Analysis of the eye developmental pathway in *Drosophila* using DNA microarrays

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Pax-6 **genes encode evolutionarily conserved transcription factors capable of activating the gene-expression program required to build an eye. When ectopically expressed in *Drosophila* imaginal discs, *Pax-6* genes induce the eye formation on the corresponding appendages of the adult fly. We used two different *Drosophila* full-genome DNA microarrays to compare gene expression in wild-type leg discs versus leg discs where *eyeless*, one of the two *Drosophila Pax-6* genes, was ectopically expressed. We validated these data by analyzing the endogenous expression of selected genes in eye discs and identified 371 genes that are expressed in the eye imaginal discs and up-regulated when an eye morphogenetic field is ectopically induced in the leg discs. These genes mainly encode transcription factors involved in photoreceptor specification, signal transducers, cell adhesion molecules, and proteins involved in cell division. As expected, genes already known to act downstream of *eyeless* during eye development were identified, together with a group of genes that were not yet associated with eye formation.**

Pax-6 genes encode evolutionarily conserved transcription factors with two DNA-binding domains acting upstream in eye developmental pathway in both vertebrates and invertebrates (1, 2). They are able to induce the expression of all the genes required to build a vertebrate camera-type eye or an insect compound eye. In *Drosophila*, retinal differentiation starts during the third larval instar, when a differentiation wave marked by an indentation of the disc epithelium, the morphogenetic furrow, traverses the eye disc from the posterior to the anterior (3, 4). Although the cells anterior to the furrow are undifferentiated and divide asynchronously, those within the furrow arrest in G_1 phase and start to differentiate. As they emerge from the furrow, cells are grouped into preclusters of five photoreceptors (R8, R2/R5, and R3/R4), whereas the other undifferentiated cells undergo an additional round of mitosis (the second mitotic wave) before differentiating into photoreceptors R1/R6 and R7, cone cells, and pigment cells (5). The *eyeless* (*ey*) gene encodes one of the two *Drosophila Pax-6* genes, both of which are expressed in the eye precursors as soon as these structures appear (6, 7). When ectopically expressed in other imaginal discs, *Pax-6* genes are able to induce the expression of all the genes required for eye formation, leading to the formation of ectopic eyes on the adult appendages (8).

The aim of our study is to gain an overview of the entire genetic cascade controlling eye morphogenesis. Here we focus on the developmental stage when the cells start differentiating into photoreceptors. To identify genes that are up-regulated when an eye morphogenetic field is induced, we used DNA microarrays to compare wild-type leg discs to others where *ey* is ectopically expressed. To validate these data, we analyzed the endogenous expression of selected genes in the eye discs.

The choice of the oligonucleotide probe sequences present on the array is critical for array-based analysis of transcription. Therefore we compared the performance of two different *Drosophila* full-genome high-density oligonucleotide arrays, roDROMEGa and DrosGenome1, which were designed independently (see *Materials and Methods*). This approach ensured cross-validation of our data and significantly increased the significance of our results. We identified a set of 371 genes transcribed in the eye imaginal discs and induced by *ey* during ectopic eye formation. As expected, genes already known to act downstream of *ey* during eye development were found together with a group of previously uncharacterized genes that were not yet associated with eye formation.

Materials and Methods

***Drosophila* Stocks.** The *Drosophila* strain $iso4^{BS}$, with an isogenized fourth chromosome, was used as wild-type strain. To enhance the ectopic expression of *ey* in the larval imaginal discs, dpp^{blink}-GAL4 (9) was recombined with UAS-GAL4 (gift from B. Hassan, Flanders Interuniversity, Institute for Biotechnology, Leuven, Belgium, and H. Bellen, Baylor College of Medicine, Houston) and crossed to UAS-*ey* (8).

DNA Microarrays. Two different high-density oligonucleotide arrays (Affymetrix, Santa Clara, CA) covering the *Drosophila* genome (10) were used in this study: roDROMEGa and DrosGenome1. roDROMEGa is a custom array of Hoffmann–LaRoche designed according to the *Drosophila* sequences deposited by Celera in the SwissProt/TrEMBL databases as of August 2000 (11). The DrosGenome1 array is based on a later release of the genome annotation and contains different oligonucleotide probes (www.affymetrix.com/analysis/index.affx).

On both arrays, some genes are represented by more than one probe set to include different splice variants. For instance, on roDROMEGa, the *longitudinal lacking* (*lola*) gene (12) is represented by five probe sets (CG12052_CDS 1–5), which reflect the five alternatively spliced transcripts described in the first release of the Genome Annotation Database of *Drosophila*, whereas on the DrosGenome1 array, the *lola* gene is represented by three probe sets derived from the sequences of CG12052, CG18376, and of the EST ld17006. We also realized that some genes are represented on only one of the two arrays. For instance, no probe set corresponding to the *teashirt* gene (13) could be found on the DrosGenome1 array, and the *appl* gene is not represented on the roDROMEGa array.

The raw data reported in this paper have been submitted to the NCBI Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo/ (accession no. GSE271).

Target Preparation. For each experiment, 100–200 imaginal discs were dissected, immediately transferred into 800 μl of Trizol (GIBCO/Life Technologies, Basel, Switzerland), and stored at −70°C. RNA extraction was performed according to manufacturer instructions. Quantity and quality of total RNA were determined by capillary electrophoresis on an RNA6000 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

To compare gene expression in control leg imaginal discs versus leg discs in which an eye field was induced [dpp^{blink}-GAL4, UAS-GAL4/UAS-*ey*], biotinylated targets were prepared from 15–20 μg of total RNA (500–700 leg discs) according to the standard Affymetrix procedure. To analyze gene expression in normal eye primordia, the eye part was separated from the antennal part of the eye-antennal disc. To reduce the number of discs required, we used a protocol involving two successive

Abbreviation: AD, average difference.

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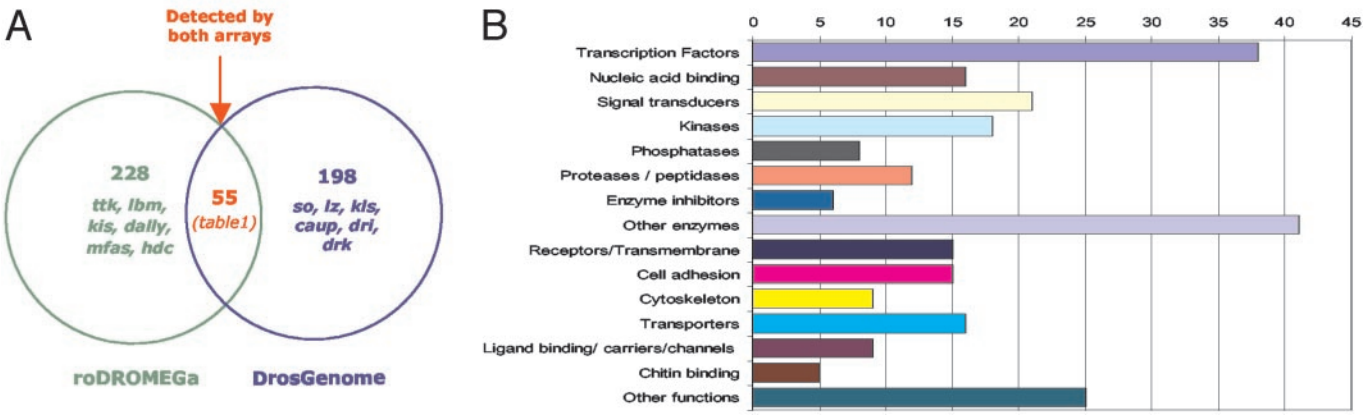

Fig. 1. (*A*) Repartition of the 371 *ey*-induced genes according to the probe array by which they are detected; examples of some genes detected by only one of the two DNA microarrays are given. (*B*) Functional classification of 254 of the 371 *ey*-induced genes to which a molecular function could be assigned.

rounds of cDNA synthesis, which allowed us to start from smaller amounts of total RNA (1–2 μg). This procedure is detailed in *Supporting Text*, which is published on the PNAS web site, www.pnas.org. In both cases, 20 μg of biotinylated antisense cRNA were ultimately fragmented and hybridized to the arrays according to the standard protocol.

Data Analysis. Expression signals were analyzed with Affymetrix MAS 4.0 software, and data were processed by using the RACE-A software (Hoffman–LaRoche) as described in ref. 14. The average difference (AD) values were used to estimate the transcript abundance. In this study, genes with AD $\geq$ 100 were considered to be expressed and, to calculate induction folds, the AD minimal value was set to 20 for all probe sets with AD $\leq$ 20. Because information about differential gene expression is not reliable without a measure of the confidence level with which the null hypothesis is rejected or accepted, we used three to five biological replicates to perform an unpaired *t* test [*t* test for independent samples with separate variance estimates (two-sided *P*)]. The *P* value of the unpaired *t* test (*P*) therefore reflects the probability at which the null hypothesis (no difference in the expression of a given gene between experimental samples) is rejected.

Results and Discussion

Screen Design and Strategy of Identification of Genes Expressed Downstream of *ey* During *Drosophila* Eye Development. To obtain a global view of the genetic program triggered by *Pax-6*, we used *Drosophila* full-genome arrays to compare RNA samples from normal leg discs versus leg discs in which an eye morphogenetic field had been induced by ectopic expression of *ey*. To enhance *ey* expression, we recombined the dpp-GAL4 driver with UAS-GAL4. The ectopic eyes generated in [dpp^{blink}-GAL4, UAS-GAL4/UAS-*ey*] flies are considerably larger than those obtained with the dpp-GAL4 driver alone (D. Felix, personal communication).

Because Ey is a transcription factor that plays a role in various tissues throughout *Drosophila* development, its ectopic expression may also induce the expression of genes that are not specifically involved in eye formation. To identify eye-specific genes, we therefore used DNA microarrays to analyze endogenous gene expression in the eye imaginal discs and combined these two criteria (ectopic induction by *ey* and expression in the larval eye imaginal disc) to discriminate between eye-specific and nonspecific gene transcription.

The same biotinylated targets were successively hybridized to the two DNA microarrays, first to roDROMEGa and subsequently to DrosGenome1, and processed by using the same standard procedure. An excess of labeled RNA was used to avoid titration of the targets during the hybridization to the second DNA microarray. In our experimental conditions, the same targets could be successively hybridized three times to DNA microarrays without any significant loss of signal (Table 3, which is published as supporting information on the PNAS web site).

Under each condition analyzed in this study (*iso4^{BS}* eye discs, *iso4^{BS}* leg discs, and [dpp^{blink}-GAL4, UAS-GAL4/UAS-*ey*] leg discs) the activity of each gene was measured independently 6–10 times, because three to five biological replicates were hybridized to the two arrays. The complete data for all probe sets of both DNA microarrays are given in Tables 4 (roDROMEGa) and 5 (DrosGenome1), which are published as supporting information on the PNAS web site (see also www.biozentrum.unibas.ch/gehring/).

Discrepancies Between the Two Microarrays. The following criteria were used to filter the data: a level of expression in the eye imaginal discs (reflected by the AD) $\geq$100 and an induction by ectopic *ey* of at least 1.5-fold with a confidence level of 95%. Only 40% of the genes ectopically induced by *ey* in the leg discs was also found to be transcribed in the eye discs. This emphasizes the importance of analyzing also the endogenous gene transcription that provides, together with the use of biological replicates, a strong validation of the data. As schematized on Fig. 1*A*, 228 and 198 genes induced by ectopic *ey* and expressed in the eye discs are detected by the roDROMEGa and DrosGenome1 arrays, respectively. This correspond to 371 unique genes (listed in Table 6, which is published as supporting information on the PNAS web site), among which 55 are detected by both microarrays according to our three-selection criteria (Table 1). If no *P* value is considered, 61% of the *ey*-induced genes detected by roDROMEGa are also detected by DrosGenome1 and, conversely, 65.5% of the *ey*-induced genes detected by DrosGenome1 are also detected by roDROMEGa (Table 7, which is published as supporting information on the PNAS web site). Because the targets hybridized to the two probe arrays were identical, these discrepancies probably reflect differences between the two DNA microarrays. Besides the statistical reasons, the different versions of the genome annotation and probe selection parameters that were used to design the two arrays, as well as the noise in the MAS 4.0 algorithm used here are possible sources of discrepancies (G. de Feo, Affymetrix, personal communication).

The *sine oculis* (*so*) gene is required downstream of *ey* during eye development (15, 16). However, no transcripts corresponding to *so* are detected by roDROMEGa in the eye discs (Table 2; AD = −8). A closer analysis of the metrics MAS 4.0 files revealed that for the five independent eye disc replicates analyzed, the number of negative probe pairs is higher or equal to the number of positive probe pairs representing the *so* gene. In contrast, the number of positive probe pairs after hybridization of the same targets to DrosGenome1 is

Table 1. 55 genes detected by both arrays as expressed in the eye discs and induced by *ey* during ectopic eye development

GENE	MOLECULAR FUNCTION	IF roDROMEGa	IF DrosGenome	GENE	MOLECULAR FUNCTION	IF roDROMEGa	IF DrosGenome
eyeless	Transcription Factor (Pax-6)	31.3	27.6	eyes absent	Epoxyde hydrolase	37.15	18.3
scratch	Transcription Factor (Zinc Finger)	71.5	11.5	CG5653	Amine Oxydase	2.4	3.8
lola	Transcription Factor (Zinc Finger)	19.9	5.7	slamdance	Membrane alanyl aminopeptidase.	14.2	3.0
rough	Transcription Factor (Homeodomain)	1.5	6.2	CG8663	Sodium/potassium-transporting ATPase	5.6	2.5
e(spl)mgamma	Transcription Factor (bHLH)	2.3	2.6	BcDNA-gh0762	Fatty-acid synthase	1.9	2.3
e(spl) mdelta	Transcription Factor (bHLH)	2.2	2.0	CG10527	Farnesoic acid O-methyltransferase	2.2	1.9
pebbled	Transcription Factor	2.3	3.2	CG15093	Mitochondrial 3-hydroxyisobutyrate dehydrogenase	2.3	1.8
bunched	Transcription Factor	2.5	2.9	CG9427	Thioredoxin like	2.6	1.8
fruitless	Transcription Factor (BTB-zinc finger)	2.5	3.3	CG14946	Oxidoreductase	2	1.6
ken and barbie	Transcription Factor (Zinc finger)	2.6	1.8	white	Pigment transporter	6.6	7.8
CG13651	pipsqueak DNA binding domain	2.2	2.8	CG8747	Chitin binding domain.	6.3	4.0
CG11798	Transcription Factor (Zinc finger)	3.3	3.2	gasp	Structural protein	6.1	3.4
CG10803	Nucleic acid binding (RNA binding domain)	2.9	1.7	quail	Actin binding	2.7	4.4
CG12238	Nucleic acid binding PHD-finger	2.0	1.7	chitinase-like	Imaginal disc growth factor	1.7	1.6
rac2	RHO small monomeric GTPase	3.3	3.9	Caf1-10	Nucleosome assembly chaperone	1.6	4.2
aplip1	Appl-interacting protein	15.9	3.3	sp2523	Homolog of *C.elegans* unc-39	11.2	6.5
unc-13	Kinase (DAG and calmodulin binding)	4.7	2.8	CG140595	No homolog, no domain	52.5	9.4
src64b	Protein tyrosine kinase	3.7	4.2	CG9335	No homolog, no domain	31.9	10.2
Tie	Protein tyrosine kinase	1.8	2.4	CG15430	No homolog, no domain	5.7	10.1
twins	Protein phosphatase 2A, regulator	9.6	2.6	CG5522	Domain CDC25 family	3.9	3.8
string	Protein tyrosine phosphatase	1.7	1.7	CG10625	No homolog, no domain	3.9	2.8
CG17124	Phosphatase inhibitor	2.1	6.4	CG5835	No homolog, no domain	2.8	2.3
roughoid	Serine-type peptidase	1.8	2.2	CG9536	No homolog, no domain	2.7	2.7
derailed	Receptor tyrosine kinase	3.3	2.4	CG8748	No homolog, no domain	2.6	3.1
fasciclin 2	Cell Adhesion	2.2	3.4	CG9170	No homolog, no domain	2.3	2.3
CG9134	Cell Adhesion (c-type lectin-like)	2.2	2.5	CG13897	No homolog, no domain	2.2	2.2
neurotactin	Cell Adhesion	2	2	CG11370	No homolog, no domain	2.0	2.1
				bg:ds01368	No homolog, no domain	1.7	1.9

Gene names are highlighted when corresponding serial analysis of gene expression (SAGE) tags were detected in eye disc libraries (35). IF, fold induction; yellow shading, IF < 1.5; orange shading, $1.5 \leq$ IF < 5; red shading, IF ≥ 5.

significantly higher (8–10; Table 3), which correlates with the positive AD for the *so* gene on this array. Conversely, the DrosGenome1 array does not detect *Sur-8*, *sprint*, or SP1173 transcripts in the eye (Table 2), although we confirmed the expression of these genes in the eye discs by *in situ* hybridization (Fig. 2).

The case of the *cyclin E* (*cycE*) gene also illustrates the differences between the two DNA microarrays. *cycE* is expressed in all the asynchronously dividing cells in S phase anterior to the morphogenetic furrow as well as in the subset of cells that will undergo the second mitotic wave, posterior to the morphogenetic furrow (17, 18). The DrosGenome1 DNA microarray does not detect any increase in *cycE* transcription after ectopic *ey* expression in the leg discs, whereas it detects the presence of the transcripts in the eye discs. In contrast, on roDROMEGa DNA microarray, *cycE* transcription in the leg discs is 16.4-fold up-regulated after ectopic *ey* expression, but *cycE* endogenous expression is not detected in the eye discs (Table 2).

Therefore, it is difficult to conclude that one microarray is more sensitive than the other because their accuracy strongly depends on the selection of the oligonucleotide sequences chosen to represent a gene, which itself depends on the genome sequence or annotation.

It is the combined use of two different DNA microarrays that allows validation of gene expression, hence considerably reducing the number of false positives and outliers.

The *ey*-Induced Genes Function High up in the Retinal Differentiation Pathway. On the basis of an AD ≥ 100, transcripts corresponding to 5,600–6,100 genes are detected in the eye discs. These genes may act in eye development upstream or in parallel to *ey*, such as *toy* and *optix* (7, 19), or may also be required for leg disc development (*Notch*, *Egfr*, and *dpp*). Therefore, despite their important role in eye development, their transcription is not significantly up-regulated by ectopic *ey*. The genes we identified here are more likely to be preferentially involved in retinal differentiation rather than being required for general morphogenesis of imaginal discs. In agreement with previous findings, the DNA microarrays detect an up-regulation of *eyes absent*, *so*, and *dachshund* (*dac*), which encode evolutionarily conserved proteins functioning together with *Pax-6* at the top of the eye developmental cascade (20). However, *dac* up-regulation occurs at only 74% of confidence (Table 2) because it is already highly expressed in leg imaginal discs in the absence of *ey*, consistent with its role in leg development (21). Because leg imaginal discs were used as the baseline for gene activity in our screen, genes more specifically required for eye rather than leg development are detected at a higher confidence level.

Table 2. DNA microarray values for the genes discussed in the text

		roDROMEGa Array					DrosGenome Array				
		AD LDc	AD LDey	IF	p	AD_ED	AD LDc	AD LDey	IF	p	AD_ED
cyclin E	Cell cycle regulator	56	918	16	0.017	<0	375	353	1	-	530
sine oculis	Transcription factor	22	548	25	0.110	<0	682	1280	1.9	0.010	687
dachshund	Nuclear protein	2184	3891	1.8	0.216	393	1418	2534	1.8	0.260	288
atonal	Transcription factor	565	893	1.6	0.065	241	435	951	2.2	0.012	425
glass	Transcription factor	80	1042	13	0.0002	52	6	462	23	0.008	1251
lozenge	Transcription factor	552	822	1.5	0.259	475	28	342	12	0.009	2129
sticky ch1	Transcription factor	55	569	10	0.104	107	277	581	2.1	0.033	410
CG4055	Transcription factor sequoia	228	529	2	0.013	305	271	161	1.7	0.176	141
net	Transcription factor; MATH6 homologue	542	1102	2	0.033	205	349	678	1.9	0.145	155
Sur-8	Ras interactor (Figure 2)	15	785	39	0.015	138	590	103	5.7	0.029	25
sprint	Ras interactor (Figure 2)	124	306	2.5	0.005	164	158	76	2.2	0.022	<0
skeletor	Nuclear protein (Figure 2)	1134	1726	1.5	0.025	1347	1207	1023	1.2	0.050	1444
CG12605	Transcription factor (Figure 2)	-199	208	1	0.008	166	35	-63	1.7	0.081	<0
CG11849	Pipsqueak DNA-binding domain (Figure 2)	-156	60	3	0.003	<0	-4	250	12.5	0.0001	1585
gh11415	mab-21 homolog (Figure 2)	135	564	4.2	0.011	71	185	537	2.9	0.010	1122
CG13532	Immunoglobulin domain (Figure 2)	107	347	3.2	0.161	239	-23	132	6.6	0.003	103
gh11973	c-type lectin-like domain (Figure 2)	31	553	18	0.004	<0	533	1461	2.7	0.005	1919
SP1173	No homolog, no domain (Figure 2)	442	1162	2.6	0.017	123	540	1485	2.8	0.005	81

Gene names are highlighted when corresponding serial analysis of gene expression (SAGE) tags were detected in eye disc libraries (35). LDc, *iso4*^{BS} leg imaginal discs; LDey, leg imaginal discs [dpp^{blink}-GAL4, UAS-GAL4/UAS-*ey*]; AD_ED, average difference in the eye imaginal discs; IF, fold induction; yellow shading, IF $<$ 1.5; orange shading, 1.5 $\leq$ IF $<$ 5; red shading, IF $\geq$ 5.

Among the 38 transcription factors found to be both induced during ectopic eye formation and expressed in the eye imaginal discs (Table 6), 18 were already associated with eye development. They are endogenously expressed in the vicinity of the morphogenetic furrow and known to be required during the first steps of photoreceptor differentiation. Among those, the *E*(*spl*) transcripts *m delta* and *m gamma* are expressed in the morphogenetic furrow (22). *atonal* is first broadly expressed in cells ahead of the advancing furrow and then undergoes successive refinements until it is expressed only in a single cell in each ommatidium, the R8 cell, which is the first photoreceptor to differentiate (23, 24). *rough* controls the differentiation of the R2 and R5 cells, which are subsequently differentiating (25, 26), and the *bunched* gene is expressed in a hedgehog-dependent stripe in the undifferentiated cells just anterior to the morphogenetic furrow (27). The genes *pebbled* and *glass* start to be expressed in the morphogenetic furrow, and their expression extends posteriorly in the differentiated photoreceptors (28, 29). *ey* also induces the ectopic expression of *lozenge*, which is expressed in all undifferentiated cells arising from the second wave of morphogenesis that give rise to the R1/R6, R7, cone, and pigment cells (30).

Known Genes Not Yet Associated with Eye Formation. Among the other 20 transcription factors up-regulated during ectopic eye formation, eight have been described to be involved in other developmental processes. For instance, the roles of *lola* (12), *sequoia* (*seq*) (31), and *stich1* (32, 33) in embryonic nervous system development were investigated on the basis on their loss-of-function phenotypes. Similarly, loss-of-function mutations in the *net* gene, which encodes the *Drosophila* homolog of MATH6, have been described to affect wing vein patterning (34). The endogenous transcription of these four genes in eye imaginal discs and their up-regulation during ectopic eye development (Tables 1 and 2) suggest a possible role during eye development. Moreover, the transcription of these genes in the developing eye was independently confirmed by serial analysis of gene expression (SAGE) transcript imaging of purified cell populations from eye imaginal discs (35); SAGE tags corresponding to *lola*, *seq*, *stich1*, and *net* were indeed detected in cDNA libraries derived from sorted populations of eye disc cells.

The *fruitless* (*fru*) and *ken and barbie* (*ken*) genes also encode transcription factors (36, 37) that are both expressed in the eye discs and induced by *ey* during ectopic eye development (Table 1). Although *ken* transcripts are present in the eye disc in several rows of cells posterior to the morphogenetic furrow (Fig. 2), no defects in eye development or morphology are described for viable mutant alleles (http://flybase.org/). One possibility is that these mutant alleles do not affect *ken* function in the eye, similar to the case of the *fru* alleles; *fru*-viable mutations cause anomalies in male courtship behavior and affect the sex-specific transcripts produced under the control of a distal promoter of the gene (38). *fru* is a multifunctional gene that encodes sex-nonspecific proteins in addition to the protein involved in male behavior (39). One or more of these proteins could be responsible for *fru* function in the eye.

Besides transcription factors, signal transducers represent an important category of genes expressed in the eye discs and up-regulated during ectopic eye formation (Fig. 1*B*). We confirmed the expression of the Ras interactors *Sur-8* (40) and *sprint* (41) as well as the *APPL-interacting protein 1* (42) in the eye discs. The specific

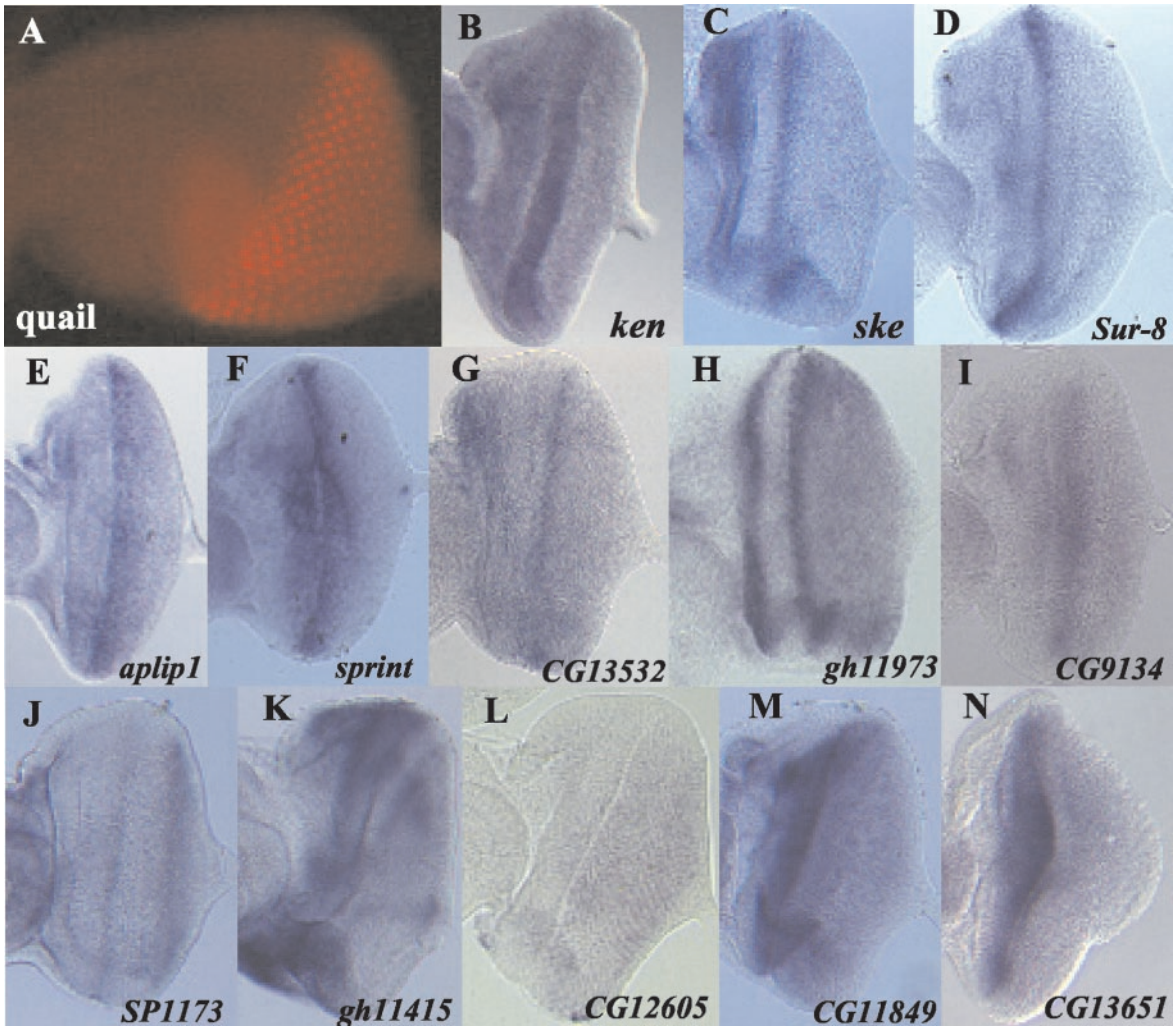

Fig. 2. Endogenous expression of the *ey*-induced genes in wild-type eye discs. (*A*) Quail protein was detected by using the 6B9 mAb developed by L. Cooley (Yale School of Medicine, New Haven, CT) and provided by the Development Studies Hybridoma Bank (Iowa City, IA). (*B–N*) *In situ* hybridization using digoxigenin-labeled antisense RNA probes derived from ESTs corresponding to the genes mentioned in each panel. Signal specificity was controlled by using sense RNA probes (not shown).

expression of these three genes in the area of the morphogenetic furrow (Fig. 2) and their significant induction during ectopic eye development (Tables 1 and 2) argues in favor of a previously uncharacterized function during eye development. Among the three *Rac* genes present in *Drosophila* (43), only *Rac2* is up-regulated during ectopic eye formation (Table 1). Rac GTPases act at various steps of development by controlling changes in cell shape (44). These modifications of the actin cytoskeleton are mediated by actin-binding proteins (Fig. 1*B*). Our data show that the QUAIL protein, which is involved in actin bundle assembly during oogenesis (45, 46) is also present in the eye discs posterior to the morphogenetic furrow (Fig. 2) and is up-regulated during ectopic eye development (Table 1).

Transcription of a number of genes required at various steps of cell division is up-regulated during ectopic eye formation; *twins* encodes the regulatory subunit of protein phosphatase type 2A involved in regulation of mitosis and expressed in imaginal discs (47). *greatwall* encodes a putative protein kinase required for chromosome condensation and mitotic progression (http://flybase.org/), and *skeletor* encodes a chromosomal protein relocalizing during mitosis (48), which was postulated to constitute a matrix for assembly of the microtubule-based spindle during prophase (49). *skeletor* is expressed in the eye disc in a discrete row of cells posterior to the morphogenetic furrow (Fig. 2), which could correspond to the cells undergoing the second wave of mitosis.

The *chit* gene encodes a chitinase-related imaginal disc growth factor synthesized by the fat body and involved in the control of imaginal disc growth (50). Here we show that *chit* is also transcribed in leg and eye imaginal discs and that its transcription is increased during ectopic eye formation (Table 1), indicating an autonomous role of *chit* in imaginal disc development and more specifically in eye differentiation. This finding is in perfect agreement with the results of the microarray analysis of genes differentially expressed in the various imaginal discs performed by Klebes *et al.* (51), where the authors found *chit* expression to be 2-fold higher in eye-antennal than in wing discs.

Previously Uncharacterized Genes Expressed During Eye Development. More than half of the 371 *ey*-induced genes identified in this study are uncharacterized. No molecular function could be assigned to 117 of them, such as SP1173 (FBgn0035710), for which no homolog nor any functional domain could be identified clearly. Interestingly, SP1173 transcripts are present in two distinct regions of the eye discs: in a band of cells located in the area of the morphogenetic furrow and at the posterior edge of the disc (Fig. 2). Transcription of three previously uncharacterized genes potentially

encoding cell adhesion molecules is also up-regulated during ectopic eye formation: CG13532, BcDNA:gh11973, and CG9134 are expressed in the area of the morphogenetic furrow (Fig. 2). CG12605 encodes a putative transcription factor similar to the pan-neural gene *scratch* and is expressed posterior to the morphogenetic furrow, where neuronal differentiation occurs (Fig. 2). CG11849 and CG13651 encode homolog proteins containing a N-terminal pipsqueak-DNA binding domain (52). Both are ectopically induced by *ey* in the leg imaginal discs and display almost identical expression patterns in the eye discs, in nondifferentiated cells anterior to the morphogenetic furrow (Fig. 2). These genes encode putative transcription factors that may represent previously uncharacterized, important regulators of eye development.

BcDNA:gh11415 encodes the homolog of the evolutionarily conserved cell fate-determining protein mab-21 identified in the nematode (53), zebrafish mouse and human. The mouse mab-21 homolog participates in cerebellar, midbrain, and eye development. In midgestation embryogenesis, it is expressed at its highest levels in the rhombencephalon, cerebellum, midbrain, and prospective neural retina. The human mab-21 homolog, CAGR1, was detected originally in a retinal cDNA library. It is expressed in several tissues, most prominently in the cerebellum (54, 55). *BcDNA:gh11415* expression anterior to the morphogenetic furrow in *Drosophila* eye imaginal discs (Fig. 2) and its ectopic induction by *ey* are consistent with an evolutionarily conserved role of mab-21 in eye development.

Conclusions

We used DNA microarrays to get an overview over the genetic cascade controlling *Drosophila* eye morphogenesis at the end of the third larval stage. By comparing gene expression in wild-type leg discs to leg discs where *ey* is ectopically expressed, we identified 371 genes that are endogenously expressed in the eye discs and up-regulated when an eye morphogenetic field is ectopically induced. Besides the genes already known to act downstream of *ey* during eye development, we identified a number of previously described genes that were not yet known to be expressed during eye formation and suggest a possible role in eye development for previously uncharacterized genes. In this regard, global transcript profiling on microarrays is a useful tool that complements genetic screens carried out to identify genes functioning in specific developmental pathways. However, the results obtained by using two different microarrays indicate that genome annotation and GeneChip design strongly influence the results.

ey mainly induces the expression of genes acting early in retinal differentiation, such as transcription factors involved in photoreceptor specification, signal transducers, actin-binding proteins, cell adhesion molecules, and proteins involved in cell division. This study provides a picture of how *ey* superimposes its action on cells by specifically activating the expression of particular members of general signaling pathways, hence generating a unique combination of gene products conferring an eye identity to the imaginal disc cells where it is expressed. This approach can be used to better understand the genetic program of *Drosophila* eye morphogenesis, from the initial establishment of an eye morphogenetic field to the final differentiation and maintenance of the compound eye. This will ultimately allow us to compare the morphogenesis of the insect eye with the morphogenesis of the camera-type eye of mammals and various other eye types found in other phyla.

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