Goosecoid-like, a gene deleted in DiGeorge and velocardiofacial syndromes, recognizes DNA with a Bicoid-like specificity and is expressed in the developing mouse brain

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The vast majority of patients with DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) have deletions of chromosomal region 22q11.2. These patients exhibit broad and variable phenotypes that include conotruncal cardiac defects, hypocalcemia, palatal and facial anomalies and developmental delay. Most of these abnormalities are thought to be due to defects in neural crest cell migration or differentiation. We have identified a homeobox-containing gene, Goosecoid-like (GSCL), that is in the region within 22q11 that is deleted most consistently in patients with DGS/VCFS. The GSCL gene is expressed in a limited number of adult tissues as well as in early human development, and is a member of a family of homeobox genes in vertebrates that includes Goosecoid and GSX. In this report, we present functional studies of the GSCL protein and determine the expression pattern of the GSCL gene in mouse embryos. We demonstrate that GSCL exhibits DNA sequence-specific recognition of sites bound by the Drosophila anterior morphogen, Bicoid. Several of these sites (TAATCCC) were found in the 5′ upstream region of the GSCL gene itself, and we present evidence suggesting that GSCL might regulate its own transcription. In situ hybridization revealed that the mouse ortholog of GSCL, Gscl, is expressed in the brain starting as early as embryonic day 9.5, and expression continues in adults. This expression pattern is consistent with GSCL having either an indirect role in the development of neural crest-derived structures or a direct role in a subset of the phenotype observed in DGS/VCFS, such as learning disorders or psychiatric disease.

INTRODUCTION

Deletion of chromosomal region 22q11.2 is associated with a variety of human disorders including DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS) and some cases of isolated and familial conotruncal cardiac defects (1). Several of the most common abnormalities observed in these patients, conotruncal cardiac defects, palatal defects, thymic and parathyroid hypoplasia or aplasia and characteristic facial features, can be explained, at least in part, by perturbation of a gene, or genes, required for neural crest cell migration or differentiation (2,3). However, several other abnormalities observed in the 22q11-deleted patients are not as clearly associated with neural crest cell migration or differentiation. For example, a significant number of patients with 22q11.2 deletions have genitourinary abnormalities and the majority of patients exhibit mild to severe developmental delay (4,5). While haploinsufficiency for a single gene could explain the broad and highly variable phenotypes seen in these patients, it is also possible that this complex disorder is due to reduced expression of several genes.

To understand the genetic basis for DGS/VCFS, we and others have identified and characterized genes within the ~2 Mb region of chromosome 22 that is deleted in >85% of patients (1). The positions of several unbalanced translocations as well as two balanced translocations suggest that the centromere proximal 250–500 kb of the deletion interval may be most critical to the phenotype (1). While numerous genes have been isolated and characterized from this region, studies performed thus far have not provided strong evidence for any one gene being directly responsible for all features seen in DGS/VCFS. For example, HIRA, LANIDD/DGCR2 and UFD1L are all located within the

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critical region; however, they are expressed ubiquitously during development of the chick and mouse (6–9). Therefore, further studies are required to determine whether these genes are required specifically for the formation of the structures that are defective in DGS/VCFS. TBX1, a member of the T-box family of transcription factors, is expressed in the first, second and third pharyngeal arches and pouches (10), making TBX1 an attractive candidate gene for DGS/VCFs. However, TBX1 maps distal to the critical region defined by most investigators (11). In addition, no mutations in TBX1 were found in the DNA of affected patients with no detectable deletions on chromosome 22 (11).

We have focused our studies on a homeobox-containing gene, Goosecoid-like (GSCL), which maps to the region that is deleted most consistently in patients with DGS/VCFs (12). GSCL is expressed in a limited number of adult tissues as well as in early human development (12). The early expression together with the known role of homeobox-containing genes in development make GSCL an excellent candidate for some of the abnormalities seen in DGS/VCFs. To understand the role that GSCL might play in DGS/VCFs, we have initiated functional analysis of the GSCL protein and characterized the expression pattern of the GSCL gene in embryos. In this report, we show that GSCL exhibits DNA sequence-specific recognition similar to that of the Drosophila Bicoid protein. Further, we show that GSCL recognizes sites found within its own promoter, suggesting that GSCL might regulate its own transcription. By in situ hybridization, we show that the mouse ortholog of GSCL, Gsc, is expressed in the brain of 9.5–15.5 day mouse embryos and in adult mouse brain. Although the observed expression pattern of Gsc is not what would be predicted for a gene responsible for the classic features of DGS/VCFs, Gsc is expressed at the time during development when the structures affected in DGS/VCFs are forming and, therefore, could play an indirect role in their development. However, a more likely interpretation of these results is that GSCL may play a role in some of the neurological phenotypes of the disease such as psychiatric illness and learning disabilities.

RESULTS

Goosecoid-like is a member of a family of paired-like homeodomain proteins

The GSCL gene encodes a 205 amino acid protein that is a member of the paired-like class of homeodomain proteins. The paired-like class, as well as other classes of homeodomain proteins, contains several smaller families of highly related proteins (15). In vertebrates, there appears to be a family of ‘goosecoid-like’ genes, goosecoid (gsc) (14), GSCL (12) and GSX (15), while in Drosophila, only a single goosecoid gene, D-gsc, has been identified (16). In addition, there appears to be a single family member in Caenorhabditis elegans (Genome Sequencing Center, personal communication). Gsc was identified originally as a cDNA expressed in the dorsal lip of Xenopus oocytes (14). It was shown subsequently to be expressed in the primitive streak in mouse embryos (17) as well as in the craniofacial region later in embryogenesis (18). The chick gene GSX was identified by low-stringency screening of a chick genomic library using Xenopus gsc as a probe and was shown to exhibit a complementary expression pattern to that of gsc in early embryos (15). Within the goosecoid-like family, there is between 68 and 87% identity in the homeodomain (Fig. 1). Outside of the homeodomain, there is little to no similarity between family members except for a heptapeptide present near the N-terminus of the protein. This heptapeptide is also present in several other homeodomain proteins and is thought to be involved in transcriptional repression (19,20). At this time, it is not known whether there is any redundancy in the function of the goosecoid-like family members, although the reported expression patterns of these genes suggest independent functions (15,17,18,21 and see below).

Genomic sequence representing the mouse homolog of GSCL, Gsc, has been described (22). The mouse and human genes display 72% identity across the entire protein and 98% identity within the homeodomain. Outside of the homeodomain, there are several smaller stretches of near identity, including one containing the heptapeptide mentioned above.

Goosecoid-like binds DNA like Bicoid

A distinguishing feature of the GSCL protein is the presence of a lysine residue at position 50 of its homeodomain (Fig. 1). A lysine at this position is rare among homeodomain proteins, the vast majority instead having glutamine (13). The Bicoid protein from Drosophila melanogaster was the first homeodomain protein identified that carries a lysine at position 50, and its binding specificity has been well characterized (23,24). Bicoid recognizes the core sequence TAATCCC, while proteins with a glutamine at position 50, such as the Antennapedia class of homeodomain proteins, recognize the core sequence TAATTTGA or TAATTAG (24). These and other studies (25,26) showed that residue 50 is part of the recognition α-helix and helps determine the DNA-binding specificity of homeodomains.

We therefore tested whether GSCL protein recognizes DNA with the same specificity as Bicoid. For these experiments, we used a sensitive in vivo transcription activation assay (24). A portion of the GSCL protein including the homeodomain (amino acids 49–201) was expressed in yeast as a fusion to the B42 activation domain (see Materials and Methods). If the fusion protein recognizes DNA, it will activate transcription through the exogenous activation domain. We tested the ability of this fusion protein to activate a lacZ reporter gene that carried Bicoid-binding sites, Antennapedia class homeodomain-binding sites or no binding sites. As shown in Figure 2A, the GSCL fusion protein
Figure 2. (A) Goosecoid-like activates transcription of LacZ reporter genes containing Bicoid consensus binding sites in the promoter region. The binding sites are as follows: Antp class sites (ATTAAATTGA TA) 6; Bicoid sites (TCTAA TCCCTA) 6; GSCL upstream sequence 5′-AGGATTA GCGCGTTCGCGGCCGGCGCTGC GGGATTA-3′. See Materials and Methods for specifics of the assay conditions. (B) Alignment of putative promoter sequences from the human and mouse GSCL genes. The conserved Bicoid consensus binding sites are boxed. Note that the consensus site, TAA TCCC, is present in this sequence on the reverse strand.

activates transcription of the Bicoid site reporter. In contrast, GSCL does not activate, above background levels (no binding site), a control reporter carrying binding sites for Antennapedia class (Gln50) homeodomains. GSCL had higher background levels of transcription compared with Bicoid, perhaps because the GSCL we used (amino acids 49–201) lacked its N-terminus. These results show that, like Bicoid, GSCL prefers the Bicoid site over a site bound by the majority of homeodomain proteins, those with a glutamine at position 50. In addition, activation of the reporter by GSCL was abolished by a mutation in the conserved TAA T core of the homeodomain-binding site (TAA TCCC→TAAA CCC) (data not shown). This result supports the conclusion that GSCL binds DNA in a manner similar to other homeodomain proteins, since both Bicoid and Antennapedia class homeodomain proteins fail to bind to sites with this mutation (24).

**Goosecoid-like may autoregulate**

We next tested whether GSCL can recognize its own 5′ enhancer. A 70 bp region that contains three strong Bicoid sites was identified upstream of the putative GSCL transcription start site (Fig. 2B). The location, orientation and spacing of these sites is conserved in the 5′ upstream region of the mouse Gscl gene (Fig. 2B). The presence of these sites was noted previously (27). Oligonucleotides matching this 70 bp sequence were synthesized and inserted upstream of a lacZ reporter gene (see Materials and Methods). Bicoid strongly recognized this GSCL enhancer–reporter gene in the yeast assay (Fig. 2A). More importantly, the GSCL fusion protein also activated this reporter gene, suggesting that GSCL may function in an autoregulatory manner (Fig. 2A). For the native GSCL protein, this might result in either activation or repression.
**Gscl is expressed in the brain**

We previously reported the expression of human GSCL in adult testis as well as in 9–10 week fetal tissue (12). RT–PCR analysis of mouse Gscl indicates that the gene is expressed in mouse embryos and in adult testis and brain but not adult kidney, liver, lung, skeletal muscle or heart (data not shown). No expression was seen on a commercial fetal mouse RNA northern blot using high stringency hybridization conditions (0.1% SDS, 0.1× SSC) (data not shown). To characterize the expression pattern of Gscl further, we performed *in situ* hybridization analysis on sectioned material from paraffin-embedded 9.5–15.5 day mouse embryos, as well as from adult mouse brain.

At 9.5 days, a narrow band of expression in the neuroepithelium of the diencephalon is seen (Fig. 3A). Examination of a complete series of transverse sections through an entire 9.5 day embryo confirms the absence of detectable expression in other locations. At 10.5 days, expression continues in the diencephalon. In addition, weak expression is detected in the anterior hindbrain (data not shown). At 11.5 days, Gscl expression in the hindbrain, in the region of the prospective pons, is much stronger and has a punctate appearance (Fig. 3B). Expression continues in the dorsal region of the diencephalon, in the region of the prospective thalamus and epithalamus (Fig. 3B).

By 12.5 days, expression in the diencephalon appears restricted to the epithalamus, a small region of the brain situated dorsal to the thalamus (Fig. 4A). To define better the location of the Gscl signal, an adjacent section was hybridized with a probe for the Gbx-2 gene, a homeobox gene that has been shown to be expressed in the dorsal thalamus but not the epithalamus in 12.5 day embryos (28). Our results indicate a striking complementarity in the expression patterns of Gbx-2 and Gscl in the dorsal diencephalon (Fig. 4B). Transverse sections at this stage hybridized with a probe to Gscl indicate that expression in the epithalamus is in the ventricular zone of the habenula (Fig. 3C). Strong expression continues in the pons, and transverse sections at this stage suggest that expression is in the region of the serotonergic nuclear complex (Fig. 3D). As was observed with the 9.5 day embryos, there was no detectable expression in tissues outside of the brain at 10.5, 11.5 or 12.5 days.

By 15.5 days, Gscl is no longer expressed in the epithalamus but there is still strong expression in the pons (Fig. 5A). The expression in the pons correlates with the position of the median and dorsal raphe nuclei, serotonergic neurons with ascending projections to the cortex. To confirm this localization, we performed immunohistochemistry on a neighboring section with an antibody to serotonin. Figure 5C shows the region of the pons exhibiting serotonin immunoreactivity. The results demonstrate an overlap between the serotonergic neurons and Gscl mRNA-expressing cells, suggesting that Gscl is expressed either in the neurons of the raphe nuclei themselves or in adjacent cells (Fig. 5B and C).

We also performed *in situ* hybridization on coronal sections through an adult mouse brain. While no expression was observed in either the pons or the habenula, expression was seen in the interpeduncular nucleus (IP) located in the ventral midbrain (Fig. 6). The IP is part of a relay system from the limbic forebrain to the midbrain (see Discussion). The expression in the adult does not derive from either of the regions of expression we observed in the embryo and, therefore, suggests a third independent site of expression for Gscl.

Interestingly, there appears to be no overlap between Gscl expression and that reported for gsc (18) at the stages we have examined. Therefore, it is unlikely that gsc and Gscl serve redundant functions at this time in development. Expression of GSX at this time of development has not been reported.
DISCUSSION

We report here functional studies of the GSCL protein and extensive expression analysis of the mouse homolog of GSCL. As a first step toward defining the molecular pathway regulated by this gene, we have investigated the DNA recognition properties of the GSCL protein. The demonstration that GSCL, which carries a lysine at position 50 of the homeodomain, recognizes Bicoid-binding sites in an in vivo assay is important for several reasons. First, it indicates that, like other homeodomain proteins, GSCL acts as a sequence-specific DNA-binding protein. Second, it suggests that GSCL target genes might be identified based on their possession of the sequence TAATCCC or close variants of it in their promoters. Indeed, we have identified the GSCL gene itself as a potential target gene for autoregulation by GSCL protein. A 70 bp element located upstream of the putative GSCL transcription start site that is conserved between human and mouse was found to contain three potential GSCL-binding sites. This element when placed in a heterologous reporter gene, is sufficient to drive GSCL-dependent expression in yeast. Further, the finding that there are multiple sites within this small element suggests that GSCL might bind DNA in a cooperative manner.

Third, work in a number of organisms suggests that homeodomain proteins that carry a lysine at position 50 are required for anterior pattern formation early in development (e.g. 29–32). Our results suggest that GSCL, which is expressed in the brain, fits this model and may play an important role in specification of anterior structures. Finally, the identification of GSCL-binding sites should enable studies aimed at determining whether GSCL is normally an activator or, as the presence of the conserved heptapeptide suggests, a repressor of gene expression.

Our studies show that in the mouse, Gscl appears to be expressed exclusively in the brain. At 9.5 days, Gscl is expressed...
in the diencephalon. At 10.5 days, expression continues in the forebrain, with faint expression being detected in the anterior hindbrain. By 12.5 days, expression is seen in two distinct regions of the brain: the epithalamus of the diencephalon and the pons of the hindbrain. This is not the expression pattern predicted for a gene involved in the ‘classic’ features of the 22q11 deletion syndrome (conotruncal cardiac defect, thymus and parathyroid gland hypoplasia and abnormal facies) because Gscl does not appear to be transcribed in neural crest cells or neural crest-derived tissues. However, this expression is consistent with GSCL playing a role in the neurological phenotypes of the disease such as psychiatric illness and learning disabilities (see below).

These results add Gscl to the growing list of homeobox genes shown to be expressed in the developing vertebrate forebrain (33). Starting at 9.5 days, the earliest time point we have examined, and continuing to 11.5 days, Gscl is expressed broadly in the diencephalon, in both the dorsal thalamus and epithalamus. By 12.5 days, expression of Gscl is restricted to the epithalamus in a pattern complementary to that of the homeobox gene Gbx-2. The latter gene is expressed throughout the dorsal thalamus but is excluded from the epithalamus (28). The epithalamus, a relatively small region of the forebrain situated dorsal to the thalamus, consists of the pineal gland, the habenula and the stria medullaris thalami. The pattern of expression seen in transverse sections of 12.5 day embryos indicates that Gscl is expressed in the ventricular zone of the habenula. In mammals, the habenula consists of two separate nuclei, the medial and lateral habenular nuclei, with distinct connections to other parts of the forebrain and the midbrain (see below). This unique expression pattern of the Gscl gene contributes to the complex pattern of homeobox gene expression in the forebrain and is likely to be important for specifying the neuronal identity of the dorsal diencephalon.

The second region of Gscl expression in the brain is in the mantle layer of the pons. This expression also was reported by Lindsay et al. (34). Expression in the pons appears to correlate with the location of the dorsal and median raphe nuclei as determined by immunostaining neighboring sections with an antibody to serotonin (this study and ref. 34). The dorsal and median raphe nuclei are groups of serotonergic neurons with ascending fibers to the forebrain (35). Interestingly, the onset of Gscl expression in the pons at mouse embryonic day 10.5 (which corresponds to embryonic day 11.5–12 in the rat), slightly precedes the time when the raphe nuclei begin to express serotonin, embryonic day 12 in the rat (36). Therefore, Gscl may be required for the specification of that group of serotonergic neurons.

The expression pattern of Gscl in the pons overlaps with the expression of Dgsi/Es2, a gene that is located immediately 3' (∼1.5 kb) to Gscl (34; W. Gong and M.L. Budarf, unpublished data). The coordinate regulation of these two genes in the pons could reflect a common regulatory element and/or a functional interaction between the two proteins. However, since Dgsi is widely expressed in the adult and embryo (34,37) while Gscl is restricted to specific regions of the brain, the function of Dgsi in most tissues is independent of Gscl. Alternatively, it is possible that the high level of expression of Dgsi in the pons reflects fortuitous increased expression of Dgsi due to the close proximity to the Gscl promoter. Further experiments are required to distinguish between these possibilities.

In the adult mouse brain, expression of Gscl is no longer seen in the raphe nuclei of the pons or in the habenula. Rather, we observe expression only in the interpeduncular nucleus (IP), a cluster of cells in the ventral midbrain. Immunohistochemical studies have demonstrated immunoreactivity for acetylcholinesterase, substance-P, enkephalins and serotonin distributed among the different subnuclei (38). We have not determined whether all or just a subset of the subnuclei express Gscl. However, all subnuclei of the IP receive major inputs from the medial habenular nuclei (38). Interestingly, the IP is one of two locations in the brain that receives major input from the habenula, the other being the dorsal and median raphe nuclei of the pons (Fig. 7) (39). The major efferents of the lateral and median habenula travel along the fasciculus retroflexus to the dorsal and median raphe nuclei and to the IP, respectively (39). In addition, the major efferent of the IP projects to the dorsal and median raphe nuclei. Therefore, the habenula, which is one of the major relay stations between the limbic forebrain and the midbrain, is the major source of input to the serotonergic neurons of the midbrain.

In addition, the dorsal and median raphe nuclei efferents project to the medial and lateral habenula providing serotonergic input to the habenula as well as to the rest of the forebrain. It is intriguing that Gscl is expressed at different times in these three interconnected locations in the brain. Functional studies will be required...
to determine whether expression of \( Gsc \) in these locations in the brain is important for establishment of this neural circuit.

Does \( Gsc \) play a role in the phenotype of DGS/VCFS patients? Based on its expression pattern, it is unlikely that \( Gsc \) is involved directly in the set of phenotypic features of DGS/VCFS patients that involve structures derived from the neural crest. However, the majority of patients with DGS/VCFS exhibit mild to severe learning disabilities (40–43). In addition, deletions of chromosomal region 22q11.2 have been found to be associated with psychiatric disorders such as schizophrenia and bipolar disorder (44–47). The regions of the brain where \( Gsc \) is expressed, the thalamus, the habenula and the raphe nuclei, play many roles in higher brain function. For example, based on studies in rats, the habenula has been proposed to be involved in a number of behaviors including regulating states of arousal, response to stress and deficits in avoidance behavior (48–50). In addition, serotonin, the major neurotransmitter of the raphe nuclei, is thought to be involved in many sensory and behavioral processes such as the control of circadian rhythms, rapid eye movement (REM) sleep and body temperature. Serotonin also has been linked to behavioral disorders such as depression and anxiety. Therefore, it is conceivable that haploinsufficiency for \( Gsc \) could lead to defects such as mental illness or learning disorders. Verification of a role for \( Gsc \) in brain development will require further functional studies or the presence of mutations in affected individuals.

**MATERIALS AND METHODS**

**In situ hybridization**

Two different probes specific for the \( Gsc \) gene were used. One probe, 486 bp in length, includes the last 14 bp of coding sequence and extends into the predicted 3′-untranslated region (3′-UTR). A second, non-overlapping probe, constructed from cDNA, contains parts of the second and third exons, including most of the homeodomain. The two probes gave identical results. A 405 bp probe specific for the \( Gbx-2 \) gene was constructed by cloning an RT–PCR product from 8 day mouse embryo RNA into the T/A cloning vector (Invitrogen). All constructs were verified by sequence analysis. No expression above background was observed using probes in the sense orientation for any of the constructs.

Embryos and adult brains were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight and embedded in paraffin. Radioactive \( in situ \) hybridization was performed largely as described (51) on 7 \( \mu \)m sections. \( ^{35} \)S-labeled RNA probes were transcribed using T7 RNA polymerase. All washes were performed at 63–64°C. Following hybridization, slides were coated in NTB-2 emulsion (Kodak), stored at 4°C and developed after 7–10 days. Slides were counterstained with Hoescht 33258.

**Antibody staining**

Immunostaining with an antibody to serotonin was performed as described by the manufacturer. Slides with transverse sections of 15.5 day paraffin-embedded mouse embryos were de-waxed in xylene (twice, for 5 min each) and rehydrated in 100% ethanol (twice, for 2 min each), 95% ethanol (2 min), 70% ethanol (2 min), PBS (2 min). Slides were incubated with 500 \( \mu \)l of blocking solution (1% goat serum, 0.1% Tween-20 in PBS) for 1 h at room temperature. Sections were covered with 400 \( \mu \)l of a pre-diluted rabbit polyclonal antibody to serotonin (Biomedia) and incubated at room temperature overnight. The next day, slides were washed for 5 min in 0.1% Tween-20/PBS, then in PBS three times for 5 min each. Sections were then incubated with 400 \( \mu \)l of a 1:100 dilution of rhodamine-conjugated goat anti-rabbit (Jackson Immunoresearch) secondary antibody for 1 h at room temperature in the dark. The secondary antibody was removed and the sections washed for 5 min in 0.1% Tween-20/PBS followed by incubation with 400 \( \mu \)l of 4',6-diamidino-2-phenylindole (DAPI; 1:1000) for 15 min in the dark. Slides were then rinsed with 0.5 ml of PBS for 15 min. Sections were mounted in Gel/Mount (Biomedia).

**Yeast expression and reporter constructs**

A region of the \( Gsc \) protein, residues 49–203, containing the homeodomain was expressed in yeast from the \( GAL1 \) promoter using plasmid pGSCL-101. This plasmid was made by PCR amplification of a 465 bp sequence from a \( Gsc \) cDNA using the following primers: 5′-GGATTCAAGACCGAGAAGCCAGAGGAGC and 5′-GAGTACGACTACCCCTTCGCGGACTTCTTGACG. The PCR product was digested with \( EcoRI \) and XhoI and inserted into the \( EcoRI \) and XhoI sites of plasmid pJG4-5 (gift of Russ Finley, Wayne State University), pJG4-Sq is identical to pJG4-5 (52) except for the addition of a polynucleotide sequence downstream of the hemagglutinin (HA) tag. The plasmid pGSCL-101 carries a 2\( \mu \) replicator and a \( TRP1 \) selectable marker, and expresses \( Gsc \) as a fusion protein consisting of an SV40 nuclear localization signal, a B42 acidic activation domain (53), an HA epitope tag and GSCL (49–203).

Bicoid was expressed in yeast from the \( GAL1 \) promoter in plasmid pDB1 which has LEU2 as a selectable marker and the 2\( \mu \) replicator, and produces an HA epitope-tagged full-length Bicoid protein (D.S. Burz, R. Rivera-Pomar, H. Jäckle and S.D. Hanes, in preparation). For Bicoid and GSCL, the \( GAL1 \) promoter was stimulated using a synthetic activator, GAL4-ER-VP16 (54), or a mutated version of this activator (GAL4-ER-VP16*), respectively. GAL4-ER-VP16 consists of the yeast Gal4 DNA-binding domain, the ligand-binding domain of the estrogen receptor and the herpes virus VP16 activation domain. The mutated version contains a point mutation (F442P) in the VP16 activation domain.

**Figure 7.** Diagrammatic representation of the connections from the habenula to the interpeduncular nucleus and the dorsal and median raphe nuclei in the adult rat brain (redrawn and adapted from ref. 39). Habenular efferents to other regions of the brain are not shown. CC, corpus callosum; DR, dorsal raphe nucleus; MR, median raphe nucleus; Cb, cerebellum.
were harvested after 5–6 h (1 GSCL fusion protein or Bicoid, the three plasmids used in the assay. To induce synthesis of the activation potential; this was used to avoid potential toxicity of

REFERENCES

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were co-transformed into yeast by the LiOAc procedure (56) and
cells were grown in complete synthetic medium lacking uracil, histidine and either leucine or tryptophan to maintain selection for the three plasmids used in the assay. To induce synthesis of the GSCL fusion protein or Bicoid, β-estradiol (Sigma) was added to

Yeast transformations, cell growth and β-galactosidase assays

Saccharomyces cerevisiae strains FY833 (MATa his3Δ200 ura3Δ52 trp1Δ63 leu2Δ1 lys2Δ202) and MGLD4-4a (MATa

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REFERENCES


