GATA-4 interacts distinctively with negative and positive regulatory elements in the Fgf-3 promoter

Akira Murakami*, Sanami Ishida and Clive Dickson1

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan and 1Imperial Cancer Research Fund, Lincoln’s Inn Fields, London WC2A 3PX, UK

Received September 10, 2001; Revised and Accepted December 11, 2001

ABSTRACT

GATA-4 binds two sites in the Fgf-3 promoter, PS4A and PS13, which function as positive and negative regulatory elements, respectively. In spite of their opposite functions, both PS4A and PS13 acted as potent enhancer elements when three copies of each were appended to a minimal tk promoter. Mutational analysis showed that the negative regulatory activity of PS13 was dependent on its close proximity to the major transcription initiation site (P3), since it was a stronger repressor when moved closer to P3, but had no significant activity when moved to more distal positions. While only the C-terminal zinc finger and the basic domain of GATA-4 were required for binding to PS13, this was insufficient for binding at PS4A. In addition to the PS4A GATA site, the presence of sequences located 10–12 bp distant was required for efficient binding. Both the sequence and location of this second site was crucial for binding and enhancer activity. Truncation deletions of GATA-4 showed that efficient binding to PS4A was dependent on both zinc fingers and the basic domain, suggesting a direct interaction between one zinc finger domain and a possible second site (AGACAA) that shows some similarity to a GATA motif. GATA-4 binding to PS4A through both zinc finger domains was essential for Fgf-3 promoter activity. The substitution in PS4A of a GATA-binding motif was essential for Fgf-3 promoter activity. The substitution in PS4A of a GATA-binding sequence similar to PS13, which only requires a single zinc finger domain, bound GATA-4 efficiently but did not activate the Fgf-3 promoter. These differences in GATA-4 binding were also reflected in DNA bending assays that suggested clear conformational differences between complexes formed on PS4A and PS13.

INTRODUCTION

Fibroblast growth factors (Fgfs) comprise a large family of related proteins that display a broad spectrum of biological activities, including the modulation of cell proliferation, differentiation, survival and motility (for reviews see 1–3). Fgf-3 was first identified as an oncogene in mouse mammary tumors, but was later found to be expressed at a variety of embryonic sites, suggesting its potential involvement in a number of developmental processes (4–7). Mice with a homozygous targeted disruption of Fgf-3 show developmental abnormalities of the inner ear and partial fusion of the caudal vertebrae, suggesting an essential role of Fgf-3 in the formation of these tissues (8).

F9 embryonal carcinoma cells can differentiate into several early cell lineages in cell culture (9). Treatment with retinoic acid and dibutyryl cAMP preferentially induces F9 cells to express markers of parietal endoderm, including Fgf-3 (10,11). Using differentiated F9 cells, we previously identified positive and negative regulatory elements within the 5’-proximal region of the Fgf-3 promoter, and concluded that an element designated PS4A was essential for promoter activity (12,13). Further analyses demonstrated that a transcription factor, GATA-4, binds PS4A and is a key regulator for induction of expression of Fgf-3 in differentiated F9 cells (14). The differentiation of F9 cells into visceral and parietal endoderm induced by retinoic acid also results in rapid induction of Gata-4 (15).

GATA-4 has an important function during early cardiogenesis, since it regulates the expression of a number of cardiac structural genes (for a review see 16). Moreover, targeted disruption of Gata-4 in mice results in early embryonic lethality due to a defect in ventral heart tube formation (17,18). In addition to its expression in the heart, Gata-4 is expressed in the primitive endoderm, proximal and distal gut, ovary, testis and liver in embryonic and fetal mice (19,20), suggesting an extensive role during mouse development. GATA-4 physically interacts with other transcription factors through the C-terminal zinc finger domain, resulting in synergistic activation of cardiac gene expression (21–25). Recently, the cofactor FOG-2 was shown to interact with the N-terminal zinc finger of GATA-4 and modulate GATA-4-mediated transcriptional activation (26–28).

GATA family proteins recognize the consensus motif (A/T)GATA(A/G) through a conserved multifunctional DNA-binding domain (29,30). While GATA factors universally recognize the GATA core motif, variants of the core sequence, such as GATT and GATC, are also recognized by some members of the family (29–31). The DNA-binding domain of GATA factors comprises two zinc fingers of the C2-C2-C2 type and an adjacent basic domain, although a number of reports have demonstrated that only the C-terminal zinc finger and basic domain are necessary for specific DNA binding. However, for a few promoters containing two adjacent GATA motifs, the N- and C-terminal zinc finger domains of GATA-1 have been shown to each interact with one of the two GATA motifs, resulting in higher affinity binding (32–35).

*To whom correspondence should be addressed. Tel: +81 75 751 4017; Fax: +81 75 751 4784; Email: amurakam@virus.kyoto-u.ac.jp
In this study we have characterized GATA-binding sites in PS4A and PS13, which were identified as positive and negative regulatory elements, respectively, within the Fgf-3 promoter. We have demonstrated that the negative effect of the PS13 GATA site is dependent on its position relative to the transcription initiation site. In addition, we show that binding of GATA-4 to PS4A involves a sequence in the central region of PS4A as well as the canonical GATA motif. The interaction of both zinc finger domains of GATA-4 with PS4A is necessary for its efficient binding and is essential for Fgf-3 promoter activity. Substitution of the GATA-binding site for one that binds GATA-4 through the C-terminal zinc finger domain alone, and is independent of an interaction with the central region, does not activate transcription.

MATERIALS AND METHODS

Electrophoretic mobility shift assay (EMSA)

The experimental conditions, preparation of nuclear extracts from F9 cells and procedures for purification of recombinant GATA-4 proteins produced using the baculovirus expression system have been described previously (14). When purified GATA-4 protein was used, BSA was supplemented in the binding mixtures at 3 μg per reaction. Antiserum to GATA-4 or GATA-6 was purchased from Santa Cruz Biotechnology. In vitro transcribed and translated GATA-4 and GATA-4 deletion mutants were synthesized from pET/GATA-4 and plasmids containing a series of deletion mutant cDNAs (see below) using the TnT T7 Quick Coupled Reticulocyte Lysate System (Promega). pET/GATA-4 was constructed by inserting the coding region of the previously described murine GATA-4 cDNA (15) into the Ndel and HindIII sites of vector pET-21b(+) (Novagen), which adds a histidine tag to the C-terminus of the protein product. A series of plasmids encoding GATA-4 deletion mutants were generated by PCR using the GATA-4 cDNA described above and synthetic oligonucleotide primers. Each PCR-generated deletion mutant cDNA was subcloned into the Ndel and XhoI sites of pET-21b(+). The N-terminal deletion mutants Δ75N, Δ131N, Δ179N and Δ255N were named to indicate the last amino acid of the deleted portion in the recombinant protein. Similarly, the C-terminal deletion mutants Δ381C, Δ325C and Δ256C were named to indicate the first amino acid deleted.

Reporter assay

The luciferase reporter plasmids ptkLuc, p(4A×3)tkLuc and pFgf-3/Luc used in the deletion experiments have been described previously (14). p(PS13×3)tkLuc, a derivative of ptkLuc containing three copies of the PS13 element in tandem, was constructed by inserting the complementary synthetic oligonucleotides with KpnI cohesive and blunt ends into the KpnI and Smal sites of ptkLuc after annealing. pFgf-3/LucPS13 lacking the PS13 element was constructed by substituting the 1.7 kb PstI–XhoI fragment of pFgf-3/Luc with that of pFgf-3/CATΔ1562–1570 (13). The pFgf-3/LucPS13ΔA, pFgf-3/LucPS13ΔB and pFgf-3/LucPS13C variants of pFgf-3/Luc containing the PS13 element located at other sites in the Fgf-3 promoter, as indicated in Figure 3B, were synthesized from pFgf-3/LucΔ13 and synthetic oligonucleotides using an in vitro site-directed mutagenesis system, GeneEditor (Promega). Using the same procedure as described above, base substitution mutations were introduced into the PS13 element (see Fig. 1B) of pFgf-3/Luc, pFgf-3/LucPS13A and pFgf-3/LucPS13A2 to generate pFgf-3/LucPS13m, pFgf-3/LucPS13Am and pFgf-3/LucPS13A2m, respectively. Similarly, point mutations were introduced into the PS4A element of pFgf-3/Luc to generate pFgf-3/Luc4AyM7, pFgf-3/Luc4AyM7zM3 and pFgf-3/Luc4A20A. Procedures for cell culture, transfection with plasmid DNAs by the calcium phosphate-DNA precipitation method, isolation of stably transfected cells, preparation of cell extracts and luciferase assays have been described previously (12–14).

DNA bending assay

Plasmids for bending assays were constructed by inserting the complementary synthetic oligonucleotides into the pBend2 vector (36). pBend2/PS4A and pBend2/PS13 contain PS4A at the XhoI site and PS13 at the XhoI and SmaI sites, respectively. The inserts plus flanking sequences were released from the vector by digestion with appropriate restriction enzymes, electrophoresed in 5% polyacrylamide gels in 1× TBE and retrieved from the gels. The purified fragments were subjected to EMSA under conditions slightly different from those described above. Samples were loaded onto 8% polyacrylamide gels (75:1 w/w acrylamide: bisacrylamide) and electrophoresed at 100 V in 0.5× TBE at room temperature for 4 h.

RESULTS

GATA-4 binds PS13 and PS4A

Previous studies identified three positive and three negative regulatory elements in the 5′-proximal region of the Fgf-3 promoter, as illustrated in Figure 1A (13). PS4A is an essential positive regulatory element for Fgf-3 transcription, which binds GATA-4 at a GATA motif (Fig. 1C) (14). Surprisingly, one of the negative regulatory elements, PS13, which is located just upstream of the major transcription initiation site P3, also contains a GATA motif (Fig. 1B). Both elements formed complexes with nuclear extracts from differentiated but not undifferentiated F9 cells (Fig. 1C, lanes 2 and 7) and mutations of the GATA motifs abrogated complex formation (Fig. 1B and C, lanes 5 and 10). The complexes formed with either the PS4A or PS13 probe were supershifted to a higher position by addition of GATA-4 antiserum, confirming the presence of GATA-4 in the complexes (Fig. 1C, lanes 3 and 8). GATA-6 was not detected in the complexes formed with either probe (Fig. 1C, lanes 4 and 9), although it is expressed in both cell lines (14). Hence, GATA-4 can form complexes with both the positive and negative regulatory elements, PS4A and PS13, respectively, in the Fgf-3 promoter.

The negative regulatory effect of PS13 is position dependent

Mutation analysis of the Fgf-3 promoter showed that PS13 is a negative regulatory element (13). However, when three copies of PS13 were joined to a minimal h promoter, they functioned as an enhancer of promoter activity (Fig. 2). Moreover, the enhancing activity of PS13 was stronger than that of PS4A placed in the same reporter construct. Thus, while PS13 can act as a positive regulatory element, in the context of the Fgf-3
promoter it has a negative regulatory activity, since its deletion causes a several-fold increase in promoter activity (pFgf-3/PS13 in Fig. 3C) (13). PS13 is well separated from the other main regulatory elements of the promoter and is located in exon 1B, just 106 bp upstream of the major transcription initiation site P3 (Fig. 1A). To test whether the relative position of PS13 in the Fgf-3 promoter influences its activity, modified promoter constructs were made where PS13 was placed either nearer or more distant to P3 (Fig. 3). Of the four modified Fgf-3 promoter constructs tested, pFgf-3/LucPS13A and pFgf-3/LucPS13B, in which PS13 is located closer to P3, showed reporter activities lower than that of the parental promoter, demonstrating an even more potent negative regulatory effect. To examine the possibility that this effect might be due to a fortuitous insertion mutation into an unknown positive regulatory element, similar constructs were tested where the GATA site in PS13 was mutated. These constructs showed that activity from the Fgf-3 promoter was fully or substantially restored to the level of activity shown by the PS13 deletion construct (Fig. 1C). This result indicates that the negative regulatory activity of PS13 in the Fgf-3 promoter is dependent on its position close to P3 and requires a functional GATA-binding site. In contrast, when PS13 was located further upstream from P3, as in the constructs pFgf-3/LucPS13C and pFgf-3/LucPS13C, the reporter activities were greater than the parental construct (Fig. 3C). However, these activities were comparable with those seen with pFgf-3/LucΔ13, where PS13 is deleted. Therefore, it appears that PS13 exerts a negative effect on promoter activity when located in the vicinity of P3, but has no significant effect when located further upstream.

**The central region of PS4A is required for stable binding of GATA-4**

PS4A is a complex regulatory element that binds multiple proteins (14). The central sequence of PS4A was shown to

---

**Figure 1.** Binding of GATA-4 protein to the PS4A and PS13 elements. (A) Schematic depiction of the regulatory elements identified within the 5′-proximal region of the Fgf-3 promoter (13). The striped blocks represent the 5′ exons, with the positions of three transcription start site clusters marked as P1, P2 and P3 (11,46). The positive and negative regulatory elements as determined in differentiated F9 cells are represented as white and black boxes, respectively. (B) Nucleotide sequences of PS4A, PS13 and the GATA motif mutants used as probes in (C). Mutated nucleotides are indicated by asterisks. The consensus binding sites for GATA proteins are boxed. For PS13, the nucleotides shown in lower case were added to the element when it was used as a labeled probe. (C) EMSA using nuclear extracts from undifferentiated (u) and differentiated (d) F9 cells. 4A and 13 indicate probes PS4A and PS13. Arrows indicate complexes formed by GATA-4 binding.

**Figure 2.** Assessment of PS13 regulatory activity using the minimal tk promoter. (A) Schematic representation of the reporter plasmids. The PS4A and PS13 elements are as shown in upper case in Figure 1. (B) Reporter assays were performed by transfecting plasmids illustrated in (A) into F9 cells and the resulting colonies of stable transfectants were pooled. Luciferase activities in the cells before and after induction of differentiation were measured and are shown with standard error bars. The promoter activities were normalized to the average plasmid copy number determined on the pooled clones by Southern blotting (14).
influence binding of GATA-4 at the GATA motif. In competition EMSA experiments, double-stranded oligonucleotides containing a mutation in the central region of PS4A or in the GATA motif were ineffective competitors of GATA-4 binding (Fig. 4B, lanes 4, 5 and 10) (14). In contrast, oligonucleotides with single point mutations located between the central region and the GATA motif successfully competed for binding. However, oligonucleotides containing three base changes, small deletions or additions (4AyM1, 4Adel and 4Aadd) again failed to compete with the PS4A probe (Fig. 4B, lanes 10–12). When 4Adel and 4Aadd were used as probes, they also failed to bind GATA-4 (Fig. 4C, lanes 2 and 3). Hence, it appears that in addition to the central region and the GATA motif, the spatial integrity of the two sites is also critical.

A possible mechanism to explain the binding requirements of GATA-4 is a cooperative interaction with another factor that binds to the central region of PS4A or in the GATA motif were ineffective competitors of GATA-4 binding (Fig. 4B, lanes 4, 5 and 10) (14). In contrast, oligonucleotides with single point mutations located between the central region and the GATA motif successfully competed for binding. However, oligonucleotides containing three base changes, small deletions or additions (4AyM1, 4Adel and 4Aadd) again failed to compete with the PS4A probe (Fig. 4B, lanes 10–12). When 4Adel and 4Aadd were used as probes, they also failed to bind GATA-4 (Fig. 4C, lanes 2 and 3). Hence, it appears that in addition to the central region and the GATA motif, the spatial integrity of the two sites is also critical.

A possible mechanism to explain the binding requirements of GATA-4 is a cooperative interaction with another factor that binds to the central region of PS4A, resulting in stable binding of GATA-4. However, in vitro transcribed/translated or purified recombinant GATA-4 alone bound to the PS4A probe (Fig. 5B, lanes 1 and 4). Although in previous experiments we had found that purified GATA-4 had yielded little complex formation with a PS4A probe (14), the complex became apparent if BSA was supplemented at a concentration comparable with that of the nuclear extracts, suggesting that a high protein concentration facilitated complex stability. Nevertheless, the binding of GATA-4 was still dependent on the integrity of the central region as well as the GATA motif (Fig. 5B, lanes 2, 3, 5 and 6). These findings suggest that the sequence in the central region forms part of the recognition sequence for GATA-4 binding. To investigate this idea further, a comparison was made with the binding of GATA-4 to other GATA-binding sites, including PS13 (Fig. 6). As predicted, probe 4Az, which encompasses the GATA motif but excluded the central region of PS4A, bound only a small amount of GATA-4 protein, while a probe containing the additional central region sequence (4Az3) restored efficient binding. Other GATA-binding sites, composed of one or two GATA motifs (TTF1 and GATAcon) bound more GATA-4 protein than 4Az, demonstrating that the GATA motif of PS4A alone is insufficient for efficient binding of GATA-4 protein. In contrast, PS13 bound a greater amount of GATA-4 protein than 4Az. As there is a one base difference between the GATA motifs of PS4A (CTATCT) and PS13 (TTATCT), we tested the effect of substituting the first C of 4Az with T, yielding the probe 4AzM3. This substitution caused a dramatic increase in bound GATA-4 protein, to almost the same level as that bound to PS13 (Fig. 6C). In addition, when this substitution was introduced into 4AyM7, which harbors a mutation in the central region of PS4A and bound little GATA-4, the resulting probe (4AyM7zM3) efficiently bound GATA-4.
bound GATA-4, indicating that binding was now independent of the central region. These results indicate that the central region of PS4A stabilizes binding of GATA-4 at the sequence CTATCT but is not required for binding at TTATCT.

The N-terminal zinc finger of GATA-4 is required for binding to PS4A

GATA-4 protein contains several distinct functional domains (37). To investigate the role of each domain of GATA-4 in binding to PS4A, a series of N- and C-terminal protein truncations were prepared and tested for their ability to bind both PS4A and PS13 probes (Fig. 7). All proteins containing the C-terminal zinc finger and the adjacent basic domains (Zn2 and B, respectively, in Fig. 7A) bound PS13, but the Δ256C mutant lacking these domains did not. Thus, consistent with previous results (37), the C-terminal zinc finger and adjacent basic domains are necessary and sufficient for binding to PS13, whereas the other domains, including the N-terminal zinc finger (Zn1) are not essential. In contrast, similar experiments using PS4A as a probe gave a different result. In addition to the Δ256C mutant, the truncated protein Δ255N, which lacks Zn1, did not bind to PS4A. Thus, not only the C-terminal zinc finger but also the N-terminal zinc finger domain is necessary for binding to PS4A. In addition, the mutated protein Δ325C, lacking a portion adjacent to the basic domain, bound to PS4A with much reduced efficiency compared with PS13. These results indicate that efficient binding of GATA-4 to PS4A requires the region adjacent to the basic domain as well as both zinc fingers.

GATA-4 binding to PS4A through both zinc fingers is essential for Fgf-3 promoter activity

Conversion of the PS4A GATA-binding site from CTATCT to TTATCT made binding of GATA-4 to PS4A independent of the interaction with the adjacent central region (compare 4AyM7 with 4AyM7zM3 in Fig. 6). As the TTATCT consensus GATA site in the context of PS13 did not require the N-terminal zinc finger for binding, we also tested the mutant PS4A for this requirement. As seen in Figure 8B, while 4AyM7 did not bind GATA-4 protein, 4AyM7zM3 containing the TTATCT GATA site was able to bind GATA-4 protein lacking the N-terminal zinc finger (Δ255N), as well as wild-type GATA-4. However, when the GATA site was changed from CTATCT to TTATCT in the Fgf-3 promoter, the mutated reporter construct, pFgf-3/Luc4AyM7zM3, showed much less activity than that of the wild-type reporter pFgf-3/Luc (see Fig. 3A). Moreover, the level of promoter activity was very similar to that of pFgf-3/Luc4AyM7 (Fig. 8C), suggesting that GATA-4 binding to the mutated PS4A derivative has little effect on transcription.

As seen in Figure 4, the central site of PS4A was also required for GATA-4 binding. Although the site does not conform to the GATA consensus recognition sequence (A/TGATA/A/G), there is a similar sequence that differs within the core region by one base, TTGTCT (AGACAA in the reverse orientation), and this might act as a site for interaction with the second zinc finger domain. We attempted to test this idea by converting the
sequence into a GATA consensus site similar to that found in PS13 by a single base substitution, to generate probe 4A20A (Fig. 8B). Despite binding GATA-4 more efficiently than parental PS4A, when this mutation was introduced into the Fgf-3 reporter construct (pFgf-3/Luc4A20A) it showed an extremely low level of activity, even less than pFgf-3/Luc4AyM7, suggesting that GATA-4 was having a negative effect on transcription. Thus, GATA-4 binding to PS4A is not necessarily sufficient, but the binding through both zinc fingers is required for Fgf-3 promoter activity.

**DNA bending by GATA-4**

The EMSA data in Figure 6 show that the mobility of probes complexed with GATA-4 was more varied than expected. For example, GATA-4 bound to PS4A migrated slightly faster

**Figure 7.** Both zinc finger domains of GATA-4 are required for binding to PS4A but not to PS13. (A) Schematic depictions of GATA-4 and deletion derivatives. Act, Zn1, Zn2 and B represent the activation, both zinc finger and basic domains, respectively. The consensus DNA sequence for GATA protein binding is boxed. (B) EMSA using the indicated probes and in vitro transcribed/translated GATA-4 or its derivative proteins illustrated in Figure 7. Procedures were as described in Figure 3.
than complexes with 4Az or PS13, despite the fact that PS4A (39 bp) is longer than 4Az (18 bp) and PS13 (20 bp). One possible explanation for the varied migration is that a complex formed through both zinc fingers has a conformation different from that engaging only the C-terminal finger. As GATA-1 has been shown to cause a conformational change in the associated DNA, resulting in a bend (38–40), the DNA-bending ability of GATA-4 was compared using PS4A and PS13 as probes. Circularly permuted probes containing PS4A or PS13 were mixed with *in vitro* transcribed/translated GATA-4 protein and analyzed by EMSA (Fig. 9). A typical mobility pattern indicating DNA bending was observed for the PS13–GATA-4 complexes. Thus, GATA-4 possesses DNA-bending ability and the bend angle of the complex was estimated as ~48°. In contrast, the PS4A–GATA-4 complexes showed a more varied mobility pattern, making the results more difficult to interpret. Nevertheless, the results suggest that there is a difference in conformation between the complexes formed on PS4A and PS13.

**DISCUSSION**

In this study we have examined the function and binding characteristics of two GATA-4 binding elements, PS4A and PS13, in the *Fgf-3* promoter (Fig. 1) (14). While PS4A was shown to be an essential positive regulatory element for promoter activity in differentiated F9 cells, paradoxically PS13 showed an even greater repressive effect on transcription. If PS13 was placed even closer to the major initiation site at position –35 or –70 it may repress transcription, as a deletion of PS13 did not abolish but rather enhances promoter activity (Fig. 3). Therefore, the mechanism of repression by PS13 seems to be different from that in those genes.

Although PS13 efficiently binds GATA-4 (compare with the other probes in Fig. 6B) and can markedly enhance the activity of a minimal tk promoter (Fig. 2), it does not appear to contribute to *Fgf-3* promoter activation. Even when PS13 was located far from P3, it did not enhance promoter activity (Fig. 3). Thus, a GATA-4-binding site alone is insufficient to confer transcriptional activation on the *Fgf-3* promoter. In contrast, the GATA-4-binding motif in PS4A is a key element of promoter activation. As two other positive regulatory elements, PS2 and PS5, were mapped in the vicinity of PS4A (Fig. 1A) (13), it is likely that GATA-4 on PS4A activates transcription in cooperation with the factors associated with these other elements. On the other hand, GATA-4 on PS13 represses transcription, but its biological significance is not clear in this study. Although *Fgf-3* is expressed in various tissues in the embryo (4–7), its expression is generally quite weak (A.Murakami, unpublished results). One possibility is that repression through PS13 might play a role to prevent too high expression of *Fgf-3*, although other possibilities are not excluded.

The binding of GATA-4 to PS4A is novel, since it requires not only the GATA motif but also a sequence located in the central region of PS4A (Figs 4 and 5). While the GATA motif CTATCT (i.e. AGATA[AG] in the opposite orientation) conforms with the known consensus recognition sequence, (A/T)GATA(A/G), it was not sufficient for effective GATA-4 binding. However, its conversion to AGATAA greatly increased GATA-4 binding (compare 4Az to 4AzM3 in Fig. 6C). Hence, judging from the efficient binding to PS13 (containing AGATAA), GATA-4 seems to prefer binding at AGATAA. The weak binding of GATA-4 to AGATAAG can be compensated for by the presence of the central region of PS4A, since addition of this region to 4Az greatly enhanced GATA-4 binding (4Az3 in Fig. 6B). Thus, this region stabilizes GATA-4 bound at AGATAAG. The mutation analysis demonstrated that binding reversal of the repressive effect was not achieved by abrogating GATA-4 binding, it is possible that binding sites for other proteins such as TBP in the promoter core region were affected by the insertion. The negative effect on transcription may be due to the GATA-4 complex on PS13 sterically interfering with assembly of a common set of general factors on the core promoter or with interaction between the assembled general factors and complexes formed on upstream elements, including PS4A. The fact that PS13 located more distantly (at –346 or –586) had little negative effect on transcription supports this hypothesis (Fig. 3). Therefore, we conclude that in the *Fgf-3* promoter the position of PS13 relative to the transcription initiation site determines whether it will act as a negative regulator of transcription.

Several genes have been shown to contain a GATA-binding site in the core promoter. For example, the rat platelet factor 4, mouse erythropoietin and chicken β-globin genes contain a GATA motif in place of the consensus TATAAA site, and GATA proteins repress their transcription by competing with the basal factors, which forms the preinitiation complex (41). Although *Fgf-3* also lacks a TATAAA sequence in the core promoter, the GATA sequence in PS13 is dispensable for transcription, as a deletion of PS13 did not abolish but rather enhances promoter activity (Fig. 3). Therefore, the mechanism of repression by PS13 seems to be different from that in those genes.
of GATA-4 to PS4A was through involvement of the N-terminal zinc finger, as well as the C-terminal zinc finger and basic domains (Fig. 7). A number of reports have shown that the C-terminal zinc finger and adjacent basic domain of GATA protein are sufficient for specific DNA binding, as demonstrated here for binding to PS13. Nevertheless, in some circumstances GATA-1 has been shown to require both zinc finger domains for high affinity binding (32,34,35). In these examples, two GATA motifs in tandem or in a palindromic structure are present, indicating that the N-terminal zinc finger associates with either of the GATA motifs. The central region of PS4A necessary for GATA-4 binding contains a sequence not too dissimilar to the consensus GATA motif, namely TTGTCT (i.e. AGACAA in the opposite orientation), although it does not contain the GATA core sequence. While GATA factors universally recognize the GATA core motif, this motif is not necessarily essential, since subtly different sequences, such as GATT and/or GATC, are recognized by GATA-2, GATA-3 and GATA-6 (29–31). Therefore, it might be possible for either zinc finger domain of GATA-4 to recognize the GATA-like sequence of the central region. Consistent with this idea is the critical distance between the two interaction sites of PS4A, since addition or deletion of 5 bp between the two sites abolished GATA-4 binding (Fig. 4C). Taken together, these findings indicate that GATA-4 binds PS4A through the association of both zinc finger domains at distinct tandem sites within the element.

GATA-4 binding to PS4A through both zinc finger domains appears to be essential for activation of the Fgf-3 promoter. This notion is supported by the mutations that permit binding of GATA-4 without the requirement of the N-terminal zinc finger, but in the context of the Fgf-3 promoter are unable to enhance transcription (Fig. 8). The PS4A–GATA-4 complex migrates faster in EMSAs than those complexes with a single binding site, such as 4A8 and PS13 (Fig. 6). Hence, binding through the two zinc fingers may result in a different DNA conformation than binding through a single site. GATA-1 has been shown to cause a conformational change in the associated DNA, resulting in a bend in the DNA (39,40), although there was little obvious difference in the bend angle reported between the two types of GATA sites (38). GATA-4 also bends DNA when it binds to PS13 (Fig. 9). However, in the case of PS4A, the circular permutation DNA bending experiments showed an unusual and complicated migration pattern that was not interpretable in terms of a simple difference in bend angles. However, some difference in the conformation of the complexes of GATA-4 with PS4A and PS13 is clearly apparent. The specific conformation of the PS4A–GATA-4 complex might be required for interaction with other trans-acting factors associated with PS2 and PS5 elements in the activation of Fgf-3 transcription.

Recently, we identified a transcription factor, SOX6, a member of the SOX family, as another PS4A-binding protein which represses Fgf-3 transcription through interaction with a co-repressor CBP2 (42). SOX6 also binds to the central region of PS4A and the recognition sequence of SOX6 almost overlaps that of GATA-4 described above. Thus, the two factors appear to act at the same site of PS4A for activation or repression of Fgf-3 transcription, although GATA-4 and SOX6 bind independently or rather competitively to PS4A (A.Murakami, unpublished results). SOX proteins have been reported to bind to the minor groove of their target DNA sequences, causing a bend in the DNA (43–45). Considering that a conformational modulation of PS4A by binding of GATA-4 was suggested (Fig. 9), certain conformational changes in PS4A may be necessary for activation or repression of Fgf-3 transcription.

ACKNOWLEDGEMENTS

We thank Dr Sankar Adhya for generously providing the pBend2 vector. This work was supported by a grant from the Human Frontier Science Program to A.M. and C.D.

REFERENCES
