Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology

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The POZ domain is a conserved protein–protein interaction motif present in a variety of transcription factors involved in development, chromatin remodelling and human cancers. Here, we study the role of the POZ domain of the GAGA transcription factor in promoter recognition. Natural target promoters for GAGA typically contain multiple GAGA-binding elements. Our results show that the POZ domain mediates strong co-operative binding to multiple sites but inhibits binding to single sites. Protein cross-linking and gel filtration chromatography experiments established that the POZ domain is required for GAGA oligomerization into higher order complexes. Thus, GAGA oligomerization increases binding specificity by selecting only promoters with multiple sites. Electron microscopy revealed that GAGA binds to multiple sites as a large oligomer and induces bending of the promoter DNA. Our results indicate a novel mode of DNA binding by GAGA, in which a large GAGA complex binds multiple GAGA elements that are spread out over a region of a few hundred base pairs. We suggest a model in which the promoter DNA is wrapped around a GAGA multimer in a conformation that may exclude normal nucleosome formation.

Keywords: BTB/POZ domain/chromatin/DNA bending/GAGA/transcription

Introduction

The *Drosophila* sequence-specific DNA-binding protein GAGA is involved in several distinct aspects of chromosome dynamics (Granok *et al.*, 1995; Read and Driscoll, 1997; Wilkins and Lis, 1997). GAGA is encoded by the essential *Trithorax-like* (*Trl*) gene (Farkas *et al.*, 1994) and was first discovered as an activator of the *Ubx* and *en* genes and by its association with heat shock and histone gene promoters (Biggin and Tjian, 1988; Soeller *et al.*, 1988; Gilmour *et al.*, 1989). Normal expression of several developmental genes, including homeotic genes, requires GAGA (Farkas *et al.*, 1994; Bhat *et al.*, 1996). Immunofluorescent staining of polytene chromosomes revealed that many euchromatic genes include binding sites for GAGA, suggesting a general role in transcription control (Tsukiyama *et al.*, 1994; Benyajati *et al.*, 1997).

How does GAGA activate transcription? A number of

studies have shown that GAGA-binding elements coincide with DNase I-hypersensitive sites at promoters, indicative of *in vivo* chromatin remodelling (Wu, 1980; Costlow and Lis, 1984; Cartwright and Elgin, 1986; Lis and Wu, 1993; Lu *et al.*, 1993; Shopland *et al.*, 1995). Biochemical studies indicate that GAGA activates RNA polymerase II transcription by counteracting chromatin repression (Croston *et al.*, 1991; Tsukiyama *et al.*, 1994; Okada and Hirose, 1998). This is likely to involve the disruption of nucleosomes harbouring GAGA-binding sites by GAGA acting together with the energy-dependent chromatin remodelling factor NURF (Tsukiyama *et al.*, 1994; Tsukiyama and Wu, 1995, 1996).

The function of GAGA is not restricted to that of a gene-specific transcriptional activator. *Trl* mutations are dominant enhancers of position-effect variegation, indicating that GAGA counteracts heterochromatic silencing (Farkas *et al.*, 1994). GAGA has also been implicated in the functioning of the polycomb response elements (Strutt *et al.*, 1997). Immunolocalization studies revealed a strong association of GAGA with the GA-rich centric heterochromatin throughout the cell cycle in early embryos (Raff *et al.*, 1994). More recent studies suggested a mitosis-specific association of GAGA with GA-rich satellite DNA (Platero *et al.*, 1998). This observation might be related to a variety of nuclear cleavage cycle defects, displayed by *Trl* mutants, that include asynchrony and failure in chromosome condensation and segregation (Bhat *et al.*, 1996). Thus, GAGA is a multipurpose protein that mediates gene-specific regulation but also plays a global role in chromosome function.

A number of distinct cDNAs have been described that encode different GAGA isoforms (Soeller *et al.*, 1993; Benyajati *et al.*, 1997). The major GAGA species is a 519 amino acid polypeptide (Soeller *et al.*, 1993) and this is the form discussed herein. The GAGA protein contains two well-conserved structural domains: a zinc finger DNA-binding domain (DBD) located within the C-terminal half of the protein, and a so-called BTB/POZ (broad complex tramtrack bric-a-brac/poxvirus and zinc finger) domain at the N-terminus (Figure 1A). Additionally, the C-terminus of GAGA is glutamine rich, a feature characteristic of a subclass of transcriptional activators. The solution structure of the DBD in complex with DNA recently has been determined (Omichinski *et al.*, 1997). The DBD of GAGA comprises a single classical C₂-H₂ zinc finger preceded by a basic helix and tail (Pedone *et al.*, 1996; Omichinski *et al.*, 1997). The GAGA DBD binds DNA as a monomer and does not introduce any major changes in the DNA structure. Although there is considerable variability in GAGA sites, optimal binding sequences contain a minimal GAGAG pentanucleotide. DNA contacts are made in the major as well as the minor groove and extend over one turn of the DNA helix.

Fig. 1. Expression and purification of GAGA polypeptides. (A) Schematic representation of full-length GAGA, a deletion mutant lacking the POZ domain (Δ POZ) and the isolated DBD. All polypeptides contained an N-terminal HA tag. The polyglutamine-rich region (PolyQ) and a basic region (++) are indicated in addition to the DBD and POZ/BTB domain. (B) These recombinant proteins were expressed in Sf9 cells, immunopurified from cell extracts using an anti-HA column and eluted with a peptide corresponding to the HA epitope. Aliquots of the eluted proteins were separated by SDS-PAGE and visualized with Coomassie Blue staining. The positions and molecular weights (kDa) of protein standards are indicated on the left.

The BTB/POZ domain, from here on referred to as the POZ domain, is an evolutionarily conserved region of ~120 amino acids present in a family of metazoan zinc finger transcription factors as well as in a restricted number of actin-binding and poxvirus proteins (Godt *et al.*, 1993; Bardwell and Treisman, 1994; Zollman *et al.*, 1994). In humans, some POZ domain transcription factors have been implicated in a number of cancers. These include BCL-6 (Ye *et al.*, 1993; Chen *et al.*, 1998), PLZF (Chen *et al.*, 1993; Dong *et al.*, 1996; X.Li *et al.*, 1997) and APM-1 (Reuter *et al.*, 1998). The PLZF POZ domain is involved in the recruitment of histone deacetylase co-repressor complexes (Grignani *et al.*, 1998; Guidez *et al.*, 1998; Lin *et al.*, 1998). The POZ domain provides a protein-protein interaction interface implicated in transcriptional activation as well as repression by POZ transcription factors (Deweindt *et al.*, 1995; Chang *et al.*, 1996; Seyfert *et al.*, 1996; Kaplan and Calame, 1997). POZ domains can associate with each other, most likely via a coiled-coil type of interaction. Binding is specific and restricted to certain combinations of POZ-containing proteins (Bardwell and Treisman, 1994; Dong *et al.*, 1996). Previously, it has been shown that the presence of the

POZ domain inhibits DNA binding (Bardwell and Treisman, 1994; Kaplan and Calame, 1997). This observation raises the question of how POZ transcription factors bind to their target promoters.

In this study, we address the function of the GAGA POZ domain in promoter recognition. Natural target promoters for GAGA typically contain multiple GAGA-binding elements. DNase I footprinting and bandshift experiments revealed that GAGA binding to these multiple sites is highly co-operative and critically depends on the POZ domain. The effects on DNA binding correlate with POZ domain-mediated multimerization of GAGA as revealed by protein cross-linking and size exclusion chromatography. We used electron microscopy (EM) to examine the effects of GAGA binding on promoter topology. Based on our DNA binding, protein cross-linking and EM experiments, we propose a model for a GAGA-promoter DNA complex. The implications of this model for GAGA-mediated chromatin remodelling are discussed.

Results

The POZ domain inhibits GAGA binding to single sites

To examine the role of the GAGA POZ domain in promoter recognition, we expressed hemagglutinin (HA) epitope-tagged full-length GAGA, a deletion mutant lacking the POZ domain (Δ POZ) and the isolated DBD in Sf9 cells infected with recombinant baculoviruses. These proteins were immunopurified to near homogeneity utilizing the HA epitope (Figure 1). We compared the DNA-binding activities of about equal molar amounts of these different polypeptides to a consensus GAGA-binding site (1 $\times$ GAGA). The bandshift assay revealed that the DBD and Δ POZ efficiently bind to a canonical single site whereas full-length GAGA binds only very weakly (Figure 2A).

DNase I footprinting analysis on a DNA fragment harbouring a single GAGA site confirmed that the presence of the POZ domain has an inhibitory effect on DNA binding (Figure 2B). However, DNA binding is not completely blocked since at the highest protein concentration, full-length GAGA does protect the canonical GAGA site against digestion (Figure 2B, lane 4). The footprinting borders of all three GAGA polypeptides are similar, confirming that the previously identified DNA-binding domain is responsible for contacting the DNA (Pedone *et al.*, 1996). From these bandshift and DNase I footprinting experiments, we estimated an ~6- to 9-fold reduction in DNA-binding affinity due to the presence of the POZ domain.

In addition, we tested the DNA-binding activity of a number of other GAGA deletion mutants. However, these experiments showed that the inhibition of DNA binding is caused solely by the POZ domain (data not shown). These observations for GAGA agree well with a previous study which demonstrated inhibition of DNA binding by the ZID POZ domain (Bardwell and Treisman, 1994). Why would a transcription factor contain a domain that inhibits DNA binding? To address this issue further, we studied the binding of GAGA to several of its natural target promoters.

Fig. 2. The GAGA POZ domain inhibits binding to single sites. **(A)** DNA binding of GAGA (lane 2), Δ POZ (lane 3) and DBD (lane 4) was compared using a double-stranded radiolabelled oligonucleotide containing a single optimal GAGA-binding site (1xGAGA; left panel). Lane 1 contains no protein. The arrows indicate the position of the free DNA and the protein-DNA complexes. The protein concentration in each binding reaction was ~15 nM, as calculated from the concentrations of the original protein stocks (see Materials and methods). To ensure that approximately equal molar amounts of protein were added to the binding reactions, aliquots were analysed by SDS-PAGE followed by Western blot analysis using the α -HA antibody (right panel). **(B)** DNase I footprinting analysis of GAGA polypeptides on a probe containing a single GAGA site (1xGAGA). The double-stranded oligonucleotide used for the bandshift assay was cloned into the Bluescript KS vector and a radiolabelled DNA probe was prepared. DNase I digestion patterns in the presence of no protein (–, lanes 1, 5, 6, 10, 11 and 15) or increasing amounts of GAGA (lanes 2–4), Δ POZ (lanes 7–9) or DBD (lanes 12–14). The concentrations of GAGA polypeptides in these reactions were increased by 3-fold increments from 15 to 135 nM (as indicated by the triangles above the lanes). Products were analysed on a 7% polyacrylamide gel in parallel with a G + A sequence reaction. The distances from the labelled end in base pairs are indicated. The black rectangle indicates the position of the GAGA element, and the protected region is indicated with a bracket.

The POZ domain mediates co-operative binding of GAGA to natural target promoters

Promoters regulated by GAGA have been identified by *in vivo* as well as *in vitro* studies. We have chosen the previously well characterized *Ubx*, *ftz*, *hsp70* and *eve* promoters to compare the binding of GAGA polypeptides. We note that all these promoters are characterized by the presence of multiple GAGA-binding sites (Biggin and Tjian, 1988; Read *et al.*, 1990; Topol *et al.*, 1991; Weber *et al.*, 1997). DNase I footprinting experiments revealed a dramatic difference in DNA-binding properties between full-length GAGA and the polypeptides lacking the POZ domain (Figure 3). The GAGA elements on the natural promoters are bound efficiently by full-length GAGA but not by equal molar amounts of either Δ POZ or DBD. The amount of GAGA required to bind the multiple promoter elements is significantly lower (>4- to 12-fold, depending on the promoter) than that required to bind a single site, indicative of co-operative DNA binding. The spacing of the GAGA elements in these different promoters varies considerably (Figure 3B). However, GAGA appears to be quite flexible and able to bind co-operatively to GAGA sites located at variable distances from each other. The *hsp70* promoter is generally GA rich and, at increasing GAGA concentrations, the footprints start to spread and

most of the promoter DNA is protected against digestion, as has been observed previously (Weber *et al.*, 1997).

In contrast to full-length GAGA, equal molar amounts of the Δ POZ or DBD polypeptides fail to bind the GAGA target promoters significantly. On the *Ubx*, *ftz* and *eve* promoters, protection of a single GAGA site by Δ POZ and DBD can be observed. As expected, these sites are the ones that most closely resemble the optimal GAGA-binding sequence. In these experiments, Δ POZ and DBD fail to bind to the weaker GAGA sites. This indicates that POZ-mediated co-operativity increases the binding affinity for these sites by at least one order of magnitude. Together, these DNase I footprinting experiments demonstrate that efficient binding of GAGA to its natural target promoters depends critically on the presence of the POZ domain, in addition to the DBD.

The promoters studied above contain weak as well as strong GAGA sites. To characterize the co-operative nature of GAGA-DNA interactions further, we synthesized a 64 bp oligonucleotide (5xGAGA) containing five optimal GAGA sites. DNase I footprinting analysis of GAGA binding to these artificial sites demonstrates strong co-operative binding that is dependent on the POZ domain (Figure 4A). In spite of the optimized binding sites in the 5xGAGA probe, we estimate that the binding affinity of

Fig. 3. Synergistic promoter recognition by GAGA requires the POZ domain. (A) DNase I footprinting analysis of GAGA binding to four natural *Drosophila* target promoters, *Ubx*, *ftz*, *hsp70* and *eve*. All fragments were labelled on the bottom strand using T4 polynucleotide kinase and were incubated with either no protein (–, lanes 1, 5, 6, 10, 11 and 15) or increasing amounts of GAGA (lanes 2–4), Δ POZ (lanes 7–9) or DBD (lanes 12–14), followed by partial DNase I digestion. The concentrations of GAGA and polypeptides in these reactions were increased by 3-fold increments from 12 to 108 nM (as indicated by the triangles above the lanes). Products were analysed on a 7% polyacrylamide gel in parallel with a G + A sequence reaction. The location of the fragments on the gel relative to the transcriptional start site (+1) and the location of the GAGA elements (black rectangles) are indicated. An open rectangle denotes a GA-rich sequence in the *hsp70* promoter. Regions protected by GAGA (brackets closest to the panel), Δ POZ and/or DBD are indicated by brackets. (B) Schematic representation of the promoters used in this study (see Materials and methods for references). The GAGA-binding elements are represented by filled ellipses.

the Δ POZ and DBD is >9-fold lower than that of full-length GAGA (Figure 4A, compare lanes 2, 9 and 14).

The close spacing of the GAGA-binding sites allowed us to use this fragment in bandshift assays. Labelled 5×GAGA oligonucleotide was incubated with increasing amounts of either full-length GAGA, Δ POZ or DBD and analysed by native PAGE (Figure 4B). Again, the DNA-binding properties of GAGA and the POZ domain-lacking polypeptides are strikingly different. At higher concentrations of Δ POZ or DBD, distinct slower migrating complexes gradually appear which correspond to increased occupancy of binding sites.

In contrast, in reactions containing GAGA, the transition from mostly free DNA to complete occupancy is quite sharp. When bound, the vast majority of probe immediately

shifts to a complex of very slow mobility, indicative of binding by multiple GAGA molecules. Together, the footprinting and the bandshift results indicate that GAGA binds multiple sites simultaneously (Figure 4A and B). It should also be noted that the amount of GAGA that produces a full shift with the 5×GAGA probe fails to bind the 1×GAGA probe significantly (compare Figure 2A, lane 2 and Figure 4B, lane 4). These results, using optimal binding sites, again demonstrate that GAGA binds DNA co-operatively.

Since the binding sites within the 5×GAGA probe are about equivalent, a shift in mobility can result from binding to any of these sites. Although the majority of DNA molecules are bound at the higher concentrations of Δ POZ or DBD polypeptides, two observations indicate

Fig. 4. GAGA binds co-operatively to multiple optimal GAGA-binding sites. **(A)** An oligonucleotide containing five closely packed optimal GAGA sites (5×GAGA) was cloned into the pBluescript KS vector. A labelled fragment was prepared and incubated with either no protein (–, lanes 1, 5, 6, 10, 11 and 15) or increasing amounts of GAGA (lanes 2–4), ΔPOZ (lanes 7–9) or DBD (lanes 12–14), followed by partial DNase I digestion. As in the previous experiment, the concentrations of GAGA polypeptides in these reactions were increased by 3-fold increments from 12 to 108 nM (as indicated by the triangles above the lanes). Products were analysed on a 7% polyacrylamide gel in parallel with a G + A sequence reaction. The location of the fragments on the gel relative to the transcription start site (+1), the position of the five GAGA elements (black rectangles), and regions protected against DNase I digestion (brackets) are indicated. **(B)** Bandshift analysis of GAGA, ΔPOZ and DBD binding to the 5×GAGA oligonucleotide. The 5×GAGA probe was incubated with either no protein (lanes 1, 8 and 15) or increasing concentrations (3.75, 7.5, 15, 30, 60 and 120 nM) of either GAGA (lanes 2–7), ΔPOZ (lanes 9–14) or DBD (lanes 16–21), and the resulting complexes were analysed by native PAGE. The numbers on the right of each panel indicate the protein–DNA complexes that correspond to the increasing occupancy of the GAGA elements. In the case of GAGA, a broad band of very slow mobility is indicated (complex) as well as a minor band that migrates like a single GAGA–DNA complex. We suspect that the latter is the result of dissociation during electrophoresis.

that a full site occupancy has not been reached in these reactions. First, not all the probe DNA is in the slowest mobility complexes, and secondly, there is no clear DNase I protection. Thus, deletion of the POZ domain results in less efficient binding to multiple binding elements even if these sites are optimal. It is pertinent to note that on natural promoters the majority of binding sites are suboptimal (Figure 3). GAGA binding to these physiological sites is even more dependent on synergism.

In conclusion, although GAGA contacts the DNA via the DBD, efficient and co-operative promoter binding depends critically on the presence of the POZ domain. How does the POZ domain mediate synergistic DNA binding?

The POZ domain mediates GAGA oligomerization

Since it seemed likely that co-operative DNA binding of GAGA involves protein–protein interactions, we next investigated the oligomeric status of GAGA. Pure GAGA or ΔPOZ polypeptides were treated with the covalent cross-linkers glutaraldehyde (GA) or, the more stringent, ethylene glycol bis(succinimidylsuccinate) (EGS) and resolved by SDS–PAGE (Figure 5A). In both GA- and EGS-treated samples, higher order oligomeric species were readily detected for GAGA whereas the ΔPOZ polypeptide did not form oligomers. Likewise, the DBD does not form higher order complexes (data not shown). The mobilities of the main cross-linked GAGA complexes suggest that they may correspond to dimers, trimers, tetramers and hexamers. Addition of a DNA fragment harbouring multiple GAGA sites did not alter the oligomeric status of GAGA, suggesting that protein–protein

interactions occur in solution and do not require DNA binding.

To determine the apparent native molecular weight of GAGA, we performed size exclusion chromatography with pure recombinant GAGA (Figure 5B). The elution profile of GAGA, as detected by protein immunoblotting, is surprisingly broad. GAGA migrates with an apparent molecular mass between ~60 and 600 kDa. This result agrees well with the cross-linking studies and suggests that GAGA is present in various oligomeric complexes ranging from monomers to very large multimers. GAGA does not seem to aggregate in an uncontrolled fashion since no GAGA eluted in the void of the column and the elution profile has distinctive borders. Together, these results suggest that POZ domain-mediated oligomerization of GAGA underlies co-operative DNA binding to multiple binding sites.

Consequences of GAGA binding for the trajectory of the *Ubx* promoter DNA

We next considered the consequences of GAGA oligomer binding to a natural promoter harbouring multiple binding elements. In particular, we were interested in the possibility that promoter binding by the GAGA complex might alter the DNA trajectory. To visualize the GAGA–promoter complex, we have employed EM. For these experiments, we have used the *Ubx* promoter that contains four GAGA-binding sites between positions –193 and –30 relative to the transcription start site (Biggin and Tjian, 1988) (Figure 3B). We used a 4 kbp DNA fragment containing a centrally located 1 kbp *Ubx* promoter sequence, with the 163 bp

Fig. 5. The POZ domain mediates GAGA oligomerization. **(A)** The recombinant GAGA and Δ POZ polypeptides were incubated without cross-linker (-), with GA (left panel) or with EGS (right panel). Experimental details are described in Materials and methods. Proteins were separated by SDS-PAGE (left panel 8%; right panel 5%) and visualized by Western blot analysis using the α -HA monoclonal antibody. GA cross-linking was performed in the absence or presence of the 5 $\times$ GAGA oligonucleotide (DNA), as indicated. The positions and molecular weights (kDa) of protein standards, GAGA monomers and Δ POZ are indicated. Arrows indicate GAGA oligomers. **(B)** Gel filtration analysis of purified GAGA by Sephacryl S-300 chromatography. Fractions collected were examined for the presence of GAGA by SDS-PAGE followed by Western blot analysis using the α -HA monoclonal antibody. The elution volume (V_e) is indicated. The column was calibrated with the following molecular mass markers (Pharmacia): BSA (B, 67 kDa), catalase (C, 232 kDa), ferritin (F, 440 kDa), thyroglobulin (T, 669 kDa) and blue dextran (D, 2000 kDa, void volume). The peak migration of these markers is indicated above the lanes.

region containing the four GAGA sites 2.3 and 1.5 kbp from the DNA ends. This DNA fragment was incubated with either GAGA or Δ POZ purified to near homogeneity. Electron micrographs of GAGA–DNA complexes reveal a roughly spherical protein mass located centrally on the DNA fragment (Figure 6C and D). This protein mass has a diameter of ~ 150 – 300 Å, consistent with the presence of a large protein complex. In contrast, DNA fragments incubated with Δ POZ were indistinguishable from naked DNA (Figure 6A and B). This observation again highlights the critical role of the POZ domain in oligomerization and promoter recognition. The specificity of GAGA binding was established further by the generation of DNA fragments in which the *Ubx* promoter sequence is positioned near the end. As expected, on these fragments, the protein mass associates with the end of the fragments (data not shown). These EM results reinforce the notion that GAGA binds multiple sites, within ~ 200 bp of DNA, as a single large oligomeric complex.

In order to analyse possible changes in the DNA trajectory in more detail, we examined GAGA in complex with shorter DNA fragments (Figure 6E–H). In these 1055 bp fragments, the *Ubx* promoter is located slightly off centre, with the 163 bp region containing the four GAGA sites 466 and 426 bp from the DNA ends. Visualization clearly indicated that in the majority of complexes the promoter DNA is bent. The observed centre of DNA bending is located close to the centre of the protein mass, and we estimate a mean bend angle of ~ 80 – 90° . Interestingly, in $\sim 19\%$ of the complexes we observed, the GAGA oligomer binds two DNA molecules simultaneously (Figure 6F). Our biochemical and EM

experiments suggest that GAGA can indeed form sufficiently large multimers to occupy the GAGA sites present in these two DNA fragments. These results point to the possibility that GAGA acts to bring distant DNA elements together.

Discussion

The POZ domain is a structural motif present in a large number of zinc finger transcription factors involved in developmental regulation and cancer. Here, we have addressed the role of the GAGA POZ domain in promoter recognition. Natural promoters regulated by GAGA are characterized by the presence of multiple GAGA sites, which are typically spread over a region of a few hundred base pairs. Moreover, *in vivo* experiments have indicated that GAGA requires multiple binding sites to stimulate transcription efficiently (Soeller *et al.*, 1993; Granok *et al.*, 1995). We have used bandshift and DNase I footprinting experiments to establish that GAGA binding to natural target promoters is highly co-operative. Synergistic DNA binding depends critically on the presence of the POZ domain. The POZ domain provides a protein–protein interaction interface that mediates GAGA oligomerization into higher order complexes as revealed by protein cross-linking and gel filtration chromatography experiments. The EM experiments directly show that GAGA binds multiple DNA elements as a large oligomer. Taken together, our results suggest that promoter recognition by GAGA requires two distinct, well separated protein domains, the zinc finger DBD that contacts the DNA and the POZ domain that directs GAGA oligomerization.

Fig. 6. Electron microscopy of the GAGA-*Ubx* promoter complex. (A and B) Representative EM fields showing a DNA fragment containing the *Ubx* promoter in the presence of Δ POZ. The DNA fragment is ~4 kbp long and contains 1 kbp from the *Ubx* gene-encompassing promoter region. The 163 bp promoter region containing the four GAGA sites (-193 to -30 relative to the transcription start site) is 2.3 and 1.5 kbp from the respective DNA ends. DNA fragments in the presence of Δ POZ are indistinguishable from naked DNA. (C and D) The same DNA fragments in complex with full-length GAGA. Two representative EM fields are shown. (E-H) Four representative images of full-length GAGA in complex with a 1055 bp *Ubx* promoter fragment. The 163 bp promoter region containing the four GAGA sites is 466 and 426 bp from the DNA ends. The protein complexes are indicated with arrows and the bars represent 100 nm. Quantitative analysis of the EM data revealed that in ~70% of the GAGA-DNA complexes (206 out of 300) the DNA is bent under an angle sharper than 100° . Although the distribution is broad, we estimate a mean bend angle of ~80–90°. A broad distribution of bend angles is expected since in EM, one dimension is lost and complexes will have different orientations on the sample grid.

Fig. 7. A speculative model for GAGA-promoter binding. The POZ domain and DBD of GAGA are indicated. Open boxes represent GAGA-binding sites. The model intends to illustrate the oligomerization of GAGA mediated by the POZ domain, the accessibility of the DNA in between the GAGA sites and the bending of the promoter DNA as a result of the GAGA complex binding. As a comparison, the promoter DNA wrapped around a histone octamer is shown. The precise stoichiometry of the GAGA multimers might vary and we suspect that not all GAGA molecules within a multimeric complex bind DNA.

A model for promoter binding by GAGA factor

What are the consequences of the binding of a GAGA oligomer for the topology of the promoter DNA? The association of a GAGA oligomer with multiple binding sites within a promoter is expected to constrain the DNA trajectory. Indeed, electron micrographs of the GAGA-bound *Ubx* promoter indicate that the DNA in the complex is bent and might be wrapped around the surface of a GAGA multimer. Since the solution structure of a GAGA DBD-DNA complex did not reveal a major distortion of the DNA (Omichinski *et al.*, 1997), we do not think that the DNA bending is caused directly by the DBD-DNA contacts. Rather, we favour the idea that DNA bending is the consequence of the DNA wrapping around the GAGA complex.

The reorganization of the *Ubx* promoter due to binding of the GAGA complex is depicted in a schematic model in Figure 7. To illustrate that the GAGA sites are spread over a distance of about one nucleosome repeat length (of ~200 bp), we have included a cartoon of the DNA wrapped around a nucleosome. It should be noted that within a nucleosome, most of the DNA is in close contact with the histones. In contrast, GAGA only contacts the GAGA elements, resulting in a more accessible DNA-protein complex. This model also provides some clues as to why the binding of GAGA to the promoter involves changes in the local chromatin structure. Since the GAGA DBD clamps almost one turn of the DNA (Omichinski *et al.*, 1997), GAGA binding to multiple sites within a nucleosome repeat length is expected to severely compromise

histone-DNA contacts. These contacts might be hampered further by DNA bending and wrapping around a GAGA oligomer. However, it is not clear whether GAGA binding leads to complete displacement of the histone core or whether some histone-DNA contacts are preserved (Tsukiyama *et al.*, 1994; Tsukiyama and Wu, 1995). In summary, after transient chromatin remodelling by NURF to allow for GAGA binding, GAGA may function as an architectural factor that reorganizes the promoter DNA and maintains it in an open conformation.

Within many GAGA target promoters, binding sites for distinct transcription factors are located between the GAGA elements. An attractive possibility is that after binding of the GAGA complex, these sites become accessible to other factors which are unable to overcome the nucleosome barrier by themselves. Indeed, there is *in vivo* evidence that such a mechanism occurs at the *hsp70* promoter where GAGA binding facilitates subsequent binding by the heat shock factor (Lis and Wu, 1993; Shopland *et al.*, 1995). POZ domain-mediated complex formation might also be important for the association of GAGA with the highly repetitive DNA sequences that form the bulk of *Drosophila melanogaster* heterochromatin (Raff *et al.*, 1994; Platero *et al.*, 1998).

The POZ domain and DNA binding

The results described here show that the GAGA POZ domain plays a critical role in promoter selectivity. The functioning of the POZ domain differs in several aspects from that of other protein-protein interaction domains, such as Leu zipper or helix-loop-helix motifs, involved in DNA binding. The latter motifs are integral parts of the DBD and mediate the binding of protein dimers to dimeric DNA sites (McKnight and Yamamoto, 1992). In contrast, multiple, well separated GAGA elements are each contacted by a single GAGA molecule that is part of an oligomer. This distinction is also reflected in the protein structure of POZ transcription factors. Within these proteins, the POZ domain is well separated from the zinc finger DBD.

Zeste, a member of the *Trithorax* group of proteins, binds DNA in a manner that is reminiscent of that of GAGA. Although lacking a POZ domain, it forms oligomers that bind co-operatively to multiple sites (Chen and Pirrotta, 1993a,b). Thus, synergistic DNA binding by higher order complexes might be a more common mechanism of DNA binding.

Why does the POZ domain hinder single site recognition by GAGA? Two, not mutually exclusive models, might explain this inhibition. First, the POZ domain may fold back and block DBD-DNA contacts. Secondly, inhibition might be a consequence of multimerization. In the absence of multiple binding sites, GAGA molecules within a complex may inhibit binding to a single site due to steric hindrance. In other words, the GAGA complex may not fit on the DNA if only one binding site is available. Since the presence of the POZ domain can also inhibit DNA binding of heterologous DBDs (Bardswell and Treisman, 1994), the notion of it specifically contacting the DBD and hampering DNA contacts seems less likely. Alternatively, as a consequence of multimerization, the DBD might be less accessible within the GAGA complex.

Multiple binding elements may be required to 'unwrap' the GAGA oligomer to allow for efficient DNA binding.

Another potential role for GAGA oligomerization might be to assist communication between distal enhancers and promoters. In our EM experiments, we frequently observed that a GAGA oligomer binds two DNA molecules simultaneously. This suggests that POZ–POZ interactions may lead to the association of enhancer- or PREs-bound GAGA with promoter-bound GAGA. Future experiments will address the potential role of GAGA in DNA looping and long-distance effects within a chromosomal context.

We propose that the POZ domains in other POZ transcription factors perform similar functions to that which it has in GAGA, and mediate co-operative DNA binding to multiple sites but inhibit single site recognition. It should be noted that POZ–POZ interactions might result in the formation of heteromeric complexes containing distinct POZ transcription factors. Several studies have demonstrated the functional importance of the POZ domain in transcriptional repression as well as activation (Deweindt *et al.*, 1995; Seyfert *et al.*, 1996; Dhordain *et al.*, 1997; Kaplan and Calame, 1997). Part of such an involvement in opposite functions may simply reflect the need for the POZ domain in order to direct the POZ transcription factors to target promoters.

Deregulated expression of BCL-6, a POZ domain transcriptional repressor, occurs in the majority of diffuse large cell lymphomas cases and a significant portion of follicular lymphomas (Ye *et al.*, 1993; Chang *et al.*, 1996; Chen *et al.*, 1998). As we have shown here, due to co-operative DNA binding mediated by the POZ domain, small changes in protein concentration can lead to dramatic differences in promoter occupancy. Likewise, it is tempting to speculate that the effects of misregulation of BCL-6 might be amplified by synergistic DNA binding, causing changes in gene expression that may promote leukaemic transformations. Translocation of the POZ domain-containing N-terminal half of the zinc finger protein PLZF to the retinoic acid receptor α (RAR α) is associated with a subset of acute promyelocytic leukaemias. Further studies have indicated that the PLZF POZ domain is responsible for the aberrant activities of the fusion protein (Chen *et al.*, 1993, 1994; Dong *et al.*, 1996; J.Y.Li *et al.*, 1997; Ruthardt *et al.*, 1997). The PLZF POZ domain is involved in the recruitment of histone deacetylase co-repressor complexes (Grignani *et al.*, 1998; Guidez *et al.*, 1998; Lin *et al.*, 1998). This function explains at least part of the dominant-negative effects of the POZ domain in the PLZF–RAR α fusion protein. Additionally, the presence of the POZ domain is likely to interfere with the binding to normal RAR α target promoters and may divert the fusion protein to other genes, resulting in an aberrant pattern of gene expression.

In summary, our findings reveal a novel role for the POZ domain in mediating co-operative DNA binding. The POZ domain is required for the formation of higher order GAGA oligomers. The GAGA complex binds target promoters by contacting multiple binding sites that are spread out over a region of a few hundred base pairs and induces bending of the promoter DNA. These results may provide a paradigm for DNA recognition by other POZ transcription factors.

Materials and methods

Expression and purification of recombinant proteins

An *NdeI* site was created at the initiating methionine of GAGA using a PCR-based strategy. The complete GAGA-coding sequence was cloned into a modified version of the pVL1392 (PharMingen) baculovirus expression vector, pVL1392HAX, containing an HA tag (YPYDVPDYA)-encoding sequence followed by the amino acids IEGRHM (factor X cleavage site). Using a similar strategy, DNA fragments encoding the Δ POZ mutant (amino acids 120–519) and the DBD mutant (amino acids 321–379) were cloned into pVL1392HAX introducing an in-frame N-terminal HA tag. All GAGA sequences derived from the pARGAGA construct (Soeller *et al.*, 1993). The integrity of all constructs was verified by DNA sequencing of both strands. pVLHAX-GAGA, pVLHAX- Δ N120 (Δ POZ) and pVLHAX- Δ (N321/C379) (DBD) were co-transfected with BaculoGold viral DNA (PharMingen) into Sf9 cells. All recombinant baculoviruses were plaque purified and amplified. Expression in Sf9 cells, extract preparation and immunopurification were all performed essentially as described previously (Chen and Tjian, 1996). Briefly, Sf9 cells were infected at an m.o.i. of ~5 and harvested 48 h post-infection. All protein procedures were carried out at 4°C using HEMG buffer (25 mM HEPES–KOH pH 7.6, 0.1 mM EDTA, 12.5 mM MgCl₂, 10% glycerol) containing 1 mM dithiothreitol (DTT), 0.2 mM AEBSF, 1 μ M pepstatin and varying amounts of KCl. Whole-cell extracts were prepared by sonication in 0.4 M KCl-HEMG containing 0.1% NP-40. After centrifugation at 10 000 g, the HA-tagged proteins were immunopurified from the supernatant using protein A–Sepharose beads (Pharmacia) covalently conjugated with anti-HA (12CA5) monoclonal antibodies (Harlow and Lane, 1988). After extensive washes, bound proteins were eluted using a peptide corresponding to the HA epitope in a buffer consisting of HEMG containing 100 mM KCl, 0.01% NP-40 and 0.2 mg/ml HA peptide (YPYDVPDYA). The protein concentrations of the final preparations were determined by Bradford assay (Bio-Rad) and Coomassie staining of protein gels using bovine serum albumin (BSA) as a standard.

Protein cross-linking and gel filtration analysis

Protein cross-linking reactions were carried out in a 20 μ l volume of HEMG containing 50 mM NaCl, 0.05% NP-40, 1 mM DTT and purified GAGA or Δ POZ to a concentration of ~0.05 μ M. Reactions were started with the addition of GA (Sigma, G-5882) to a final concentration of 0.01% or EGS (Sigma, E-3257) to a final concentration of 10 mM. Reactions were terminated after 4 or 8 min (EGS) or after 8 min (GA) by the addition of 6 μ l of 2 $\times$ SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS). After quenching of the reactions, samples were analysed by SDS–PAGE on 8 or 5% gels. Proteins were transferred to nitrocellulose, probed with the α -HA mouse monoclonal antibody and visualized using the alkaline phosphatase staining reaction following standard procedures (Harlow and Lane, 1988). Gel filtration analysis was performed on a Pharmacia HiPrep 16/60 S-300 Sephacryl column equilibrated and developed with HEMG buffer containing 0.1 M NaCl, 0.01% NP-40 and 1.5 mM DTT on a Biologic HR system (Bio-Rad). The column was calibrated with native protein standards according to instructions provided by the suppliers (Pharmacia). About 1–1.5 μ g of purified recombinant GAGA was applied to the column; 1 ml fractions were collected throughout the runs and analysed for the presence of protein by SDS–PAGE followed by Western immunoblotting with the α -HA monoclonal antibody.

DNA binding assays

The following probes were used in DNA binding experiments: for bandshift assays, double-stranded oligonucleotides harbouring a single GAGA site (1 $\times$ GAGA: 5'-AAGAGAGCGCAAGAGC-3') or five artificial GAGA sites (5 $\times$ GAGA: 5'-GAGAGAGCAAAGGCCTC-TCGTTCATTGCTCTCTGTCTAGAAACAGAGAGCTTAGCTCTC-3') were used. These double-stranded oligonucleotides contained 5' overhangs compatible with an *EcoRI* and *BamHI* site, respectively, and were cloned into the *EcoRI*–*BamHI* sites of pBluescript KS. For DNase I footprinting experiments, *Asp718*–*SacII* fragments of each of the above constructs were labelled on the top strand using the T4 polynucleotide kinase. The following probes were used for DNase I footprinting on the natural promoters: *Ubx*, a *NruI*–*MluI* fragment corresponding to promoter sequences –203 to +115 relative to the transcription start site of the *Ubx* promoter (Biggin and Tjian, 1988), derived from pAK100 (containing a 1 kbp *Ubx EcoRI*–*HindIII* fragment comprising the region –600 to +400; A.Kal, unpublished data); *ftz*, an

EcoRI–*BamHI* fragment corresponding to promoter sequences –445 to +40 of the *ftz* promoter (Topol *et al.*, 1991) derived from pBlue-*ftz* (containing *ftz* promoter sequences –445 to +40 flanked by *EcoRI* and *BamHI* restriction sites introduced by using a PCR-based strategy); *hsp70*, an *EcoRI*–*HindIII* fragment corresponding to promoter sequences –190 to +84 of the *hsp70* promoter derived from *hsp70*-190XBS (Weber *et al.*, 1997); *eve*, an *EcoRI*–*BamHI* fragment corresponding to promoter sequences –449 to +135 of the *eve* promoter (Read *et al.*, 1990) derived from pBlue-*eve* (containing *eve* promoter sequences –449 to +135 flanked by *EcoRI* and *BamHI* restriction sites introduced using a PCR-based strategy). The *eve* and *ftz* fragments in the pBlue-*eve* and pBlue-*ftz* plasmids were generated by PCR from the plasmids p48-4.7 (G.Struhl, unpublished data) and pFZPacP8 (H.Francis-Lang, unpublished data) respectively. All promoter fragments were end-labelled on the bottom strand using T4 polynucleotide kinase. Probes were prepared and purified using standard procedures (Sambrook *et al.*, 1989). Bandshift and DNase I footprinting experiments were performed essentially as described previously (Verrijzer *et al.*, 1995). Briefly, for bandshift assays, double-stranded oligonucleotides were end-labelled with T4 polynucleotide kinase. Binding reactions were carried out in a reaction volume of 20 µl of 0.5× HEMG buffer containing 50 mM NaCl, 50 µg/ml BSA, 0.05% NP-40, ~60 fmol of double-stranded labelled probe, 75 ng of poly(dIdC)–poly(dIdC) and the indicated amounts of polypeptide. All binding reactions were carried out for 30 min on ice and were analysed on 5% polyacrylamide gels run in 0.5× Tris–glycine/0.01% NP-40 buffer at room temperature. For DNase I footprinting reactions, ~50 ng of probe was used with the appropriate polypeptide in 60 µl reactions in 0.5× HEMG buffer containing 50 mM NaCl, 2% polyvinyl alcohol, 0.05% NP-40, 30 µg/ml BSA and 50 ng of poly(dIdC)–poly(dIdC). After binding for 45 min at room temperature, samples were processed and analysed on a 7% sequencing gel.

Electron microscopy

DNA fragments used in EM analysis were derived from plasmid pAK100 generated by cloning a 1 kbp *EcoRI*–*HindIII* fragment from the *Ubx* gene into pBluescript KS. The 4 kbp fragment was generated by linearizing pAK100 with *SacI* digestion. The 163 bp promoter region containing the four GAGA sites (–193 to –30 relative to the transcription start site) is 2.3 and 1.5 kbp from the respective DNA ends. A 1055 bp DNA fragment encompassing the *Ubx* promoter was generated by digestion of pAK100 with *HindIII* and *SacI*. The 163 bp promoter region containing the four GAGA sites is 466 and 426 bp from the DNA ends. DNA was purified by Qiagen column chromatography according to instructions provided by the supplier. All DNA binding reactions were performed as described above. Immediately after binding, protein–DNA complexes were fixed in 0.2% GA for 20 min at room temperature. Next, a small (4 µl) drop of sample material was deposited upon a carbon-filmed nickel grid made hydrophobic by high-voltage glow discharge. After 30 s absorption followed by 3–4 washes with 2–5 mM magnesium acetate, the samples were positively stained with 2% aqueous uranyl acetate as described (Spiess *et al.*, 1987). Electron micrographs were obtained with a Jeol 1200WEXII electron microscope at 50 000× magnification.

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