serum (FCS; Life Technologies, Gaithersburg, MD). Approximately 2×10^6 cells per dish (at a density of 5×10^4 cells p/cm^2) were transfected by adding to the culture medium (10 ml volume) pSupFG1 DNA (5 μg) premixed with cationic liposomes (50 μg) (DOTAP; Boehringer Mannheim, Indianapolis, IN). After 12 hours, the cell monolayers were washed three times and fresh medium containing the selected oligonucleotide at a concentration of 2 μM was added.

8. The oligonucleotides were not conjugated to any mutagen but were modified to resist nuclease-mediated degradation by incorporation of either a 3′ propylamine group or phosphorothioate internucleoside linkages. Similar results were obtained with either modification.
9. Isolation of the SV40 vector DNA and transformation of bacteria for genetic analysis of the *supFG1* gene were performed as described (*5*).
10. Sequencing of the *supFG1* gene mutations was performed directly from the plasmid vector DNA with a primer complementary to a region in the β-lactamase gene adjacent to the *supFG1* gene, as described (*5*).
11. XPA cells from patient XP12BE were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ; repository number GM04429E). These cells were SV40-transformed fibroblasts from a patient with XPA. CSB cells (CS2BE; repository number GM01098B) were SV40-transformed fibroblasts from a patient with CSB. Normal fibroblasts (repository number GM00637F) were SV40-transformed cells from an apparently normal donor. Transformed XPA fibroblasts from patient XP2OS and the XP2OS cells transfected with a vector expressing XPA cDNA (XP2OS-pCAH19WS) were obtained from K. Kraemer (NIH) (*13*). The cells were grown in DMEM supplemented with 10% FCS (Life Technologies).
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15. The CSB cells, at a density of 1×10^6 cells in 75-cm^2 flasks, were transfected with 20 μg of pSupFG1 and 10 μg of pSLME6(+) DNA (*14*) premixed with cationic liposomes (100 μg) (DOTAP; Boehringer Mannheim). After 12 hours, the monolayer was washed with phosphate-buffered saline and fresh medium containing the selected oligonucleotides at 2 μM was added. Forty-eight hours later, the vector DNA was isolated for analysis as described (*5, 10*). Extracted DNA was subjected to digestion with Sal I to eliminate any persisting pSLME6 plasmid DNA and with Dpn I to eliminate any unreplicated pSupFG1 vector molecules. Plasmid DNA was analyzed from all of the white colonies to confirm that the colonies arose from pSupFG1 mutants and not from pSLME6(+).
16. Transcription of *supF* gene sequences in COS cells was examined by Northern blot analysis of total cellular RNA by the method of Chirgwin *et al.* [J. H. Chirgwin, D. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]. RNA was resolved by electrophoresis through formaldehyde agarose gels and transferred to nylon filters. Synthetic oligonucleotides of 30 bp, matching the sequences of the sense and antisense strands at the 5′ end of the *supF* gene, were used as probes. Hybridization was done at 42°C in 20% formamide, 5× standard saline citrate (SSC), 1× Denhardt's solution, 0.05 M sodium phosphate (pH 7.2), 0.1% SDS, and 1 mg/ml of salmon sperm DNA, followed by autoradiography.
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19. HeLa cell–free extracts were prepared essentially as described in Glazer *et al.* [P. M. Glazer, S. N. Sarkar, G. E. Chisholm, W. C. Summers, *Mol. Cell. Biol.* **7**, 218 (1987)], and repair synthesis assays were done essentially as described (*17*), with slight modifications. Supercoiled pSupFG1 plasmid DNA at 2×10^{-9} M plus pUC19 DNA at 5×10^{-8} M (as an internal control) were preincubated with the oligonucleotides at 1×10^{-6} M for 2 hours at 37°C in 10 mM tris (pH 7.4) and 10 mM $MgCl_2$. The DNA samples were added to HeLa cell extracts containing 10 to 15 μg/μl of protein and [α-^{32}P]dCTP, additionally supplemented as described (*17*), and incubated for 3 hours at 30°C. The DNA was extracted with phenol-chloroform, concentrated by filtration with a Centricon 100 filter (Amicon, Beverly, MA), and linearized with Eco RI. The samples were analyzed by 0.7% agarose gel electrophoresis, ethidium bromide staining, and autoradiography.
20. In vitro transcription reactions were performed in HeLa cell extracts (*19*) under conditions described in G. S. Read and W. C. Summers [*Proc. Natl. Acad. Sci. U.S.A.* **79**, 5215 (1982)]. Template DNA (pSupFG1) was linearized with Sca I and incubated with selected oligonucleotides in triplex binding buffer (*5*) at 37°C for 2 hours. Oligonucleotide-plasmid samples containing 1 μg of template DNA were incubated in 40-μl reactions containing 5 μl of cell extract (*19*) and supplemented with [α-^{32}P]guanosine 5′-triphosphate at 30°C for 1 hour. RNA products were visualized by electrophoresis on a 4% denaturing polyacrylamide gel containing 7 M urea, followed by autoradiography.
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Cell Killing by the *Drosophila* Gene *reaper*

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The *reaper* gene (*rpr*) is important for the activation of apoptosis in *Drosophila*. To investigate whether *rpr* expression is sufficient to induce apoptosis, transgenic flies were generated that express *rpr* complementary DNA or the *rpr* open reading frame in cells that normally live. Transcription of *rpr* from a heat-inducible promoter rapidly caused widespread ectopic apoptosis and organismal death. Ectopic overexpression of *rpr* in the developing retina resulted in eye ablation. The occurrence of cell death was highly sensitive to the dosage of the transgene. Because cell death induced by the protein encoded by *rpr* (RPR) could be blocked by the baculovirus p35 protein, RPR appears to activate a death program mediated by a *ced-3*/ICE (interleukin-1 converting enzyme)–like protease.

Programmed cell death, or apoptosis, is an active, gene-directed process that seems to have been conserved throughout animal evolution (*1*). Apoptosis in *Drosophila* is ultrastructurally and biochemically similar to apoptosis in mammals and is controlled by many of the same signals (*2*). The *reaper* (*rpr*) gene appears to play a key role in regulating apoptosis in *Drosophila* (*3*). This gene is expressed in cells that are doomed to die, and a deletion that includes *rpr* eliminates all cell death in the *Drosophila* embryo. *rpr* may be a switch; when *rpr* is expressed in a cell, that cell undergoes apoptosis. The gene may activate downstream cell death effectors or suppress protective functions, which in turn prevent the activation of constitutively expressed effectors.

The *rpr* gene encodes a small (65–amino acid) peptide that shares limited amino acid similarity with the "death domains" of the mammalian tumor necrosis factor receptor (TNFR) family, which includes the Fas antigen and a number of interacting proteins (*4*). TNFR 1 and Fas induce cell death when activated by ligand binding or when overexpressed, and this killing requires the death domain (*5, 6*).

To investigate the ability of RPR to kill cells that normally live, we generated transgenic flies in which *rpr* was both overexpressed and expressed ectopically. Initially, we used a transgene that expressed an *rpr* complementary DNA (cDNA) under the control of the *hsp70* promoter (*hsrpr*) (*7*).

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Upon heat shock (*8*), massive ectopic cell death was induced throughout embryos carrying one or two copies of *hsrpr* (Fig. 1, C and D). Embryos expressing other transgenes from the same promoter did not show any excessive cell death after a similar treatment (Fig. 1A) (*9*).

The *rpr* open reading frame (ORF) gene also induced ectopic apoptosis when ectopically expressed (*hsrprORF*) (*10*) (Fig. 1E), indicating that the ORF encodes *rpr* function. The amount of death induced by this transgene was less than that seen with the whole cDNA. The small *rprORF* transcript may be less stable than the cDNA, or regions outside the ORF may contain additional sequences necessary for optimal *rpr* production. As a further control, we generated a mutant form of the *rpr* ORF by introducing a 1–base pair insertion immediately after the AUG triplet, leading to an unrelated ORF of similar size to *rpr* (*hsrprORFmut*). This mutant form of the ORF did not induce any ectopic cell death after heat shock (Fig. 1B).

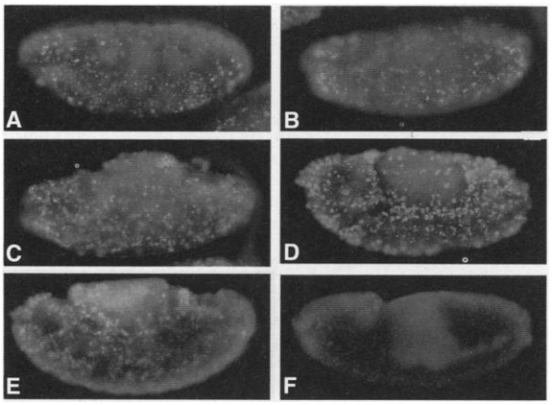

Fig. 1. Ectopically expressed *rpr* induces cell death in a large number of embryonic cells. All embryos were given a 1-hour heat shock and stained with acridine orange 1 hour later (*8*). The brightly staining dots represent apoptotic cells. (**A**) Control embryo transgenic for *hsdisco*, which affects morphogenesis in the embryo but does not rapidly induce ectopic cell death. (**B**) Embryo transgenic for the mutant *hsrprORF*. Expression of this construct does not induce ectopic apoptosis. (**C** and **D**) *hsrpr* embryos. This transgene induces much ectopic cell death in both wild-type and H99 mutant embryos. Apoptotic cells could be seen throughout the embryo, and the morphology of the embryos was severely disrupted. Embryos carrying two copies of *hsrpr* that were also homozygous for H99 were indistinguishable from those not carrying this deletion. (**E**) *hsrprORF* embryo. This construct also induces ectopic cell death and abnormal morphology in both wild-type and homozygous H99 embryos, which indicates that the activity of the *rpr* cDNA is due to the ORF. (**F**) H99 embryos after heat shock do not show increased cell death.

hsrpr was also able to induce a large amount of apoptosis in embryos homozygous for Df(3L)hidH99 (H99), which removes *rpr* and *head involution defective* (*hid*), another gene implicated in *Drosophila* cell death (*11*). This deletion blocks both normally occurring cell death and ectopic death induced by developmental defects and low levels of x-rays (*3*). Ectopic expression of other genes did not cause apoptosis in H99 homozygous embryos (*9*). Apoptosis was also induced by *hsrprORF* in H99 homozygous embryos, although at lower levels. Thus, *rpr* expression alone is sufficient to kill a large number of cells in the embryo, and this killing does not require the activity of the *hid* gene.

In the embryo, the fate of individual cells after *rpr* induction cannot be easily followed. To examine whether *rpr* could kill all cells in a particular tissue, we examined the effect of *rpr* overexpression in the *Drosophila* compound eye. The *rpr* cDNA was placed under the control of an eye-specific promoter (*12*), and the resulting construct, pGMRrpr, was used to generate transgenic flies (*13*). This transgene ablated the eye in a dose-dependent manner. Flies carrying a single copy of pGMRrpr had overall normal eyes (Fig. 2A). Two copies resulted in a "rough" eye, which was also reduced in size (Fig. 2B). Three and four copies resulted in a very small eye and an eyeless fly, respectively (Fig. 2, C and D). Only the bristles of the eye appeared to be spared. This may reflect a lack of expression of the transgene in the bristle cells, or these cells may be resistant to *rpr* killing.

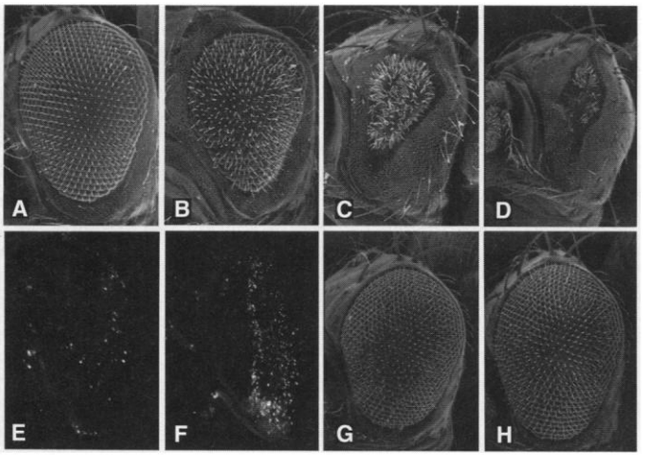

Fig. 2. Expression of *rpr* leads to the death of cells that would normally live. Transgenic flies that carry increasing copies of pGMRrpr have increasingly small eyes (*23*), as shown here in scanning electron microscope images. (**A**) Eyes of animals carrying one copy of pGMRrpr are apparently normal. (**B**) Two copies of pGMRrpr cause the occurrence of a rough eye. (**C**) Three copies decrease the size of the eye substantially. (**D**) The eye is eliminated by four copies of the pGMRrpr transgene. This phenotype is a result of ectopic apoptosis. (**E**) Wild-type third instar eye disc labeled with the TUNEL technique (*24*). (**F**) Massive amounts of TUNEL labeling can be seen after the morphogenetic furrow in third instar eye discs from larvae carrying four copies of the pGMRrpr transgene. The effect of this transgene can be blocked with the baculovirus antiapoptotic gene p35, which indicates that cell death alone can account for the defects in these flies. (**G**) The pGMRp35 transgene causes a slightly rough eye (*12*). (**H**) pGMRp35 with two copies of pGMRrpr.

The reduction in eye size seen in the pGMRrpr transformants could be the result of activating apoptosis or an effect on the development of the retina. We therefore used the TUNEL (*14*) technique to visualize the DNA breaks that are characteristic of apoptotic cells. Third instar eye discs from wild-type larvae and from larvae carrying four copies of pGMRrpr were assayed (Fig. 2, E and F). A small number of apoptotic cells were visible in wild-type discs. In contrast, large numbers of apoptotic cells could be seen behind the morphogenetic

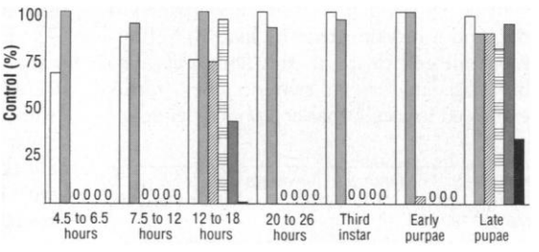

Fig. 3. Some developmental stages are resistant to killing by *hsrpr*. Embryos were heat-shocked at various stages of development (*18*), and the number of transgenic animals who survived to adulthood was scored as a percent of the control. Lines 37 (white) and 79 (light gray) carry the mutant form of *hsrprORF*, 57 (diagonal hatching) and 79 (horizontal hatching) carry the wild-type form of *hsrprORF*, and 46 (dark gray) and 53 (black) carry the *hsrpr* cDNA. Late embryos and late pupae are resistant to *rpr*-induced killing. The cDNA construct proved more effective at killing than the ORF alone.

furrow in transgenic larvae. Thus, the eyeless phenotype of these animals is due to ectopic apoptosis.

Overexpression of a gene may induce cell death directly through its normal function in the cell death pathway or indirectly as a result of an insult to the general physiology or developmental program of the cell. We therefore investigated, by coexpressing *rpr* and the antiapoptotic p35 baculovirus gene, whether ectopic *rpr* expression had effects on eye development in the absence of apoptosis. The p35 protein has been shown to block cell death in vertebrate and invertebrate cells (*12*, *15*), apparently by specifically inhibiting the *ced-3*/ICE (interleukin-1 converting enzyme) family of proteases (*16*). Expression of p35 in the eyes of pGMRrpr transgenics abrogated the effects of *rpr*; the eyes of the flies carrying both pGMRrpr and pGMRp35 transgenes were indistinguishable from those of flies carrying pGMRp35 alone (Fig. 2, G and H) (*17*). Thus, *rpr* expression does not have general effects on eye development, differentiation, or pattern formation, but rather acts specifically and directly in the cell death pathway. In addition, the ability of p35 to block *rpr*-induced death implicates a *ced-3*/ICE-like protease in both ectopic and endogenous *rpr* killing. This type of analysis underscores one benefit of an in vivo system, as it is often difficult to differentiate direct and indirect effects of gene overexpression in cultured cells. When cell death is blocked in these systems, it is generally not possible to tell if a cell is returned to a "normal" state.

To assess the effect of ectopic *rpr* expression at other stages of development, we tested the ability of *hsrpr* to kill flies (Fig. 3) (*18*). A single heat pulse applied at a variety of developmental stages killed flies carrying a single copy of the *hsrpr* or *hsrprORF* transgene. Two stages appeared resistant to killing by *rpr*: late embryos and late pupae. Both of these stages are also resistant to killing by x-rays, perhaps because of a slower rate of cell division (*19*). This suggests that killing by *rpr* may be more efficient in dividing cells. Alternatively, at these stages cells may be highly protected against cell death or may lack essential apoptotic effector functions.

Adult flies can also be killed by *hsrpr* (*20*). Four to 6 days after a 1-hour heat shock at 39°C, about half of the flies carrying the *hsrpr* or the *hsrprORF* transgene died rather suddenly. However, more than 85% of their nontransgenic siblings survived, as did the non–heat-shocked transgenics, a result reminiscent of the response of adult flies to radiation (*19*).

Our results indicate that *rpr* expression can kill many different cell types at many stages of development, which supports the idea that *rpr* is sufficient to activate apoptosis. Because killing by *rpr* is unusually dose-dependent, constructs such as pGMR-rpr could be used as sensitive reporters to identify genes involved in *rpr*-mediated killing. Given the homology with the death domain of TNFR, which is required for both killing and multimerization of the receptor (*6*, *21*), the described dosage dependency may reflect the need of *rpr* to form multimers for cell killing. Such a model is attractive because it would provide a mechanism for safeguarding against occasional errors in the control of *rpr* expression. Interestingly, recombinant *rpr* multimerizes in vitro (*22*), but the functional significance of this finding has not been tested in vivo.

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7. The *hsrpr* transgene was made by inserting a 0.9-kb Eco RI fragment containing the 13B2 *rpr* cDNA (*3*) into a pCaSpeR vector that contained the *hsp70* promoter (*11*). Two independent lines containing a single insertion of the transgene were tested for their ability to induce apoptosis.
8. Embryos were collected from flies of the genotypes *w; hsrpr46/+; H99/+* and from the cross *w hsrpr53/Y; H99/+* X *w; H99/TM3*, for 6 hours at 22°C and aged for 16 hours at 18°C. Embryos were heat-shocked on egg collection plates submerged in a water bath at 39°C, allowed to recover at 25°C for 1 hour, and stained with acridine orange as described [J. M. Abrams, K. White, L. I. Fessler, H. Steller, *Development* **117**, 29 (1993)]. Although levels of *rpr* expression were not directly assessed, expression of the *hsp70* promoter is considered to be moderately strong but within the range of strongly expressed endogenous genes.
9. Three heat shock cDNA constructs were tested previously for their ability to rapidly induce apoptosis after heat shock (*11*). The effects of these heat shock constructs were examined in both wild-type and H99 backgrounds. No excessive cell death was seen in the wild-type background (for example, Fig. 1A), although these constructs do induce abnormalities in embryonic morphology upon heat shock. In addition, these transgenes do not induce apoptosis in homozygous H99 embryos.
10. The transformation constructs that contained the *rpr* ORF (*hsrprORF*) were generated by polymerase chain reaction (PCR), with the *rpr* 13B2 cDNA as a template. The 5′ primer for the wild-type version contained an R1 site and *rpr* sequence from 20 bases upstream of the AUG triplet to 11 bases downstream. The mutant form (*hsrprORFmut*) contained the insertion of an A residue directly after the AUG triplet, resulting in a predicted translation product of 67 amino acids. The 3′ primer contained an Xba I site and extended from 10 bases downstream of the *rpr* stop codon to 18 bases within the coding region. A single base change was included downstream from the ORF to act as a stop codon for the mutant form. The PCR products were cut with Eco RI and Xba I and inserted into pCaSpeR-hs cut with Eco RI–Xba I [V. Pirrotta, in *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, R. L. Rodriguez and D. T. Denhart, Eds. (Butterworths, Boston, 1988), pp. 437–456]. Embryos from flies of the genotypes *w; hsdisco C/+, w; hsrprORFmut37A/+*, *hsrprORF71/+*, and *w; hsrprORF57/+; H99/+* X *w; H99/TM3* were treated and stained as described (*8*).
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17. Flies carrying four pGMRrpr insertions were crossed with flies homozygous for pGMRp35 (*12*), and the phenotype of the progeny was scored.
18. Embryos were collected from crosses of *yw67c23* to *w;hsrpr46/+*, *w;hsrpr53/+*, *w;hsrprORF57/+*, *w;hsrpr0RF71/+*, *w;hsrprORFmut37/+*, and *w;hsrprORFmut79/+*. Embryos were aged at 18° or 22°C until the developmental stage indicated in Fig. 3 and heat-shocked in a water bath for 1 hour at 39°C. The flies were then allowed to develop to adulthood, and *w+* (transgenic) and *w* siblings were counted soon after hatching.
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20. Young adult progeny from the crosses described above were heat-shocked in a water bath for 1 hour at 39°C, and then placed in vials and scored by counting the *w* and *w+* (transgenic) survivors every other day. Flies were placed on fresh food after scoring.
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23. Young adult flies were prepared for scanning electron microscopy as described (*11*).
24. Eye discs were dissected from climbing third instar larvae from *yw67c23* and *pGMRrpr 4 copy* stocks, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and washed in PBS for 15 min. The tissue was dehydrated in methanol for 30 min and rehydrated through 75% methanol and 25% PBT (PBS with 0.1% Tween 20), 50% methanol and 50% PBT, and 25% methanol and 75% PBT for 5 min each, washed in PBT, and treated with 10 μg/ml of proteinase K for 5 min. The tissue was then fixed for 20 min in 4% paraformaldehyde, washed, treated with 2:1 ethanol:acetic acid for 10 min at −20°C, washed, and equilibrated for 1 hour in equilibration buffer from the Apoptag fluorescein kit (Oncor). The tissue was incubated in the working strength terminal transferase mixture with the addition of 0.3% Triton X-100 for 3 hours at 37°C, washed in stop buffer for 2 hours at 37°C (both from the Apoptag kit), blocked in PBS with 2 mg/ml of bovine serum albumin, 0.3% Triton X-100, and 5% goat serum, and incubated overnight with preabsorbed fluorescein isothiocynate–conjugated anti-digoxygenin antibody (Oncor), diluted 1:1 with blocking solution. Final washes were done in PBS with 0.3% TritonX-100, and the samples were visualized on a Bio-Rad confocal microscope.

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