Deeper insight into the nature of the proton transfer to water could doubtless be achieved if neutron scattering studies were made on progressively dehydrated (or hydrated) samples of HSAPO-34.

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Mutagenesis in Mammalian Cells Induced by Triple Helix Formation and Transcription-Coupled Repair

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When mammalian cells were treated with triplex-forming oligonucleotides of sufficient binding affinity, mutations were specifically induced in a simian virus 40 vector contained within the cells. Triplex-induced mutagenesis was not detected in xeroderma pigmentosum group A cells nor in Cockayne's syndrome group B cells, indicating a requirement for excision repair and for transcription-coupled repair, respectively, in the process. Triplex formation was also found to stimulate DNA repair synthesis in human cell extracts, in a pattern correlating with the inhibition of transcription in such extracts. These findings may have implications for therapeutic applications of triplex DNA and raise the possibility that naturally occurring triple helices are a source of genetic instability.

Triple helices can be formed when oligonucleotides bind in the major groove of duplex DNA at polypurine-polypyrimidine sequences (*1*). Triple helix formation has been used to block transcription initiation and elongation (*2*) and to cleave DNA in vitro (*3*). We have explored the use of triplex-forming oligonucleotides (TFOs) as a mechanism to deliver a tethered mutagen to a selected gene for the site-specific introduction of DNA damage and consequent mutations within cells (*4*, *5*). In the course of this investigation, we observed that TFOs have the potential to induce mutations in vivo even in the absence of a tethered mutagen.

Three oligonucleotides (AG10, AG20, and AG30) were designed to bind as third strands in the antiparallel triple helix motif (*1*) to part or all of a 30–base pair (bp) polypurine-polypyrimidine target site in the *supFG1* reporter gene within the simian virus 40 (SV40) vector, pSupFG1 (*5*, *6*). These oligonucleotides were tested for their ability to induce mutations in the vector within monkey COS cells (Table 1). The cells were first transfected with SV40 vector DNA (*7*). After 12 hours to allow chromatinization of the vector, the oligonucleotides were added to the growth medium at a concentration of 2 μM (*8*). Two days later, the vector DNA was harvested from the cells for analysis of *supFG1* gene mutations (*9*). Oligonucleotide AG30 generated mutations in the target gene at a frequency of 0.27%, 13 times above the spontaneous background in the assay. In contrast, AG10 and AG20, which show weaker third-strand binding to *supFG1*, were less effective in producing mutations. As an additional control, an oligonucleotide of 30 nucleotides consisting of a mixture of all four bases (Mix30) (*5*, *6*) was also tested. This oligonucleotide did not form a detectable triple helix with *supFG1* (*5*) and it did not generate any mutagenesis above the background. The *supFG1* mutations generated

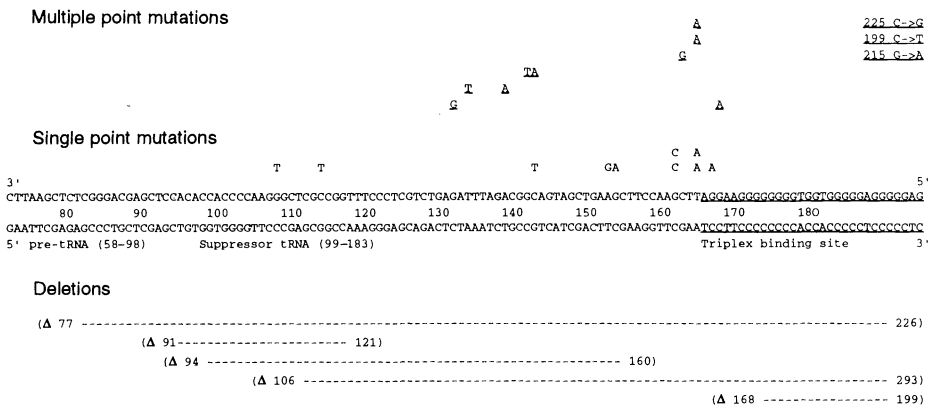

Fig. 1. Sequences of *supFG1* mutations induced by triple helix formation in COS cells. Three classes of mutations were observed, including single point mutations, deletions, and multiple, simultaneous point mutations, as indicated. The base substitutions listed above the corresponding *supFG1* gene sequence represent changes with respect to the upper strand. The multiple point mutations are underlined, with each set of simultaneous changes presented on a separate line. Point mutations occurring outside of the listed sequence are indicated by position numbers, with the involved base changes given. The deletions are presented below the gene sequence, with the deletion end points indicated. The triplex target site at base pairs 167 to 196 of the *supFG1* gene is indicated.

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by AG30 in COS cells were examined by DNA sequence analysis (*10*) (Fig. 1). Mostly point mutations, along with some deletions, within and around the triplex binding site were observed. Among the point mutations, there were several occurrences of multiple base substitutions.

To investigate a possible role for DNA repair in triplex-induced mutagenesis, we examined the ability of TFOs to cause mutations within both repair-deficient and repair-proficient human cells (*11*) (Table 2). In the xeroderma pigmentosum group A (XPA) cells, which have a defect in the DNA damage recognition protein that is a component of a ternary repair endonuclease complex (*12*), and are almost completely deficient in nucleotide excision repair, no triple helix–targeted mutagenesis was observed. The same result was obtained in two independent XPA cell lines (XP12BE and XP2OS). When one of the XPA lines (XP2OS) was corrected by transfection of a vector expressing the wild-type XPA gene complementary DNA (cDNA) (*13*), the ability of oligonucleotide AG30 to induce mutations in the vector was restored. These data suggest that excision repair is a necessary component of the triplex-induced mutagenic pathway.

Triplex-induced mutagenesis was also absent in the Cockayne's syndrome group B (CSB) cells (Table 2), which are defective in transcription-coupled repair of active genes (*14*). They can carry out excision repair but are unable to recruit the repair apparatus to sites of stalled transcription (*14*). Cotransfection of a vector expressing the CSB gene cDNA (*15*) partially restored the triplex-induced mutagenesis (up to a frequency of 0.12% from 0.04% in the parental cells; Table 2). Because these results implied an association of the triplex-induced mutagenesis and transcription-coupled repair, we investigated whether the *supF* gene region is transcribed in the SV40 vector. Although *supF* itself is a bacterial gene, Northern (RNA) blot analysis (*16*) showed that both strands of the *supF* gene are, in fact, transcribed in COS cells (Fig. 2), presumably as a result of read-through transcription in both directions from the SV40 promoter in the vector.

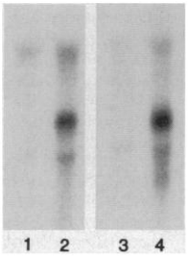

Fig. 2. Transcription of *supF* gene sequences in COS cells as determined by Northern blot analysis. RNA was prepared from either COS cells alone (lanes 1 and 3) or COS cells transfected with vector (lanes 2 and 4) (*18*). Duplicate samples were analyzed by hybridization to labeled single-stranded oligonucleotide probes corresponding to either the sense (lanes 1 and 2) or antisense (lanes 3 and 4) strand of the *supF* gene, followed by autoradiography.

Table 1. Mutagenesis induced by triple helix formation within monkey COS cells. The equilibrium dissociation constant (K_d) is given for triple helix formation, as determined by gel mobility-shift assay (*25*). The values in the third and fourth columns indicate the frequency of mutations detected in the pSupFG1 SV40-based shuttle vector after transfection of the vector DNA into COS cells, subsequent treatment of the cells with the indicated oligonucleotides at a concentration of 2 μM, and extraction of the vectors for genetic analysis in bacteria 48 hours later.

Oligonucleotide	K_d for third-strand binding (M)	Mutants/total	Mutation frequency (%)
None		9/44,850	0.02
Mix30	No detectable binding	5/16,590	0.03
AG10	$\geq 3 \times 10^{-5}$	10/14,475	0.07
AG20	3×10^{-7}	11/10,399	0.11
AG30	2×10^{-8}	148/54,899	0.27

To test whether a triple helix can stimulate repair of otherwise undamaged DNA, we examined the ability of the TFOs to stimulate repair synthesis on the vector template in HeLa cell–free extracts (Fig. 3) (*17, 18*). The supercoiled pSupFG1 vector DNA was incubated with the indicated oligonucleotides in extracts (*19*) that were supplemented with [α-^{32}P]deoxycytidine 5′-triphosphate ([α-^{32}P]dCTP) to detect induced DNA synthesis. As an internal control, pUC19 plasmid DNA, which lacks the triplex target site, was included with pSupFG1 DNA in all samples. After incubation in the extracts, the plasmids were linearized by digestion with Eco RI and analyzed by agarose gel electrophoresis. The quantities of the pSupFG1 and pUC19 DNAs were essentially constant in all of the samples (Fig. 3A).

Incorporation of the labeled nucleotide into the plasmid DNA was determined by autoradiography (Fig. 3B). AG30 and, to a lesser extent, AG20 stimulated labeling of pSupFG1 but not of pUC19, which is consistent with high-affinity, sequence-specific triplex formation at the binding site in pSupFG1 by these two TFOs. Neither AG10, which binds weakly to pSupFG1, nor Mix30, which does not bind at all, induced any repair synthesis above the background. Hence, repair activity in the HeLa extracts is induced by a tightly bound third strand on the SV40 vector, leading to repair synthesis and label incorporation. Stimulation of label incorporation occurs with oligonucleotides that are substituted at

Table 2. Mutagenesis induced by triple helix formation in human repair-deficient and repair-proficient cell lines. The cell lines are as follows: XPA, fibroblasts from patients with xeroderma pigmentosum, complementation group A, either XP12BE or XP2OS as indicated; XPA corrected, XP2OS cells transfected with a vector expressing wild-type XPA gene cDNA; CSB, fibroblasts from a patient with Cockayne's syndrome, group B; CSB corrected, CSB cells transfected with a vector expressing wild-type CSB cDNA. AG30 binds strongly as a third strand to the target *supFG1* gene ($K_d = 2 \times 10^{-8}$ M), whereas Mix30 shows no detectable binding. The values in the third and fourth columns indicate the frequency of mutations detected in the pSupFG1 SV40-based shuttle vector after transfection of the vector DNA into the indicated cells, subsequent treatment of the cells with the indicated oligonucleotides at a concentration of 2 μM, and extraction of the vectors for genetic analysis in bacteria 48 hours later.

Cell line	Oligonucleotide	Mutants/total	Mutation frequency (%)
Normal fibroblasts	None	6/16,550	0.04
	Mix30	4/13,400	0.03
	AG30	70/31,700	0.22
XPA (XP12BE)	None	13/48,329	0.03
	Mix30	14/42,500	0.03
	AG30	9/32,750	0.03
XPA (XP2OS)	None	6/18,600	0.03
	Mix30	3/14,800	0.02
	AG30	7/20,125	0.03
XPA (XP2OS) corrected	None	2/6,354	0.03
	Mix30	2/9,900	0.02
	AG30	65/21,250	0.31
CSB	None	8/36,725	0.02
	AG30	43/112,050	0.04
CSB corrected	None	3/13,539	0.02
	Mix30	3/17,404	0.02
	AG30	32/27,067	0.12

the 3′ end with a propylamine group (*8*); therefore, the possibility that the oligomers simply serve as primers can be ruled out.

We next investigated whether the stimulation of repair activity could be correlated with the ability of the triple helices to inhibit transcription and thereby potentially trigger the transcription-coupled repair pathway. The pSupFG1 DNA was used as a template for in vitro transcription assays in the HeLa cell extracts (*20*) in the presence or the absence of selected oligonucleotides (Fig. 4). The DNA was linearized to provide discrete transcription end points. Several run-off transcripts were generated from the pSupFG1 template. In the presence of AG30 and AG20, the 1.1-kb transcript is truncated to just 600 nucleotides, consistent with the inhibition of transcript elongation that occurs at the triplex binding site. Neither AG10 nor Mix30 blocked transcription. Thus, the oligonucleotides that induced mutagenesis and stimulated repair were precisely the ones that were able to inhibit transcription.

The mechanism by which the triplex-mediated transcription inhibition not only triggers repair but also induces mutagenesis can be extrapolated from the model of Hanawalt (*21*), who has suggested that the transcription-repair coupling pathway of a cell may trigger "gratuitous repair" when transcription stalls at natural pause sites, even in the absence of a classical DNA lesion. This gratuitous repair may lead to repetitive attempts at transcription and reiterative generation of repair patches. Such hyperactivity theoretically has the potential to introduce mutations into the DNA template. In yeast cells, the rate of spontaneous mutation at the *lys2* locus was increased when the gene was highly transcribed (*22*). Inactivation of the *rev3* gene, which has been implicated in error-prone repair in response to DNA damage (*23*), lessened the effect of transcription on mutagenesis (*22*).

We propose that formation of the triple helix blocks transcription at the site, triggering gratuitous and potentially error-prone repair even in the absence of chemical damage to the DNA. Furthermore, multiple cycles of attempted transcription, triplex-mediated inhibition, and stimulated repair can occur in a single vector molecule, inasmuch as the continued presence of the TFOs in the nucleus can lead to repetitive triplex formation. Even if the basic repair pathway is not particularly error-prone, each repetition of this process may increase the probability of a mutagenic event. In addition, in light of the results from yeast experiments (*22*), it is also possible that there is an error-prone repair pathway in mammalian cells that involves *rev3*-like functions that may be associated with transcription-coupled repair and triplex-induced mutagenesis.

The scattered spectrum of mutations that we observed is consistent with our proposed mechanism, which includes occasional repair errors on an otherwise undamaged template. These errors would be expected to occur in the region of the triple helix, but not necessarily at a specific base pair. We predict that such errors should occur in a quasi-random manner, perhaps influenced by sequence context effects. That most of the mutations were detected on just one side of the triplex binding site may simply be due to the fact that mutations downstream from base pair 183 in the *supFG1* reporter gene do not, by themselves, cause a detectable phenotype change and so are not scored in the assay. However, a directional bias in the mutagenic pathway cannot be ruled out. Also, the probability of finding a mutation is greatest at base pairs near the triplex site and diminishes with increasing distance, which suggests a possible gradient effect.

Although significantly above background, the frequency of the triplex-induced mutations reported here (in the range of 0.2 to 0.3%) is still somewhat lower than the targeted mutation frequency observed when TFOs are used to deliver a tethered psoralen molecule (2.1%) (*5*). This is not surprising, as psoralen is a highly reactive mutagen when photically activated. However, genetic manipulation with mutagen-conjugated TFOs requires either control over the reactivity of the mutagen (as with photoactivation) or entails the risk of nonspecific reactivity of the tethered reagent. The ability of oligonucleotides without any tethered reactive group to generate mutations in a target gene overcomes these drawbacks and may be advantageous for genetic manipulation. On the other hand, the fact that triple helix formation can lead to mutations may be an important consideration in the use of oligonucleotides in research and as therapeutics. Triplex-forming oligonucleotides designed to block transcription and even antisense oligonucleotides meant to prevent translation may have unintended and unexpected mutagenic effects.

Endogenous intramolecular triple helices as well as sequences conducive to triplex or tetraplex formation have been detected in the chromosomes of mammalian cells (*24*). Our findings raise the question of whether intramolecular triple helices, tetraplex DNA, and other possible non–duplex chromosome structures trigger repair and mutagenesis and thus constitute endogenous sources of genomic instability.

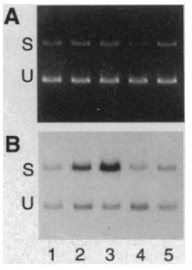

Fig. 3. Repair synthesis stimulated by triple helix formation in HeLa cell extracts. Supercoiled plasmids pSupFG1 (S) and pUC19 (U) were co-incubated for 2 hours at 37°C with selected oligonucleotides to allow triplex formation: AG10 (lane 1), AG20 (lane 2), AG30 (lane 3), Mix30 (lane 4), and no oligonucleotide (lane 5). The DNA was then added to HeLa cell extracts supplemented with [α-^{32}P]dCTP (*21*). After 3 hours at 30°C, the plasmid DNA was isolated, linearized by Eco RI digestion, and analyzed by agarose gel electrophoresis. (**A**) Visualization of the plasmid DNA by ethidium bromide staining. (**B**) Autoradiogram of the same gel showing labeled nucleotide incorporation indicative of repair synthesis.

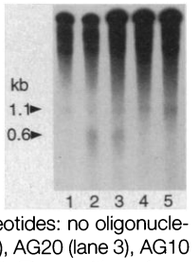

Fig. 4. Inhibition of transcription by triple helix formation in HeLa cell extracts. Linearized pSupFG1 DNA was used as a template for in vitro transcription reactions in HeLa cell extracts (*22*) in the presence of selected oligonucleotides: no oligonucleotide (lane 1), AG30 (lane 2), AG20 (lane 3), AG10 (lane 4), and Mix30 (lane 5). The position of one band representing a run-off transcript from the SV40 promoter is indicated (1.1 kb), along with the position of the truncated transcript resulting from the presence of the TFOs (0.6 kb).

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6. Oligonucleotide sequences were AG10, 5′-AGGAAGGGGG-3′; AG20, 5′-AGGAAGGGGGGGGTGGTGGG-3′; AG30, 5′-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-3′; and Mix30, 5′-AGTCAGTCAGTCAGTCAGTCAGTCAGTCAG-3′. Oligonucleotides were synthesized by J. Flory (W. M. Keck Biotechnology Resource Center, Yale University) with materials from Glen Research (Sterling, VA). The polypurine triplex target site was base pairs 167 to 196 of *supFG1* (see Fig. 1) (*5*).
7. Monkey COS cells were obtained from the American Type Culture Collection (Rockville, MD; 1651-CRL) and were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf

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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