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Specific interaction between the *XNP*/*ATR-X* gene product and the SET domain of the human EZH2 protein

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Mutations in the *XNP* gene result in different inherited disorders, including the ATR-X syndrome which is characterized by mental retardation (MR) associated with α-thalaessemia. Amino acid sequence analysis revealed that the XNP protein is a new member of the SNF2-like family, which comprises numerous members involved in a broad range of biological functions: transcriptional regulation, DNA repair and chromosome segregation. Since experiments on fibroblasts from ATR-X patients have provided no evidence for either a DNA repair defect or abnormal chromosome breakage or segregation, it seems more likely that the XNP protein is somehow involved in regulation of gene expression. Recent genetic and biochemical studies have led to the emerging concept that SNF2-like proteins are components of a large protein complex which may exert its functions by modulating chromatin structure. To investigate whether XNP could mediate the activity of gene-specific activators through chromatin remodelling, we performed a yeast two-hybrid analysis using XNP and several human heterochromatin-associated proteins. We found a specific interaction between the XNP and the EZH2 proteins. In light of these observations, we discuss how the XNP protein may regulate gene transcription at the chromatin level.

INTRODUCTION

The *XNP* gene in Xq13.3 recently has been shown to be mutated in 15 patients with a characteristic form of syndromal mental retardation (MR) with α-thalaessemia (ATR-X syndrome). We also described a mutation segregating in a large family with the Juberg–Marsidi syndrome, another X-linked severe MR syndrome, as well as two mutations associated with an ATR-X like phenotype but without α-thalaessemia (1–4). Very little functional data are available on the *ATR-X* gene product and they essentially result from cDNA sequence, expression analysis or genotype–phenotype correlations. Indeed, we found the *XNP* transcript to be ubiquitously expressed and particularly abundant in human and mouse brain. In the developing mouse brain, the gene is highly expressed in areas where neural proliferation is occurring.

Sequence analysis and database comparison of the *XNP* cDNA show that it encodes a putative 2492 amino acid protein which displays a modular shape and can be divided into distinct regions (5): a putative DNA-binding domain composed of three multi-cysteine zinc finger motifs, a large 3074 bp long exon, a polyglutamic acid stretch, the seven conserved ‘helicase’ motifs found in DNA-stimulated ATPase and DNA helicase (6), and a C-terminus glutamine-rich domain. The characteristics of the helicase domains make the XNP protein a new member of the SNF2/SWI DNA helicase family.

Recent findings suggest that, in higher eucaryotes, the epigenetic regulation of transcription by chromatin remodelling plays a critical role in the controlled expression of developmental programmes and the stability of the cellular determined state (7–9). For example, many experiments have emphasized the role of chromatin structure in regulating α-globin gene expression (10). In addition, recent observations have revealed interactions between the heterochromatic protein mHP1 and several transcriptional factors (11). Finally, it has been demonstrated that the yeast SNF2 and SNF5 transcriptional activators regulate gene expression by altering chromatin structure. This led us to hypothesize that, in a similar way, XNP could regulate gene expression by direct interaction with heterochromatin-associated proteins, and we therefore set up a yeast two-hybrid assay to evaluate such interactions. We report here the demonstration of a specific interaction between XNP and the chromatin-associated EZH2 protein (12). We show that this interaction is mediated by the SET domain of the EZH2 protein and is independent of the yeast two-hybrid assay since we have been able to confirm this result using an *in vitro* binding assay.

RESULTS

Demonstration of the XNP–EZH2 interaction

Given the organization and the very large size of the XNP protein, we decided to fractionate the cDNA into different fragments to

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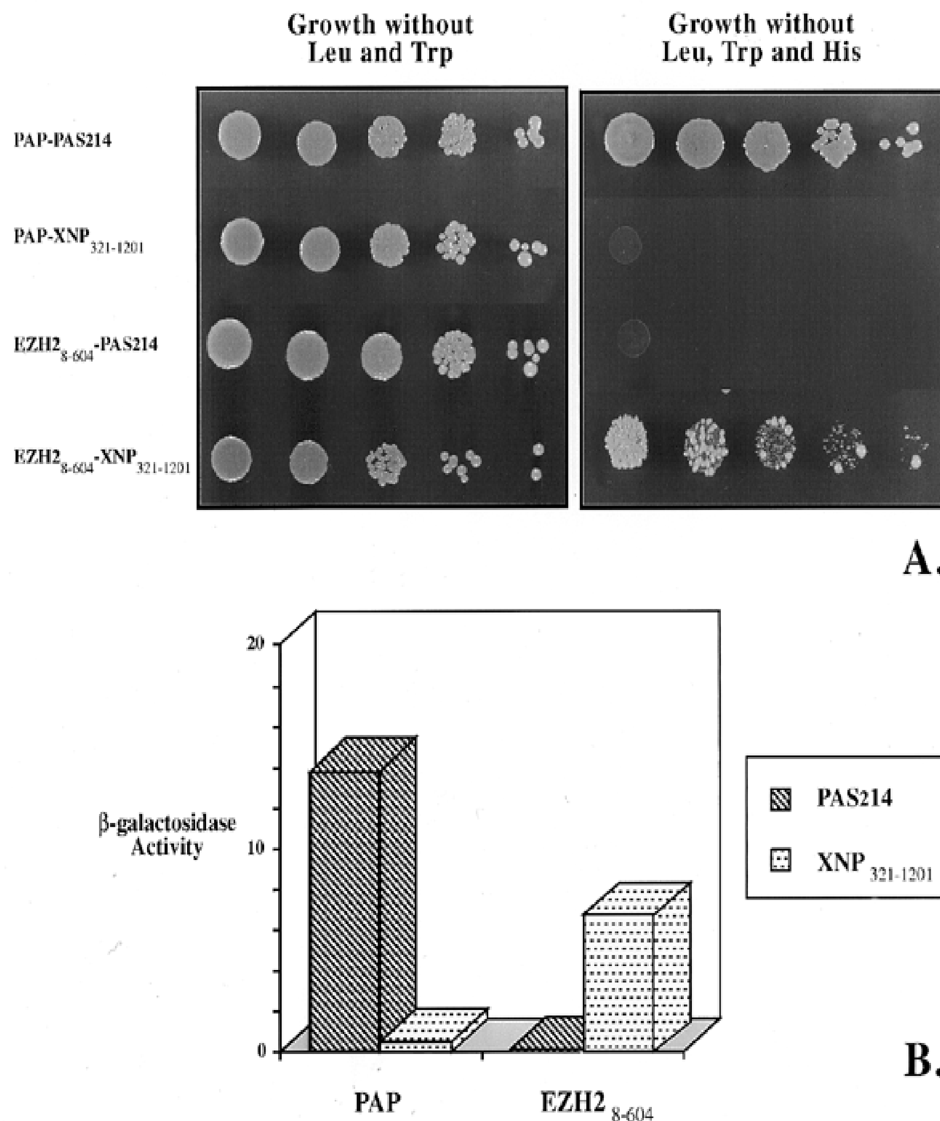

Figure 1. Results of the yeast two-hybrid assays. (**A**) Diploids were first selected on synthetic complete (SC) dropout medium lacking leucine and tryptophan and then tested by a serial dilution assay for their ability to growth on SC dropout medium without histidine. The pAS214 and PAP constructs are controls. (**B**) Diploids were grown on minimal medium, and β-galactosidase activity was measured using ONPG as substrate. The β-galactosidase units were calculated according to Miller (16).

explore putative interactions with chromatin proteins. A first set of hybrid proteins was constructed which fused cDNA fragments corresponding to amino acid residues 1–338, 321–1201 or 865–2492 of XNP to the GAL4 DNA-binding domain. The second set were hybrids between the GAL4 activation domain and various cDNAs corresponding to the HP1 human protein (13) and members of the Polycomb group of (PcG) gene products: EZH2 (also called ENX1), BMI1 and MEL18 (12,14,15). Constructs containing non-related proteins were also made to provide positive and negative controls for the specificity of the interaction. When expressed alone, none of these constructs can transactivate the *HIS3* reporter gene. In contrast, we found that the simultaneous expression of $XNP_{338–1201}$ with the EZH2 protein results in the ability of the yeast cells to grow without histidine (Fig. 1A). No activation of the reporter gene was detected with any other combination. This result was confirmed by the β-galactosidase assay (Fig. 1B). Only yeast cells harbouring the XNP and the EZH2 constructs showed LacZ activity. Therefore, our data demonstrate that, in yeast, the human EZH2 protein is able to associate with the *ATR-X* gene product.

Delineation of the interaction domains

Since both XNP and EZH2 contain several defined sequence motifs, we next sought to determine which of these motifs are responsible for the interaction. We therefore generated a set of XNP deletion constructs prepared in the GAL4 DNA-binding domain vector and a set of EZH2 deletion constructs prepared in the GAL4 activating domain plasmid. Yeast were co-transformed with different combination of plasmids and assayed for their ability to grow on medium without histidine. As shown in Figure 2A, we found that the XNP interaction domain is located between residues 475 and 734, a region of the protein with no remarkable features. Similarly, we found that the C-terminal region of EZH2

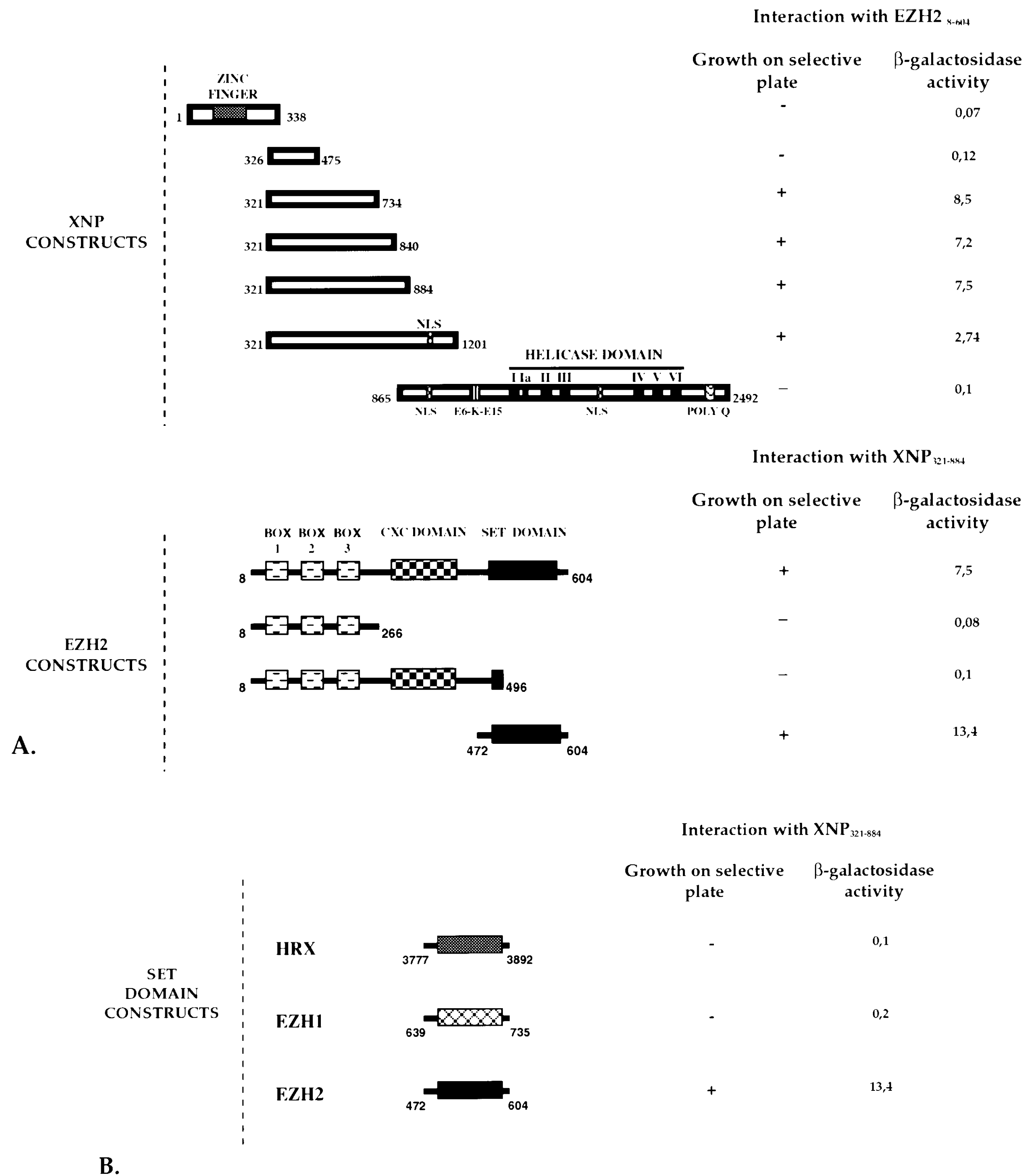

Figure 2. (**A**) Delineation of the XNP and EZH2 interacting domains. Different portions of the XNP and EZH2 proteins were cloned into the GAL4 fusion protein vectors and transformed into the yeast two-hybrid reporter strains. The structural motifs of XNP and EZH2 are indicated: NLS, nuclear localization sequence; E6-K-E15, polyacidic stretch; POLY Q, glutamine-rich domain for XNP; BOX 1, 2 and 3, conserved sequences between EZH2 and the *Drosophila E(z)* gene; CXC, the cysteine-rich and the SET domain for EZH2. The interactions between the respective protein domains were monitored by measuring the activity of the reporter genes *HIS3* and *LacZ*. +, ability to grow on medium lacking His; –, no growth. The results of the corresponding β-galactosidase activity measurements by ONPG assays are reported on the right. (**B**) cDNA fragments corresponding to the SET domain of the human HRX and EZH1 proteins were cloned in the GAL4 activation domain vector and tested for their ability to interact with the $XNP_{321–884}$ protein.

(residues 472–604) is necessary and sufficient to bind XNP (Fig. 2A). Interestingly, this part of the protein contains a well-conserved structural motif: the SET domain (17) for which biological function has never been demonstrated. Our data therefore provide the first evidence that this domain is a protein–protein interaction domain.

Since this domain is found in many proteins from fungi to insects and vertebrates (18), we wondered wether the interaction

was specific of the EZH2 SET domain or would be positive for another protein with a SET domain. We thus performed similar experiments with the two most closely related sequences. Fusion proteins were constructed which correspond to the human HRX SET domain (19) and the human EZH1 (also called ENX2) SET domain (20). However, as shown in Figure 2B, no evidence of transactivation of the reporter *HIS3* gene or of the *LacZ* gene was obtained, suggesting that the interaction between XNP and EZH2 is highly specific.

In vitro binding between XNP and EZH2

To obtain additional evidence for the interaction between XNP and EZH2, we performed a series of *in vitro* binding assays. Various recombinant glutathione *S*-transferase (GST) fusion proteins (GST–EZH2$_{8–604}$, GST–XNP$_{321–884}$ and GST–XNP$_{1668–2492}$), and polyhistidine-tagged fusion proteins (HIS-EZH2$_{472–604}$, HIS-XNP$_{321–884}$ or HIS-control) were produced in *Escherichia coli* and monitored by SDS–PAGE and Coomassie staining (data not shown). Lysates from cells expressing HIS-XNP were mixed with either GST–EZH2 or control proteins, and purified using affinity chromatography on glutathione–agarose beads. Following washes, bound GST fusion protein and any associated proteins were dissociated, and analysed by SDS–PAGE and immunoblotted with an anti-polyHIS antibody. As shown in Figure 3, we found that the HIS-XNP$_{321–884}$ protein is able to bind the GST-EZH2$_{8–604}$ protein but not a control GST fusion protein. In addition, GST–EZH2$_{8–604}$ protein is unable to bind a polyHIS-tagged control protein. Similarly, we found specific binding of the EZH2 SET domain (HIS-EZH2$_{472–604}$) to immobilized GST–XNP$_{321–884}$. Our results are therefore in complete agreement with those obtained using the yeast two-hybrid assay.

Spatial and temporal distribution of XNP and EZH2 transcripts

To obtain additional evidence that the interaction detected in yeast between XNP and EZH2 may actually occur *in vivo*, and because anti-XNP antibodies are not available presently, we performed *in situ* hybridization analysis with EZH2- or XNP-specific probes on adjacent saggital sections from rat embryos at different stages. The specificity of the hybridization reaction was demonstrated by the results obtained with the two corresponding sense probes which showed no detectable signals (not shown). Preliminary results are shown in Figure 4. In E12.5 animals, the XNP transcript was detected in the whole germinal layer including the prosencephalon, the diencephalon, the rhombencephalon and the spinal cord (Fig. 4A). In the peripheral nervous system, a signal was detected in the dorsal root ganglia (data not shown). Outside the nervous system, a signal was detected in the tongue and the jaw. At the same stage, the EZH2 signal was also detected in the whole germinal layer, the dorsal root ganglia, the tongue and the jaw (Fig. 4B). In 18.5 animals, XNP transcript was detected in the CNS, including the spinal cord and the olfactory epithelium, and to a lesser extent in the tongue (Fig. 4C). Outside the head, XNP was only detected in the spinal cord (Fig. 4E). At the same stage, the CNS and the tongue were also labelled with the EZH2 riboprobe (Fig. 4D). Also outside the head, a diffuse EZH2 signal was detected in the whole body including the spinal cord (Fig. 4F).

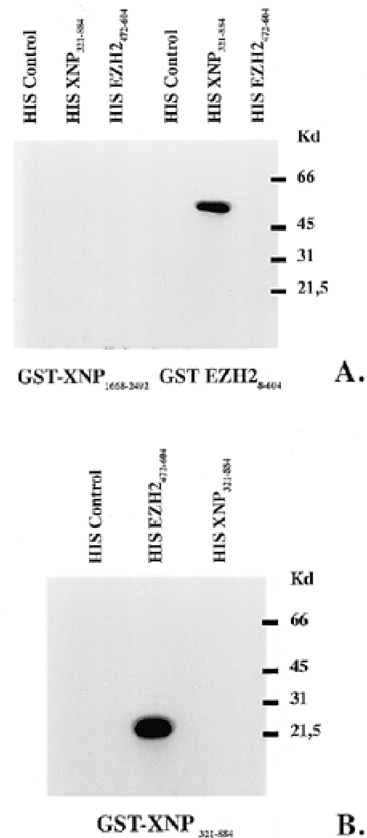

Figure 3. *In vitro* interaction between XNP and EZH2 proteins. (**A**) Binding of various polyhistidine-tagged proteins to columns containing immobilized GST–EZH2 fusion protein (GST–EZH2$_{8–604}$) or a control XNP fusion protein (GST–XNP$_{1668–2492}$). Following binding and washes, proteins complexed to the GST-fused proteins were separated by SDS–PAGE and immunoblotted with a monoclonal anti-polyHIS antibody. The position of protein size standards in kilodaltons are indicated (MW). (**B**) A similar experiment was performed to test binding of various polyHIS-tagged proteins to a column loaded with the GST–XNP$_{321–884}$ protein.

DISCUSSION

Using the yeast two-hybrid assay system, we have demonstrated that the XNP protein is able to interact with the SET domain of the EZH2 protein. These results obtained in yeast were confirmed further by *in vitro* binding experiments since we demonstrated that a recombinant HIS-XNP protein from *E.coli* was retained selectively by GST–EZH2 linked to glutathione–agarose. However, the definitive demonstration that the interaction between the two proteins actually occurs in mammalian cells will require additional experiments (such as co-immunoprecipitation) showing that both proteins are actually part of the same complex. Antibodies raised against the XNP protein presently are being developed to detect such a complex in cultured cells and animal tissues. We note, however, that additional data support the hypothesis of an XNP–EZH2 association. Firstly, both proteins have been reported to be located predominantly in the nucleus (2,12). Secondly, data from *in situ* hybridization experiments revealed several embryonic regions where XNP and EZH2 transcripts are expressed concomitantly: the brain, the spinal cord and the dorsal root ganglia. These results suggest that the XNP–EZH2 association may play an important role in neuronal development. Interestingly, we also

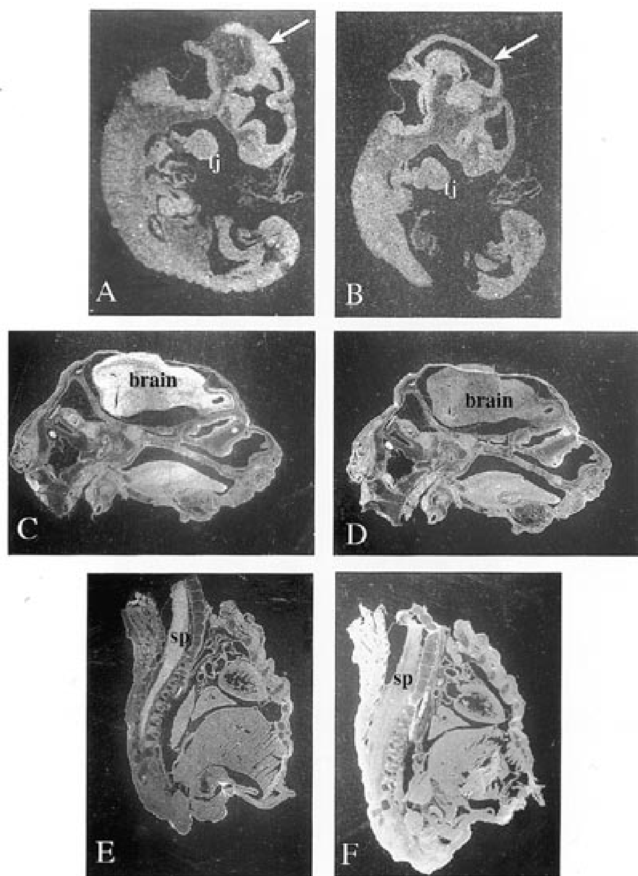

Figure 4. Partial co-distribution of XNP and EZH2 transcripts in E12.5 and E18.5 rat embryos. *In situ* hybridization of saggital sections of the whole embryo at E12.5 revealed with XNP riboprobes (**A**) and EZH2 riboprobes (**B**). Note that the entire germinal layer (arrow) as well as the tongue jaw blastema (tj) are labelled with both probes. E18.5 head saggital sections (**C** and **D**) and body saggital sections (**E** and **F**) hybridized with XNP riboprobe (C and E) and EZH2 riboprobe (D and F). With both probes, the brain (C and D) and the spinal cord (Sp) (E and F) were labelled.

found a few sites of XNP expression where the EZH2 transcript is not detected. This suggests that in these cells, the XNP protein may fulfil its biological function by interacting with another SET domain-containing protein. We presently are performing an extensive double hybrid screen using $XNP_{321–884}$ as bait to isolate such proteins.

Our data provide the first insight into the function of XNP as well as the pathogenesis of ATR-X, since they demonstrate that it is able to associate with the PcG protein EZH2. Since previous experiments have demonstrated that EZH2 (amino acids 67–318) interacts with the VAV1 proto-oncogene product (13), we propose that the VAV1–EZH2–XNP complex may play an important role during haematopoiesis. An important question raised by this work is how the interaction between an SNF2-like protein and a PcG protein involved in formation of repression complexes leads to the activation of α-globin gene transcription. One possibility is that XNP, via its putative helicase activity, may counteract EZH2-mediated chromatin repression by maintaining the α-globin locus in an open chromatin conformation. This could facilitate binding of erythroid-specific factors to their target sequence and result in α-globin gene activation. Alternatively, XNP could sequester EZH2 into an inert complex negatively regulating EZH2 function. Finally, since the *XNP* gene is also expressed in lymphocytes, it could repress erythroid-specific loci in this lineage.

Another significant finding is that this interaction is mediated by the EZH2 SET domain. Recent observations on the *Drosophila E(z)* gene suggest that molecular interactions through the SET domain determine whether the E(z) protein acts as either a trithorax-like or a Polycomb-like gene (21), and that this process is regulated during development and is tissue-specific. One can speculate, therefore, that in erythroid cells, this interaction results in the recruitment of the EZH2 protein in a trithorax-like complex. Interestingly, this interaction seems specific for the EZH2 SET domain since no interaction was detected with the HRX or EZH1 SET domains. Because of the strong similarity between the EZH2 and EZH1 SET domains (only eight different amino acids out of 128), we suggest that the variant positions (which include two prolines) might be critical residues for the interaction. Due to the complementarity of the EZH2 and EZH1 expression profile during development, distinct functions for these two proteins has already been suggested (20). Our results demonstrate that the primary structure of the SET domain may provide such functional specificity.

The last point regards our results and their relation to pathology. So far, all mutations associated with α-thalaessemia are localized within the helicase domain, and none involve the EZH2–XNP interaction domain. It is likely, therefore, that mutations in this domain result either in no phenotype, or more likely in embryonic lethality. Finally, EZH2 recently has been mapped to 21q22.2 (22). Although its participation in some features of Down syndrome cannot be ruled out or proven, an interesting deletion syndrome, the del21q22 syndrome, involves the same region of chromosome 21. Since clinical presentation of this syndrome shows intriguing similarities with ATR-X (MR, dysmorphic features and sexual ambiguities), the potential role of the EZH2 gene in this pathology is under study.

MATERIALS AND METHODS

Yeast two-hybrid assay

cDNA fragments corresponding to various parts of the XNP N-terminal domain (residues 1–338, 321–884 and 321–1201) were cloned in-frame into the *Nde*I site of the GAL4 DNA-binding domain of the pAS2.1 vector (Clontech) and transformed into *Saccharomyces cerevisiae* Y187 (MATα, *leu2-3,112, ura3-52, trp1-901, his3-D200, ade2-101, gal4Δgal80Δ, URA3::GAL-lacZ*). cDNAs corresponding to the different chromoproteins were cloned in-frame into the *Bam*HI site of the GAL4 activation domain of the pACT2 vector (Clontech) and transformed into Y190 (MAT**a**, *leu2-3,112, ura3-52, trp1-901, his3-D200, ade2-101,. gal4Δgal80Δ, URA3::GAL-lacZ, LYS2::GAL-HIS3, cyc^{r}2*). These constructs were tested for protein expression by immunoblot analysis and, except for BMI-1, we could detect a significant amount of hybrid proteins (data not shown). The control plasmids pAS2.14 and PAP are pAS2.1 and pACT2 derivatives which encode yeast proteins that interact. Yeast transformation and mating were performed according to Gietz *et al.* (23). Diploids were selected on synthetic complete (SC) dropout medium lacking leucine and tryptophan and then tested for their ability to growth on SC dropout medium without histidine; 25 mM 3-aminotriazole was added to the selection medium plates to suppress growth of transformants containing non-interacting pro-

teins. β-Galactosidase assays were performed using *O*-nitrophenyl β-d-galactopyranoside (ONPG) as substrate.

In vitro protein–protein interaction assay

GST fusion proteins were obtained by cloning the *EZH2* cDNA from amino acids 8 to 604 (GST–EZH2_{8-604}), the *XNP* cDNAs from amino acids 321 to 884 (GST–$XNP_{321-884}$) or 1770 to 2492 (GST–$XNP_{1770-2492}$) into the pGEX-KT vector (Pharmacia). Polyhistidine fusion proteins were obtained by cloning the *XNP* cDNA fragments from amino acids 321–890 (HIS-$XNP_{321-890}$), the *EZH2* cDNA fragment from amino acid 472 to 604 (HIS-EZH$2_{472-604}$) or a control unrelated cDNA fragment (HIS-control) into the pRSET vector (Invitrogen). After transformation into *E.coli* BL21(DE3), protein expression was induced for 4 h at 30°C with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell lysates were prepared from a 20 ml induced culture under native conditions in phosphate-buffered saline (PBS). HIS-protein extracts corresponding to 5 ml of culture were first pre-absorbed on agarose beads at 4°C for 1h, clarified by centrifugation at 8000 *g* for 5 min and then incubated with agarose beads containing GST–EZH2 or other fusion proteins for 2 h at 4°C. The resin was pelleted and washed three times with PBS buffer, and the remaining bound proteins were eluted by boiling in SDS sample buffer, separated on a 12% SDS–poyacrylamide gel and transferred onto nitrocellulose membrane. Filters were incubated with monoclonal Anti-Xpress antibody (Invitrogen) according to the manufacturer's instructions, followed by an anti-mouse secondary antibody conjugated to peroxidase.

In situ hybridization procedure

Preparation of embryos and embryonic sections, their fixation and hybridization were performed according to Timsit *et al*. (24). The EZH2 probe is a rat cDNA fragment corresponding to nucleotides 960–1590 of the human cDNA sequence. The XNP probe is a rat cDNA fragment corresponding to nucleotides 5129–5989 of the human cDNA sequence. Single-stranded sense and antisense [^{35}S]UTP-labelled RNA probes were prepared using the Riboprobe kit (Promega Corp., Madison, WI) and hydrolysed in 0.4 M Na_2CO_3 at 60°C to generate fragments of ~150 bp. After hybridization, slides were coated with Kodak NTB2 film emulsion and exposed for 6–12 days.

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