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Positive autoregulation of the glial promoting factor glide/gcm

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Fly gliogenesis depends on the glial-cell-deficient/glial-cell-missing (glide/gcm) transcription factor. *glide/gcm* expression is necessary and sufficient to induce the glial fate within and outside the nervous system, indicating that the activity of this gene must be tightly regulated. The current model is that glide/gcm activates the glial fate by inducing the expression of glial-specific genes that are required to maintain such a fate. Previous observations on the null *glide/gcm*$^{N7-4}$ allele evoked the possibility that another role of *glide/gcm* might be to maintain and/or amplify its own expression. Here we show that *glide/gcm* does positively autoregulate *in vitro* and *in vivo*, and that the glide/gcm^{N7-4} protein is not able to do so. We thereby provide the first direct evidence of both a target and a regulator of *glide/gcm*. Our data also demonstrate that *glide/gcm* transcription is regulated at two distinct steps: initiation, which is *glide/gcm*-independent, and maintenance, which requires *glide/gcm*. Interestingly, we have found that autoregulation requires the activity of additional cell-specific cofactors. The present results suggest transcriptional autoregulation is a mechanism for glial fate induction.

Keywords: autoregulation/differentiation/fly/glide–gcm/gliogenesis

Introduction

Cell fate determination relies on positional cues laid during the early stages of development. Since such cues are provided transiently within the cell, the assignment of a stable fate identity involves the maintenance and/or the amplification of the initial information, a step that often requires autoregulation. A typical example is provided by the segmentation genes, which establish cell fates along the antero-posterior axis in *Drosophila melanogaster*. Direct and indirect feedback loops have been described in both gap genes (Warrior and Levine, 1990) and pair-rule genes, transiently expressed activators and repressors that delimit the antero-posterior expression of homeotic genes (Hiromi and Gehring, 1987; Frasch *et al*., 1988; Lawrence and Johnston, 1989; Pick *et al*., 1990; Jiang *et al*., 1991). In addition, autoregulation is also required for the maintained expression of segment polarity genes (Siegfried *et al*., 1992; Yoffe *et al*., 1995) and that of the homeotic genes, although in this case gene expression is stable throughout development (Kuziora and McGinnis, 1988; Chouinard and Kaufman, 1991; Thuringer *et al*., 1993; for a review see Bienz, 1994). Interestingly, the control of gene activity through direct or indirect autoregulation is also used in pathways as diverse as those involved in sex determination (Bell *et al*., 1991; Keyes *et al*., 1992), cell cycling and proliferation (for examples see Wu *et al*., 1993; Johnson *et al*., 1994; Shan *et al*., 1994; Yin *et al*., 1994; for reviews see Pines, 1992; Lam and La Thangue, 1994), and circadian rhythms (Carter and Murphy, 1996; Foulkes *et al*., 1996; for a review see Sassone-Corsi, 1998). The use of such a strategy in these pathways suggests that the reinforcement of a primary signal is a crucial step in the establishment of a new cell state.

Glial fate determination depends on the expression of *glial cell deficient/glial cell missing* (*glide/gcm*), a gene that is necessary and sufficient to activate the glial fate (Hosoya *et al*., 1995; Jones *et al*., 1995; Vincent *et al*., 1996; Bernardoni *et al*., 1998; for reviews see Anderson, 1995; Pfrieger and Barres, 1995; Giangrande, 1996; Klambt *et al*., 1996). glide/gcm is a transcription factor that binds to an octamer sequence called the glide/gcm binding site, or GBS (Akiyama *et al*., 1996; Schreiber *et al*., 1997). Previous observations on *glide/gcm*$^{N7-4}$, a loss-of-function mutation, suggested that glide/gcm activity might possibly be controlled by autoregulation. In this mutant, *glide/gcm* RNA initially accumulates at levels that are indistinguishable from those observed in wild-type embryos; however, at late developmental stages *glide/gcm* transcripts cease to accumulate and are extinguished earlier than in the wild-type cells (Jones *et al*., 1995; Bernardoni *et al*., 1997).

In this paper we show that the *glide/gcm*$^{N7-4}$ allele carries a point mutation in the DNA-binding domain (DBD) which abolishes DNA binding and transactivating activities. We demonstrate that five GBSs exist within the proximal 6.5 kb of the *glide/gcm* promoter sequence and that the binding of glide/gcm to these sites activates transcription. In addition, we show that the five sites contribute differentially to the promoter activity. Finally, we demonstrate that *glide/gcm* autoregulates *in vivo*. By characterizing the autoregulation of *glide/gcm* we have defined the first direct target and regulator of glide/gcm. The role of positive autoregulation as a mechanism to control glide/gcm activity during glial differentiation is discussed.

Results

To test the possibility that *glide/gcm* expression is controlled by positive autoregulation during development, we undertook three approaches. First, we characterized the *glide/gcm*$^{N7-4}$ mutation and assessed how it may affect

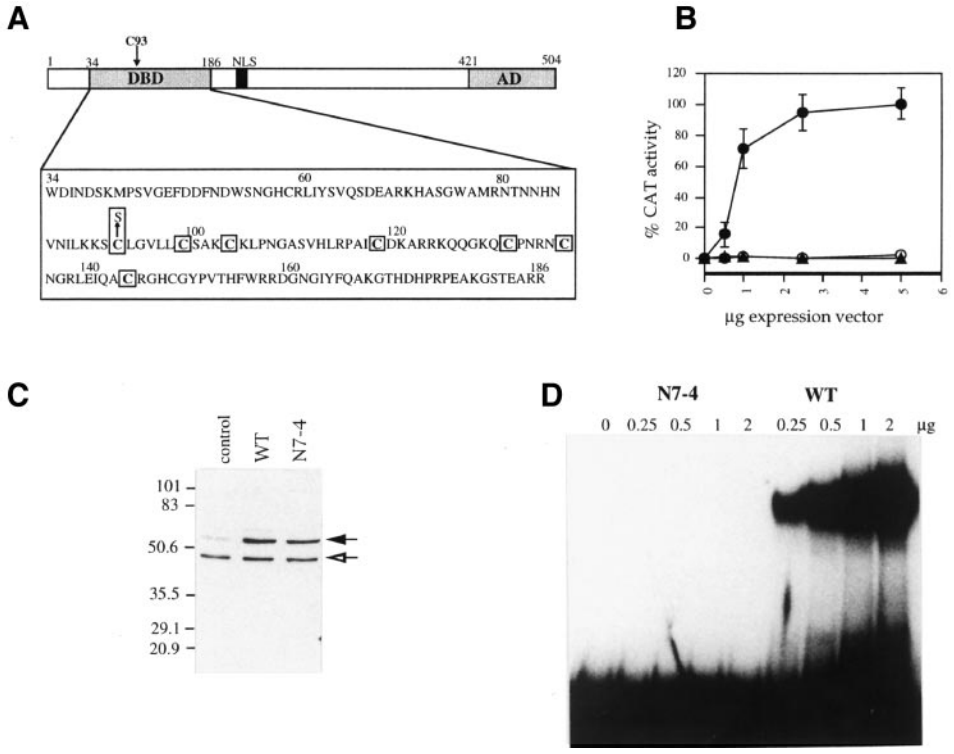

Fig. 1. glide/gcm$^{N7\text{-}4}$ is defective in transactivation and DNA binding activity. (**A**) Schematic representation of the glide/gcm protein, which shows a conserved region contained in the DBD (Schreiber *et al*., 1997), a nuclear localization signal (NLS) and an activation domain (AD). The sequence of the DBD is shown below it, with the conserved cysteines indicated in boxes. The *glide/gcm$^{N7\text{-}4}$* allele consists of a point mutation within the DBD, in which the first conserved cysteine (position 93) is converted into a serine. (**B**) Relative abilities of increasing amounts of the expression vectors pPAC-*glide/gcm* (●) and pPAC-*glide/gcm$^{N7\text{-}4}$* (○) to activate transcription from 1 µg of the reporter pBLCAT5-GBS in transfected S2 fly cells. (△) CAT activity measured upon co-transfection of increasing amounts of pPAC-*glide/gcm* with a reporter carrying a non-specific sequence instead of the GBS, pBLCAT5-NS. (**C**) Western blot analysis of extracts from S2 cells transfected with 5 µg of either pPAC alone (control), pPAC-*glide/gcm* (WT) or pPAC-*glide/gcm$^{N7\text{-}4}$* (N7-4). Anti-actin was used as a loading control. Filled and open arrows indicate the bands corresponding to glide/gcm and actin products, respectively. Molecular weight is indicated in kDa. (**D**) Relative abilities of increasing amounts of purified glide^{DBD} fusion proteins (0–2 µg) to bind to a labeled 30mer containing the consensus GBS. Wild-type is shown on the right and glide/gcm$^{N7\text{-}4}$ on the left.

glide/gcm activity. Secondly, we determined whether glide/gcm-binding sites exist in the *glide/gcm* promoter and if its transcription is *glide/gcm*-dependent. Thirdly, we determined whether autoregulation occurs *in vivo*.

glide/gcm$^{N7\text{-}4}$ is a point mutation in the DNA binding domain which abolishes binding and transactivation

glide/gcm$^{N7\text{-}4}$, a null mutation in which *glide/gcm* transcript levels decrease more rapidly than in wild-type embryos (Jones *et al*., 1995; Bernardoni *et al*., 1997), has been induced by DEB treatment (Lane and Kalderon, 1993), which suggests the presence of a point mutation. Using PCR amplification and sequence analysis on genomic DNA from mutant embryos we found that this mutation consists of a single base change (G→C) within the *glide/gcm* transcribed sequence. This results in the conversion of the cysteine residue at position 93 to a serine (Ser93) (Figure 1A). Cys93 constitutes the first of seven cysteine residues conserved among all vertebrate homologs (Akiyama *et al*., 1996; Altshuller *et al*., 1996; M.Kammerer and A.Giangrande, in preparation) and lies within the DBD of glide/gcm (Akiyama *et al*., 1996; Schreiber *et al*., 1997).

Binding-site selection assays have shown that glide/gcm binds to the DNA consensus sequence 5′-AT(G/A)CGGG(T/C)-3′ (Akiyama *et al*., 1996; Schreiber *et al*., 1997) and activates transcription from this binding site in transient transfection assays (Schreiber *et al*., 1997). To assess the ability of glide/gcm$^{N7\text{-}4}$ protein to activate transcription, we synthesized a 30mer carrying the consensus GBS 5′-ATGCGGGT-3′ in the center and cloned it into a chloramphenicol acetyl transferase (CAT) reporter plasmid which contains a thymidine kinase (tk) promoter preceding the CAT coding sequence (pBLCAT5). We then tested the ability of wild-type and mutant glide/gcm to activate transcription from this fragment by cotransfecting the reporter construct with an expression vector containing the wild-type or the *glide/gcm$^{N7\text{-}4}$* cDNA in the S2 *Drosophila* cell line and by determining the relative amounts of CAT activity as compared with the same reporter containing a 30mer of non-specific DNA. Using increasing amounts of expression vector, we observed that the wild-type protein transactivates gene expression from the GBS in a dosage-dependent manner. The reporter containing non-specific DNA was inactive even in the presence of the highest levels of transfected glide/gcm. Strikingly, the glide/gcm$^{N7\text{-}4}$ protein is completely unable to transactivate, even when high amounts of expression vector are used (Figure 1B). Western blot analysis showed that both wild-type and mutant glide/gcm proteins are expressed at the same levels in transfected S2 cells (Figure 1C). Because of the position of the mutation, we then assessed whether the defect in transactivation is due

to the inability of glide/gcm^{N7-4} to bind to DNA. To this end, we conducted gel-shift assays using as probes the 30mer containing the consensus GBS tested in transfection experiments. Purified recombinant GST fusion proteins carrying the first 202 aa of wild-type or mutant glide/gcm were tested for DNA binding activity. This region of the protein includes the DBD (as defined by Akiyama *et al*., 1996). Figure 1D shows that the wild-type GST–glide^{DBD} protein specifically forms complexes with the GBS while DNA binding is completely abolished when the Ser93 GST–glide^{DBD} fusion protein is used, illustrating the importance of the cysteine residue at position 93. This result is also in agreement with the recent finding that a homologous mutation in one of the mouse *GCM* genes, the replacement of cysteine at position 76 with a serine, completely abolishes binding (Schreiber *et al*., 1998). We therefore conclude that glide/gcm^{N7-4} is unable to transactivate the reporter in the CAT assay because it is defective in its DNA-binding activity. Thus, the decay in transcript levels in *glide/gcm^{N7-4}* embryos is probably due to lack of direct or indirect autoregulation rather than to defects in RNA stability.

glide/gcm *positively regulates glide/gcm transcription in vitro*

As a second step in defining an autoregulatory loop for *glide/gcm*, we sequenced the *glide/gcm* promoter and searched for sites to which the protein would bind. Our sequence analyses revealed the presence of five GBSs scattered throughout 6.5 kb upstream of the *glide/gcm* transcription start site (Figure 2A and B) whereas none were found in >5 kb of sequence downstream of it. One of the GBSs corresponds to the octamer consensus sequence (site C), while the four other sites display a nucleotide change at the seventh (site A) or at the sixth position (sites B, D and E; Figure 2B). Interestingly, the promoter of *reverse polarity* (*repo*), a putative target of *glide/gcm*, also contains several GBSs (Akiyama *et al*., 1996). Two of the 11 binding sites correspond to the consensus, while all the others display a mismatch at one of the eight positions.

The presence of mismatches in a binding site may induce a change in the DNA binding and in the transactivation abilities compared with those observed with the consensus sequence. For example, mutations at positions 2, 3, 6 and 7 in the GBS have a strong impact on the binding of mouse GCM (Schreiber *et al*., 1998). We therefore proceeded to analyze *in vitro* the function of the five GBSs. We synthesized 30mers carrying the different sites and cloned them individually into pBLCAT5. We then tested the ability of glide/gcm to activate transcription from each of these fragments in cotransfection experiments as above (Figure 2C and D). The five sites found in the *glide/gcm* promoter do indeed display different activation potentials. Sites C and A induce the highest levels of CAT activity, 100- and 25-fold, respectively, compared with the activity observed with control reporter plasmids not containing the GBS or containing a 30mer of random DNA sequence. Sites B, D and E are poorly active, their levels of CAT activation being 2.4-, 5.2- and 2.2-fold, respectively, compared with the control. From these data we conclude that sites C and A are the main sites for transcriptional activation, and that this activation is mediated through the activity of the glide/gcm protein.

We have observed that sites A and D reside in the opposite orientation with respect to that of B, C and E. Interestingly, sites present in the opposite orientation were also observed in three of the 11 GBSs found in the *repo* promoter. To determine whether activation of transcription is dependent on the orientation of the GBS, we tested the transactivation potential of each site in its reverse orientation relative to the reporter gene. The results in Figure 2B show that the level of glide/gcm-mediated transcriptional activation varies only slightly depending on the orientation of the binding site.

Binding properties of the glide/gcm *binding sites in the glide/gcm promoter*

In order to further analyze the role of the five GBSs found in the *glide/gcm* promoter we also determined their relative binding affinity. Binding assays were performed using the purified recombinant GST–glide^{DBD} fusion protein and the six 30mers already tested in transfection experiments. Figure 3A shows that GST–glide^{DBD} forms complexes with notably different affinities for the five sites. Site C, which corresponds to the consensus site as defined by binding site selection (Akiyama *et al*., 1996; Schreiber *et al*., 1997), displays the highest affinity. The glide^{DBD} fusion protein binds to site A with ~9-fold lower affinity than to site C. Interestingly, site A contains an A instead of a G, a change observed in only 2% of the cases in the binding-site selection assay. Sites B, D and E, which present a mismatch at the sixth position, display very poor (sites E and D) or no (site B) affinity for glide^{DBD}. This is in agreement with the observations that the glide/gcm binding sites identified through site selection assays always contained a G residue at the sixth position and that sites mutagenized at this position are inactive (Schreiber *et al*., 1998). Interestingly, site A (ATGCGGAC) is still active, while a site that introduces a T at the seventh position is not (Schreiber *et al*., 1998), suggesting that the activity of a GBS also depends on the type of residue at a given position.

The specificity of the binding was confirmed by challenging the glide/gcm–DNA complex with increasing amounts of specific and non-specific DNA competitors. In these experiments, the specific competitor was the 30mer used as probe. The protein–DNA complex between glide/gcm and site C is affected upon the addition of an equal amount of cold specific competitor DNA and nearly completely disrupted upon the addition of 10-fold excess competitor (Figure 3B). In addition, the complex is able to withstand the addition of up to 100-fold of non-specific competitor. Therefore, the glide/gcm protein displays a very high affinity for its consensus binding site. Similar results were obtained using the A-binding site, but in this case binding was completely abolished by adding an equal amount of specific competitor due to the lower affinity of site A for glide/gcm (Figure 3B).

To eliminate the possibility that the differences in affinity arise from the use of a truncated glide/gcm protein, we also tested the entire protein. To this end, DNA probes were incubated with nuclear extracts from a stably transformed S2 cell line in which *glide/gcm* expression is under the control of a metal-inducible promoter. DNA-

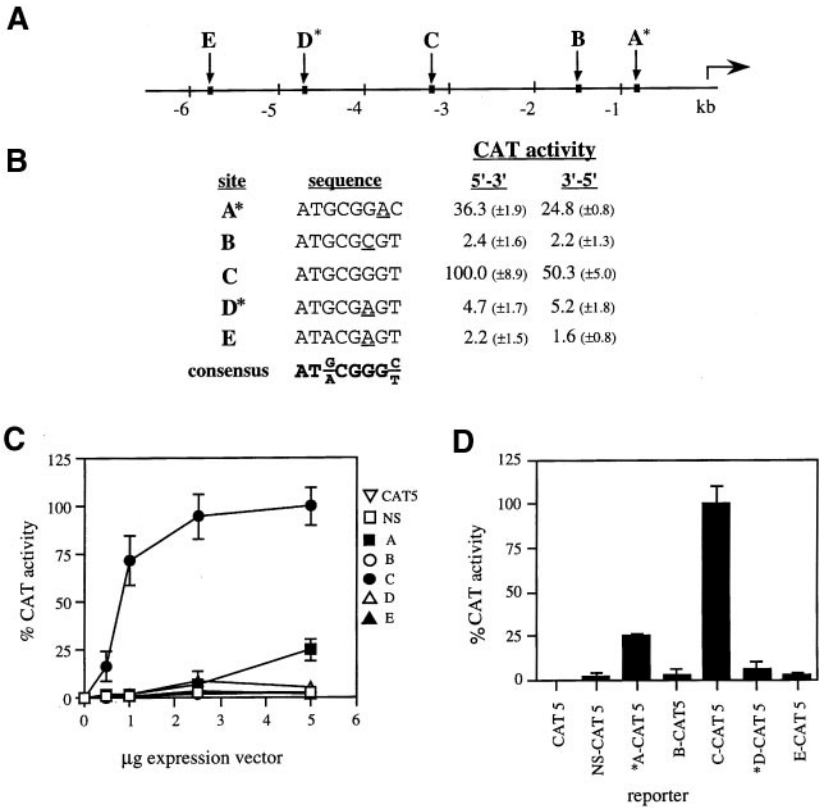

Fig. 2. *glide/gcm* positively autoregulates *in vitro*. (**A**) Schematic depiction of the five glide/gcm binding sites (GBS, sites A through E) found within 6.5 kb of the *glide/gcm* promoter. Horizontal arrow indicates the transcription start site. (**B**) Sequence of the five GBSs (left column) and relative ability to support transactivation when cloned into a pBLCAT5 reporter [as determined by relative CAT activity (right columns)]. Each GBS was tested for transactivation potential in both orientations. Sites indicated by an asterisk are oriented 3′–5′ in the 6.5 kb promoter. (**C**) Relative amounts of CAT activity upon cotransfection of increasing amounts of pPAC-*glide/gcm* and the CAT5 reporter construct containing either one of the five GBSs, non-specific DNA (NS) or no additional cloned sequences (CAT5). Symbols corresponding to each reporter are shown on the right.
(**D**) Histogram depicting results for maximum CAT activity observed for each site in the presence of 5 µg pPAC-*glide/gcm*. Only the orientation found in the *glide/gcm* promoter is shown in (C) and (D).

binding assays were performed under both inducing and non-inducing conditions (Figure 3C). Expression of glide/gcm under inducing conditions was verified by Western blot analysis (data not shown). For the consensus GBS (site C), a specific band appears upon incubation with induced nuclear extracts that is not present in non-induced samples, or when induced nuclear extracts are incubated with a 30mer containing non-specific DNA. As in the case of the glide^{DBD} fusion protein, the five GBSs display different binding affinities for the full-length glide/gcm protein, with site C being the strongest followed by weak binding observed for site A. Sites B, D and E are totally inactive in this test (Figure 3C; data not shown). Importantly, the binding affinity of either the full-length glide/gcm or a fusion protein containing only its DBD for the GBSs corresponds well to the degree of their transactivation abilities revealed in the above CAT assays (compare with Figure 2C and D).

The specificity of the binding was also confirmed by adding specific and non-specific competitor DNA (Figure 3D). The addition of 10-fold excess of specific competitor completely eliminates the protein–DNA complex (low mobility band), while this same complex is not affected by the addition of 10-fold excess of non-specific competitor.

glide/gcm-dependent activation of the glide/gcm promoter

In order to determine the contribution of each GBS to autoregulation within the context of the *glide/gcm* promoter, we assessed the transactivation potential of the entire 6.5 kb fragment. Since this fragment carries the *glide/gcm* promoter and the transcription start site, a CAT reporter vector devoid of tk promoter sequences (pBLCAT6) was used instead of pBLCAT5. The 6.5 kb fragment induces a basal level of CAT activity in the absence of transfected *glide/gcm* (Figure 4A). This activity is due to the sequences present in the 6.5 kb because no activity was observed for the pBLCAT6 reporter vector itself (Figure 4A). Such basal activity may depend on the presence of positive transcription factors that act on the *glide/gcm* promoter and/or to the presence of endogenous glide/gcm, since anti-glide/gcm antibodies detect a product in non-transfected S2 cells in Western blot assays (Figure 1C). The intensity of the signal detected in non-transfected cells, however, is much weaker than that detected upon transfection with a *glide/gcm* expression vector. In agreement with this, we have found that a 3.3-fold increase of CAT activity takes place upon cotransfection of the 6.5 kb reporter vector with a *glide/gcm* expression vector while cotransfection with the expression

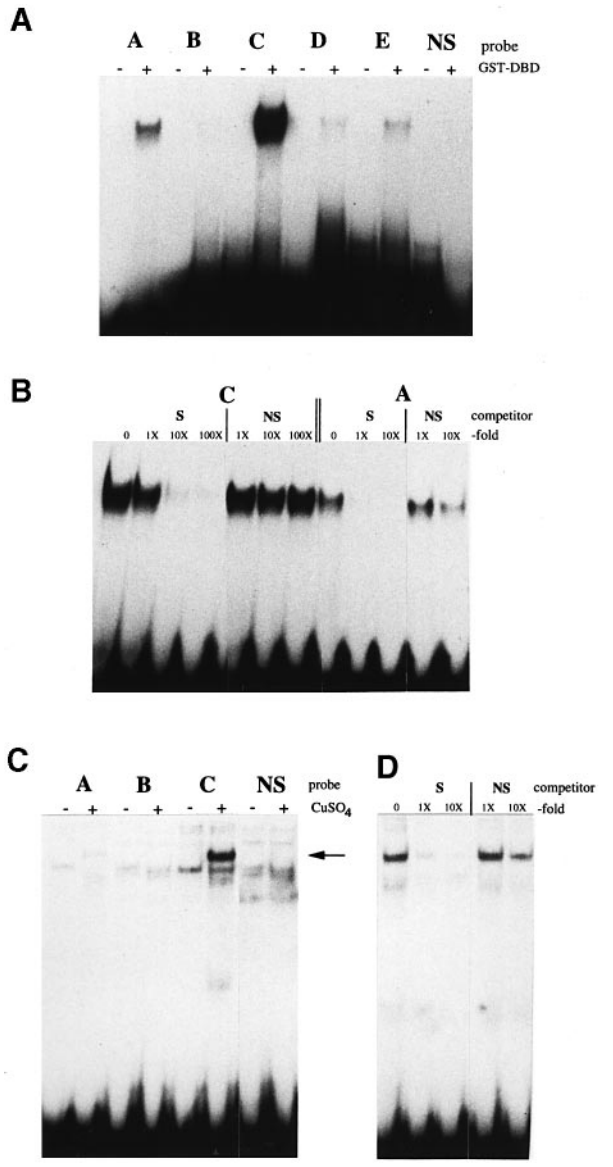

Fig. 3. glide/gcm displays different affinities for the five GBSs found within 6.5 kb of the *glide/gcm* promoter. (**A**) Gel-shift assay to determine the ability of purified glide^{DBD} fusion protein to bind to labeled 30mers containing the five different GBSs or non-specific DNA. Site C, which contains the consensus GBS, displays the highest level of binding, followed by site A, with ~9-fold lower affinity. '–' and '+' indicate the absence and presence of the fusion protein, respectively. (**B**) Competition gel-shift assay on sites C and A. One- to one-hundred-fold excess of non-specific (NS) or specific (S) cold DNA competitor was preincubated with 500 ng of purified glide^{DBD} fusion protein, followed by incubation with labeled DNA. (**C**) Gel-shift assay to determine the ability of full-length glide/gcm to bind to the A, B and C GBSs or to non-specific DNA. Nuclear extracts from S2 cells stably transformed with glide/gcm were prepared after treatment in both inducing (+) and non-inducing (–) conditions. One microgram of extract was incubated with labeled 30mers as in (A). Arrow indicates the band which is specific for glide/gcm binding. Note that full-length glide/gcm displays the same relative affinities for the five GBSs as the purified glide^{DBD} fusion protein. (**D**) Competition gel-shift assay on site C using induced nuclear extracts from stably transformed cells, performed as in (B).

vector devoid of the *glide/gcm* cDNA or carrying the *glide/gcm*$^{N7\text{-}4}$ sequences does not result in any increase in enzymatic activity (Figure 4A).

To determine the contribution of each GBS to overall transcriptional activation in the context of the entire 6.5 kb

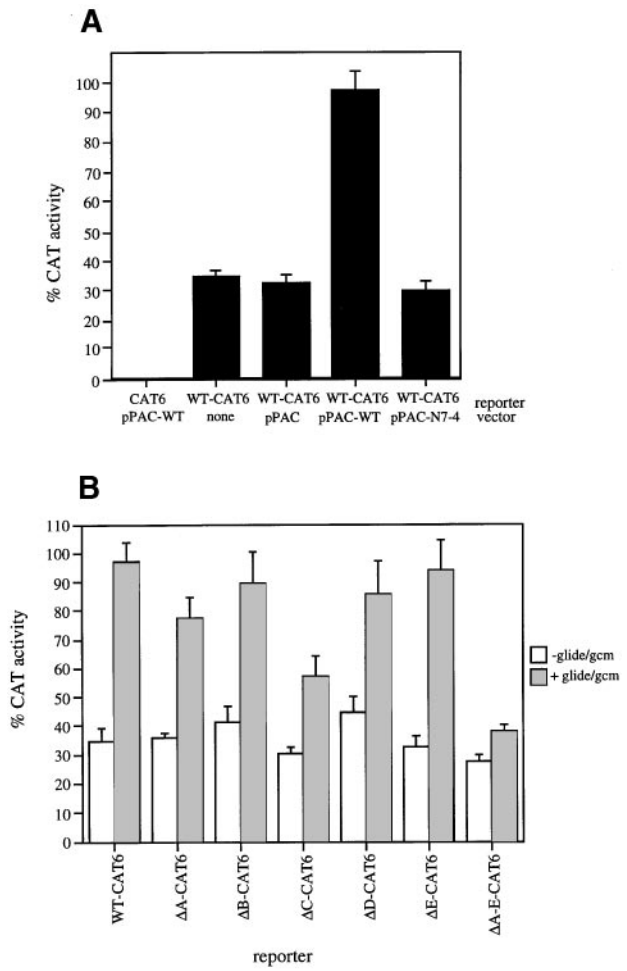

Fig. 4. glide/gcm activates transcription from the *glide/gcm* promoter *in vitro*. (**A**) A reporter construct containing 6.5 kb of *glide/gcm* promoter sequence (WT-CAT6) was tested for transactivation potential when co-transfected in S2 cells with 5 µg of the following: carrier DNA (none, column 2), pPAC (column 3), pPAC-*glide/gcm* (pPAC-WT, column 4) or pPAC-*glide/gcm*$^{N7\text{-}4}$ (pPAC-N7-4, column 5). CAT6 was co-transfected with 5 µg of pPAC-*glide/gcm* as a control (column 1). The relative CAT activity for each reporter is shown. (**B**) Reporter constructs with individual (ΔA–CAT6 through ΔE–CAT6, grey columns 2–6) or all (ΔA–E-CAT6, grey column 7) GBSs abolished by mutation were tested for transactivation potential when co-transfected with 5 µg of pPAC-*glide/gcm*. The degree of activation of the wild-type promoter (WT-CAT6, grey column 1) is arbitrarily assigned 100%. The basal activity was also evaluated for each reporter (–glide/gcm) and is represented in the white columns.

promoter, we created five reporter constructs (ΔA through ΔE), each with one GBS replaced by the sequence AGGG-AAAC (Figure 4B). Gel-shift assays previously showed that glide/gcm is not capable of binding to this sequence alone (Figure 3). The mutation of sites E and B did not significantly affect the transactivation potential of the 6.5 kb promoter, which we arbitrarily define as 100% (Figure 4B). In contrast, mutation of site C resulted in a decrease in transcriptional activation to 43%, whereas a decrease in activity to 67% was observed upon the mutation of site A (Figure 4B), once the basal activity is taken into account. Site D behaved like site A, although with higher variability (see standard deviation bars in Figure 4B). We also tested a reporter with all five GBSs mutated (ΔA–E) and a construct that contained mutations in sites A, B and C (ΔA–C). Most strikingly, the ability

to respond to cotransfected glide/gcm is almost completely abolished when the ΔA–E construct is used, with an observed CAT activity of 16% compared with the wild-type promoter (Figure 4B). Similar results were obtained with the ΔA–C construct (data not shown). These data altogether clearly demonstrate that (i) the activation of *glide/gcm* promoter depends on autoregulation; (ii) the most important GBSs for autoregulation are C, the consensus site, and to a minor extent, sites A and D; and (iii) *glide/gcm* activation through either site C or A takes place independently from the other site.

The ΔA–E construct still retains some *glide/gcm*-dependent transcriptional activation, despite the absence of any site through which glide/gcm can directly mediate its effects (Figure 4). This could imply that transfected or endogenous glide/gcm activates downstream genes, which in turn would positively regulate the *glide/gcm* promoter at sites other than the GBSs. Alternatively, glide/gcm may act in concert with other factors to positively regulate its transcription. The role of glide/gcm in this activation is also in agreement with the observation that the ΔA–E construct is less active in the absence of transfected *glide/gcm*. Finally, part of the basal activation observed in the absence of the GBSs and in the absence of transfected *glide/gcm* may depend on the sole activity of factors acting on other *cis* elements present throughout the *glide/gcm* promoter.

glide/gcm autoregulates in vivo

The previous results indicate that direct autoregulation of *glide/gcm* takes place in a cell line. To demonstrate that *glide/gcm* regulates its own expression *in vivo*, we used two approaches. First, we analyzed the accumulation of the glide/gcm product in *glide/gcm*$^{N7\text{-}4}$ embryos. Whilst in wild-type embryos *glide/gcm* is expressed in all glial precursor cells (Figure 5A and B), in mutant embryos its expression is limited to a few cells at the periphery of the ventral cord (Figure 5C and D). In agreement with the *in situ* hybridization results and as predicted from a mutation affecting autoregulation, the difference in the pattern of *glide/gcm* expression between wild-type and mutant embryos was not detectable at early developmental stages (data not shown). Together with the data obtained *in vitro*, this result strongly suggests that autoregulation does take place during glial differentiation.

As a second approach, we expressed *glide/gcm* ectopically and determined whether this can induce autoregulation using the original enhancer trap line, *rA87*, in which the lacZ gene is under the control of the *glide/gcm* promoter (Hosoya *et al*., 1995; Jones *et al*., 1995; Vincent *et al*., 1996), to follow *glide/gcm* expression. We employed the upstream activating sequences (UAS)-GAL4 system (Brand and Perrimon, 1993) to express *glide/gcm* ectopically and determined whether this would induce ectopic β-galactosidase (β-gal) expression in a *rA87* background. *rA87* was therefore crossed with *UAS-glideM21G* (*M21G*) (Bernardoni *et al*., 1998), a transgenic line which carries *glide/gcm* under the control of the UAS, the targets of the GAL4 transcription factor (Fischer *et al*., 1988). The *rA87/+*; *M21G/+* progeny were then crossed with a *scabrous-GAL4* (*sca-GAL4*) driver which mimicks the *scabrous* profile of expression in the whole neurogenic region (Mlodzik *et al*., 1990; data not shown). Ectopic *glide/gcm* expression does indeed promote activation of the endogenous *glide/gcm* promoter since massive β-gal labeling was observed throughout the ventral cord (Figure 6a). We and others have shown previously that ectopic glide/gcm leads to ectopic activation of the glial differentiation program (Hosoya *et al*., 1995; Jones *et al*., 1995; Bernardoni *et al*., 1998). The colocalization between the glial-specific repo product and β-gal indicates that *glide/gcm* autoregulation takes place in cells that take the glial fate at ectopic positions.

To determine whether autoregulation can only take place within the neurogenic region, we crossed the *twi-GAL4* line, which expresses GAL4 in mesodermal cells (Baylies and Bates, 1996), with *rA87/+*; *M21G/+* flies (Figure 6b), which also results in ectopic *repo* expression. Strikingly, autoregulation does not occur in the majority of the cells expressing *glide/gcm* ectopically. Indeed, very few cells show colocalization between β-gal and repo. Similar results have been obtained by expressing *glide/gcm* in the dorsal ectoderm (data not shown). This clearly indicates that *glide/gcm* autoregulation requires the presence of positive cofactors or the absence of negative cofactors that are tissue-specific.

Discussion

glide/gcm is a direct target of glide/gcm

During development, cell differentiation depends on the activity of transcription factors that impose a specific cell fate in response to cell autonomous decisions and/or cell–cell interactions. The role of such transcription factors is to transduce a signal so that an irreversible commitment is made by a given cell. In flies, the glide/gcm transcription factor is necessary and sufficient for the induction of the glial fate, indicating that its regulation must be tightly controlled (Hosoya *et al*., 1995; Jones *et al*., 1995; Vincent *et al*., 1996; Bernardoni *et al*., 1998). In order to determine the molecular cascade involved in glial differentiation it will be extremely important to identify the regulators, the targets and the mode of action of *glide/gcm*. In this paper we identify the first direct target and regulator of *glide/gcm* and provide compelling evidence for autoregulation as a mechanism that controls glide/gcm activity.

First, a mutation that renders the glide/gcm protein inactive abrogates autoregulation *in vivo*. Secondly, we have found several GBSs in the *glide/gcm* promoter. Some of these sites are sufficient to activate transcription from an heterologous promoter in a *glide/gcm*-dependent manner. Thirdly, the abolishment of GBSs almost completely abolishes the ability of glide/gcm to activate transcription from its own promoter. Fourthly, ectopic glide/gcm activity induces the expression of the endogenous gene *in vivo*. These data, along with the finding that β-gal expression driven by the *glide/gcm* promoter decreases in mutations that lower the amount of glide/gcm active product (Vincent *et al*., 1996), strongly support the hypothesis that a positive feedback loop takes place during glial differentiation. Therefore, the role of *glide/gcm* in glial differentiation is twofold. On one hand, it amplifies the signal that dictates the fate choice through positive autoregulation; on the other hand, it activates the glial differentiation program, probably by inducing the glial-specific genes *pointed* (*pnt*), *tramtrack* (*ttk*), *repo* and *prospero* (*pros*) (*pros*: Doe *et al*.,

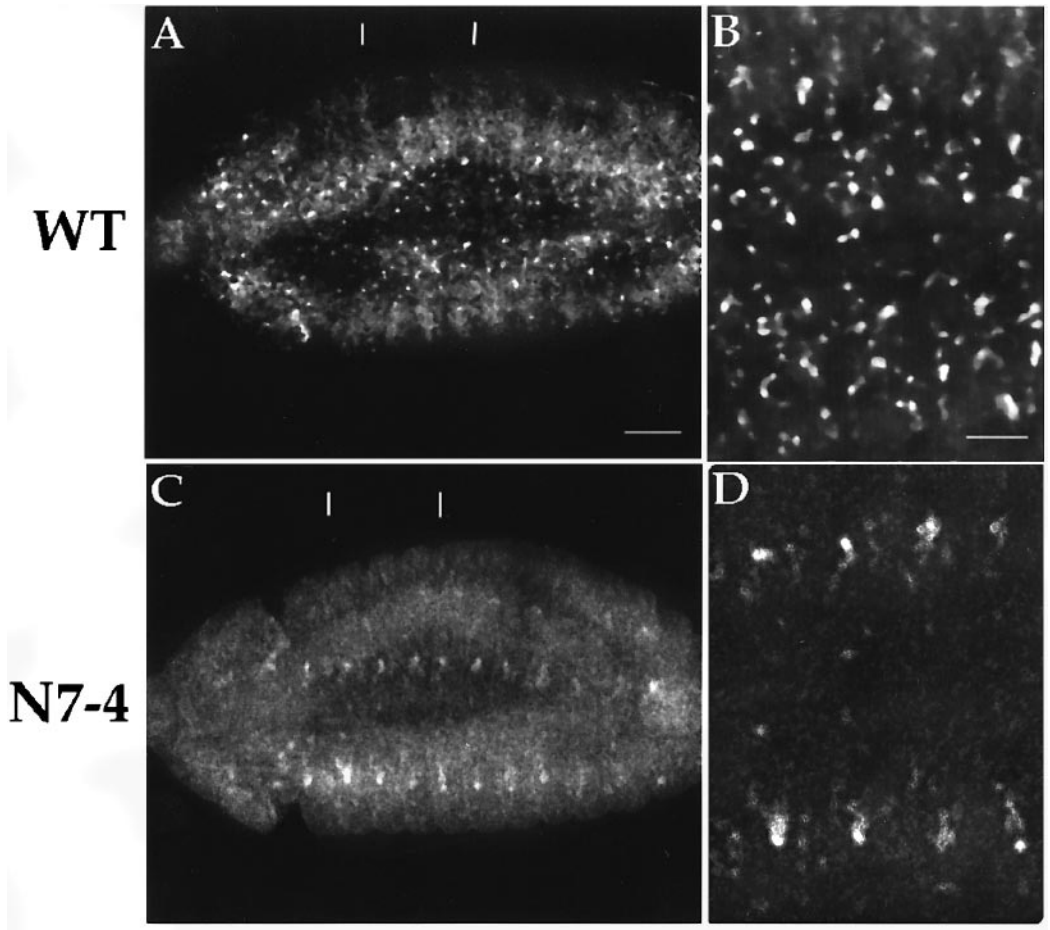

Fig. 5. Expression of glide/gcm protein is defective in *glide/gcm*$^{N7-4}$ mutant embryos. Ventral views of late stage 12 embryos, total projections of confocal images. Anti-glide/gcm labeling in wild-type (**A** and **B**) and mutant embryos (**C** and **D**). Vertical white lines in (A) and (C) indicate region that is shown in (B) and (D), respectively. Bar: 25 μm in (A and C); 50 μm in (B and D).

1991; Vaessin *et al*., 1991; *pnt*: Klambt, 1993; Klaes *et al*., 1994; *repo*: Campbell *et al*., 1994; Xiong *et al*., 1994; Halter *et al*., 1995; Akiyama *et al*., 1996; *ttk*: Harrison and Travers; 1990; Giesen *et al*., 1997).

We have observed that several GBSs are present in the *glide/gcm* promoter. Amongst them, the site containing a perfect consensus sequence shows the highest affinity in terms of binding and activation while the four sites presenting one mismatch show intermediate (mismatch at position seven) or very low (mismatch at position six) affinity. The presence of multiple binding sites for its own product is a feature shared by the promoter of several genes that are able to autoregulate. For example, the promoters of invertebrate and vertebrate genes such as *fushi-tarazu* (*ftz*), *deformed* (*dfd*), *even-skipped* (*eve*) and *Pit-1* all contain multiple binding sites for their own product. Some of these show high affinity, while others show intermediate or low affinity (Jiang *et al*., 1991; Regulski *et al*., 1991; Schier and Gehring, 1992; DiMattia *et al*., 1997). Interestingly, one of the ftz-binding sites with intermediate affinity present in the *ftz* promoter abuts a binding site for Ftz-f1, a cofactor that cooperates with Ftz to activate transcription (Guichet *et al*., 1997; Yu *et al*., 1997). It is probable, therefore, that the optimal activity of some GBSs requires the presence of cofactors assisting *glide/gcm* for binding and transactivation. In the future, it will be important to determine the mode of action and the precise role *in vivo* of the consensus GBS and that of the sites presenting a mismatch.

Role of autoregulation in the maintenance of glide/gcm expression

A crucial step in *glide/gcm* regulation occurs at the level of transcriptional initiation since ectopic glide/gcm is sufficient to override the endogenous differentiation programs and to promote glial differentiation within and outside the nervous system (Hosoya *et al*., 1995; Jones *et al*., 1995; Bernardoni *et al*., 1998). Subsequent to the first wave of *glide/gcm* transcription, however, there is a second level of regulation which involves the maintenance of glide/gcm activity. The requirement for this maintenance was strongly suggested by the phenotype of *glide/gcm*$^{N7-4}$ embryos, in which the *glide/gcm* transcript rapidly decays. We show that this mutation is due to a single amino acid substitution that renders the glide/gcm protein unable to bind DNA and transactivate, eliminating any positive feedback loop.

It is reasonable to assume that the maintained expression of certain essential regulators is necessary for the irreversible commitment of cells to a particular tissue phenotype. Indeed, direct and/or indirect autoregulation has been observed in other genes involved in the determination of a specific cell fate. Because the activity of early acting genes may be transient, autoregulation may be used

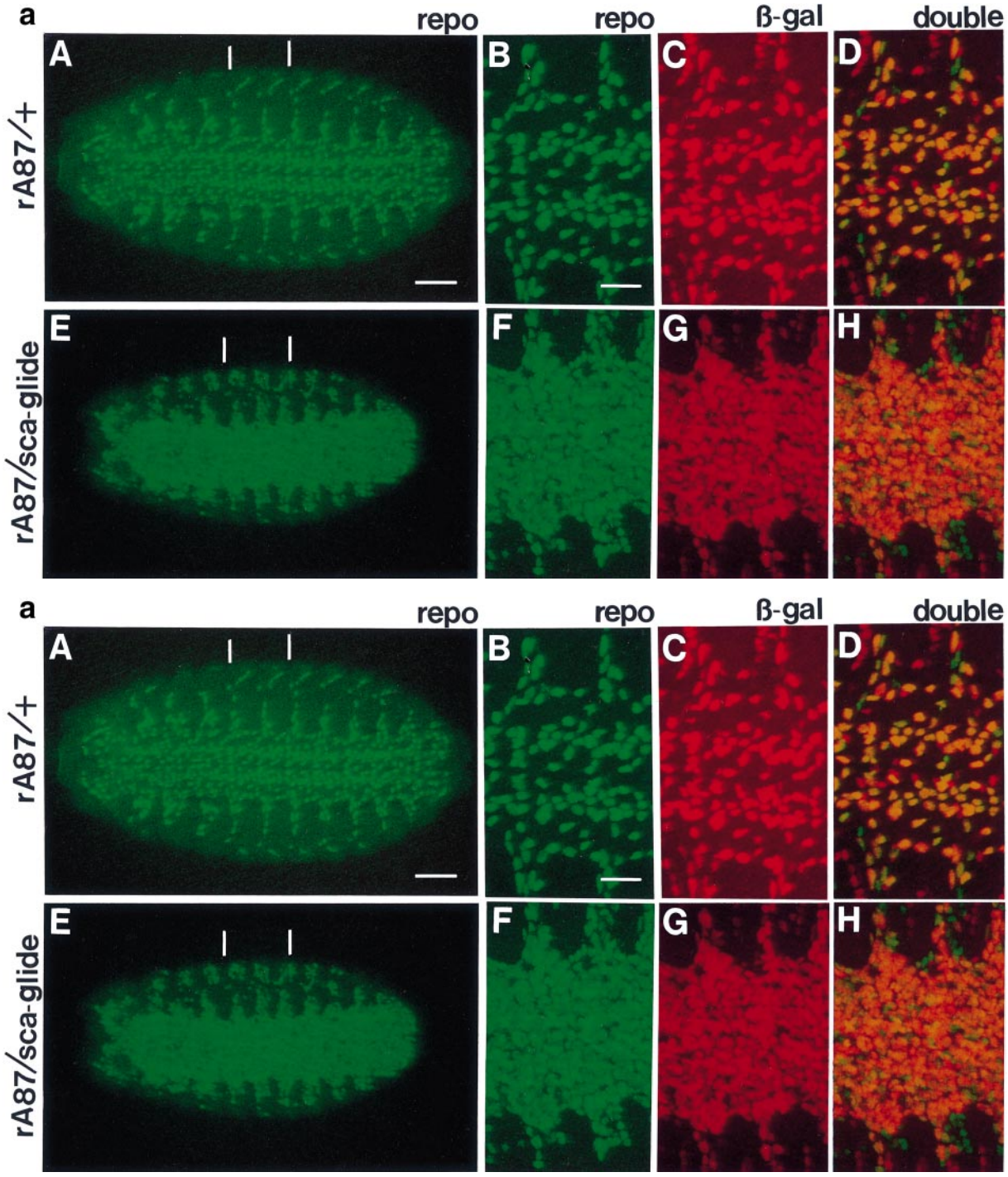

Fig. 6. *In vivo* analysis of *glide/gcm* autoregulation. (**a**) Positive autoregulation occurs in the neurogenic region. Ventral views of stage 14 embryos, total projections of confocal images. Anti-repo labeling is shown in green (A, B, E and F) and anti-β-gal labeling is shown in red (C and G); double labeling is shown in (D) and (H). (A–D) show *rA87/+* embryos, and (E–H) show *sca-GAL4/rA87; M21G/+* (rA87/+; sca-glide) embryos. Vertical white lines in (A) and (E) indicate the region shown in (B–D) and (F–H), respectively. Note in (H) that ectopic expression of *glide/gcm* leads to repo-positive cells which are also β-gal-positive (yellow nuclei), which indicates the activation of the endogenous *glide/gcm* promoter. Bar: 25 μm in (A) and (E); 50 μm in (B–D) and (F–H). (**b**) Positive autoregulation of *glide/gcm* does not take place in the mesoderm. Dorso-lateral views of stage 14 embryos, total projections of confocal images. Anti-repo labeling is shown in green (A–C and F) and anti-β-gal labeling is shown in red (D and G); double labeling is shown in (E) and (H). (A) and (C–E) show *rA87/+* embryos, (B) and (F–H) show *twi-GAL4/rA87; M21G/+* (rA87/+; twi-glide) embryos. Vertical white lines in (A) and (B) indicate the region shown in (C–E) and (F–H), respectively. Cells which appear yellow in (E) and (H) express both *repo* and β-gal. Note in (E) the presence of two nuclei which are both repo- and β-gal-positive (arrows). These nuclei belong to the dorsal-most peripheral glial cells. Only a few ectopic repo-positive cells in (H) display activation of the endogenous *glide/gcm* gene (as revealed by β-gal expression). Cells in (D–H) that are β-gal- but not repo-positive correspond to the hemocytes and to the stripes of ectodermal cells already described in Vincent *et al*. (1996) and in Bernardoni *et al*. (1997). Bar: 25 μm in (A and B); 50 μm in (C–H).

to transduce short-lived signals into stable patterns of expression. For example, *ftz* provides information for subdivision of the embryos into metameric units. The autoregulatory binding sites in the promoter of pair-rule genes are necessary to maintain the striped pattern of expression (Hiromi and Gehring, 1987; Frasch *et al*.,

1988; Lawrence and Johnston, 1989). Similarly, the dfd sites in the *dfd* promoter enable the dfd homeobox-containing protein to maintain segment identity (Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990; Lou *et al*., 1995). Finally, the vertebrate POU-domain *pit-1* gene, which governs the specification of three anterior pituitary cell lineages (Li *et al*., 1990), positively autoregulates during development (Chen *et al*., 1990).

Strikingly, glide/gcm activity seems to require several controls, as evidenced by its tight transcriptional regulation (Hosoya *et al*., 1995; Jones *et al*., 1995; Bernardoni *et al*., 1997, 1998), its ability to autoregulate (this paper) and its dependence on the cellular redox potential (Schreiber *et al*., 1998). Moreover, the presence of additional motifs, a PEST sequence and an instability element in the 3′ untranslated region (UTR) (Hosoya *et al*., 1995), as well as the presence of potential phosphorylation sites (Schreiber *et al*., 1997) predict further levels of regulation. This strongly suggests that glide/gcm activity imperatively must be shut off if not required, and that only the combination of transcriptional and post-transcriptional controls will ensure the strict regulation necessary for proper development.

Positive autoregulation of glide/gcm in vivo

We have determined that *glide/gcm* positively autoregulates *in vivo* by analyzing the *glide/gcm*$^{N7\text{-}4}$ mutation and by ectopically expressing *glide/gcm* in different tissues. This confirms and extends the results obtained *in vitro*. Strikingly, autoregulation occurs at much higher levels in the neurogenic region than in the mesoderm or in the dorsal ectoderm. Since the *UAS-glide/gcm* line used for ectopic expression in the different tissues is the same, it is likely that one or more cofactors necessary for *glide/gcm* to autoregulate are differentially expressed in the embryo. Interestingly, a similar situation has been observed in the case of the *ftz* autoregulatory element. Indeed, the upstream element in the *ftz* promoter depends on ftz activity and acts to enhance the striped expression in the ectoderm. However, the same element does not exhibit ftz-dependent enhancer activity in the CNS, another tissue in which *ftz* is normally expressed and required (Hiromi and Gehring, 1987). We speculate that the ability to support *glide/gcm* autoregulation reflects the competence of a given cell to adopt the glial fate. Cells of the nervous system may be loaded with cofactors which allow autoregulation, which make them more competent to take the glial fate than other cell types. Alternatively, cells outside the nervous system express inhibitory factors that do not allow autoregulation. The absence of the right combination of cell-specific factors can be compensated by high levels of glide/gcm such as those obtained using the GAL4 system, which renders all cells competent to adopt the glial fate even in the absence of autoregulation. Indeed, although no autoregulation takes place in the mesoderm, *glide/gcm* expression driven by the *twi* promoter does activate the glial fate in this derivative (Bernardoni *et al*., 1998). The need for cofactors may constitute an additional level of regulation of gene activity, in order to ensure that a given fate is adopted only in the proper cells.

The regulation of *glide/gcm*, as for other genes promoting specific cell fates (for reviews see Weintraub *et al*., 1991; Botas, 1993; Duffy and Gergen, 1994; Vervoort *et al*., 1997; Crews, 1998), undoubtedly will prove to be finely tuned and highly complex. By identifying a key component of the regulation of the *glide/gcm* locus we have begun the important task of determining the factors involved in the expression of this gene and analyzing the mode of action of such factors. Finally, in characterizing the autoregulation of *glide/gcm* we have defined the first direct target and regulator of *glide/gcm in vitro* and *in vivo*. Vertebrate homologs have recently been found in both humans and mice (Akiyama *et al*., 1996; Altshuller *et al*., 1996; Kammerer and A.Giangrande, in preparation). It will be interesting to determine whether these homologs display similar mechanisms of regulation.

Materials and methods

Stocks

The wild-type stock was *Sevelen*. *glide/gcm*$^{N7\text{-}4}$ was generated by Lane and Kalderon (1993). The *rA87* enhancer trap line, a gift from C.Goodman, is described in Vincent *et al*. (1996). The 'blue balancer' *CyO twi-lacZ* was used to recognize homozygous mutant embryos. The *UAS-glide/gcm* line is described in Bernardoni *et al*. (1998). *twi-GAL4* (Baylies and Bates, 1996) was provided by M.Bates, and *sca-GAL4* by M.Mlodzik.

Identification of the glide/gcm$^{N7\text{-}4}$ mutation

Zero to fifteen hour embryos from *glide/gcm*$^{N7\text{-}4}$*/CyO twi-lacZ* flies were collected and stained for β-gal activity using a standard X-GAL staining protocol (Ashburner, 1989). Genomic DNA was extracted from homozygous mutant embryos, recognizable by the absence of β-gal expression. PCR amplification of four different 450–500 bp fragments covering the entire *glide/gcm* cDNA sequence (containing the coding region and 400 bp of 3′ UTR) was performed and the PCR products were cloned into the *Bam*HI site of pBluescript II SK (SK). Four clones from each of the four fragments were sequenced to determine the location and nature of the mutation.

Cloning of glide/gcm wild-type and mutant expression vectors

The full-length wild-type *glide/gcm* cDNA was sub-cloned into SK (Stratagene) (A.Verdeil and A.Giangrande, unpublished results) and into the expression vector pPAC5C (Krasnow *et al*., 1989) (gift from C.Thummel, which we refer to as pPAC). pPAC-*glide/gcm*$^{N7\text{-}4}$ was generated by double-stranded mutagenesis (Clontech) on a recombinant p513 plasmid containing the wild-type cDNA using the mutagenic primer 5′-GAATATCCTAAAGAAGAGCTCCCTGGGAGTGCTCCTCTGC-3′ to create the N7-4 mutation. The plasmid was verified by sequencing and the resulting *glide/gcm*$^{N7\text{-}4}$ cDNA was subcloned into pPAC in the same manner as the wild-type cDNA.

Construction of reporter constructs, cell transfection and CAT activity assay

Overlapping fragments covering 6.5 kb of DNA sequence 5′ to the coding sequence of *glide/gcm* were isolated from λ6, a recombinant phage carrying genomic sequences of the *glide/gcm* locus (J.Reed and A.Giangrande, unpublished results). The 6.5 kb were subcloned into SK and sequenced. Oligonucleotides corresponding to the glide/gcm binding sites were designed to contain the GBS octamer (underlined) or non-specific DNA (for a negative control) flanked by four bases of surrounding DNA, followed by *Bam*HI sites (in bold) as follows:

GBS-E: 5′-GCGC**GGATCC**CAGGATACGAGTGGAG**GGATCC**GCGC-3′;
GBS-D: 5′-GCGC**GGATCC**ACTCACTCGCATTCTA**GGATCC**ATAT-3′;
GBS-C: 5′-GCGC**GGATCC**TGCAATGCGGGTATCT**GGATCC**ATAT-3′;
GBS-B: 5′-GCGC**GGATCC**TCCTATGCGCGTGCTA**GGATCC**ATAT-3′;
GBS-A: 5′-GCGC**GGATCC**TAATGTCCGCATTAAA**GGATCC**ATAT-3′;
Non-specific (NS): 5′-GCGC**GGATCC**CCAACATTGACACCGC**GGATCC**ATAT-3′.

These oligonucleotides were annealed to their complements, digested with *Bam*HI and cloned into the reporter plasmid pBLCAT5 (Boshart *et al*., 1992).

The 6.5 kb *glide/gcm* promoter fragment contains both promoter sequences and the transcription start site. It was therefore cloned into pBLCAT6, a CAT reporter vector devoid of tk promoter sequences.

Mutation of each GBS was performed by double-stranded mutagenesis (Clontech) on pBLCAT6-6.5kb using the following mutagenic primers (replacement mutation underlined):
ΔE: 5′-CTGAAGGACTCGCAGGAGGGAAACGGAGTTATACTTGTAG-3′;
ΔD: 5′-CGAGGTGAATGCACGTTTCCCTTCTAATAGTGCTCATC-3′;
ΔC: 5′-GTTTTCAAAGGACATGCAAGGGAAACATCTCTTCATGGATTG-3′;
ΔB: 5′-CCGCCGTAATCCTTTGAGTTCCTAGGGAAACGCTACGATCCTGATC-3′;
ΔA: 5′-GATTTAGCAGCTTTAATGTTTCCCTAAAAAAGTCTTAAAGCC-3′.

The 6.5 kb reporter containing ΔA was then used as a template to create a reporter lacking all five GBSs, ΔA–E, by performing a double-stranded mutagenesis in which the reaction mixture contained the four oligonucleotides corresponding to ΔB through ΔE. Using this mutagenesis protocol we also obtained the ΔA–C clone. Each mutation was verified by sequencing.

Transient transfection of the *Drosophila* cell line S2 (Schneider, 1972) was performed according to DiNocera and Dawid (1983) with 15 μg DNA containing the following: 1 μg pCMV-lacZ, 500 ng reporter DNA, 0–5 μg pPAC-*glide/gcm* and SK as carrier DNA. Cells were harvested 48 h after transfection, normalized for β-gal activity and assayed for CAT activity as described in Sambrook *et al*. (1989).

Western blot analysis

The S2 cells were transfected as above with 5 μg of pPAC alone, pPAC-*glide/gcm* or pPAC-*glide/gcm* $^{N7\text{-}4}$. Cell lysates were prepared as above. One hundred microliters of these lysates (containing ~25 μg total protein) were precipitated with 10% TCA and analyzed by Western blot with a mixture of a 1:500 dilution of a rabbit polyclonal anti-glide/gcm antibody (Bernardoni *et al*., 1997) and a 1:200 dilution of a rabbit polyclonal anti-actin antibody (Sigma) as an internal control. Detection was performed with a goat-anti-rabbit antibody conjugated to horseradish peroxidase and ECL substrates (Amersham).

DNA-binding assay

An *Eco*RI fragment containing the sequence corresponding to the first 202 amino acids of glide/gcm was generated by PCR using the template SK-wt cDNA. This fragment was cloned into pGEX4T3 (Pharmacia) to create a fusion protein of 49 kDa, GST–glide^{DBD}. Purified fusion protein was generated using GST-conjugated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The GST–glide^{DBD} containing the Ser93 mutation was produced in the same manner, except the template used for the PCR was p513-*glide/gcm*$^{N7\text{-}4}$. All constructs were verified by DNA sequencing.

As probes, the 30mers containing the five GBS sites were end-labeled with [γ-^{32}P]ATP and purified on a polyacrylamide gel. The 30mer used to mutagenize the C site (see above) was also labeled and used as a negative control. Five hundred nanograms of purified GST–glide^{DBD} (wild-type or mutant) were incubated with 5000 c.p.m. of the ^{32}P-labeled probe as described in Akiyama *et al*. (1996). For competition assays, the fusion protein was pre-incubated for 20 min with unlabeled competitor DNA (specific competitor was the same 30mer as the probe, non-specific competitor was the 30mer described above) before adding labeled DNA. The reaction mixtures were loaded on an 8% polyacrylamide gel and run at room temperature. Gel-shift assays using extracts from the stably transformed S2 cell line (see below) were conducted in the same manner as above, using 1 μg of nuclear extract prepared according to Andrews and Faller (1991).

Stable transformation of wild-type glide/gcm cDNA in S2 cell lines

Stable transformation was performed using the DES expression system (Invitrogen) as follows. A 2.0 kb *Eco*RI fragment containing the wild-type cDNA was cloned into the expression vector pMT/V5HisC in order to place control of transcription under the inducible pMT promoter. S2 cells were cotransfected with 19 μg of this plasmid (pMT-*glide/gcm*) and 1 μg of the selection plasmid, pCoHYGRO. Stable transformants were selected according to the manufacturer's protocol. The presence of stably inserted *glide/gcm* was verified by Western blot of nuclear extracts from cells treated with or without 500 μM $CuSO_4$.

Immunohistochemistry

Antibody labeling on embryos was performed as in Vincent *et al*. (1996). *sca-GAL4/rA87; M21G/+* and *twi-GAL4/rA87; M21G/+* embryos were double labeled with 1:500 rabbit anti-repo (gift from A.Travers) and 1:1000 mouse anti-β-gal (Sigma). The signal was revealed using 1:500 Oregon Green (Molecular Probes) and Cy3 (Jackson Laboratories) conjugated secondaries. *glide/gcm*$^{N7\text{-}4}$ embryos were devitellinized by hand and labeled with 1:500 rat anti-glide/gcm raised against a fusion protein within the conserved N-terminal region of the glide/gcm protein. The signal was revealed as above. Embryos were observed with a confocal microscope (Leica DMRE).

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