

Our findings show that there is no measurable *rep*E mRNA in vegetatively growing cells or at the onset of development. However, *rep*E mRNA begins to be expressed during the aggregation stage of development, increases to a maximum by the time the cells have entered aggregates and stays at this steady-state level for the remainder of development. The level of *rep*E mRNA is very low even at its maximum during development. The pattern of developmental expression of *rep*E is reminiscent of that reported for the *Drosophila haywire* gene (XPB homolog), where lower levels of mRNA were found in embryos than in the larva and the adult stage (40). Our finding that *rep*E mRNA accumulates during development demonstrates that the expression of the gene is developmentally regulated.

The *rep*E gene is highly homologous to the *UV-DDB*/*XPE* gene of primates. A *Drosophila* homolog has been found but no yeast equivalent has been reported. The *Dictyostelium* gene encodes a protein with a predicted molecular weight of 127 kDa, which is identical to that of the primate UV-DDB protein. The primate *UV-DDB*/*XPE* gene encodes a nuclear protein which preferentially binds to UV-damaged DNA (primarily 6-4 pyrimidine dimers) as well as to DNA damaged by chemical agents (4,41–43). There is now considerable evidence indicating that the UV-DDB protein functions as a two subunit complex of p127 and p48 (5). The activity of the UV-DDB protein is missing in some XPE patients (4,6,44–46). It has been suggested that cells from XPE patients which do not exhibit loss of DNA binding activity of p127 may lack either the other subunit of the complex or have a defect in the domain of the XPE protein which is responsible for the protein–protein interaction, but not the DNA binding domain. Microinjection of *UV-DDB* mRNA (47) or purified DDB protein (48) have been shown to increase unscheduled DNA synthesis in XPE cells. However, no sequence analysis of the gene from a human XPE patient has been done in order to unambiguously correlate an aberrant gene with the XPE phenotype. The identification of *rep*E in *Dictyostelium* may help to shed light on the role of this protein in repair and/or other processes, as it will allow us to generate specific mutations in the gene and analyze the resultant developmental and repair phenotypes.

The predicted *rep*E gene product contains a leucine zipper motif and associated basic region which suggest that it plays a regulatory role and that it may be localized to the nucleus (49,50). There is a 64% identity and 82% similarity around this region between RepE, the primate and *Drosophila* UV-DDB sequences, indicating the functional importance of this region. Moreover, there is a 40% homology to the *Escherichia coli* UvrB protein. The latter protein functions in a complex with UvrA ($UvrA_2B$) to enable binding to damaged DNA (the recognition step in prokaryotic NER). The similarity to this protein is very intriguing. Although it has been shown that eukaryotic NER performs the same functions as the prokaryotic pathway, there is little homology on the primary sequence level between the protein components of the two NER pathways. The homology in the region of the leucine zipper between the RepE, UV-DDB and the prokaryotic UvrB proteins suggests an evolutionary relation between these proteins. The regulatory role of RepE suggested by the presence of the leucine zipper is consistent with our data showing that the level of expression of *rep*E is very low and that its expression is under developmental regulation.

The presence of leucine zippers in proteins is often an indication that the protein functions as a dimer. A recent study

showed, using the yeast two-hybrid system, that the human UV-DDB protein, which they named X-associated protein (XAP-1), interacts with the hepatitis B virus X protein in order to initiate viral multiplication *in vivo* (51). It was hypothesized that the interaction of the X protein with the DNA repair protein may modify cellular transcription processes. This protein–protein interaction demonstrates a regulatory function for the UV-DDB/XPE protein separate from its involvement in DNA repair, similar to many other components of the NER pathway which have dual functions. We are now trying to identify the protein partner of the *repE* gene product in *Dictyostelium*.

The *rep*E gene product has another striking structural feature, three extended stretches of poly(Asp). These poly(Asp) regions are absent in the primate homolog. The function of these regions in the predicted *Dictyostelium* RepE protein is not known and they may be acting merely as spacers between discrete functional domains of the protein. However, they may have specific functional roles in this organism. A search of the GenBank for proteins containing poly(Asp) regions resulted in the identification of numerous proteins whose function involves interaction with DNA. Some of these include: topoisomerase II (52), RNA polymerase I (53) and the RNA polymerase III large subunit (54) from *Plasmodium falciparum*; Suppressor of Zeste from *Drosophila* (55); the yeast DNA binding protein BAF1 (56) and transcription factor ADR6 (57); and the *Dictyostelium* RNA helicase (Mahal,B. and Nellen,W., unpublished, s49262.) and G box binding protein (58). Thus the presence of the poly(Asp) regions in RepE may further indicate that this protein is involved in interaction with DNA. Interestingly, the poly(Asp) regions are reminiscent of the poly(Gln) regions found in the proteins associated with neurodegenerative diseases such as Huntington's disease. In these diseases the length of these poly(Gln) regions increases in succeeding generations and the increase in length appears to be responsible for the phenomenon of genetic anticipation (59). Moreover, there is evidence that these long poly(Gln) regions can form polar zippers with other proteins (60). These newly discovered protein–protein interactions may be responsible for the pathology of these diseases. It is possible that the poly(Asp) regions in the *rep*E gene product may be involved in protein–protein interactions similar to the poly(Gln) polar zippers. The precise roles of the poly(Asp) regions are directly testable because of the ease with which homologous gene replacement can be performed in *Dictyostelium*.

The results presented here show that expression of the *rep*E gene is intimately tied to development and encodes motifs that suggest that the protein has a regulatory role. Efforts are now underway to use this system to elucidate the precise roles of *rep*E and other XP-related genes in morphogenesis and cell differentiation in this organism.

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