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Drosophila Goosecoid requires a conserved heptapeptide for repression of Paired-class homeoprotein activators

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SUMMARY

Goosecoid (Gsc) is a homeodomain protein expressed in the organizer region of vertebrate embryos. Its *Drosophila* homologue, D-Gsc, has been implicated in the formation of the Stomatogastric Nervous System. Although there are no apparent similarities between the phenotypes of mutations in the *gsc* gene in flies and mice, all known Gsc proteins can rescue dorsoanterior structures in ventralized *Xenopus* embryos. We describe how D-Gsc behaves as a transcriptional repressor in *Drosophila* cells, acting through specific palindromic HD binding sites (P3K). D-Gsc is a 'passive repressor' of activator homeoproteins binding to the same sites and an 'active repressor' of activators binding to distinct sites. In addition, D-Gsc is able to strongly repress transcription activated by Paired-class homeoproteins through P3K, via specific protein-protein interactions in what we define as 'interactive repression'. This form of repression requires the short conserved GEH/eh-1 domain, also present in the Engrailed repressor. Although the GEH/eh-1 domain is necessary for rescue of UV-ventralized *Xenopus* embryos, it is dispensable for ectopic induction of *Xlim*-1 expression, demonstrating that this domain is not required for all Gsc functions in vivo. Interactive repression may represent specific interactions among Prd-class homeoproteins, several of which act early during development of invertebrate and vertebrate embryos.

Key words: *Drosophila*, *goosecoid*, Homeodomain, Transcriptional repression

INTRODUCTION

The homeodomain (HD) found in many developmental regulators mediates most of the functional specificity of the homeoproteins in which it is embedded (reviewed by Duboule, 1994). However, little or no differences exist in the DNA binding specificity of these proteins since they bind to similar DNA sequences (Desplan et al., 1988; Gehring et al., 1994). Therefore, these precise developmental regulators must rely on mechanisms other than DNA binding to ensure targeting to the correct promoters. Homeodomain proteins have been proposed to achieve functional specificity through interaction with other homeoproteins (Hayashi and Scott, 1990) mainly via dimerization and/or cooperative DNA binding (for review see: White, 1994; Wilson et al., 1993). In addition, homeoproteins may require cofactors (for review see: Wilson and Desplan, 1995), possess additional DNA binding domains (Treisman et al., 1991; Voss et al., 1991), protein-protein interaction domains, *e.g.* LIM domains (Taira et al., 1994) and may function to modulate transcription either as activators or repressors (Han et al., 1989; Han and Manley, 1993b; Jaynes and O'Farrell, 1991).

We were interested in a class of homeoproteins (Paired-class) which can cooperatively dimerize on palindromic binding sites containing two core TAAT HD sites (P3 sites). Heterodimerization on dimeric sites could result in preferential interactions between activator and repressor homeoproteins, leading to combinatorial control. A specific sub-class within the Prd-class of homeoproteins contains a lysine residue at the critical position 50 of their homeodomains (K_{50}), a residue also found in the *Drosophila* anterior morphogen Bicoid (Bcd; Berleth et al., 1988). All K_{50} homeoproteins recognize the same binding sites with high specificity and, except for Bcd, all belong to the Prd-class (Treisman et al., 1989; Hanes et al., 1989). These homeoproteins, which include Bcd, Orthodenticle (Otd and its vertebrate homologues Otx), Goosecoid (Gsc) and vertebrate Ptx 1 (Lamonaire et al., 1996), are most often required in the most anterior part of the *Drosophila* embryo, suggesting that they may be part of a network of interactions.

Gsc was first identified as a marker of the organizer region of the *Xenopus* embryo able to induce secondary axis formation when its mRNA is injected into the ventral side of the embryo. Based on misexpression studies in *Xenopus*, *gsc* is thought to activate some genes (*chordin*, *Xlim 1*, *otx*; Blitz and Cho, 1995; Sasai et al., 1994; Taira et al., 1994) and to

repress others (*e.g. BMP-4*; Fainsod et al., 1994). However, these interactions have not been shown to be direct. Analysis of Gsc protein expression patterns and phenotypes in both vertebrates (Cho et al., 1991; Rivera-Perez et al., 1995; Yamada et al., 1995) and *Drosophila* (Goriely et al., 1996; Hahn and Jäckle, 1996), has led to the conclusion that the precise developmental role of these proteins may have diverged considerably, despite their functional homology and conserved position within a regulatory network of developmental genes (Goriely et al., 1996). The sequence homology between *Drosophila* and vertebrate Gsc proteins is mainly restricted to the HD, but both *gsc* and *D-gsc*, rescue UV-ventralized *Xenopus* embryos (Goriely et al., 1996). The inability of *bcd* and *otd* to rescue ventralized embryos (Goriely et al., 1996; Pannese et al., 1995), despite sharing the landmark K_{50} (Bcd and Otd) and Prd-class HDs (Otd), strongly suggests that the effect of *D-gsc* in this assay is dependent on the Gsc HD and not solely on the presence of K_{50} (Goriely et al., 1996). In addition to the HD, all Gsc molecules share a conserved 7 amino acid stretch at the N terminus, the GEH (Goosecoid Engrailed Homology) domain, known as the eh-1 domain in the homeoprotein Engrailed (En) (Hemmati-Brivanlou et al., 1991; John et al., 1995; Logan et al., 1992). En is capable of repressing transcription both in vitro and in vivo (Han and Manley, 1993a; Jaynes and O'Farrell, 1988; John et al., 1995). The repression domain of En has been mapped in vitro to a 55 amino acid long alanine rich domain (Han and Manley, 1993a). Studies using En as a dominant transcriptional repressor in the *Drosophila* embryo, however, suggested that this domain may not be sufficient for repression and that, in addition, the eh-1 domain is required (John et al., 1995). In this study we present evidence that regions other than the HD contribute to Gsc molecular function.

The mechanisms of transcriptional repression are less well understood than those leading to activation. Considering that only 7% of the genome is transcribed in a typical eukaryotic cell, repression was originally reasoned as the default state (Johnson, 1995). However, it has recently become apparent that, during development, repression is paramount not only to control spatial gene expression but also to ensure timely downregulation. In the best studied example of spatiotemporal regulation in *Drosophila*, it is both activation (by Bcd and Hunchback) and repression (by Krüppel (Kr) and Giant) that lead to the formation of the *even-skipped* (*eve*) stripe 2 expression during segmentation (Small et al., 1991). Molecular characterization of En, Eve and Kr has reinforced the idea that repressor molecules are, like activators, modular in structure with a repressor domain usually distinct and separable from the DNA binding domain (Han and Manley, 1993a; Han and Manley, 1993b; Licht et al., 1990; Sauer and Jäckle, 1995). Repressors are thought to block transcription by competing with activators for the same binding sites ('passive repression', Levine and Manley, 1989), by interacting with the transcriptional machinery ('direct repression', Jaynes and O'Farrell, 1988; Johnson, 1995; Sauer et al., 1995) or by quenching. Quenching describes a situation where the repressor masks activation domains when positioned within 100 bp of the activator binding sites (Gray and Levine, 1996). Local interaction between repressor and activator molecules allows multiple enhancers to act autonomously within complex promoters. Whether a repressor will act through quenching or direct repression will depend on the position and the nature of its binding sites. Kr, for example, can both repress activators through quenching and direct repression (Gray et al., 1994; Sauer et al., 1995), depending on whether the Kr binding sites are within 100 bp of the activator or the transcription start site (Gray and Levine, 1996; Gray et al., 1994). In both cases Kr can be classified as an 'active repressor' since it is not competing for binding with the activator (Jaynes and O'Farrell, 1991).

Here we show that D-Gsc represses transcription by different mechanisms depending on the nature of the activator protein and the nature of the binding sites. D-Gsc acts as a 'passive repressor' successfully competing for binding with other K_{50}-bearing activator homeoproteins. For this function, D-Gsc requires its HD and a separate repression domain. Moreover, D-Gsc very efficiently represses transcription activated by Prd-class homeoproteins which can cooperatively dimerize on P3K sites. We call this 'interactive repression' since it is based on protein-protein interactions and heterodimerization which are dispensable for passive repression. Interactive repression requires the GEH/eh-1 domain in D-Gsc and may reflect a specific mechanism of interaction among the members of the large Prd-class of homeoproteins which are involved in intricate regulatory networks during early development. In addition, we show that D-Gsc represses activators that bind to distinct sites and thus behaves as an 'active repressor' (Jaynes and O'Farrell, 1991).

MATERIALS AND METHODS

Cloning and site-directed mutagenesis

Expression plasmids used in transfection assays were made by cloning the appropriate cDNA (*D-gsc and otd*) or genomic fragment (*bcd*) into pPAC (Krasnow et al., 1989). *pPAC-Dgsc* was generated by blunt-end cloning a 2.1 kb *Hin*dIII-*Eco*RI fragment containing the *D-gsc* ORF, into a unique *Eco*RV site in pPAC. Blunt-end insertion of the 3.8 kb *Eco*RI fragment comprising the full-length *otd* cDNA into the *Eco*RV site in pPAC, yielded pPAC-*otd*. Bcd producer was made by blunt-end cloning of the genomic *Sal*I-*Xba*I fragment into the *Eco*RV site in pPAC (Bellaïche et al., 1996). Responder plasmids were generated by cloning either one or three copies of the Bcd site (TAATCCC) and either one or three copies of the P3K oligo (top strand: GATCCTGAGTCTAATCCGATTAGTGTACA; bottom strand: GACTCAGATTAGGCTAATCACATGTCTAG), into the *Bam*HI site in pD33-CAT (Ronchi et al., 1993; Wilson et al., 1993) upstream of the minimal dADH (distal alcohol dehydrogenase) promoter driving the CAT (chloramphenicol acetyl transferase) reporter gene, to yield $(P3K)_1$-CAT, $(P3K)_3$-CAT, 1×BcdBS-CAT and 3×BcdBS-CAT. GR producer was a kind gift from J. Jaynes. GR responders that could also respond to D-Gsc had $(P3K)_3$ inserted either 11 bp upstream (cloned into the *Xho*I site of pT3D-33CAT) or 13 bp downstream (cloned into the *Xba*I site of pT3D-33CAT) of the glucocorticoid response element (GRE, a.k.a. T3) to yield $(P3K)_3$-GRECAT or GRE-$(P3K)_3$CAT. For details of construction of pT3D-33CAT see Jaynes and O'Farrell (1991). $(P3K)_3$-700bpGRECAT was constructed by cloning an unrelated 700 bp *Xho*I/*Sal*I fragment into the *Xho*I site of pT3D-33CAT. $(P3K)_3$ was subsequently cloned into the *Xho*I site generated 700 bp upstream of the GRE.

Mutations into either the *D-gsc* or *otd* cDNA were introduced by oligonucleotide-mediated site-directed mutagenesis using the Kunkel method (Sambrook et al., 1989). *D-gsc* cDNA was first subcloned into pKSII+ (Stratagene) for the production of single stranded DNA. The

otd cDNA in pBS+ was generously supplied by R. Finkelstein. The sequences of the oligonucleotides used for mutagenesis are as follows:

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ΔGEH       5′GCCTCCACCCTGTTGGCGGGATCCGAGAGACGCGGCAGCGGC 3′
ER43-Dgsc 5′CTTGAACCACACCTCAACGCGTCGTTCTTTGAGATCCACCTT 3′
SR43-otd   5′AAGATCAATCTGCCCGAACGTAGAGTACAGGTGTGGTTC 3′
IK28-otd   5′TTTGGCAAGACCCGTTATCCCGACAAATTCATGCGCGAAGAAGTG 3′
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All mutants were diagnosed by the introduction or deletion of restriction sites and were confirmed by sequencing. The full-length GST-Otd fusion construct was generated by partial *Stu*I digestion of the *otd* cDNA followed by cloning into the blunted *Eco*RI site of pGEX-3X (Pharmacia). GST-HD constructs were made by cloning PCR amplified HDs in frame with GST into the *Eco*RI site of pGEX-2T. The sequences of primers used to amplify the D-Gsc and Otd HD are as follows:

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D-Gsc HD:  5′CGGAATTCATGGACCTCCGCCGAAG 3′
           5′CGGAATTCGCTCCTGCTCCTCGCGCTT 3′
Otd HD:    5′CGGAATTCCAGGCGTCAACACACGA 3′
           5′CGGAATTCCTGCTGCTGCTGCTGCAA 3′
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Xenopus laevis ventralization and RT-PCR

Ovulation of females and in vitro fertilization were carried out as described by Condie and Harland (1987). Embryos were dejellied by treatment with 3% L-cysteine in 0.1× modified Ringer's saline pH 7.8 with 1 M NaOH (Hemmati-Brivanlou et al., 1990) and UV treated using a Stratalinker before the first cleavage (optimal dose was 1200 mJ). RNA was made using SP6 message machine (AMBION) from sP64T vector containing the *Hin*dIII-*Eco*RI fragment of either the wild-type (wt) or *ΔGEH-Dgsc* cDNA. Injections (50 pg/blastomere) were carried out as described by Goriely et al. (1996) and embryos were allowed to develop at 18°C, until controls (non-UV treated) had reached tadpole stage. To assay for *Xlim* 1 expression, dorsal and ventral marginal zone explants were dissected from embryos that had been injected in the VMZ at the 2-cell stage once these embryos had reached the early gastrula stage. Processing of explants and RT-PCR was performed essentially as described by Wilson and Hemmati-Brivanlou (1995). Sequences of the primers used to amplify *Xlim 1* are: 5′CCCTGGCAGCAACTATGA 3′ and 5′GGTGGGTGTGACAAATGG 3′.

Protein preparation

GST fusion proteins were expressed in *E.coli* strain BL21 as described by Ausubel et al. (1991). Protein was not eluted from the beads and protein/beads slurries were kept at 4°C.

Proteins for EMSA and protein-protein interaction experiments were synthesized in vitro using the coupled transcription/translation rabbit reticulocyte system (Promega) and labeled with [^{35}S]methionine (Amersham) which enabled their quantification following separation in SDS-PAGE (8% gel) using a PhosphorImager and ImageQuant software.

EMSA

The P3K probe was labeled using DNA polymerase as it possesses 5′GATC overhangs. Electrophoresis mobility shift assays were performed as described by Wilson et al. (1993), with the following exceptions: the reaction volume was 10 μl, and 50 μg/ml of herring sperm DNA was added to each reaction. The α-D-Gsc polyclonal antibody used was raised against a 113 aa peptide (this region does not encompass the HD) fused to GST. Serum from rabbits injected with the purified fusion peptide was run through glutathione agarose bead columns, to which GST-D-Gsc had been previously coupled, to affinity purify the antibody. A 1:20 dilution of the α-D-Gsc antibody was used. Specific competitor was 20× excess of unlabeled P3K and non-specific competitor was 20× excess of PNPal (top strand: GATCCTGAGTCTAATAGCATTAGTGTACA, bottom strand: GACTCAGATTATCGTAATCACATGTCTAG; (Wilson et al., 1993). Underlined bases are those different from P3K.

Cell culture and transfections

Drosophila Schneider cells (Schneider line 2; Schneider, 1972) were cultured in Shields and Sang M3 Insect medium (Sigma) supplemented with 12% fetal bovine serum (Geminii Bioproducts). Cells were transfected by the calcium phosphate precipitation method as described previously (Jaynes and O'Farrell, 1988; Wilson et al., 1993). A total of 5 μg of DNA containing 0.25 μg of hsp82-lacZ as reference gene and 2 μg of responder were used in each transfection. The amounts of DNA per transfection were equalized using empty pPAC vector. Quantification of acetylated and non-acetylated chloramphenicol was done with a PhosphorImager using the integrate volume function.

Protein-protein interactions

All washes, reagents and incubations were done at 4°C. Approx. 5 μg GST-Otd (quantification was done by Bradford assay) was washed 2× 2minutes in 0.1 M NaCl HEMG (25 mM HEPES at pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$ and 10% glycerol) and incubated in with the appropriate ^{35}S-labeled protein ($1/10^{th}$ reaction) with and without 200 ng of P3K oligo O/N. Samples were washed 6× 2minutes in 0.1 M NaCl HEMG and then split into two. Half the samples were washed for a further 6× 2minutes in 0.1 M NaCl HEMG whilst the other half was washed 6× 2minutes in 0.4 M NaCl HEMG. Samples were analyzed on 10% SDS-PAGE and visualized with PhosphorImager.

RESULTS

Passive and active repression

Gsc proteins are homeodomain transcription factors belonging to the Prd-class. This class of homeoproteins is unique, since their HDs are able to cooperatively homo- and heterodimerize on P3 palindromic sequence motifs composed of two characteristic TAAT HD core sequences arranged as a palindrome with a 3 bp spacing (Wilson et al., 1993). Depending on which residue occupies position 50 (Q_{50}, S_{50} or K_{50}), the Prd-class HDs recognize P3 sites with different levels of cooperativity and with different spacer sequences between the TAATs. For K_{50} HDs such as the Gsc or Otd proteins, the optimal sequence is TAAT **CCG** ATTA and is called P3K (Wilson et al., 1993). Although Bcd also bears a K_{50} HD, its HD does not belong to the Prd-class and binds DNA as a monomer (Yuan et al., 1996). The optimal binding sequence defined for Bcd is the non-palindromic TAAT**CCC** site, which corresponds to a P3K half site (Driever and Nüsslein-Volhard, 1989; Ronchi et al., 1993). In order to test whether D-Gsc could modulate transcription, we carried out co-transfection experiments in S2 *Drosophila* Schneider cells (Jaynes and O'Farrell, 1988; Krasnow et al., 1989; Han and Manley, 1993b). Initially, we used a reporter construct containing a single copy of the monomeric Bcd binding site (BcdBS: TAAT**CCC**) through which Bcd could drive CAT expression (Ronchi et al., 1993; Fig.1A) as the simplest site to measure D-Gsc activity. Following co-transfection of the 1×BcdBS-CAT reporter and an expression plasmid driving *D-gsc* (pPAC-*Dgsc*), CAT activity levels remained indistinguishable from background. These data suggest that D-Gsc was not an activator of transcription, providing the protein was expressed and could bind to the Bcd site. To test whether D-Gsc acts as a repressor of transcription we used expression plasmids for the transcriptional activators Bcd and Otd. Both proteins could activate transcription through the Bcd site in co-transfection

assays (Fig. 1A). We chose an amount of activator expression plasmid which resulted in near saturation levels of transcription, as measured by reporter gene activity. This amount was used in all subsequent repression assays in which only the amount of pPAC-*Dgsc* was varied. When both pPAC-*bcd* and pPAC-*Dgsc* were co-transfected, Bcd-driven CAT activity was down regulated and brought down to basal levels with increasing amounts of pPAC-*Dgsc* (Fig. 1A). The same situation was observed when pPAC-*Dgsc* was co-transfected with pPAC-*otd*. When equimolar amounts of *D-gsc* and either *otd* or *bcd* expression plasmids were co-transfected, CAT activity decreased by approximately 50%. These data, together with gel retardation assays using nuclear extracts from cells transfected with either D-Gsc, Otd or Bcd producer plasmids and P3K as a probe (data not shown), suggest that the amount of each protein present in transfected cells correlates with the amounts of expression plasmid transfected. Thus, when using the monomeric Bcd site, the down regulation of Otd and Bcd-activated transcription by D-Gsc appears to occur through passive competition for binding (passive repression, Jaynes and O'Farrell, 1991).

D-Gsc repressed both Otd- and Bcd-driven activation when a reporter containing three copies of the Bcd binding site (3×BcdBS-CAT) was used (Fig. 1B). However, in this case, the

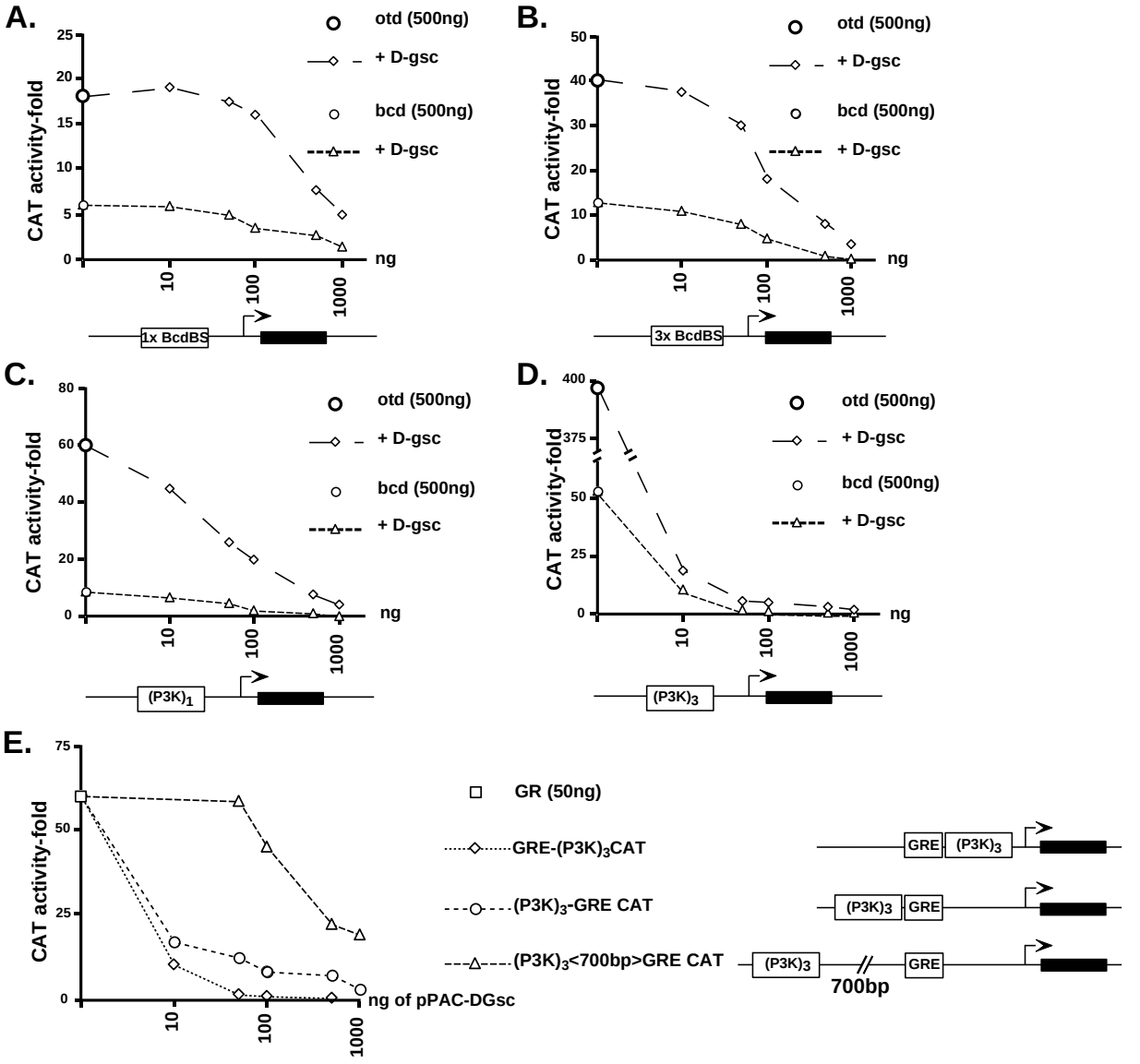

Fig. 1. Repression of activated transcription by D-Gsc. Schneider cells were co-transfected with either (A) 1× BcdBS-CAT, (B) 3× BcdBS-CAT, (C) $(P3K)_1$-CAT, or (D) $(P3K)_3$-CAT reporter plasmid and pPAC expression vectors driving *D-gsc*, *otd* and *bcd*. (E) 2 μg of either GRE-$(P3K)_3$CAT, $(P3K)_3$-GRE-CAT or $(P3K)_3$-700bpGRE-CAT were co-transfected with 50 ng of GR produced plasmid and increasing amounts of pPAC-*Dgsc*. GR was induced to bind to the GRE in the presence of 0.1 μM DEX (Sigma) 12-24 hours before harvesting. Diagrams illustrate the reporter constructs used in each experiment. The black boxes represent the CAT gene ORF. All transfection experiments were repeated at least twice. Plasmids were transfected at various ratios keeping the activator (pPAC-*otd*, *bcd*) amount constant with increasing amounts of pPAC-*Dgsc*. A 1:50 ratio, e.g. 10 ng of *D-gsc* vs. 500 ng of *otd*, is defined as a 'low level' in reference to the low amount of D-Gsc producer plasmid transfected. Consequently, ratios of 1:5 or 1:1 are defined as a 'high level' of D-Gsc.

downregulation of Otd- and Bcd-driven activation could not be explained by simple competition for binding, since 50% repression was achieved when 100 ng of pPAC-*Dgsc* were co-transfected with 500 ng of either *bcd* or *otd* expression plasmids (see Fig. 1B). Bcd has been reported to bind cooperatively to multiple copies of its monomeric site (Ma et al., 1996). Thus, we were expecting Bcd to prevent repression by D-Gsc by effectively competing with D-Gsc for binding to 3×BcdBS. This data could be explained if D-Gsc was acting as an active repressor (Jaynes and O'Farrell, 1991), in which case a single D-Gsc molecule bound to any one of the three Bcd sites would be sufficient to block transcriptional activation driven by either Otd or Bcd bound to the remaining sites. Alternatively, D-Gsc could bind cooperatively to the 3×BcdBS, with higher cooperativity than Bcd, effectively competing for binding with Bcd. However, D-Gsc has a Prd-class HD known to bind cooperatively only to dimeric palindromic sites (Wilson et al., 1993).

D-Gsc could also act as a repressor through a single dimeric P3K site. Co-transfection of $(P3K)_1$-CAT and expression plasmids for either activator (pPAC-*otd* or pPAC-*bcd*) and for *D-gsc* (Fig. 1C) showed that low levels of D-Gsc efficiently repressed Bcd when acting through $(P3K)_1$ (for definition of low and high levels see legend of Fig. 1). This could be due to active repression (one D-Gsc molecule binding to the P3K half site blocking activation of a Bcd molecule bound to the other half), or to the ability of D-Gsc to efficiently compete with Bcd by binding cooperatively as a homodimer to P3K (Wilson et al., 1993). Significant repression of Otd at low D-Gsc levels was also observed. This was unlikely to be due to active repression since both homeoproteins are capable of dimerizing on P3K (Wilson et al., 1993; see below). However, D-Gsc could compete for binding more efficiently as a dimer and/or recruit Otd into an inactive heterodimer in a mechanism other than active or passive repression.

Repression of Otd- and Bcd-driven activation was most dramatic when using $(P3K)_3$-CAT, a reporter construct with three tandem copies of P3K (Fig. 1D). When pPAC-*Dgsc* (10 ng) and pPAC-*otd* (500 ng: an amount able to provide a 400-fold activation) were co-transfected, Otd-driven CAT activity was dramatically down regulated (20-fold) and brought down to basal levels with increasing amounts of pPAC-*Dgsc* (Fig. 1D). The stronger effect of D-Gsc on Otd-driven activation when using multiple copies of (P3K) could be due to active repression.

Active repression of a heterologous activator

An additional assay to confirm that D-Gsc is capable of repressing transcriptional activators other than by competition for binding sites involved the use of an activator that binds to sites other than P3K. The reporter construct used contains three copies of the glucocorticoid response element (GRE; Jaynes and O'Farrell, 1991; Yoshinaga and Yamamoto, 1991). Three copies of P3K were inserted both 13 bp downstream or 11 bp upstream of the GRE to yield GRE-$(P3K)_3$CAT and $(P3K)_3$-GRE CAT respectively. The rat glucocorticoid receptor (GR) could activate transcription 60-fold through binding to GRE, following induction by dexamethasone (DEX; Fischer et al., 1988). Addition of pPAC-*Dgsc* (at 1:5 ratio) repressed GR-driven activation by 6-fold through GRE-$(P3K)_3$ (Fig. 1E). The repressive effect of D-Gsc was less pronounced (4-fold) when $(P3K)_3$-GRE was used, suggesting that, at least in part, repression by D-Gsc may be due to steric hindrance when its binding sites are positioned between those of the activator and the promoter. D-Gsc repression was dependent on the presence of P3K, since addition of D-Gsc was of no consequence in its absence (data not shown). To test whether D-Gsc could repress GR at a distance, we used the $(P3K)_3$-700 bp GRE reporter in which the P3K sites are located 700 bp upstream of the GRE. Higher levels of D-Gsc were required to repress GR-driven activation than when the P3K sites were immediately adjacent to the GRE, suggesting that repression by D-Gsc is, at least in part, distance dependent. For maximum repressor activity D-Gsc may require close proximity to the activator and/or the promoter.

Different domains within D-Gsc mediate repression of different activators

Since D-Gsc is able to repress several activator molecules through different mechanisms, we asked which protein domains were required for each type of repression. First, we could observe that the D-Gsc HD as well as its specific binding sites were required for all its repressor activities (data not shown).

Sequence comparison of all known Gsc proteins revealed that, in addition to the HD, there was another highly conserved region located at the N-terminus of the vertebrate proteins, the GEH/eh-1 domain (Goriely et al., 1996). This domain, known as eh-1 in the Engrailed protein, has been implicated in its repression activity (Smith and Jaynes, 1996).

In order to identify the potential repression domain within D-Gsc, we performed systematic N-terminal deletions of D-Gsc. D-GscΔ1 (Δ1) removed 98 amino acids (aa) at the N-terminal end of D-Gsc. This region is absent from the vertebrate Gsc molecules. D-GscΔ2 (Δ2) removed these 98 aa and the following 120 aa, including the GEH/eh-1 domain. D-GscΔ3 (Δ3) removed the N terminus of the protein, up to the HD (277 aa; see Fig. 2A). We also deleted the core seven aa of the GEH/eh-1 domain, (ΔGEH construct). We then tested the ability of Δ1, Δ2, Δ3 and ΔGEH to repress activation driven by Otd, Bcd or GR (Fig. 2B-D). All deletions, Δ1–3 and ΔGEH were still able to repress Bcd- and Otd-driven activation through passive repression, indicating that these D-Gsc constructs were able to compete for the binding sites in an equimolar ratio of repressor/activator and were therefore produced and folded properly (Fig. 2B,C). The Δ1 construct was able to repress transcription driven by the three activators essentially as efficiently as the full length D-Gsc (Fig. 2B). Deletions Δ2 and Δ3, however, were unable to repress Otd, Bcd and GR activation at low levels, indicating that these constructs no longer acted as active repressors (Fig. 2D). These results suggest that a general repression domain is present in D-Gsc between aa98 and aa218, in the region between the GEH/eh-1 domain and the HD.

The ΔGEH deletion could still repress Bcd and GR mediated repression (Fig. 2C-D), indicating that the GEH/eh-1 domain does not serve as a general active repression domain. However, ΔGEH had lost one specific function: its ability to repress Otd activation at low levels (see Fig. 2B). This argues that repression of Otd-mediated activation does not happen via passive competition for binding, or via active repression alone. It must occur, in addition, via another mechanism that requires the presence of the GEH/eh-1 domain. We have called this mode of repression 'interactive repression' as it appears to

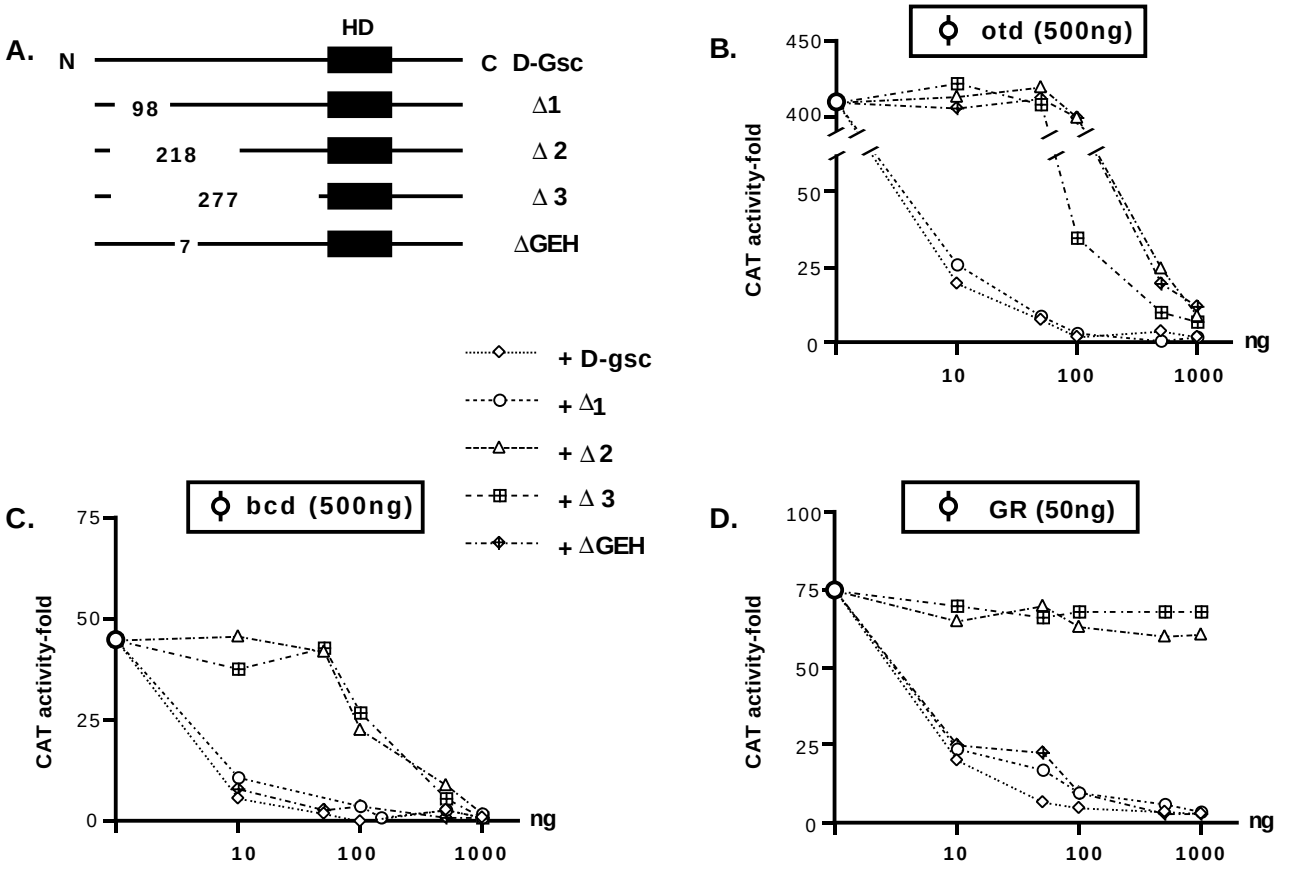

Fig. 2. Mapping of the D-Gsc domains involved in repression. (A). Diagram representing the full-length 418 aa long D-Gsc protein and the various mutants. The number of deleted aa (98, 218, 277, 7) are shown for each mutant. Deletions started at the same position (aa 6) and ended at aa 104 (Δ1), aa 224 (Δ2) and aa 233 (Δ3). The 7 aa comprising the GEH/eh-1 domain were at position 110-116. (B,C,D). Cotransfection assays (see Fig. 1 legend) using (B) Otd, (C) Bcd, and (D) GR as activators, wt D-Gsc, Δ1, Δ2, Δ3 and ΔGEH-DGsc as potential repressors and either $(P3K)_3$-CAT (B,C) or GRE-$(P3K)_3$-CAT (D) as reporter plasmids. Symbols apply to all three graphs. The amount of each activator used is indicated above. Deletion of the GEH/eh-1 domain from D-Gsc only affected the very efficient repression of Otd activation.

involve protein-protein interactions between Otd and D-Gsc (see below). We verified that the inability of ΔGEH to repress Otd activation was not due to lower levels of protein, or of DNA binding activity. Gel retardation assays showed that the binding activity *ΔGEH* is similar to that of wt D-Gsc (Fig.3A). Furthermore, ΔGEH was as efficient as wt D-Gsc at repressing GR and Bcd activation (Fig. 2C,D).

Thus, D-Gsc appears to repress transcription in three different ways. As a passive repressor, when its HD competes for the same binding sites with other K_{50} homeoproteins *(*i.e. repression of Bcd activation); as an active repressor by binding to neighboring binding sites (i.e. repression of GR activation); and as an interactive repressor when competing for binding with other Prd-class homeoproteins *(*i.e. repression of Otd activation). We show that the region between aa98-218 is required for all types of repression by D-Gsc while the interactive repression specifically requires the presence of the GEH/eh-1 domain.

The GEH/eh-1 domain is involved in protein-protein interactions with Prd-class homeoproteins

Although Otd, Bcd and D-Gsc all recognize P3K, the Bcd HD binds as a monomer (Yuan et al., 1996) whereas the Otd and D-Gsc HDs bind cooperatively as dimers (Wilson et al., 1993). Thus, the repression mechanism used by D-Gsc for repressing activation by Bcd when acting through palindromic sites such as P3K, is likely to be competition for the same sites. For Otd activation, however, it could involve formation of heterodimers between D-Gsc and Otd. Interestingly, D-Gsc was also able to repress transcriptional activation driven by another Prd-class K_{50} homeoprotein that does not share any sequence similarity with Otd outside the HD, a mutated version of the *Drosophila* Paired (Prd) protein ($PrdK_{50}$, whose S_{50} has been replaced by K_{50}). ΔGEH was unable to repress activation driven by $PrdK_{50}$ (data not shown). Although the HD is sufficient to provide cooperative dimerization (Wilson et al., 1993), our data argues that the GEH/eh-1 domain somehow affects heterodimer formation, since its removal from D-Gsc only impaired repression of activation driven by homeoproteins that can dimerize on P3K sites (Prd and Otd). An alternative explanation may be that the GEH/eh-1 domain interacts with a factor from the general transcription machinery contacted by an activation domain present on both Otd and Prd, but distinct from the activation domains present in Bcd or GR.

Using a GST pull-down assay, we showed that the ability of ΔGEH to interact with other Prd-class homeoproteins appears

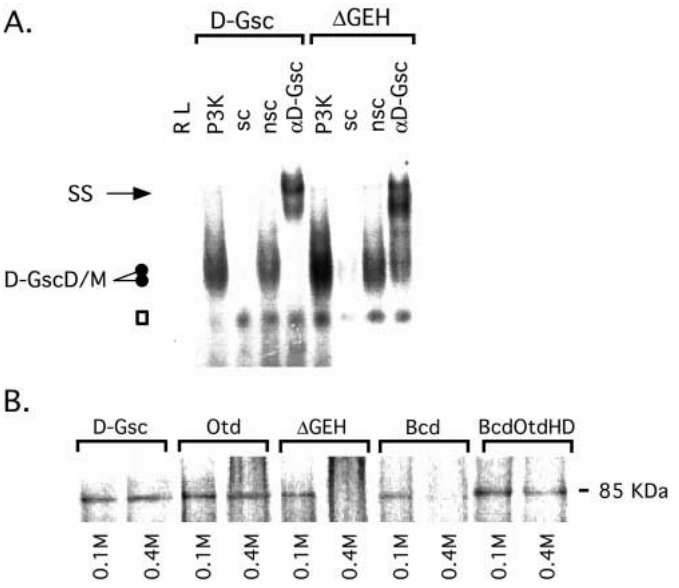

Fig. 3. The GEH/eh-1 domain is required for heterodimer formation. (A) EMSA using in vitro transcribed and translated full length D-Gsc and ΔGEH. Brackets group samples in which the same protein (but different competitors) was used. The arrow marks the position of supershifted complexes following addition of α-D-Gsc antibody. The two circles point to the D-Gsc monomer and dimer bands. The square marks the non-specific band present in primed reticulocyte lysate; RL unprimed reticulocyte lysate. (B) Protein-protein interaction assay analyzed by SDS-PAGE. Full length GST-Otd was incubated with in vitro synthesized, [^{35}S]methionine labeled, D-Gsc, Otd, ΔGEH Bcd and BcdOtdHD. The NaCl concentration at which the samples were washed is shown below the gel.

to be impaired (Fig. 3B). At a salt concentration of 0.4 M, GST-Otd interacted with D-Gsc, Otd and BcdOtdHD (a Bcd molecule with an OtdHD, see below), but not with ΔGEH and Bcd. These interactions were enhanced in the presence of 200 ng of P3K (data not shown), indicating that cooperative DNA binding is important for dimer formation (Wilson et al., 1993). Therefore, there appears to be a requirement for the presence of both the GEH/eh-1 domain and a Prd-class HD for the interaction between D-Gsc and Otd to occur. This may be important when D-Gsc is competing for binding with another homeoprotein (Otd) that has the same potential for occupying P3K, a situation that is likely to occur in the early *Xenopus* and *Drosophila* embryos where both proteins are co-expressed (Blumberg et al., 1991; Goriely et al., 1996).

The GEH/eh-1 domain is necessary for rescue of ventralized *Xenopus* embryos

To test the importance of the GEH/eh-1 domain for the *in vivo* function of D-Gsc, we used *Xenopus* embryos in which we had previously shown that *D-gsc* is able to partially rescue UV ventralization (Goriely et al., 1996). In this system, *Xenopus gsc (X-gsc)* is also able to rescue the dorsoventral axis of the embryo (Goriely et al., 1996) and has been shown to induce *Xlim 1* expression when misexpressed in the ventral marginal zone (VMZ; Taira et al., 1994). *Xlim 1* is expressed only in the dorsal marginal zone (DMZ) in wt embryos (Taira et al., 1992; Fig. 4B). *D-gsc* mRNA rescued UV-ventralized embryos to a dorsoanterior index (DAI; Kao and Elinson, 1988) of 1.4 (Fig. 4A), as visualized by the appearance of dorsoanterior structures. When injected into the ventral marginal zone, it also induced *Xlim 1* expression, as assayed by RT-PCR (Fig. 4B). In contrast, *ΔGEH* was unable to rescue UV-ventralized embryos (Fig. 4A), the DAI (0.45) remaining essentially equal to that of control embryos (0.3). However, *ΔGEH* was still able to induce *Xlim 1* expression (Fig. 4B), suggesting that *D-gsc* mediates rescue of dorsoanterior structures and of *Xlim 1* induction via distinct mechanisms. The HD was required for both functions since no UV rescue (data not shown; Cho et al., 1991) or *Xlim 1* expression in the VMZ was observed with a mutant form of *D-gsc* lacking the HD sequence (*ΔHD*, Fig. 4B).

Dimerization is required for interactive repression

The crystal structure of the Prd HD dimers bound on DNA suggests that the nature of the amino acids at positions 28 and 43 is critical in determining whether a HD can dimerize on DNA (Wilson et al., 1995). Bulky amino acids at these positions, very often found in non-Prd-class HDs, prevent dimerization but do not affect binding as a monomer (Wilson et al., 1995). We mutated E43 in the D-Gsc HD to R43 to create a full-length ER43-DGsc. This mutant was only able to repress Otd-driven activation through $(P3K)_3$ when present at high levels (i.e. through passive repression), confirming that HD dimerization is essential for interactive repression (compare Figs 5A and 1D). We expected repression of both Bcd and Otd mediated activation to be impaired, as ER43D-Gsc is only able to bind P3K as a monomer and therefore, should bind less efficiently to this site than the wt D-Gsc protein. A D-Gsc

A. UV Rescue

	lacZ	X-gsc	D-gsc	ΔGEH	UI	WT
DAI	0.3	1.2	1.4	0.45	0.1	5
n embryos	45	60	40	42	60	50
% survival	82	80	71	79	85	95

B. *Xlim 1* Expression

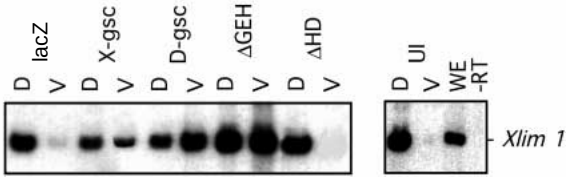

Fig. 4. The GEH/eh-1 domain is necessary for the rescue of UV-ventralized *Xenopus* embryos by *D-gsc*. (A) Table representing the dorsoanterior index (DAI, 0 = ventralized embryo; 5 = wild type) of embryos injected at the 2-4 cell stage with synthetic capped mRNA for *lacZ*, *X-gsc*, *D-gsc* and *ΔGEH* or uninjected (UI), following UV-irradiation. (B). *Xlim 1* expression in dorsal (D) and ventral (V) marginal zone explants, as assayed by RT-PCR following injection of *lacZ*, *X-gsc*, *D-gsc*, *ΔGEH* or *ΔHD* mRNA into the ventral blastomeres of the 2-4 cell stage wild type embryos. UI, uninjected embryos; WE, whole embryo; -RT, mock reverse transcriptase reaction to which no enzyme was added.

molecule that can no longer dimerize behaves essentially as wt D-Gsc on multiple copies of the monomeric Bcd site (3×BcdBS; Fig. 1B). Active repression per se, however, should not be affected when disrupting dimerization. Indeed, ER43-DGsc could still repress GR-driven activation (though less efficiently than wt D-Gsc; Fig. 5A). This is in agreement with the presence in D-Gsc of a bona fide repressor domain that is targeted to the promoter via the HD.

Similarly, disrupting Otd dimerization by either the S43R or I28K mutation (Wilson et al., 1996) transformed the protein to a less effective activator. Mutant Otd behaved essentially as Bcd (compare Fig. 5B with 2B,C). As a consequence, Otd molecules could now be effectively repressed by ΔGEH on $(P3K)_3$ (Fig. 5B), presumably through active and passive repression. We confirmed by EMSA that the mutant Otd and Gsc homeodomains could no longer cooperatively dimerize on DNA (Fig. 5C). In this experiment, we also observed that cooperative dimerization of the wt D-Gsc HD on P3K appears stronger than that of the Otd HD. Thus, when present at low levels, D-Gsc may efficiently repress Otd-driven transcription by driving Otd into a transcriptionally inactive heterodimer both through cooperative HD heterodimerization and via protein-protein interactions mediated by the GEH/eh-1 domain.

Finally, we used a Bcd-variant containing the Otd HD instead of its own HD (BcdOtdHD) to asses the importance of a dimeric homeodomain for interactive repression. In contrast to Bcd, BcdOtdHD interacted well with Otd in the GST pull down assay (see above), suggesting that the nature of the HD contributes to the in vitro interaction. BcdOtdHD-driven activation was efficiently repressed by wt D-Gsc. In addition, ΔGEH/eh-1 did not efficiently repress BcdOtdHD suggesting that the HD swap which replaced the Bcd HD with that of Otd is sufficient to render Bcd 'Otd-like' (compare Figs 2C and 5D). These data argue that the GEH/eh-1 domain enables D-Gsc to efficiently repress Otd at low levels, possibly by interacting with the Otd HD. Our data suggests that the interaction of the GEH/eh-1 domain with Prd-class HDs is crucial to D-Gsc ability to act as an interactive repressor as it enhances heterodimer formation mediated by its strongly cooperative HD.

DISCUSSION

Transcriptional repressors have become the focus of intense research as a consequence of their important regulatory function (Johnson, 1995). Homeoprotein repressors are of particular interest since they are key developmental regulators that must achieve functional specificity despite their highly related DNA binding specificity. In this paper, we show that the D-Gsc homeoprotein acts as a strong repressor of transcription by three distinct mechanisms: passive, active and interactive repression (Fig. 6). Passive and active repression by homeoproteins have been previously described, in particular with reference to En (Han and Manley, 1993a; Jaynes and O'Farrell, 1991). Repression of Bcd-driven activation by D-Gsc through the monomeric Bcd site (1×BcdBS) is likely to involve passive repression i.e. competition for binding sites (Fig. 6A). Repression of Bcd-driven activation thorough multiple copies of the monomeric Bcd site (3×BcdBs) could be due to active or passive repression depending on how D-Gsc binds to this site. We favor active repression by non-cooperative D-Gsc binding to 3×BcdBS based on our previous analysis of Prd-class HD binding (Wilson et al., 1993). When using the dimeric palindromic P3K site, cooperative dimerization of D-Gsc on this site (Wilson et al., 1993) would render it a very effective competitor of the Bcd monomer, resulting in efficient repression of Bcd-driven activation. Consistent with this model, repression of Bcd activation is significantly reduced when using the monomeric Bcd binding sites or a D-Gsc molecule unable to dimerize. Further evidence that D-Gsc could act as an active repressor is its ability to strongly repress Otd-driven transcription through multiple copies of P3K. Considering both homeoproteins can cooperatively dimerize on P3K, competition for binding is

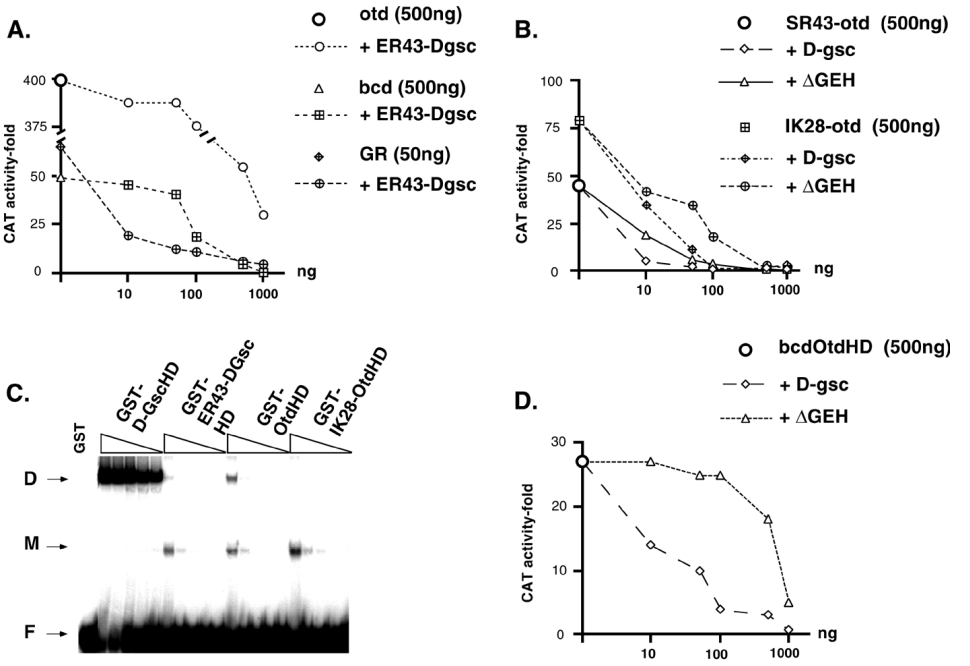

Fig. 5. Repression by D-Gsc requires cooperative dimerization. (A) Transfection assay using $(P3K)_3$-CAT and $pT3(P3K)_3$-CAT reporter and expression plasmids driving *otd*, *bcd*, *GR* and *ER43-Dgsc*, a full-length *D-gsc* mutant with a mutation in the HD which renders it unable to dimerize. (B) Transfection assay using $(P3K)_3$-CAT and pPAC driving *SR43-otd*, *IK28-otd*, (*otd* mutants unable to dimerize), full length *D-gsc* and *ΔGEH*. (C) EMSA comparing the abilities of the wild type D-Gsc HD, the ER43 mutant D-Gsc HD, the wild type Otd HD and the IK28 mutant Otd HD to cooperatively dimerize on P3K. The HDs were produced as GST fusions and purified on glutathione beads. Arrows point to the position of the HD monomer (M) and dimer (D) and free probe (FP). (D) A Bcd protein whose HD has been replaced by the Otd HD is efficiently repressed by D-Gsc. This repression is dependent on the GEH/eh-1 domain.

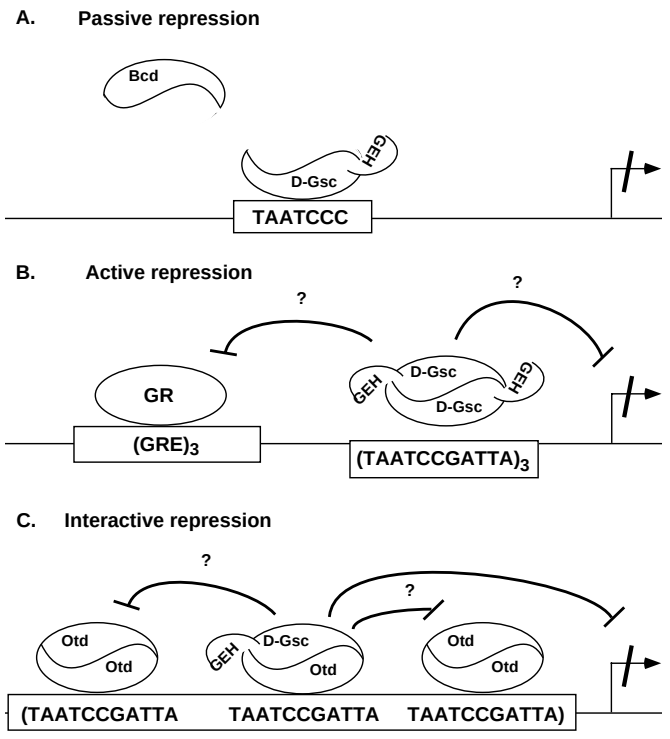

Fig. 6. Model of passive, active and interactive repression. (A) Passive repression. When the monomeric TAATCCC Bcd site is used, repression of Bcd by D-Gsc is only observed at 1:1 DNA ratios. When dimeric P3K sites are used, cooperative binding by D-Gsc allows it to efficiently compete for binding with Bcd allowing D-Gsc to repress even at low levels (not shown). (B) Active repression does not involve competition for binding. D-Gsc and GR bind to distinct sites. Nonetheless, D-Gsc can repress activation by GR. D-Gsc can also actively repress homeoprotein activators when using multimerized sites (C). The presence of a D-Gsc:Otd heterodimer allows D-Gsc to repress Otd-driven transcription when present at low levels. The GEH/eh-1 domain is essential for interactive repression which appears to also depend on the presence of dimeric HDs.

unlikely to yield effective repression when D-Gsc is present at very low levels. The strong repression observed could be explained by D-Gsc binding to a single copy of P3K (either as a homodimer or in a Otd:D-Gsc heterodimer when present at low levels, see below) and repressing transcription from the remaining copies of P3K.

Active repression is the *bona fide* mechanism by which D-Gsc represses GR-driven activation since GR and D-Gsc bind to different sites (Fig. 6B). There are two general views of how active repression might work. One is that the repressor contacts the transcriptional machinery directly or via a general cofactor, and thus blocks transcription irrespective of the activator (direct repression; Johnson, 1995). Alternatively, the repressor could interact with the activator, either directly or indirectly through a cofactor, and thus block the effect of that particular activator (quenching, Gray et al., 1994). In both cases, the repressor does not act by competing for binding sites. D-Gsc effectively represses GR-driven activation when both activator and repressor sites are in close proximity to each other and the promoter, suggesting that D-Gsc could act both through quenching and/or through direct contact with the transcriptional machinery (Gray and Levine 1996). Consistent with short-range repression by D-Gsc, its repressor activity is impaired once P3K is 700 bp away from the GRE. However, significant repression of GR-driven activation is observed with high levels of D-Gsc when using the $(P3K)_3$-700bpGRE reporter indicating that, at high enough levels, D-Gsc will interfere with transcription irrespective of the position of its binding sites.

We refer to interactive repression as a form of repression that involves protein-protein interactions between repressor and activator homeoproteins. This type of repression is illustrated by the repression of Otd activation by D-Gsc, which depends on the presence of the HD and GEH/eh-1 domain in D-Gsc, and on the presence of a Prd-class HD in the activator protein.

The conservation of the eh-1 domain which is similar to the GEH/eh-1 domain but is found in other classes of homeoproteins such as En, has led to the proposal that it mediates protein-protein interactions. However, it is not clear how this domain is involved in repression (Smith and Jaynes, 1996). We propose that, in the case of D-Gsc, this conserved domain acts to enhance the formation of HD-dependent heterodimers between D-Gsc and Prd-class homeoprotein activators, leading to an increased ability of D-Gsc to repress transcription.

A model for interactive repression is shown in Fig. 6C. D-Gsc and Otd can exist either as homo- or heterodimers. Our data suggest that D-Gsc:Otd heterodimer formation is favored over homodimer formation in the presence of low D-Gsc levels. This is similar to what is observed in the case of c-Fos and c-Jun where the presence of even small amounts of c-Fos dramatically shift the equilibrium from c-Jun homodimers to c-Jun:c-Fos heterodimers (Halazonetis et al., 1988). The enhanced heterodimerization between Otd and D-Gsc depends on HD dimerization, as well as protein-protein contacts between the GEH/eh-1 domain and the Otd HD. A similar interaction has been observed for another homeodomain protein containing a eh-1 domain: the eh-1 domain of NK-3 or NK-4 interacts with HDs of the NK-2 class homeoproteins. However, in this case the eh-1-HD interaction does not appear to be required for the inhibitory activities of the proteins in cell culture (Cheol-Yong Choi and Yongsok Kim et al., personal communication). The D-Gsc:Otd heterodimer may have increased affinity for DNA and it may even use the activator (Otd) to efficiently target the repression domain of D-Gsc to the promoter. In these circumstances, a single heterodimer bound to one copy of P3K may be sufficient to block activity of Otd homodimers bound to the remaining two copies of P3K in $(P3K)_3$.

The eh-1 domain has been shown to be an important determinant of the ability of En to repress transcription in vivo. The En eh-1 is neither the only region that contributes to En repressor activity, nor is it required for all En functions (Han and Manley, 1993a; John et al., 1995; Smith and Jaynes, 1996). Similarly, the interactions mediated by the GEH/eh-1 domain in D-Gsc are unable to account for active repression: ΔGEH is as efficient as wt D-Gsc in repressing GR and Bcd-driven activation and requires a general repression domain present between aa98 and 218 in D-Gsc. The fact that ΔGEH is unable to rescue UV-ventralized *Xenopus* embryos but can still induce *Xlim 1* expression, reinforces the notion that the GEH/eh-1 domain is only required for a subset of Gsc functions in vivo.

The GEH/eh-1 domain is present in divergent classes of homeoproteins (*e.g.* En, Msh, NK; Smith and Jaynes, 1996), suggesting that this domain is ancient, and must have been present before the duplication events that resulted in the various HD classes in which eh-1 has been identified. The presence of this domain in a subset of homeoproteins may be a necessary additional feature they possess to achieve increased specificity. This might also be true for another short amino acid stretch, the conserved heptapeptide that mediates interactions between the Hox and En homeoproteins and the Pbx/Exd family of homeoproteins (Chan et al., 1994; van Dijk and Murre, 1994; Chang et al., 1995; Peers et al., 1995; Peltenburg and Murre, 1996; Phelan et al., 1995). Despite mediating interactions with the same family of proteins (Pbx/Exd), the heptapeptides of Hox and En proteins are only distantly related. Thus, it is possible that the GEH/eh-1 domain of En and D-Gsc (which are different from the hexapeptide), may have distinct functions, having evolved independently from each other, but in parallel with the factor with which they interact. Thus, the D-Gsc GEH/eh-1 domain may have evolved to interact with Prd-class homeoproteins as shown by the inability of ΔGEH to repress activation brought about by Otd, $PrdK_{50}$ and BcdOtdHD.

Interestingly, both Gsc and Otd/Otx are co-expressed in flies and vertebrates (Goriely et al., 1996; Pannese et al., 1995) and are thought to belong to a conserved regulatory network (Goriely et al., 1996). There is indirect evidence that Gsc may act as both an activator and repressor of Otd in vivo (Pannese et al., 1995). In *Drosophila*, although *otd* regulates the early anterior expression pattern of *D-gsc* (Goriely et al., 1996), any effect of *D-gsc* on *otd* targets must be redundant, since *D-gsc* mutant embryos, unlike *otd* mutant embryos, develop normal head structures (Hahn and Jäckle, 1996). Other Prd-class homeoproteins may also be expressed early in development and their activator function be affected by Gsc repression (Lamonaire et al., 1996).

The HD and the GEH/eh-1 domains are the only common features among Gsc proteins. All forms of repression by D-Gsc, however, require a general repression domain outside these conserved regions which shares no homology in primary sequence with other Gsc molecules. We have narrowed the repressor domain in D-Gsc to 120 aa between the GEH/eh-1 domain and the HD. The repressor function of D-Gsc appears to be conserved amongst Gsc proteins, despite the lack of sequence homology of the repressor domain, since *X-gsc* is also a transcriptional repressor behaving similarly to *D-gsc* in our assay (data not shown).

Differences in homeoproteins potency to activate or repress transcription may partially account for their functional specificity. Although the HD itself does not appear to mediate repressor function (Jaynes and O'Farrell, 1991), it substantially contributes to the targeting and protein-protein interactions which potentially define homeoprotein function. The Prd-class HDs can interact with other HDs of the same class to cooperatively dimerize (Wilson et al., 1993). It can also interact with other protein domains to mediate repression (this study; Johnson, 1995) or activation (Grueneberg et al., 1992) of gene expression. Heterodimeric transcription factor complexes offer several advantages. First, they allow for more elaborate regulation of transcription as production of each partner can be independently regulated. Second, their interactions allow a small number of proteins to generate a large number of combinations of transcription factors with different binding specificities and functions. This strategy would be particularly useful during development when multiple regulators are co-expressed.

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