Functional domain analysis of glass, a zinc-finger-containing transcription factor in *Drosophila*

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ABSTRACT   The glass gene is required for proper photoreceptor differentiation during development of the *Drosophila* eye. glass codes for a DNA-binding protein containing five zinc fingers that we show is a transcriptional activator. A comparison of the sequences of the glass genes from two species of *Drosophila* and a detailed functional domain analysis of the *Drosophila melanogaster* glass gene reveal that both the DNA-binding domain and the transcriptional-activation domain are highly conserved between the two species. Analysis of the DNA-binding domain of glass indicates that the three carboxyl-terminal zinc fingers alone are necessary and sufficient for DNA binding. We also show that a deletion mutant of glass containing only the DNA-binding domain can behave in a dominant-negative manner both *in vivo* and in a cell culture assay that measures transcriptional activation.

The glass gene encodes a polypeptide that contains five zinc fingers of the Cys-His class near the carboxyl-terminus (1). glass protein binds DNA (2), and here we show it functions as a transcriptional activator. glass gene function is required in all photoreceptors for proper differentiation in the *Drosophila* eye imaginal disc, a monolayer epithelium which gives rise to the adult eye. During development, an indentation called the morphogenetic furrow sweeps across the monolayer and is followed shortly by cell-fate determination. The neuronal fate of photoreceptors is determined at this time. Several hours after neuronal cell fate has been established in the eye disc, photoreceptor-specific differentiation begins (for review, see ref. 3). It is at this stage that glass is required, since in eye imaginal discs mutant for glass, cells undergo neuronal fate determination but never express photoreceptor-specific genes (1). Although glass protein is expressed by all cells in and posterior to the morphogenetic furrow, it is required only in photoreceptors (1). The activity of glass is negatively regulated in the nonneuronal cells of the eye disc by another transcription factor that binds adjacent to it in certain enhancer elements (4).

Structure/function studies of transcription factors have revealed a remarkably modular structure. Domains have been identified that are sufficient to confer either DNA-binding or transcriptional-activation activity when fused to heterologous proteins (for review, see ref. 5). Several classes of highly conserved DNA-binding motifs, such as the homeodomain, the Ets domain, and zinc fingers, have been identified by using these techniques (6). Although such studies have also allowed the demarcation of transcriptional-activation domains, the level of amino acid conservation observed between them has been much lower. Proline-rich, glutamine-rich, isoleucine-rich, and acidic activation domains have been described (7–11), but the precise positioning of residues does not appear to be very well conserved phylogenetically, as is the case with DNA-binding domains. In only a few instances have any conserved sequences been identified, and these consist of short stretches

of low amino acid similarity (12–14). To learn more about transcriptional-activation domains, as well as other aspects of transcription factor function, we undertook a detailed study of the glass protein.

We began by cloning and sequencing the glass gene from *Drosophila virilis*, a *Drosophila* species that diverged from *Drosophila melanogaster* 60 million years ago (15). Homologous genes from these two species retain a higher degree of sequence conservation in regions coding for important functional domains of proteins compared with other domains (16, 17). We tested the functionality of the conservation observed between the two glass genes in two ways. First, we demonstrate that the *D. virilis* glass gene can provide glass activity in *D. melanogaster in vivo*. Next, in order to determine whether the conserved regions were required for specific transcription factor functions, we tested deletion mutants of the *D. melanogaster* gene for activity in both DNA-binding and transcriptional-activation assays. Further analysis of the zinc-finger domain of glass has revealed that the three carboxyl-terminal fingers are required for DNA binding, while the two amino-terminal fingers are dispensable, though they do serve to increase affinity. Interestingly, data obtained in the DNA-binding studies have allowed us to define a mutant of glass consisting of only the DNA-binding domain that has a dominant-negative phenotype both *in vivo* and in our *in vitro* cell culture assay for transcriptional activation.

MATERIALS AND METHODS

Isolation of the *D. virilis* glass Gene. A λ EMBL3 *D. virilis* genomic library (gift from M. Scott, Stanford University) was screened at reduced stringency with a 2.2-kb *Bst*BI fragment from the *D. melanogaster* glass cDNA 3-2 (1). Filters were hybridized at 50°C in 5× SSCP (1× SSCP = 20 mM sodium phosphate, pH 6.8; 15 mM sodium citrate; 120 mM sodium chloride), 5× Denhardt's solution (1× Denhardt's = 0.02%, Ficoll, polyvinylpyrrolidone, and bovine serum albumin) (18), 0.5% SDS/10 mM EDTA/100 µg of sonicated herring sperm DNA per ml. Filters were washed at 55°C in 1× SSC (0.15 M NaCl/0.015 M sodium citrate)/1% SDS and then in 0.2× SSC/0.1% SDS. Four positive clones were detected on duplicate filters from 120,000 plaques screened. These clones were purified and found to have identical restriction maps. An 8-kb *Bam*HI fragment that hybridized to the 2.2-kb *Bst*BI *D. melanogaster* fragment was identified by DNA blotting and inserted into pBluescript (pBS; Stratagene). Sonicated fragments of this plasmid were inserted into M13mp10 for DNA sequencing. Templates were selected on the basis of hybridization to either *D. melanogaster* cDNA probes to cover the coding regions or small *D. virilis* genomic fragments to cover the intronic regions. Sequences were assembled and analyzed by using IntelliGenetics software.

Generation of Plasmids. The plasmid pP[vir-glass] was generated by ligating an 8-kb BamHI restriction fragment containing *D. virilis* glass (19) into the BamHI restriction site of pW8 (20). The plasmid pgl₃BCAT was constructed by inserting three tandem copies of the annealed oligonucleotides KMgl1 and KMgl2 (2) into Sal I-digested pBCAT (21).

An Nde I restriction site was introduced by standard *in vitro* mutagenesis at the initiating methionine of the open reading frame of the 3-2 glass cDNA (1). The plasmid pTβglass was generated by using a three-part ligation with Nde I/BamHI-digested pTβstop (22) as the vector and the 400-bp Nde I–Ava I and 1660-bp Ava I–BamHI restriction fragments from the modified glass cDNA. The Nde I–Ava I fragment was sequenced to confirm that no other changes had been introduced during *in vitro* mutagenesis. Fragments were generated for the amino-terminal deletion mutants by exonuclease III digestion from the 5′ HindIII restriction site of pTβglass, followed by S1 nuclease digestion and repair with Klenow DNA Polymerase and then digestion with BamHI. After gel purification, these fragments were ligated into HincII/BamHI-digested pTβstop. Candidate mutants were sequenced to identify those fused in the correct reading frame. A similar cloning scheme was used to generate inserts for carboxyl-terminal and internal deletion mutants, except that exonuclease III digestion was started from the 3′ BamHI restriction site, and the enzyme used for the second digestion was HindIII. For carboxyl-terminal deletions, inserts were ligated into HindIII/HincII-digested pTβstop, which contains stop codons in all three reading frames just after the HincII restriction site. For internal deletion mutants, inserts were ligated into pTβN432 which had been digested with Nco I, end-filled with Klenow DNA polymerase, and then digested with HindIII. Candidate internal deletion mutants were sequenced to identify those fused in the correct reading frame. Wild-type and mutant forms of glass were subcloned from the appropriate pTβstop derivative by insertion into pP_ACUbx+Nde I (23) as Nde I–BamHI fragments.

The plasmid pP[glass-N432] contains the Sal I restriction fragment from pBSglass-N432 inserted into the Xho I site of pW8. pBSglass-N432 is derived from a vector carrying a 10-kb Sal I restriction fragment of genomic DNA containing the glass gene, into which an Nco I recognition site was introduced at the initiating ATG codon of glass. The sequence between this Nco I site and the BamHI site downstream of the glass stop codon was then replaced with the 1.2-kb Nco I–BamHI fragment from pTβN432.

Generation of Transgenic Flies and Scanning Electron Microscopy. Transgenic flies were generated by injecting appropriate plasmids into w^{1118} or w^{1118} gl^{60j} flies according to standard procedures. Individual flies carrying a single copy of each *P* element were used to generate stocks. In the case of P[glass-N432], standard genetic crosses were also used to generate flies carrying up to four copies of the *P* element.

Scanning electron microscopy was performed as described (24).

Assays for DNA-Binding and Transcriptional-Activation Activities. Transfections and chloramphenicol acetyltransferase (CAT) assays were performed essentially as described (25). Briefly, 5×10^6 *Drosophila* S2 cells were split into 6-cm plates and transfected 24 h later. Cells were harvested and lysates were prepared 48 h after transfection. Unless otherwise stated, 100 ng of the indicated expression plasmid was cotransfected along with 2 μg of pgl₃BCAT and 3 μg of pUC118 by the calcium phosphate method. *In vitro* transcription/translation and gel mobility-shift assays were performed as described (26), by using the oligonucleotides BO28 (GATC-CCAGTGGAAACCCTTGAAATGCCTTTAA) and BO29 (GATCTTAAAGGCATTTCAAGGGTTTCCACTGG) (4) as the probe.

RESULTS AND DISCUSSION

Glass Is a Transcriptional Activator. We began our biochemical studies by developing an assay to test the ability of glass protein to activate transcription of templates containing glass recognition sites. A glass-binding site has been identified in the promoter of the major opsin gene of *D. melanogaster* (2). We used this binding site to generate a reporter construct that could be used in cotransfection assays to study the transcriptional-activation properties of glass. Three tandem copies of this glass-binding site were inserted upstream of the core E1B promoter driving expression of the CAT gene. A second construct was generated in which the glass gene could be expressed at high levels under the control of the *D. melanogaster* actin 5C promoter. When these two plasmids were cotransfected into *D. melanogaster* tissue culture cells, levels of CAT activity averaging between 50- and 200-fold higher than
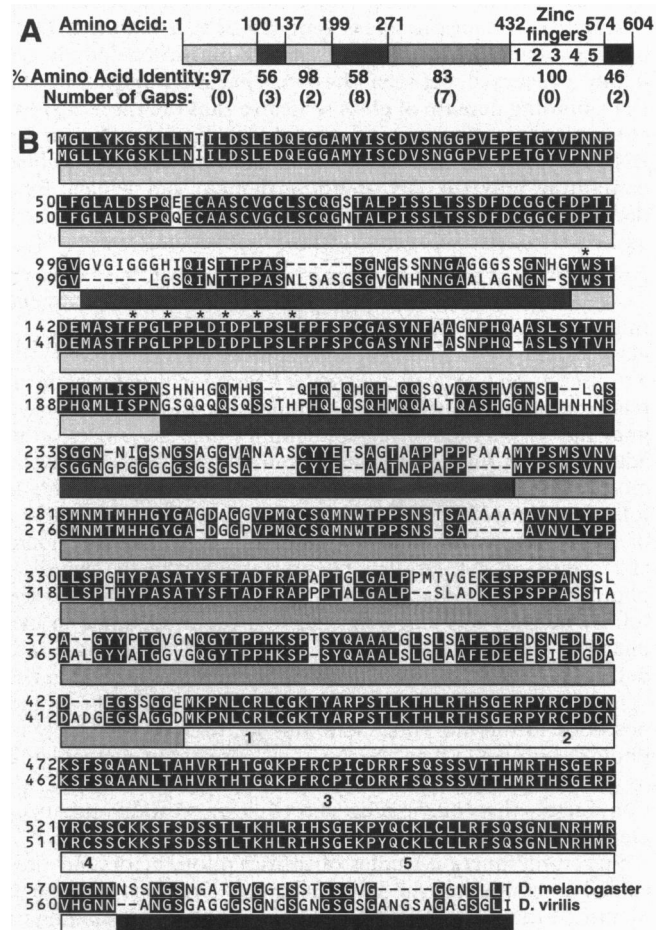

FIG. 1. Comparison of the amino acid sequences of the glass genes from *D. melanogaster* and *D. virilis*. (*A*) Schematic diagram of the *D. melanogaster* glass protein. The numbers above the diagram refer to amino acid number. The numbers below the diagram indicate the percent amino acid identity for each region of the protein relative to the *D. virilis* glass gene and the number of gaps it was necessary to introduce in order to maximize identity between aligned residues. (*B*) The amino acid sequence of the *D. melanogaster* glass gene is shown on the top line, while the deduced amino acid sequence of the *D. virilis* gene is on the bottom line. Amino acid identities are shown with white letters on black. Numbering of amino acids is shown on the left. Dashes indicate the location of gaps that were introduced to maximize amino acid identity in the alignment. The variously shaded boxes under the sequence correspond to the regions of identity indicated in the diagram shown in *A*. Zinc fingers 1 through 5 are indicated by a number under the first cysteine residue of each finger. Hydrophobic residues indicated by an asterisk are the ones that were changed to alanines for a portion of this study (see text).

control transfections were observed. This experiment demonstrates that glass is a potent transcriptional activator.

Cloning and Testing the Functional Conservation of the glass Gene from *D. virilis*. We cloned the glass gene from the related *Drosophila* species *D. virilis*, in order to identify conserved domains that might be important for glass function. A *D. virilis* genomic λ phage library was screened at low stringency with a probe derived from the *D. melanogaster* glass gene. The portion of the phage clone that had sequence homology to glass was sequenced, and a putative intron/exon structure was deduced by comparison with the *D. melanogaster* gene (19). A comparison of the amino acid sequences of the two genes revealed that in addition to the 100% identical zinc-finger domain near the carboxyl-terminus, there are two regions of high identity (97–98%) in the amino-terminal half

of the protein (Fig. 1). Overall the amino acid identity was 78%. Interspersed regions of high and low amino acid identity are a common feature when *D. virilis* and *D. melanogaster* genes have been compared (17, 27–30). Previous comparisons between transcription factor homologues have revealed that, as is the case for glass, the DNA-binding domain corresponds to the region with highest degree of amino acid identity (17, 27, 29). The functions of the other regions of high identity in these transcription factors were not determined.

To test the functionality of the sequence conservation observed for glass, we generated transgenic flies that express the *D. virilis* glass gene in *D. melanogaster*. This transgene is largely able to rescue the glass phenotype (Fig. 2C), indicating that the *D. virilis* gene retains domains necessary for glass function in *D. melanogaster*. Since evolutionary processes
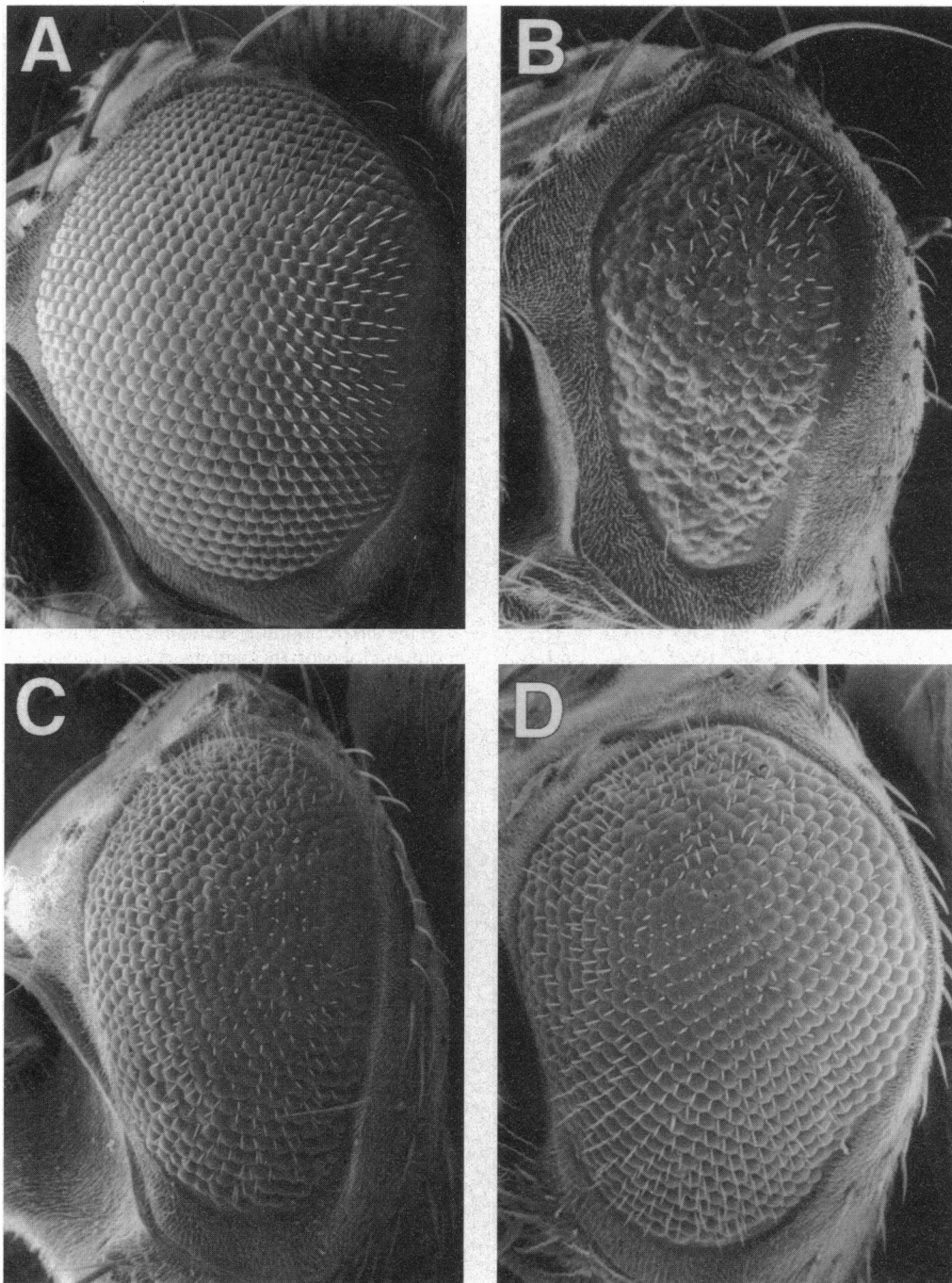

FIG. 2. Scanning electron micrographs showing the compound eye of flies described in this study. Genotypes of the flies are as follows: wild type (Canton S) (*A*), glass (gl^{60j}) (*B*), w^{1118} gl^{60j} P[virilis-glass] (*C*), w^{1118} P[glass-N432] (four copies) (*D*). (×187.)

select for conservation of important functional domains, it is likely that the three regions of high amino acid identity between the two *Drosophila* species correspond to domains important for glass function.

Testing the Function of Conserved Domains. To obtain direct evidence for functional conservation of the homologous domains, we generated an extensive series of deletion mutants of the *D. melanogaster* glass gene, which were then tested for both DNA-binding and transcriptional-activation activities. These studies allowed us to define minimal domains of the glass protein sufficient for each of these two activities that are critical for transcriptional regulation.

To eliminate any bias, the deletion end points in the mutants were generated randomly with exonuclease III. Two sets of mutants were generated initially by deleting from either the 5' or 3' end of the glass cDNA to generate amino-terminal or carboxyl-terminal deletion mutants, respectively. Truncated protein products were then generated from each mutant by *in vitro* transcription/translation, and the DNA-binding activity of each mutant was assayed by gel mobility-shift assays using a probe containing a glass-binding site (2). A schematic diagram summarizing the DNA-binding activity of the mutants is shown in Fig. 3. As expected, the zinc-finger domain was required for DNA binding. Interestingly, our DNA-binding

studies revealed that mutants with larger amino-terminal deletions bound to the probe more tightly than either full-length protein or smaller deletions, even when equimolar amounts of *in vitro* translated proteins were used. We further observed that the three carboxyl-terminal zinc fingers were critical for DNA binding, while the amino-terminal fingers were dispensable, though they did serve to increase affinity significantly (Fig. 4). Another zinc-finger containing protein, PRDI-BF1, has also been reported to require only the amino-terminal two of five zinc fingers for correct DNA-binding specificity (31).

Next, with the goal of identifying a minimal transcriptional-activation domain, we tested each of the amino- and carboxyl-terminal deletion mutants for its ability to enhance transcription in the cotransfection assay. The data obtained are summarized in Fig. 3. The amino-terminal deletion mutants allowed us to establish that the first 131 amino acids of glass are dispensable for transcriptional activation. Interestingly, one of the three sequences highly conserved between *D. melanogaster* and *D. virilis* is contained within these 131 residues. This conserved domain is apparently not absolutely required for transcriptional activation, although it does stimulate activation by about 3-fold. Transcriptional-activation activity is not abolished in the amino-terminal deletion series until the second region of high identity with *D. virilis* is removed.

The carboxyl-terminal deletion mutants generated were not informative for delimiting a minimal activation domain, since the zinc fingers, located near the carboxyl terminus of the protein, are deleted in this series of mutants. We therefore generated a series of internal deletion mutants which left the zinc fingers intact, and then deleted residues progressively further upstream of them. These mutants allowed us to define residue 214 as the carboxyl-terminal boundary of the activation domain. However, the putative minimal activation domain consisting of residues 131–214 was not sufficient to provide transcriptional-activation activity when fused directly to the DNA-binding domain. However, we were able to detect weak transcriptional activation with a minimal activation domain consisting of residues 131–262.

This small domain, sufficient for transcriptional activation, contains one of the amino-terminal sequences that is highly conserved between the *D. melanogaster* and the *D. virilis* glass genes. Within this domain there are seven hydrophobic residues in a pattern that resembles a motif also found in the

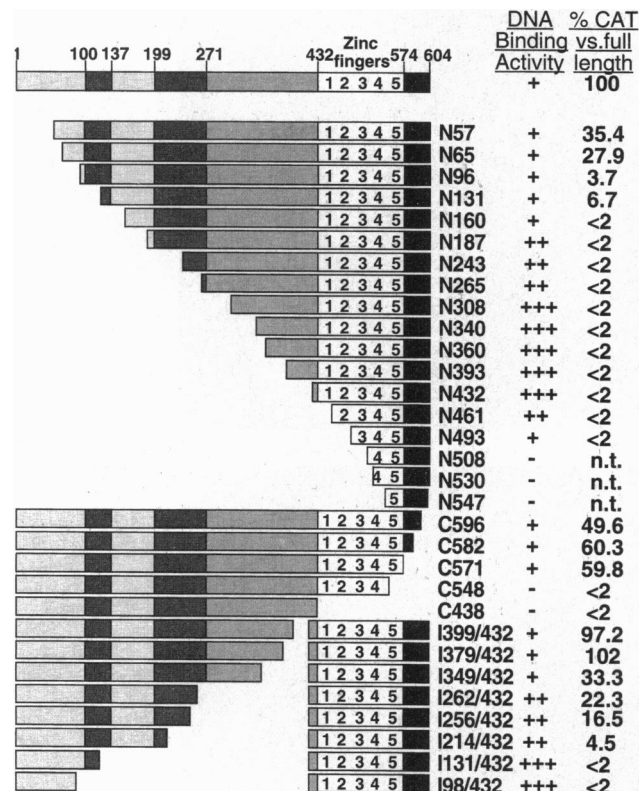| Construct	DNA Binding Activity	% CAT vs. full length
(full length)	+	100
N57	+	35.4
N65	+	27.9
N96	+	3.7
N131	+	6.7
N160	+	<2
N187	++	<2
N243	++	<2
N265	++	<2
N308	+++	<2
N340	+++	<2
N360	+++	<2
N393	+++	<2
N432	+++	<2
N461	++	<2
N493	+	<2
N508	−	n.t.
N530	−	n.t.
N547	−	n.t.
C596	+	49.6
C582	+	60.3
C571	+	59.8
C548	−	<2
C438	−	<2
I399/432	+	97.2
I379/432	+	102
I349/432	+	33.3
I262/432	++	22.3
I256/432	++	16.5
I214/432	++	4.5
I131/432	+++	<2
I98/432	+++	<2

FIG. 3. The DNA-binding and transcriptional-activation activities of deletion mutants of glass. A description of the diagram of full-length glass at the top of the figure can be found in the legend to Fig. 1. Below the diagram of full-length glass are shown the end points of the amino-terminal (N), carboxyl-terminal (C), or internal (I) deletion mutants that were used in this study. The number(s) in the name of each mutant correspond to the residue immediately amino-terminal to the deletion end point. In the right column is the DNA-binding activity of the various mutants, as estimated by gel mobility-shift assay. One "+" indicates the amount of binding observed for full-length glass. Many of the amino-terminal mutants bound DNA more tightly than full-length glass in this assay. The transcriptional-activation activities of the deletion mutants of glass are shown as a percentage of the activity observed for the full-length protein. "n.t." indicates that a particular mutant was not tested for activity since it had previously been shown to be unable to bind DNA.
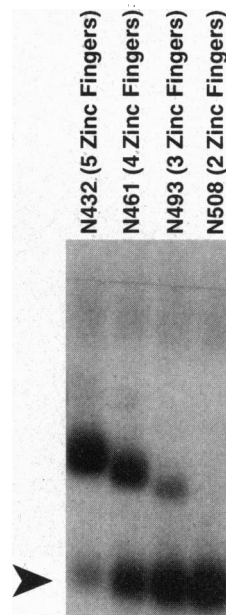

FIG. 4. Deletion mutants of glass missing either one or two of the amino-terminal zinc fingers can still bind DNA. A gel mobility-shift assay performed with equimolar amounts of each of the indicated mutant proteins is shown. The oligonucleotide probe used contains the glass-binding site from the promoter of the major rhodopsin gene of *D. melanogaster*. The arrowhead indicates unbound probe. The name of each mutant and the number of zinc fingers remaining are indicated.

activation domains of several other transcriptional activators, including p53 (32), VP16, and SP1 (33). Detailed analyses of the activation domains of these three proteins have revealed that point mutations in one or more of the bulky hydrophobic residues severely impair transcriptional activation (32–34). To test the importance of this motif in glass, we generated four mutants in this domain that change one or two of the seven hydrophobic residues to alanine (see Fig. 1). These point mutants were then tested in the transcriptional-activation assay. Surprisingly, none of the mutants reduced activation to a significant extent. Perhaps if all seven of the residues were mutated simultaneously, an effect would be detectable. Apparently, the hydrophobic residues are not as important in the glass-activation domain as they are in Sp1, VP16, and p53, suggesting that another motif in this conserved domain is responsible for mediating transcriptional activation.

It is also surprising that removal of the first domain of high homology in the amino-terminus reduced activation by only two-thirds. Perhaps the only function of this domain is to stimulate the transcriptional-activation activity of the other highly conserved domain in the amino-terminal half of glass. Alternatively, this domain might have some other as yet unidentified role critical to glass protein function. For example, it might be required for interacting with the factor that negatively regulates glass activity in nonphotoreceptor cells (4).

Characterization of a Dominant-Negative Form of glass *in Vitro* and *in Vivo*. Since we have observed that a mutant containing only the five zinc fingers bound DNA more tightly than the full-length protein in our gel mobility-shift assay, we tested this construct for its ability to act as a dominant-negative mutant in the transcriptional-activation assay. We found that when a mutant containing the zinc fingers alone is transfected together with an equal amount of full-length expression construct plasmid, transcriptional activation of the reporter construct is down to between 3% and 7% of the activity of the same amount of full-length expression construct DNA alone (see Fig. 5). Thus, this mutant has a dominant-negative effect in the transfection assay. We further showed that increasing the amount of mutant DNA transfected increased the amount of inhibition over 100 fold. A mutant containing only the four most carboxyl-terminal zinc fingers also was able to act as a dominant negative, although the effect was weaker and only detectable when higher amounts of DNA were transfected. These data suggest that the DNA-binding domain alone is able

to effectively compete with the full-length protein for binding sites, thus reducing the amount of transcriptional activation.

We were also able to show that a mutant containing just the five zinc fingers (N432) is able to confer a dominant-negative phenotype *in vivo*, since flies carrying multiple copies of a transgene expressing N432 under the control of glass regulatory sequences exhibit a rough eye phenotype (Fig. 2D). Of three independent *P* element insertion lines generated which express this transgene, two appeared to have wild-type eyes, while one line had only mild defects. It was not until flies were generated carrying three or four copies of the transgene that the dramatic phenotype shown in Fig. 2D was observed. Transgenic flies expressing single copies of this form of glass will be useful in experiments where modulating the genetic does of glass is important. For example, investigators have made use of a system where a gene of interest is expressed in the eye under the control of glass-binding sites (35), resulting in a rough eye phenotype. The severity of such phenotypes can be reduced by coexpression of this dominant negative form of glass (Bruce Hay and G.M.R., unpublished observations).

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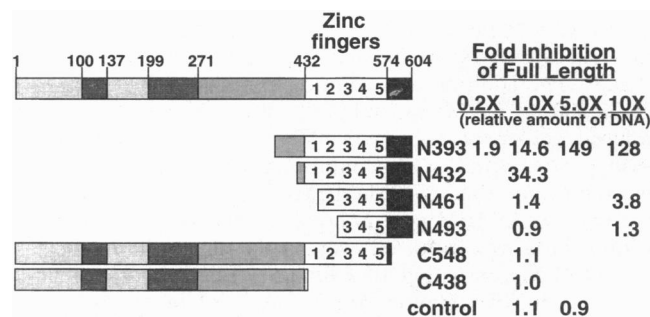
FIG. 5. An amino-terminal mutant of glass containing only the DNA-binding domain can dominantly inhibit transcriptional activation by full-length glass. Schematic diagrams are described in the legend to Fig. 2. In each case, *Drosophila* S2 cells were cotransfected with 100 ng of pP$_{AC}$Glass and 20 ng (0.2×), 100 ng (1.0×), 500 ng (5.0×), or 1 μg (10×) of the indicated mutant in pP$_{AC}$Ubx+NdeI. The fold inhibition of full-length glass was determined by dividing the amount of activity observed with transfection of pP$_{AC}$Glass alone by the amount observed by cotransfection of pP$_{AC}$Glass with the indicated amount of each mutant-containing plasmid.