Identification of the *Drosophila* interband-specific protein Z4 as a DNA-binding zinc-finger protein determining chromosomal structure

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Summary

The subdivision of polytene chromosomes into bands and interbands suggests a structural chromatin organization that is related to the formation of functional domains of gene expression. We made use of the antibody Z4 to gain insight into this level of chromosomal structure, as the Z4 antibody mirrors this patterning by binding to an antigen that is present in most interbands. The *Z4* gene encodes a protein with seven zinc fingers, it is essential for fly development and acts in a dose-dependent manner on the development of several tissues. Z4 mutants have a dose-sensitive effect on w^{m4} position effect variegation with a haplo-suppressor and triplo-enhancer phenotype, suggesting Z4 to be involved in chromatin compaction. This assumption is further supported by the phenotype of Z4 mutant chromosomes, which show a loss of the band/interband pattern and are subject to an overall decompaction of chromosomal material. By co-immunoprecipitations we identified a novel chromo domain protein, which we named Chriz (Chromo domain protein interacting with Z4) as an interaction partner of Z4. Chriz localizes to interbands in a pattern that is identical to the Z4 pattern. These findings together with the result that Z4 binds directly to DNA in vitro strongly suggest that Z4 in conjunction with Chriz is intimately involved in the higher-order structuring of chromosomes.

Key words: Chromatin domains, Chromosomal borders, Interbands

Introduction

For several structural and functional reasons the enormous size of eukaryotic genomes has to be folded into chromatin in a hierarchical fashion. While the architecture of the nucleosome at the low end of this hierarchy is known in detail, this knowledge is progressively lacking if one considers higher-order chromatin structures like the 30 nm fiber or looped domains.

The looped domain concept was proposed to explain several cytological observations and biochemical findings when studying higher-order chromatin. It basically states that stretches of chromatin form topologically discrete units, chromatin loops which may be further compacted according to their state in gene expression. It is also proposed in this model that the bases of the loops are formed by the local interaction of sequences which are far apart in the linear DNA. Such sequences, also called boundary sequences, may have certain physiological functions, like that of insulator or blocking elements to shield the regulatory influence of flanking domains. Boundary elements also were proposed to have a role for the attachment of looped domains to an as yet ill-defined nuclear skeleton.

Cytologically, looped domains were first observed as the large lampbrush loops of meiotic prophase chromosomes of certain amphibia (Callan, 1987) and *Drosophila* (Hess and Meyer, 1963). Correlates of looped domains were further found by the biochemical and electron microscopic analysis of salt extracted chromosomes (Benyajati and Worcel, 1976; Paulson and Laemmli, 1977; Adolph, 1980; Vogelstein et al., 1980; Lebkowski and Laemmli, 1982). In this respect, the characteristic and reproducible pattern of alternating bands and interbands of polytene chromosomes is very suggestive to represent the chromosomal structure defining looped domains. This becomes evident on puff formation when certain polytene bands locally decondense in response to endogenous or environmental signals to allow transcription of their resident genes. The classical studies of Benyajati and Worcel (Benyajati and Worcel, 1976) suggested a looped domain organization of *Drosophila* diploid interphase chromosomes, which is shared by chromatin of other organisms as well (Igo-Kemenes and Zachau, 1978). The looped domains observed in all of of these studies are building blocks of chromatin in the order of several tenthousand basepairs of DNA, and the same organization may be common to many – if not all – eukaryotic organisms.

More importantly, looped domains are also units of chromosomal function. The most well known examples are the globin loci in vertebrates, in particular the chicken *β-globin* locus. In chicken red blood cells the globin genes are coordinately regulated within a 33 kb chromosomal domain, which differs by its chromatin structure from the flanking chromatin already early in development. The domain is flanked by two constitutive hypersensitive regions, HS4 and 3′HS, whose function as boundary elements was shown by their ability to act as enhancer blockers and by conferring position-

independent expression of transgenes in vertebrates and invertebrates. In addition, the CTCF protein, which also was identified as an essential boundary element factor in other species, is needed for their function. Upstream, the HS4 element blocks the spreading of a 16 kb heterochromatic chromatin region and inhibits the regulatory crosstalk with the strong folate receptor gene enhancer. The downstream element keeps the expression of the globin genes independent from that of the immediate flanking odorant receptor gene, which is normally expressed in olfactory epithelia and certain neurons only (for reviews, see Bulger et al., 2002; Bell et al., 2001).

A very similar organization is met at the *Drosophila* heat-shock locus in 87A7. The two divergently transcribed *hsp70* genes are flanked by strong DNAseI hypersensitive sites which are part of the so called specialized chromatin structure elements *scs* and *scs′*, respectively (Udvardy et al., 1985). On heat-shock induction these elements mark the edges of the decondensed puff formed at this site. Both *scs* and *scs′* elements function as boundary elements. They confer position-independent expression on *Drosophila* transgenes and are functional as enhancer blockers in transgene expression (Kellum and Schedl, 1991; Kellum and Schedl, 1992). The insulating activity of the *scs* element is mediated by the Zeste-white 5 protein (Zw5) (Gaszner et al., 1999), whereas the scs′ activity is mediated by the boundary element associated factor 32 (BEAF-32) (Zhao et al., 1995). More recently, it was shown that Zw5 and BEAF-32 proteins interact. Results from ChIP experiments using *Drosophila* cell lines provided evidence that the *scs* and *scs′* elements are close in space to each other in nuclei in vivo, although they are separated by 15 kb of intervening sequence on linear DNA (Blanton et al., 2003). This is consistent with a role of these elements in forming the base of a looped domain. Besides its binding to 87A7, the BEAF-32 protein is localized at numerous interbands on polytene chromosomes, suggesting a similar boundary function at these sites as well. Similarly, Zw5 is found at over 100 sites on polytene chromosomes, although not as widespread as BEAF-32. The general organization of genes into functional chromosomal domains is substantially supported by recent findings that in *Drosophila* co-expressed genes tend to be organized in genomic clusters, differing significantly from a random distribution (Hager and Miller, 1991; Spellman and Rubin, 2002; Ueda et al., 2002; Boutanaev et al., 2002).

An important role of interbands in the formation of looped domains is suggested by the finding that other *Drosophila* proteins involved in chromosomal domain organization like Suppressor of Hairy wing [Su(Hw)] (Spana et al., 1988; Harrison et al., 1993) and Mod(mdg4) also bind to numerous interbands (Dorn et al., 1993; Gerasimova and Corces, 1998; Büchner et al., 2000). In addition, by high resolution in situ hybridization DNA sequences involved in boundary formation have been localized to the interband 3C6-3C7 preceding the *Notch* locus (Rykowski et al., 1988). These sequences confer position-independent expression of transgenes in *Drosophila* and show enhancer blocking activity (Vasquez and Schedl, 2000). Their deletion in the recessive hypomorphic *Notch* allele *facet strawberry* (fa^{swb}) results in inappropriate expression of the *Notch* gene, which may be explained by the unhindered crosstalk of regulatory elements from genes adjacent to the *Notch* promoter. (Keppy and Welshons, 1977; Welshons and Welshons, 1985).

Although interbands so far are defined cytologically only, we have reason to believe that they reflect a common structural motif of interphase chromosomes. The same pattern of band/interband structure can be reproducibly observed in tissues of lower polyteny up to a level when the chromosomes become to fragile to be spread by the available methods. Structurally, interband chromatin is less condensed than chromatin of adjacent bands. Correlating the unit length of bands and interbands with DNA sequences precisely localized to the same interval by high resolution in situ hybridization, Rykowski et al. (Rykowski et al., 1988) proposed that DNA in interbands at the *Notch* locus fold into a 10 nm fiber, whereas chromatin in bands would be at least six times more compacted as a 30 nm fiber. Intuitively, this difference in compaction between bands and interbands suggests the existence of boundaries between the two elements. Whether interbands in general provide boundaries of chromosomal domains has not been proven experimentally so far. Also, we do not currently have a model regarding how the decondensed state of interbands is maintained. The isolation of the tandem kinase Jil-1 was recently reported, which, besides its function in dosage compensation, plays a role in the maintenance of interbands (Jin et al., 1999; Jin et al., 2000), since removing Jil-1 function resulted in highly compacted polytene chromosomes lacking any banded appearance.

The accumulating evidence for a functional involvement of interbands in the formation of a higher-order chromatin organization prompted us to use our library of monoclonal antibodies against nuclear proteins of *Drosophila* to identify proteins with a specific localization to the interbands. We describe the cloning of the *Z4* gene encoding an interband-specific zinc finger protein. We show that Z4 binds directly to DNA in vitro and is involved in the establishment or maintenance of the alternating band/interband pattern. The chromosomal phenotype of *Z4* mutants in conjunction with a dose-dependent influence on w^{m4} position effect variegation uncovers Z4 to constitute an essential component in the functional organization of chromosomes.

Materials and Methods

Drosophila stocks

Fly strains were maintained at 22°C on standard *Drosophila* cornmeal medium with the addition of dry yeast, soy bean meal and molasses. Oregon-R was used as a wild-type stock. The EP(3)0756 stock was obtained from the Szeged Drosophila Stock Centre (University of Szeged, Hungary) and w^{m4h} was kindly provided by G. Reuter (University of Halle, Germany). The GAL4-lines used for ectopic expression were T80-GAL4 (Wilder and Perrimon, 1995), ey-GAL4 (Hazelett et al., 1998) and G61-GAL4 (kindly provided by U. Hinz, University of Cologne, Germany). As a genomic source of transposase the strain P[ry^{+} Δ2-3]99B was used (Robertson et al., 1988). Genes, chromosomes and symbols are described by Lindsley and Zimm (Lindsley and Zimm, 1992).

DNA cloning

For the complementation analysis of *Z4*-mutants a 7.4 kb genomic sequence between the *Sac*I and the *Bam*HI sites encompassing the complete transcription unit of *Z4* and the first five exons of the gene *CG12974* (see Fig. 2) was cloned into the P transformation vector Casper (Pirrotta, 1988). At the 3′-end of the *Z4* coding sequence a restriction site was introduced by the insertion of a PCR fragment that

was generated with appropriately designed primers. Into this restriction site a $(myc)_3$-H_6 epitope (Aagaard et al., 1999) was inserted to generate an in-frame fusion with the *Z4* gene. Further details of the cloning procedure are available on request. The construct was injected into w^{1118} embryos to establish the transgenic line P[Z4myc].

To overexpress *Z4* in *Drosophila*, the *Z4* cDNA from clone LD15904 (obtained from Research Genetics, Huntsville, AL) was cloned into the P element transformation vector pUAST (Brand and Perrimon, 1993) to generate the vector pUAST-Z4, which was injected into w^{1118} embryos to establish the homozygous viable transgenic lines P[pUAST-Z4]. For the verification of the interband localization of Chriz, the Chriz cDNA was cloned in-frame 3′ of the $(myc)_3$-H_6 epitope in pUAST. This vector was injected into w^{1118} embryos from which the transgenic line P[pUASTmycChriz]33.5 was established.

The vector P[BJ1(1-547)GFP] was constructed by inserting a PCR product amplified from the BJ1 cDNA (Frasch, 1991) into the vector pEGFP-1 (Clontech, Palo Alto, CA) to generate an in-frame fusion of BJ1 with GFP. The fusion construct was excised as an *Eco*RI-*Not*I-fragment and inserted into the *Eco*RI and *Not*I sites of pUAST.

Identification of Z4 cDNAs

A λgt11 *Drosophila* embryonic library (Hovemann et al., 1991) was screened with the Z4 antibody (Saumweber et al., 1980) using standard procedures (Sambrook et al., 1989). Four overlapping clones were isolated and sequenced using the sequencing service of the Department of Genetics at the Humboldt University, Berlin. The programs BLASTN and BLASTP at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) were used to search for homologous sequences.

Germline transformation

To generate germline transformants, P transposons with a concentration of 500 μg/ml together with 100 μg/ml of helper plasmid pπ25.7wc (Karess and Rubin, 1984) were injected into *Drosophila* embryos of the w^{1118} strain according to Rubin and Spradling (Rubin and Spradling, 1982).

P element excision and complementation of mutants

Mutant alleles of *Z4* were generated by imprecise excisions of the EP transposon (Rorth et al., 1998) in *w/Y;EP(3)0756/P[ry*$^+$*;Δ2-3]99B* heterozygous males. These males were crossed with *w/w;TM3,Sb/TM6,Tb* females and progeny with white eyes were mated in single crosses to *w;TM3,Sb/TM6,Tb* males or females. *Sb* siblings from the next generation were crossed inter se from which a total of ten recessive lethal lines were obtained. The lines *w;Z4-1.3/TM3,Sb*, *w;Z4-2.1/TM3,Sb* and *w;Z4-7.1/TM3,Sb* (named *Z4-1.3*, *Z4-2.1*, *Z4-7.1*, respectively) were selected for further analysis.

The ability of the *Z4* gene to complement the *Z4* mutations was tested with the 7.4 kb genomic sequence present as a transgene in *P[Z4myc]* transgenic flies. *w/Y;P[Z4myc]/P[Z4myc];TM3,Sb/TM6,Tb* males were crossed with *w/w;Z4-1.3/TM3,Sb* females to obtain *w;P[Z4myc]/+;Z4-1.3/TM3,Sb* males and females. These flies were crossed inter se and the progeny was scored for *Sb*$^+$ adults. The same crossings were used to complement the mutations of the independent lines *Z4-2.1* and *Z4-7.1*.

Modification of w^{m4} PEV by *Z4* mutants

w^{m4}/w^{m4} females were crossed with w^{1118}/Y;*Z4-1.3/TM3,Sb* males and the eyes of w^{m4}/Y;*TM3*/+ males were scored and compared with the eyes of w^{m4}/Y;*Z4-1.3*/+ males derived from the same cross. The crosses with the lines *Z4-2.1* and *Z4-7.1* were analogous to those with *Z4-1.3*. Males of the genotype w^{m4}/Y;*e,st,spo*/+ derived from the cross of w^{m4}/w^{m4} females with w^{1118}/Y;*e,st,spo/TM3,Sb* males served as a reference control for the measurement of eye pigments.

To test the effect of three copies of *Z4* on w^{m4} PEV, w^{m4}/w^{m4} females were crossed with w^{1118}/Y;*P[Z4myc]/CyO* males and the w^{m4}/Y;*P[Z4myc]*/+ males were scored for their eye phenotypes and compared with the eye phenotypes of their w^{m4}/Y;*CyO*/+ siblings.

Eye pigments were quantified as described by Negeri et al. (Negeri et al., 2002).

Immunohistochemistry

Cells of the *Drosophila melanogaster* cell line Kc and embryos from the Oregon R stock were stained with the Z4 antibody as described previously (Frasch and Saumweber, 1989). Polytene chromosomes were prepared from wandering third instar larvae, fixed and stained with primary and secondary antibodies essentially as described (Saumweber et al., 1980). As primary antibodies Z4 (Saumweber et al., 1980) and the anti-myc antibody 9e-10 (Errede and Ammerer, 1989) were used; the secondary antibody was a rhodamine conjugated goat anti-mouse antibody (Dianova).

For the staining of chromosomes in situ salivary glands were isolated from 3rd instar larvae in PBS and fixed in 4% paraformaldehyde for 30 minutes. The glands were washed three times in PBT (PBS with 0.3% Triton X-100) for 10 minutes and incubated in PBT supplemented with 5% fetal calf serum. The glands were incubated with the Z4 antibody for 16 hours at 4°C, washed three times in PBT for 20 minutes and incubated with a rhodamine conjugated goat anti-mouse antibody (1:5000 in PBT, 5% fetal calf serum) for 1 hour at room temperature. The glands were washed three times in PBT for 20 minutes and the chromosomes were stained with Sytox Green (1 μg/ml in PBS; Molecular Probes, Eugene, OR) and mounted in 85% glycerol, 3% n-propylgalleat for microscopy.

Chromosomes were viewed with a Zeiss Axiophot microscope equipped with a Pro Series high-performance CCD camera. For high-resolution analysis chromosomes were viewed with a Deltavision Spectics optical sectioning microscope (Deltavision, Issaquah, WA). Chromosomes stained with Sytox Green were analyzed with a confocal laser scanning microscope equipped with an Argon/Krypton laser. Images were electronically processed with the Image Pro program or with Deltavision Softworx Software.

Western blot and immunoprecipitations

Protein extracts were prepared from *Drosophila* Kc cells and dechorionated embryos as described previously (Reim et al., 1999). Proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels and blotted to a nitrocellulose membrane as described (Frasch and Saumweber, 1989). The membrane was incubated with 10-20 μg/ml Z4 antibody overnight and further processed as previously described (Frasch and Saumweber, 1989). Immunoprecipitations and MALDI-TOF mass spectrometry of proteins extracted from SDS-polyacrylamide gels were carried out as described by Reim et al. (Reim et al., 1999).

Electrophoretic mobility shift assay

Two fragments from the 5′-region of *Notch* were amplified from genomic wild-type DNA by PCR. The primers Notch F4 (5′-TGAAAACTAAGAACGTATTGCG) and Notch R4 (5′-ATTC-GACAATGTAAGATTCGTAG) were used to amplify the fragment N1 and with primers Notch F3 (5′-GATTTATACACTCG-AATCTAATTCTATTC) and Notch R3 (5′-CGTTCTTAGTTT-TCATTTTCCG) the N2 fragment was amplified. After amplification the DNA was digested with *Hin*fI and end-labeled by Klenow

polymerase in a fill-in reaction of the DNA-ends in the presence of [α-^{32}P]dATP. The labeled DNA was separated from unincorporated nucleotides by centrifugation through a column filled with 1 ml of Sephadex-G50 (Amersham Pharmacia) and stored at –20°C.

To express the full-length Z4 protein in *Escherichia coli* the coding region of *Z4* was amplified with suitable primers from clone LD15904 (obtained from Research Genetics) and cloned into the expression vector pGEX4T-2 (Amersham Pharmacia) to generate pGEX-Z4. This clone was transformed into the *E. coli* strain BL21 and the GST-Z4 fusion protein was purified from the cells using gluthatione agarose beads according to the manufacturer's instructions (Amersham Pharmacia).

Binding of Z4 to the DNA was performed in 20 μl by incubating 5 ng of the labeled DNA-probe with 0.5 μg of purified Z4 in binding buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 0.1 mM $ZnCl_2$, 0.2 mg/ml BSA, 4% Ficoll 400) either without or with varying amounts of competitor DNA for 20 minutes at 4°C. The reaction mixtures were electrophoresed at 4°C with 10-15 V/cm on 6% polyacrylamide gels in 45 mM Tris-borate (pH 8.0). The gels were dried and exposed to an X-ray film at –70°C.

Results

Identification of *Z4* encoding a chromosomal interband protein

Among the collection of monoclonal antibodies against *Drosophila* nuclear proteins, several antibodies were shown to stain preferentially the interbands of salivary gland polytene chromosomes. Within this class of mAb, Z4 showed the most prominent localization to interbands (Saumweber et al., 1980). A detailed re-examination of its chromosomal distribution showed an exclusive staining of many interbands and a concomittant absence within bands and the heterochromatic chromocenter (Fig. 1A). Transcriptionally active loci, as exemplified by the *hsp70* heat-shock puffs at cytological location 87A and 87C, are not stained by the Z4 antibody. Instead, Z4 localizes at one edge of each puff (Fig. 1A,d), suggesting an involvement of Z4 in the definition of a functional chromosomal domain.

With the exception of the chromocenter, the pattern of Z4-

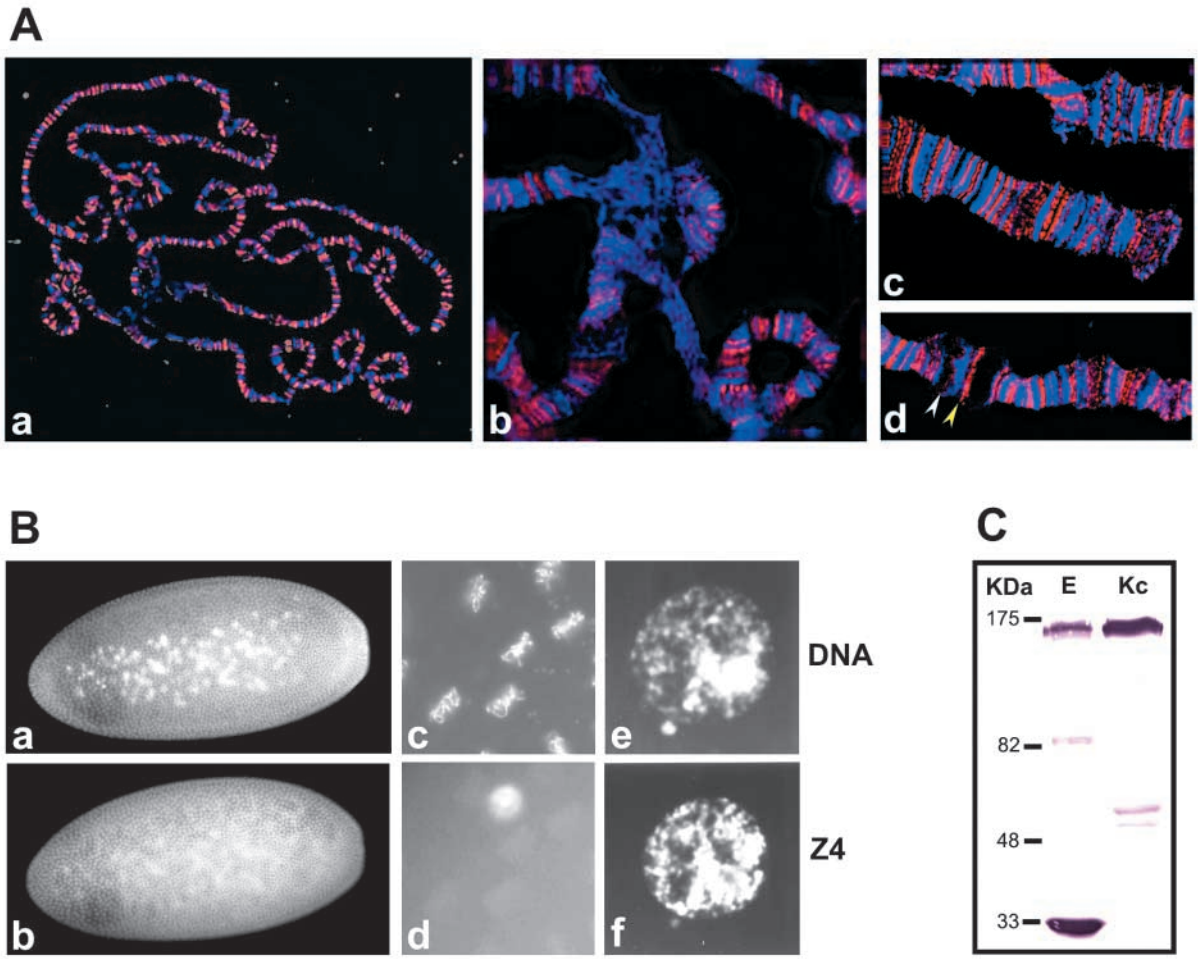

Fig. 1. Z4 expression and localization to chromosomal interbands. (A) Polytene chromosomes of wild-type 3rd instar larvae were double labeled with DAPI (blue) and the Z4 antibody (red). Z4 stains the euchromatic arms of the chromosomes (a) and is absent from the heterochromatic chromocenter (b). Within euchromatin, Z4 stains exclusively the interbands (c). In heat-shocked wild-type larvae Z4 demarcates the distal edge of the 87A *hsp70* heat-shock puff (d, marked by the white arrowhead) and the proximal edge of the *hsp70* heat-shock puff in 87C (yellow arrowhead). (B) Early embryos from wild-type flies (a-d) and Kc cells (e,f) were double labeled with DAPI (a,c,e) and the Z4 antibody (b,d,f). In the early embryo Z4 is ubiquitously expressed. The staining of Kc cells shows that Z4 is chromosomally associated during interphase (e,f). By contrast, Z4 does not associate with mitotic chromosomes of early embryos but disperses within the cell (c,d). (C) Western blot of nuclear proteins from wild-type embryos (E) and Kc cells (Kc) with the Z4 antibody. In addition to antigens that are uniquely present in Kc cells or embryos, the antibody recognizes the 170 kDa Z4 protein, which is present in both fractions.

staining of diploid interphase cells is similar to the pattern of DNA-staining, showing that the Z4 antigen is not concentrated in distinct foci within the nucleus and that it is mainly chromosomally associated (Fig. 1B). The chromosomal localization is lost during mitosis, where Z4 distributes diffusely within the mitotic spindle region (Fig. 1B, c and d). On western blots of nuclear extracts prepared from Kc cells and 0-18-hour-old embryos the Z4 antibody recognizes a major protein with an apparent size of 170 kDa (Fig. 1C). Additional bands at sizes of 85 and 33 kDa are present in the embryo fraction only and two minor bands in the range of 55-60 kDa in the Kc cell fraction. To identify the corresponding gene(s) we screened an expression library containing *Drosophila* embryonic cDNA with the Z4 antibody. Four cDNA clones were isolated and sequenced. A comparison of these sequences with the Berkeley Drosophila Genome Project database (http://www.fruitfly.org/) revealed that they are overlapping clones identical to the 3′-end of the putative gene *CG7752*, which we name *Z4*. A map depicting the genomic organization of the *Z4*-region as predicted from the Drosophila Genome Project is shown in Fig. 2. The 4 kb *Z4* transcript contains two introns and can be translated into a conceptual 996 amino acid protein with a calculated molecular mass of 113 kDa.

Analysis of the amino acid sequence with conventional software tools reveals the presence of seven zinc fingers of the classical C_2H_2-type. They are arranged in two groups with three closely spaced fingers and a single isolated finger between amino acids 239 and 515. Beyond these zinc fingers Z4 doesn't have significant homology neither to other well known protein motifs nor to any other protein sequence contained in the databases. To verify that the isolated *Z4* gene in fact encodes the protein present in the interbands of salivary gland chromosomes we generated transgenic flies expressing a myc-tagged version of the *Z4* gene. The tag was fused to the 3′-end of the *Z4* coding sequence. This modified *Z4* gene, together with 5′ and 3′ genomic regions (see Fig. 2), including regulatory regions sufficient to direct the endogenous expression of *Z4*, was transformed into *Drosophila* embryos. From 3rd instar larvae of a transgenic strain polytene chromosomes were fixed and stained with a monoclonal α-myc-antibody. As can be seen in Fig. 2, the antibody stained the interbands as exemplified for the X-chromosome. This result clearly shows that the *Z4* gene encodes a zinc finger protein that localizes to chromosomal interbands.

Generation of *Z4* mutants

In our search of the Flybase for mutations concerning *Z4* we identified the P element insertion line EP(3)0756 in which the EP element (Rorth et al., 1998) had inserted 106 bp 5′ of the transcription start of *Z4*. The line is homozygous viable lacking an obvious mutant phenotype. To obtain mutants for *Z4* we mobilized the P element in line EP(3)0756 in a standard cross with the Δ2-3 strain, which stably expresses transposase (Robertson et al., 1988), and screened the white-eyed progeny for recessive lethality. From this series of P element excisions we obtained the lines *Z4-1.3* and *Z4-2.1*, which are embryonic lethal, and the line *Z4-7.1*, which is pupal lethal. By PCR-amplification of genomic regions surrounding the site of the EP-element insertion and sequencing of the PCR products, lines *Z4-1.3* and *Z4-7.1* were found to be affected by imprecise excisions of the EP element. The extent of genomic deletions is shown in Fig. 2. Line *Z4-1.3* has a deletion of 800 bp, removing the transcription start site and 143 bp encoding the 5′-UTR of the *Z4* gene. In the pupal lethal line *Z4-7.1* 1500 bp of genomic sequence flanking the site of the EP-element insertion are deleted, but the transcription start site of *Z4* is unaffected. In both lines the putative neighboring gene *CG12974* is affected by deletions encompassing two or three exons of the alternative transcripts CG12974-RA and CG12974-RB, respectively. The lethal mutations of these lines can be complemented by the 7.4 kb

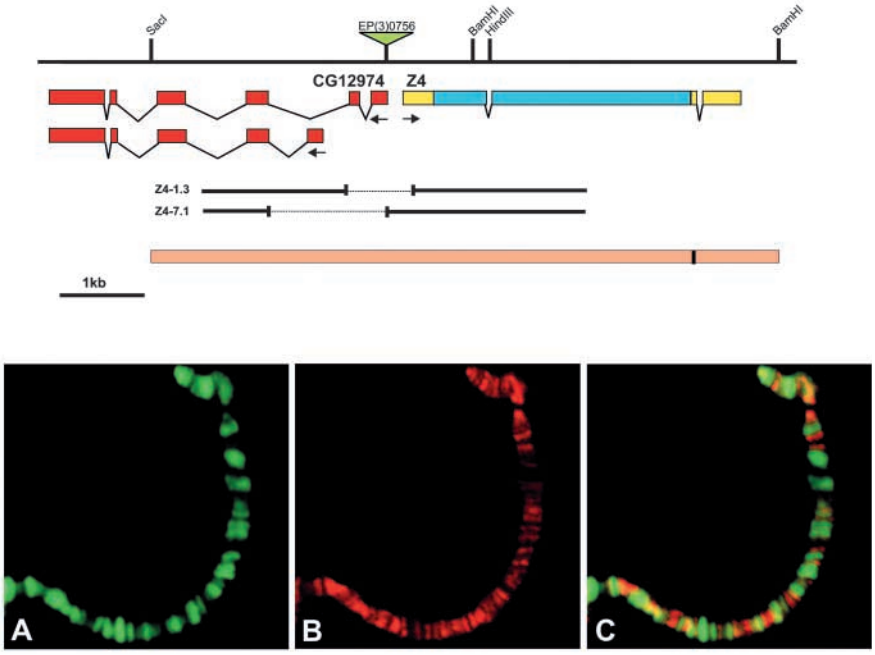

Fig. 2. Generation and verification of *Z4* mutations. The genomic region with the two alternatively spliced exons of the gene *CG12974* (red bars) and the transcript of *Z4* depicting its translated regions (blue bars) and untranslated regions (yellow bars) is shown. Arrows indicate the direction of transcription. The site of the EP-element (green triangle) insertion in line EP(3)0756 is shown, from which deletions were generated by imprecise excision to generate the homozygous lethal lines *Z4-1.3* and *Z4-7.1*. The extent of the deletions is indicated by the broken lines. In line *Z4-1.3* the deletion encompasses 800 bp of DNA including the 5′-transcription start of *Z4* and the 5′-region of the gene *CG12974* encoding two exons of the transcript CG12974-RA. In line *Z4-7.1* 1800 bp to the left of the EP-element are deleted, which removes part of the transcription units of gene *CG12974*, but leaves the coding region of *Z4* unaffected. The bar below indicates the genomic region that complements the lethal mutation of lines *Z4-1.3* and *Z4-7.1*. Within this bar, the vertical line shows the position of the myc-tag that was fused in frame to the 3′-end of the *Z4* coding region. Chromosomes from larvae transgenic for the tagged genomic region were stained with DAPI (A) and a monoclonal anti-myc antibody (B). The composite image (C) reveals the localization of the tagged Z4 protein to the chromosomal interbands.

genomic region encoding the complete *Z4* gene with a 3′ myc-tag (see Fig. 2), resulting in viable adults. As the genomic rescue construct encompasses the complete *Z4* gene but not the gene *CG12974*, for which the 3′-ends of both transcripts are not included, the lethality is complemented due to the expression of *Z4*. Furthermore, the T80-Gal4-induced expression of the *Z4* cDNA in *Z4-1.3* homozygous mutants rescued the embryonic lethality and mutants were able to develop into viable adults. Although it is likely that in the *Z4-1.3* mutant the function of the neighboring gene *CG12974* is also affected, these results nevertheless strongly indicate that failure to express *Z4* leads to embryonic lethality and further suggest that the embryonic lethal lines *Z4-1.3* and *Z4-2.1* are null mutants of *Z4* and that the pupal lethal line *Z4-7.1* represents a hypomorphic *Z4* allele.

Z4 has a dose-dependent effect on w^{m4} position effect variegation

Variegated expression of *white* from the w^{m4}-allele is a very sensitive test system that has extensively been used to uncover various components directly or indirectly influencing the organization of chromatin. The presence of Z4 within the interband regions could be directly connected to a functional role that Z4 might have in the determination and establishment of characteristic band/interband chromatin structures. Although a detailed understanding of the chromatin structures that establish and distinguish a band from an interband is still missing, it is nevertheless widely accepted that chromatin building a band is more compacted than chromatin contained within an interband. Loss of an essential interband determinant may be expected to promote the compaction of chromosomes, which might be uncovered as an enhancement of the variegated expression of w^{m4}. We therefore used this genetic system to test an effect of Z4 on chromatin structure.

w^{m4}/w^{m4} females were crossed with w^{1118}/Y; $Z4^{-}$*/TM3,Sb* mutant males and the eyes of the male progeny were scored for *white* expression (Fig. 3A). Variegated expression of *white* in the *TM3,Sb* control progeny manifested in few red patches of ommatidia in a mainly white background, whereas all three *Z4* mutant chromosomes had a dominant suppressing effect on w^{m4} PEV, resulting in an increase of the amount of red ommatidia within the eye. To quantify the Su(var)-effect of the *Z4*-mutants we extracted the eye-pigments from samples of 20 male heads and measured their concentration photometrically (Fig. 3B). Compared with the control flies the amount of pigments was increased by a factor of 5 in the pupal lethal line *Z4-7.1* and even by 11-fold in the embryonic lethal lines *Z4-1.3* and *Z4-2.1*. Therefore, surprisingly, a reduction of the *Z4* dose enhances the expression of w^{m4}, which categorizes *Z4* as a haplo-suppressor of w^{m4} PEV. Previously, some of the haplo-suppressors of PEV were shown to have a triplo-enhancer effect (see Henikoff, 1996). The influence of three copies of *Z4* on w^{m4} PEV could be investigated with the line *P[Z4myc]2A*, which expresses a transgenic *Z4* gene with a myc-tag (see Fig. 2). This line has homogenous yellow eyes due to the expression of a mini-white gene contained within the P element construct. Nevertheless, the variegated expression of w^{m4} concomittant with the mini-white expression is clearly visible as a scattering of red ommatidia within a yellow background. When w^{m4}/w^{m4} females were crossed with w^{1118}/Y; *P[Z4myc]2A/CyO* males, the w^{m4}/Y; *P[Z4myc]2A/+* male progeny had a significantly reduced amount of red ommatidia compared with the w^{m4}/Y; *CyO/+* males derived from the same cross (Fig. 3C). The expression of w^{m4} was further reduced in the presence of two wild-type and two

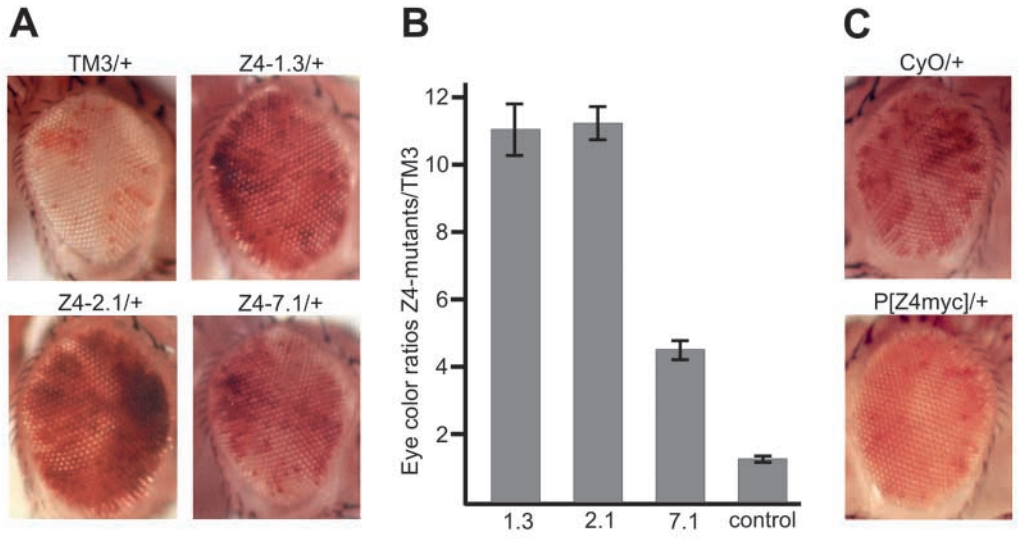

Fig. 3. Dose-dependent effect of *Z4* on *wm4* position-effect variegation. (A) Representative eye phenotypes of males derived from a cross between homozygous *wm4* females and males heterozygous for the *Z4* mutants balanced over *TM3, Sb*. Compared with the males with the *TM3* chromosomes, the eyes of sibling males mutant for *Z4* have increased numbers of red ommatidia. (B) Quantitative measurement of eye pigments of the phenotypes shown in (A). Eye pigments were extracted from the heads of 20 representative individual males for each cross and their absorbance at 480 nm was determined. The columns show the ratios of absorbances between the *Z4* mutants and their *TM3* siblings derived from the identical cross. The control shows the ratios obtained from w^{m4}*/Y;e,st,spo/+* and w^{m4}*/Y;TM3,Sb/+* siblings. (C) Eye phenotypes of males from a cross between w^{m4}/w^{m4} females and w^{1118}*/Y;P[Z4myc]/CyO* males. Males with three copies of the *Z4* gene have significantly reduced numbers of red ommatidia compared with their sibling males with two doses of *Z4*.

transgenic copies of *Z4* (data not shown). This result shows that *Z4* has a dose-dependent opposite effect as a haplo-suppressor and triplo-enhancer on w^{m4} PEV and indicates a possible involvement of Z4 in the establishment or maintenance of 'closed' rather than 'open' chromatin structures.

A *Z4* mutant affects the band/interband organization of polytene chromosomes

The pupal lethal line *Z4-7.1* could be used to visualize possible effects that loss of Z4 might have for the structure of polytene chromosomes. Homozygous 3rd instar larvae of line *Z4-7.1* stay an extensively prolonged time in this phase of development, but they eventually pupariate and die in the early pupal phase. Homozygous mutant Tb^+ 3rd instar larvae were collected from the *Z4-7.1/TM6,Tb* stock and chromosomes from salivary glands were squashed and stained with DAPI. Besides chromosomes with a nearly wild-type appearance many aberrant chromosomes were present, showing a progressive loss of the band/interband structure (Fig. 4A-C). A progressive disintegration of the chromosomal structure due to the loss of Z4 is suggested by the fact that the severity of disintegration of the banded structure was correlated with the time of development from early to late 3rd instar larvae. Mutant chromosomes were not compacted in size; instead, those regions that had lost the band/interband organization had a decompacted and cloudy appearance. This indicates a disintegration of the more compact chromatin bands in the *Z4* mutant rather than condensation of the interbands with a concomitant fusion of neighboring bands, which would result in shortened compact chromosomes. To verify that the altered morphology of the *Z4* mutant chromosomes was not an artefactual result of the squashing procedure, we investigated the chromosomes in whole-mount preparations of salivary glands. In these preparations, wild-type chromosomes show a clear band/interband organization when stained with the DNA dye Sytox Green, and this organization is also obvious by staining with the Z4 antibody (Fig. 4D,G). By contrast, Z4 mutant larvae transheterozygous for the alleles *Z4-7.1* and *Z4-1.3* have strongly reduced levels of Z4 for which an interband localization is no longer discernable. The chromosomes of these mutants have lost the characteristic band/interband structure and are disorganized, showing an overall decompaction of the chromosomal material (Fig. 4E,H). To further analyze the mutant chromosomes in vivo without the need for fixation, we used a transgenic line expressing the chromosomal protein BJ1 fused to GFP. BJ1 binds to the chromosomal bands (Frasch, 1991), which is complementary to the interband localization of Z4. In transgenic lines the fusion protein BJ1-GFP localizes to chromosomal bands as well (H.E. and H.S., unpublished), revealing the banded organization of the chromosomes in native salivary glands (Fig. 4F). When native glands were analyzed from larvae transheterozygous for the mutant alleles *Z4-1.3* and *Z4-7.1*, the GFP signal was diffusely distributed in the nuclei, indicating the loss of chromosomal structure in vivo (Fig. 4I). In conclusion, the analysis of the structure of polytene chromosomes affected in *Z4* mutants by several independent methods revealed Z4 as an important factor in the maintenance of distinct chromatin structures as exemplified by the repetitive band/interband pattern.

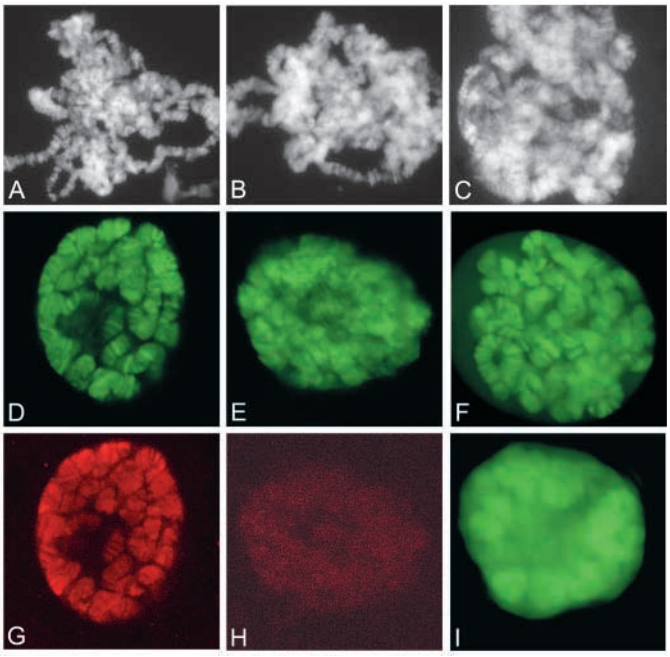

Fig. 4. Morphology of chromosomes mutant for *Z4*. Tb^+ homozygous mutant 3rd instar larvae were collected from the *Z4-7.1/TM6,Tb* stock and chromosomal squashes were stained with DAPI (A-C). Whole mount salivary glands from wild-type (D,G) and *Z4* mutants transheterozygous for the alleles *Z4-1.3/Z4-7.1* (E,H) were double stained with Sytox Green (D,E) and the Z4 antibody (G,H). Unfixed salivary glands dissected from larvae expressing the fusion construct BJ1-GFP (F) or from *Z4-1.3/Z4-7.1* mutant larvae transgenic for BJ1-GFP (I) were analyzed by CLSM. In *Z4* mutants a pertubation of chromosomes with the appearance of decondensed chromatin is evident (C,E,I).

As mentioned above and shown in Fig. 4, the squashed chromosomes from Z4 mutant 3rd instar larvae show varying chromosomal structures ranging from nearly wild-type to chromosomes that have completely lost the band/interband organization. Similar to this variability in chromosome structure mutant chromosomes exhibit a range of staining intensities for the Z4 protein. Besides this quantitative variability we noticed a remarkable qualitative change in the localization of Z4 in mutant chromosomes. In many chromosomal squashes Z4 was no longer present in most interbands but concentrated at a limited number of interbands and some but not all telomeres (Fig. 5A). Because of the poor chromosome morphology, sites of strong Z4 staining could not unambiguously be mapped to defined chromosome regions in the *Z4-7.1* mutant. A similar concentration of Z4 at telomeric sites was obvious in the *Z4-1.3* homozygous mutants that were able to develop to larval stages by expression of the *Z4* cDNA. In these mutants, the telomere of the X-chromosome and the telomere of the chromosomal arm 2L are strongly stained by Z4 (Fig. 5,B,C). We interpret this staining pattern to possibly reflect high-affinity chromosome binding sites of Z4, which might be preferentially occupied under conditions of limiting amounts of Z4.

To investigate a possible opposite effect of Z4 for the structure of chromosomes, we overexpressed the *Z4* cDNA with the UAS-Gal4 system in the salivary glands of transgenic

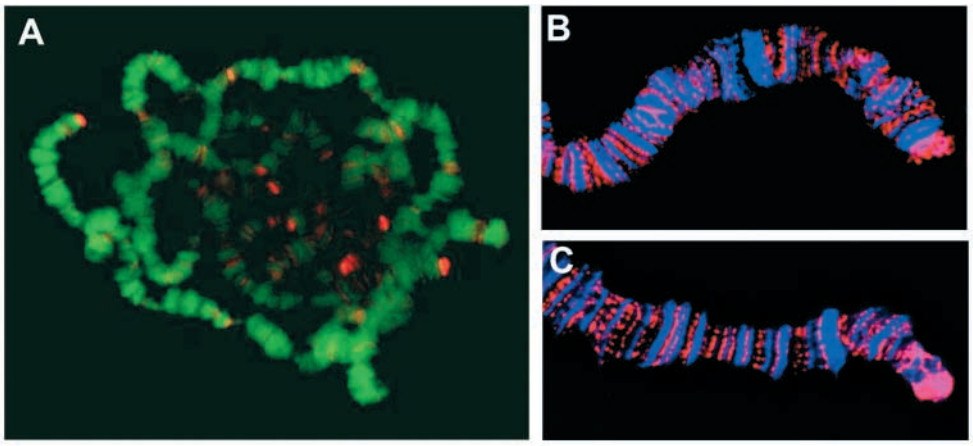

Fig. 5. Presence of Z4 in chromosomes of *Z4* hypomorphic mutants. (A) Chromosomes were squashed from 3rd instar larvae homozygous mutant for *Z4-7.1*. The DNA of the squashed chromosomes was stained with Sytox Green (green) and with the Z4 antibody (red). The composite image shows the localization of Z4 to some telomeres in addition to a few internal chromosomal sites, which are still complementary to the DNA staining. (B,C) Chromosomes from homozygous mutant larvae of the line *Z4-1.3* that were rescued by the expression of the *Z4* cDNA were squashed and stained with DAPI (blue) and the Z4 antibody (red). Compared with its staining of the interbands, Z4 is concentrated on the telomere of the X chromosome (B) and on the telomere of the left arm of chromosome 2 (C).

flies. 3rd instar larvae expressing *Z4* under control of the salivary gland specific Gal4 line *G61* developed glands which were substantially reduced in size compared with wild-type (data not shown). Unfortunately, chromosomes for the investigation of their band/interband structure could not be prepared from these glands. Nevertheless, the impairment of organ development following overexpression of Z4 underscores the dose dependence in the function of Z4 for tissue development. An interference with the normal development was also observed when Z4 was overexpressed in other tissues. Overexpression in the eye driven by eye-Gal4 resulted in small-sized eyes, and ubiquitous overexpression with the T80-Gal4 line was lethal to affected individuals. Therefore, *Z4* is a dose-dependent factor essential for the normal development of many tissues in *Drosophila*.

Z4 binds to DNA in vitro without sequence specificity

The fact that Z4 localizes to most of the chromosomal interbands, together with the finding that *Z4* mutants loose this banded organization, suggests that *Z4* is directly involved in the formation or maintenance of the alternating band/interband pattern. A specificity for interbands could be the result of a direct binding of Z4 to one or several sequence motifs characteristic for interbands. To investigate this possibility we used DNA fragments from the 5′-region of *Notch* for electrophoretic mobility shift assays. By high resolution in situ hybridization the 5′-*Notch*-region has been cytologically mapped to the interband region between polytene bands 3C6 and 3C7. In the fa^{swb} mutant, a *Notch* allele in which 900 bp from the 5′-region of *Notch* are deleted, this interband is missing. Thus, the fa^{swb} sequence of *Notch* is one of a few cases where a sequence has been classified to constitute interband DNA. From the fa^{swb} region the overlapping fragments N1 and N2 (see Fig. 6A) were amplified by PCR, digested with *Hin*fI, end labeled by a fill-in reaction with Klenow and incubated in the presence or absence of bacterially expressed and purified Z4. The products were resolved by native PAGE. From probe N1 two unshifted fragments could be seen, corresponding to the uncut fragment of 225 bp and the *Hin*fI-digested fragment of 208 bp. Probe N2 was separated into two fragments consisting of 144 and 136 bp (Fig. 6B, lanes 1 and 8). Following the addition of Z4 the fragments of both probes were unable to enter the gel and were completely retained in the loading slots (Fig. 6B, lanes 2, 7, 9 and 14). The retention was relieved by the addition of the competitor poly(dI-dC) to the incubation mixture (Fig. 6B, lanes 3-6 and 10-13). Randomly chosen plasmid DNA was as effective as poly(dI-dC) in this competition (data not shown). The retention of the probes in the gel slots indicates that Z4 binds to many sites of the N1-sequence as well as to many sites of both N2-fragments, yielding complexes too large to enter the gel. However, this interaction is not DNA-sequence specific, as several double-stranded DNA sequences effectively competed for the binding of Z4. Nevertheless, Z4 can directly bind to DNA, possibly reflecting its interaction with chromosomes in vivo and its specificity for interband DNA might only be established within the chromatin context, which is not reproduced in an in vitro assay.

Z4 interacts with a novel chromo domain protein

In addition to Z4, several other proteins are expected to be involved in the formation of higher-order chromatin structures that distinguish bands from interbands. To identify such proteins we performed co-immunoprecipitations of Kc cell extracts with the Z4 antibody. SDS-PAGE and Coomassie staining of the immunoprecipitated proteins revealed the expected Z4 protein of 170 kDa and a major protein with a relative molecular mass of 160 kDa (Fig. 7). The analysis of this protein by MALDI-TOF mass spetrometry revealed that it is encoded by the putative gene *CG10712* of unknown function. This protein contains a single chromo domain as a conserved protein motif, and we therefore named this novel

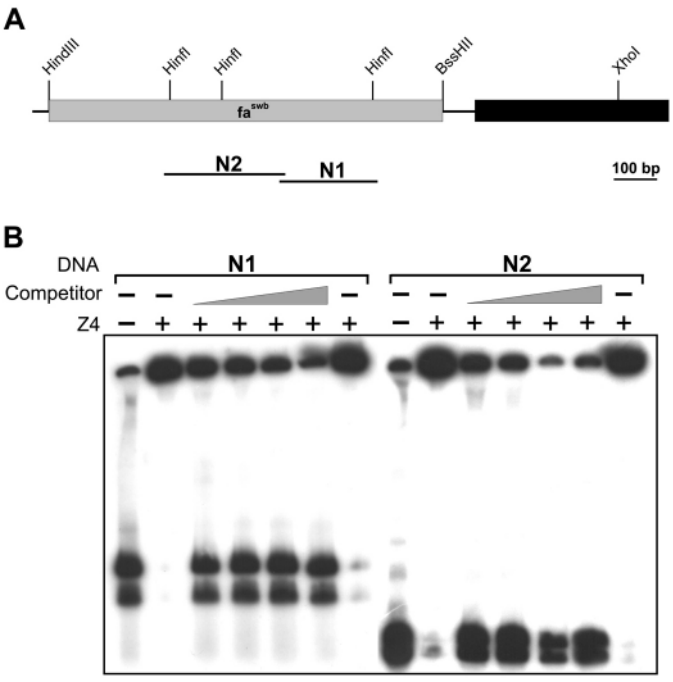

Fig. 6. Binding of Z4 to *Notch* sequences in vitro. (A) 5′-region of Notch. The black bar represents the transcribed region of *Notch*, and the gray bar indicates the region deleted in the fa^{swb}-allele. The position of the N1 and N2 fragments that were used for in vitro binding to Z4 are shown below. Both fragments were digested and endlabeled at the *Hin*fI-sites indicated. (B) Electrophoretic mobility shift assay with 0.5 μg of purified Z4 (omitted in lanes 1 and 8), 5 ng of the end-labeled DNA fragments N1 (lanes 1-7) or N2 (lanes 8-14) and a 100-, 200-, 400- or 800-fold excess of poly(dI-dC) competitor DNA (lanes 3-6 and 10-13) as indicated on the top.

gene *Chriz* (Chromo domain protein interacting with Z4). To investigate the binding of Chriz to chromosomes we generated a transgenic line expressing the myc-tagged Chriz protein in salivary glands. An antibody against myc stained most of the interbands of polytene chromosomes from this line (Fig.7), which is identical to the pattern of staining obtained by the Z4 antibody. The colocalization on chromosomes together with the co-IP of both proteins from Kc cells is strong evidence for an in vivo interaction between Z4 and Chriz. With respect to higher-order chromatin structures, the association of Z4 with a chromo domain protein is very significant, as diverse proteins containing a chromo domain have been shown to participate in the maintenance of epigenetically determined chromatin states.

Discussion

Influence of Z4 on chromatin structure

To gain insight into the protein components involved in the structuring of chromosomes we used the Z4 antibody, which stains most if not all interbands to identify the corresponding protein. The clones isolated from an embryonic expression library contained a unique gene encoding a protein with seven zinc fingers. The identity of the Z4 protein with the antigen present in the interband regions was confirmed by expression of the myc-tagged Z4 protein in transgenic flies and staining of polytene chromosomes with an anti-myc antibody, which showed the same interband specificity. Z4 is an essential protein that is ubiquitously present in all tissues during embryonic and larval development and exerts its function

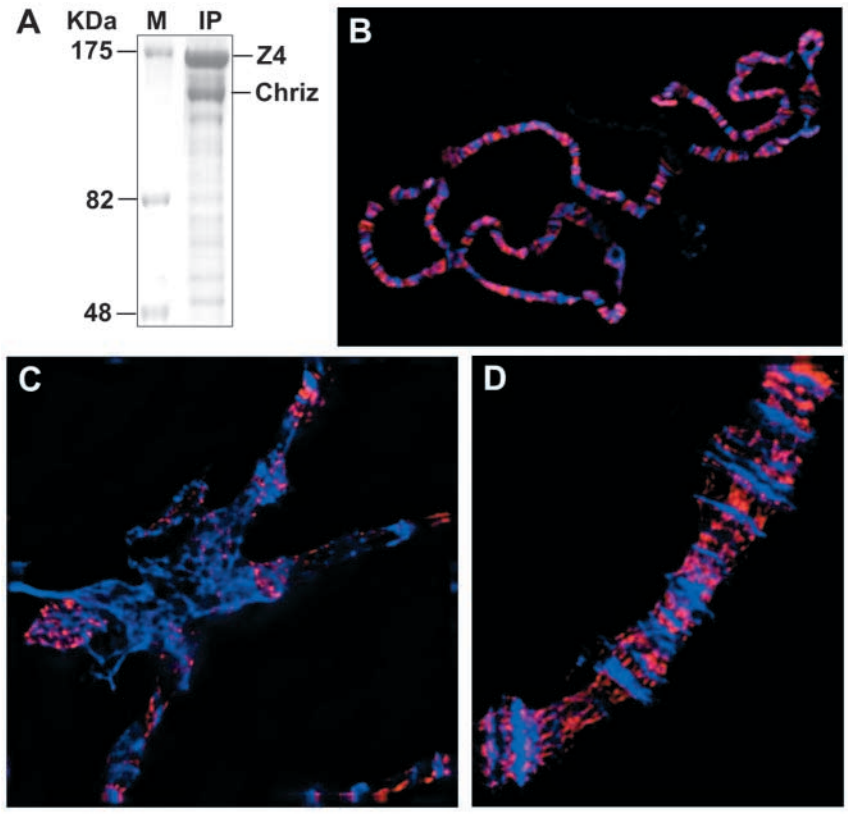

Fig. 7. Identification of a chromodomain protein interacting with *Z4*. (A) Proteins were immunoprecipitated from nuclear extracts prepared from Kc cells with the anti-*Z4* antibody, resolved by SDS-PAGE on a 8% gel and visualized by Coomassie staining. The major band at a relative molecular weight of 150-160 kDa was eluted from the gel and analyzed by MALDI mass spectrometry. The band labeled Chriz is the protein encoded by the gene *CG10712*. (B-D) Polytene chromosomes of a transgenic line that expressed a myc-tagged Chriz protein in salivary glands were stained with an antibody against the myc-tag. The DNA staining (blue) is complementary to the staining of myc-Chriz (red), showing its interband localization on all chromosomes (A). myc-Chriz is restricted to the euchromatic parts of the chromosomes and does not bind to the heterochromatic chromocentre (C). At higher resolution the localization of myc-Chriz to a high number of interbands is obvious from the optical section shown in (D). It is identical to the interband localization of Z4.

within the interphase chromosomes, as it does not bind to the chromosomes during mitosis.

The zinc finger protein motif has been shown to be functionally important for many transcription factors and RNA-binding proteins, qualifying Z4 with its seven zinc fingers to be potentially involved in transcription or RNA metabolism. However, the localization of Z4 to all interbands and the concomittant absence from transcriptional active loci as represented by the puffed regions strengthens the view that Z4 predominantly participates in the formation of particular chromatin structures. Several different chromatin components with an impact on chromatin structure have been identified by their dose-dependent effect on the expression of the variegating w^{m4}-allele. In particular, the genes *Su(var)2-5* (Eissenberg et al., 1990), *Su(var)3-7* (Cléard et al., 1997) and *Su(var)3-9* (Tschiersch et al., 1994) with a haplo-suppressor and triplo-enhancer phenotype were shown to encode proteins associated with heterochromatin. The localization to heterochromatin is in accordance with the presumed function of these proteins to influence the expression of w^{m4} at the euchromatin/heterochromatin border by variably establishing highly compacted repressive chromatin structures. In contrast to these proteins, Z4 does not bind to heterochromatin but is distributed exclusively within the euchromatic part of chromosomes in the interbands. Although the detailed structure of chromatin constituting bands and interbands is unknown, it is generally accepted that DNA contained within an interband is less compacted than DNA contained within a band (Zhimulev, 1996). Therefore, reducing the dosage of *Z4* was expected to favor chromatin compaction, resulting in an enhancement of w^{m4} PEV. Conversely, the overexpression of *Z4* was expected to favor 'open' chromatin structures and lead to a suppression of w^{m4} PEV. Surprisingly, *Z4* in contrast to these expectations turned out to have a haplo-suppressor and a triplo-enhancer effect. This result indicates that Z4 structures chromosomes by supporting the condensation of chromatin. This conclusion is further substantiated by the analysis of chromosomes from 3rd instar larvae mutant for a hypomorphic allele of *Z4*. In these animals chromosomes are evident which have lost the organization into bands and interbands and altogether appear as a less compact mass of chromatin. The loss of chromosomal structure could be the result of an unpairing of the chromosomal fibres that are oriented in parallel bundles in polytene chromosomes. However, we find it rather unlikely that *Z4* might have a primary function in the pairing of chromatids. A null-allele of *Z4* is embryonic lethal, which exhibits an essential function of *Z4* in diploid cells unrelated to chromatid pairing. A possible role of *Z4* could involve the establishment of chromosomal borders that separate chromatin domains of different compaction levels and determine the extent of interband formation. The exact length of DNA included within interbands is still unclear, but has been estimated to range from a few hundred to a few thousand base pairs of DNA (Rykowski et al., 1988; Zhimulev, 1996). Furthermore it is unknown whether Z4 proteins cover the whole length of interbands or are present only at the borders of bands and interbands to exert a classical boundary function. The latter is supported by the finding that within the *hsp70* heat-shock puffs Z4 localizes exactly at one of the borders of each structural domain. This is very reminiscent to the localization of two proteins involved in insulator function, Zw5 and BEAF, to the proximal and distal edges of the 87A puff, respectively (Zhao et al., 1995; Blanton et al., 2003) and suggests common functions in the definition of structural chromosomal domains.

Proteins localizing to chromosomal interbands

In addition to Z4, several different proteins have been shown to localize to the interbands of polytene chromosomes. JIL-1, a protein with two conserved serine/threonine kinase domains is present in hundreds of interbands, with a twofold enrichment on the male X-chromosome compared with autosomes, suggesting an involvement of JIL-1 in the hyperactivation of X-chromosomal genes in the male for dose compensation (Jin et al., 1999; Jin et al., 2000). Hypomorphic mutants of JIL-1 have decreased levels of histone H3Ser10 phosphorylation and chromosomes are highly condensed due to the loss of the euchromatic interbands (Wang et al., 2001). These results provided evidence for a role of JIL-1 in the establishment or maintenance of an open chromatin structure correlated with the interbands to facilitate gene transcription. Quite evident from the chromosomal phenotypes of the corresponding mutants, Z4 and JIL-1 have opposite effects on chromosomal structure, despite the fact that both proteins localize to interbands. This indicates that different activities contribute to the formation of the banding pattern. Although the function of JIL-1 seems to be tightly linked to the modulation of chromatin in interbands to achieve a more decondensed state, the function of Z4 could be primarily associated with the establishment of chromosomal borders influencing the chromatin structure of the chromosomal bands as well.

A correlation of transcription taking place in the interbands is supported by the finding that the elongating form of RNA *Pol*II is found in hundreds of interbands (Kaplan et al., 2000; Armstrong et al., 2002). In addition, transcription factors like Spt5 and Spt6 (Kaplan et al., 2000), CHD1 (Stokes et al., 1996) and the chromatin remodeling complex including Brahma (Armstrong et al., 2002) localize to the less compacted interband regions. An involvement of Z4 in the promoter-selective transcription and/or chromatin remodeling is suggested by the recent finding that Z4 is a component of a macromolecular complex containing the TBP-related factor TRF2, DREF, ISWI and NURF-55 (Hochheimer et al., 2002). However, the chromosomal localizations of the factors involved in general or promoter-selective transcription differs from the localization of Z4 in that the latter is present in nearly all the interbands, whereas the former are found at only a subset of interbands at a few hundred sites. Owing to this difference Z4 is assumed to perform a unique function which is fundamental to the repetitive organization of chromatin into bands and interbands, which in a subset of interbands is possibly used by the transcriptional machinery. Whether this function of Z4 is related to the formation of boundaries is currently unknown. Proteins that bind to boundary or insulator sequences and are distributed in a subset of the interbands in *Drosophila* have been identified with the BEAF-32 (Zhao et al., 1995), Su(Hw) (Spana et al., 1988) and Mod(mdg4) proteins (Dorn et al., 1993). Su(Hw) and Mod(mdg4) are involved in the nuclear organization of about 500 insulator sequences into 20 to 30 insulator bodies, organizing the chromatin fibre into looped domains (Gerasimova and Corces, 1998; Gerasimova et al., 2000). A similar organizing capacity

is not evident for Z4, as Z4 shows a more uniform distribution in Kc cells, lacking a pronounced concentration in a small number of discrete foci. However, owing to the greater number of sites bound by Z4, the number of nuclear foci organized by Z4 could exceed those formed by Su(Hw) and Mod(mdg4) and remain undetected in a low resolution analysis of nuclei stained for Z4.

Specificity of Z4 for interband chromatin

Regardless of the precise chromatin composition that differs between a band and an interband, a primary distinction can be expected to act at the level of the DNA sequence. In this respect the interband DNA should contain one or more sequence motifs that are specifically recognized by one ore more proteins, and Z4 with the seven zinc finger motifs is a potential candidate to exert this function. In vitro, Z4 bound to the interband sequence derived from the 5′ region of *Notch* without sequence specificity. Possibly, the accumulated general affinity of the seven zinc fingers for DNA obscured the specific interaction of one or a few of the fingers with its target site in vitro. Still, the question remains regarding how the targeting of Z4 to the interbands is achieved in vivo. This question is especially relevant as a comparison of the few cases of DNA sequences that were unambiguously mapped to the interband regions revealed that these sequences did not contain a single characteristic shared sequence motif (Demakov et al., 1993). A possible explanation could be given by the capability of Z4 to bind to a variety of consensus sequences, each specifically recognized by single zinc fingers and/or different combinations of the fingers, as has been shown for the vertebrate zinc finger protein CTCF (Filippova et al., 1996).

Alternatively, or in addition to the interaction with DNA, Z4 could bind to a target protein present in interbands. This requires one or a few proteins covering all the chromosomal binding sites of Z4. Until now the novel protein Chriz is the only candidate displaying a chromosomal localization identical to Z4. Significantly, Chriz contains a chromo domain, a motif that has been found in many chromosomal proteins participating in the maintenance of diverse chromatin conformations (Cavalli and Paro, 1998; Eissenberg, 2001). Therefore, Z4 and Chriz seem to be central for the modulation of the higher-order chromatin states distinguishing bands from interbands.

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