Molecular Analysis of the Drosophila EGF Receptor Homolog Reveals That Several Genetically Defined Classes of Alleles Cluster in Subdomains of the Receptor Protein

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ABSTRACT

Mutations in the *torpedo* gene, which encodes the fruitfly homolog of the epidermal growth factor receptor (DER), disrupt a variety of developmental processes in Drosophila. These include the survival of certain embryonic ectodermal tissues, the proliferation of the imaginal discs, the morphogenesis of several adult ectodermal structures and oogenesis. *torpedo* is genetically complex: a number of alleles of the gene differentially affect the development of specific tissues, such as the eye, wing, bristles and ovary. In addition, *torpedo* mutations exhibit interallelic complementation. Molecular analysis of 24 loss-of-function mutations in the *torpedo* gene provides insights into the mechanistic basis of its genetic complexity. We observe an intriguing correlation between molecular lesions and mutant phenotypes. Alleles that differentially affect specific developmental processes encode receptors with altered extracellular domains. Alleles that fully or partially complement a wide range of embryonic and postembryonic *torpedo* mutations encode receptors with altered intracellular domains. From these findings we conclude the following. First, the *torpedo* protein may be activated by tissue-specific ligands. Second, the *torpedo* receptor tyrosine kinase may phosphorylate multiple substrates. Third, signal transduction by *torpedo* appears to require the physical association of receptors. Finally, the extracellular domain of the Torpedo protein may play an essential role in mediating receptor-receptor interactions.

RECEPTOR tyrosine kinases (RTKs) form a family of signal transduction molecules that has been conserved in metazoans for at least 800 million years. Tyrosine kinases that are structurally related to the epidermal growth factor receptor (EGF-R), for example, mediate cell-cell communication in organisms ranging from nematodes (AROIAN *et al.* 1990) to dipterans (LIVNEH *et al.* 1985; WADSWORTH *et al.* 1985) to humans (ULLRICH *et al.* 1984; COUSSENS *et al.* 1985; YAMAMOTO *et al.* 1986; KRAUS *et al.* 1989; PLOWMAN *et al.* 1990, 1993). The EGF-R protein-tyrosine kinase is a transmembrane protein with a single membrane-spanning domain. The extracellular (amino-terminal) portion of the receptor contains four subdomains (SI–SIV), of which SII and SIV are cysteine-rich, and SI and SIII are relatively cysteine-poor. The intracellular (carboxyl-terminal) portion of this protein consists of a short juxtamembrane domain, a tyrosine kinase domain and a carboxyl-terminal tail (see YARDEN and ULLRICH 1988).

Much of our understanding of the biochemistry of signal transduction by EGF-R and its relatives comes from vertebrate tissue culture studies (reviewed in ULLRICH and SCHLESSINGER 1990; SCHLESSINGER and ULLRICH 1992). This work has lead to a model for EGF-R function that is depicted in Figure 1. In the absence of ligand, EGF-R preferentially exists as a monomer. Upon ligand binding, which is mediated by the extracellular portion of the protein, the receptor forms dimers. Oligomerized receptors undergo phosphorylation on tyrosine residues located in the carboxyl terminus of the molecule by a *trans*-molecular mechanism. This autophosphorylation is thought to allow the specific binding of substrate and coupling molecules—via their SH2 domains—to phosphorylated tyrosine residues in the carboxyl-terminal portion of the receptor. Receptor activation involving autophosphorylation appears to promote receptor-substrate interactions and may stimulate substrate phosphorylation. The physical association with and/or phosphorylation of cellular proteins by EGF-R then initiates a biochemical cascade resulting in the transmission of a signal into the cell.

Cell culture studies have shed light on the role of EGF-R in the control of cell proliferation, differentiation and survival. Studies using ligands for EGF-R in organ culture and in whole organisms similarly have provided insights into the role of receptor function in specific tissues [see CLIFFORD and SCHÜPBACH (1992) for references]. Gene disruption provides another means of analyzing the role of this receptor in vertebrate development. This technique has been used to investigate the role of transforming growth factor-α, an activator of EGF-R (DERYNCK *et al.* 1984), in mouse development (LUETTEKE *et al.* 1993; MANN *et al.* 1993).

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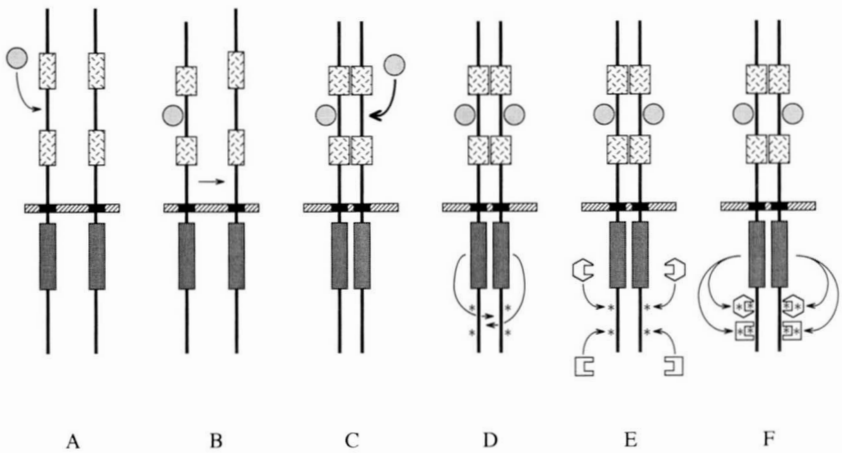


FIGURE 1.—Simplified model for signal transduction by receptor tyrosine kinases of the EGF receptor subfamily (adapted from SCHLESSINGER and ULLRICH 1992). In the absence of ligand, the receptor chiefly exists as a monomer. (A) In the monomeric form, the receptor binds ligand with low affinity (thin arrow). (B) Ligand binding stimulates receptor dimerization. Ligand binding by one receptor may be sufficient for dimer formation (see DISCUSSION). (C) In the dimeric form, receptors display high affinity ligand binding (heavy arrow). (D) Dimerized receptors autophosphorylate on tyrosine residues located in the carboxyl-terminal domain of the molecule through a *trans*-molecular mechanism. (E) Cellular substrates bind to specific phosphotyrosines via their SH2 domains. (F) Bound substrates are phosphorylated by the activated receptor.

Studies in vertebrate systems have been complemented by the genetic analysis of receptor function in Drosophila and Caenorhabditis. The versatile genetics of the worm and fly facilitate examination of the developmental roles of these receptors through phenotypic analyses, the structure/function analysis of these molecules through molecular genetics, and the identification of additional components of the signalling pathway through the isolation of suppressor and enhancer mutations.

The *torpedo* gene encodes the *Drosophila melanogaster* homolog of the vertebrate epidermal growth factor receptor, DER (PRICE *et al.* 1989; SCHEJTER and SHILO 1989). Over 40 mutant alleles of the gene, including both loss-of-function and two gain-of-function lesions, have been isolated (see LINDSLEY and ZIMM 1992). Phenotypic characterization of these mutations has shown that the *torpedo* receptor tyrosine kinase performs multiple functions in Drosophila development.

In the embryo *torpedo* is necessary for the establishment of cell identity in the ventral ectoderm, the survival of amnioserosa and ventral ectodermal cells, the formation of the central nervous system, germband retraction and the production of denticles, hooklike outgrowths of the ventral epidermis. Temperature-shift studies show that the *torpedo* product is required at several points in embryonic development (CLIFFORD and SCHÜPBACH 1992; RAZ and SHILO 1992, 1993). *torpedo* does not appear to regulate the major phases of cell proliferation in the Drosophila embryo (CLIFFORD and SCHÜPBACH 1992).

During the larval and pupal stages of development, the *torpedo* product is needed in the imaginal discs for the proliferation or viability of cells. A partial loss of gene activity results in a severe size reduction in several imaginal discs (CLIFFORD and SCHÜPBACH 1989). Clones of cells lacking functional *torpedo* product are recovered in adult animals less frequently than clones of genetically wild-type cells. Moreover, mutant clones generally are smaller than wild type, which indicates that the growth of mutant tissue is impaired (BAKER and RUBIN 1989; DÍAZ-BENJUMEA and GARCÍA-BELLIDO 1990; XU and RUBIN 1993; our unpublished observations). In the developing retina, gain-of-function mutations in *torpedo* lead to a great increase in the number of cells entering S phase (BAKER and RUBIN 1992; ZAK and SHILO 1992), but few of these cells undergo mitosis (BAKER and RUBIN 1992).

torpedo is required in the pupa for imaginal disc morphogenesis. A variety of abnormalities are seen in the ectodermal tissues of adult flies suffering a partial loss of *torpedo* gene activity. These include roughened compound eyes, shrunken or missing ocelli, the deletion of certain wing veins, the duplication or elimination of sensory bristles and the elimination of tarsal claws (CLIFFORD and SCHÜPBACH 1989). Clones of ectodermal tissue possessing reduced *torpedo* activity show similar developmental abnormalities (DÍAZ-BENJUMEA and GARCÍA-BELLIDO 1990).

If the *torpedo* gene encodes a single activity, it should be possible to arrange all alleles in a simple hypomorphic series progressing from weak to intermediate to complete loss-of-function mutations. Roughly half of the loss-of-function *torpedo* alleles, however, cannot be fit into such a series. These mutant alleles show complementation behavior consistent with the idea that they differentially affect specific gene functions. Certain lethal mutations fully or partially complement (CLIFFORD and SCHÜPBACH 1989; RAZ *et al.* 1991), suggesting that the proteins encoded by these alleles are defective in different receptor activities. Other alleles appear to dif-

ferentially affect gene activities required for specific developmental processes such as oogenesis and imaginal disc patterning and/or morphogenesis (BAKER and RUBIN 1989; CLIFFORD and SCHÜPBACH 1989). The remaining mutations, in contrast, behave genetically as if they reduce all gene activities in a uniform fashion (CLIFFORD and SCHÜPBACH 1989).

To gain insight into the mechanistic basis of *torpedo*'s genetic complexity, we have identified the presumptive molecular lesions of 24 loss-of-function alleles of *torpedo*. The examined mutations fall into four phenotypic categories. Class I alleles disrupt all gene activities in a uniform fashion, class II mutations are embryonic lethal lesions that fully or partially complement a variety of embryonic and pupal lethal *torpedo* alleles and the class III and class IV mutations differentially affect the development of specific tissues (CLIFFORD and SCHÜPBACH 1989). From this analysis we find a striking correlation between phenotypic classes and molecular lesions. Tissue-preferential alleles (with one exception) alter single amino acids in the extracellular domain of the receptor. The class II alleles, on the other hand, alter the intracellular portion of the molecule. We interpret the results of our molecular genetic study within the framework of a current biochemical model for EGF-R function.

MATERIALS AND METHODS

Nomenclature: Mutations in the gene encoding the Drosophila epidermal growth factor receptor homolog have been isolated independently by a number of workers. Consequently, the locus has been named *Ellipse* (BAKER and RUBIN 1989), *faint little ball* (NÜSSLEIN-VOLHARD *et al.* 1984), *torpedo* (SCHÜPBACH 1987) and *l(2)57DEFa* (O'DONNELL *et al.* 1989). On the basis of the homology of its product to the vertebrate EGF receptor, the gene has been designated DER (LIVNEH *et al.* 1985), DEGFr (DÍAZ-BENJUMEA and GARCÍA-BELLIDO 1990) and *Egfr* (LINDSLEY and ZIMM 1992). In this work, we refer to all loss-of-function alleles of the gene as *torpedo* mutations, and the protein product of the locus as Torpedo.

The *torpedo* gene encodes two proteins which are approximately 90% identical and differ only at their amino termini (SCHEJTER *et al.* 1986; SCHEJTER and SHILO 1989) (Figure 2). Since all mutations characterized in this study affect residues common to both isoforms of the receptor, we refer to both protein products of the gene as Torpedo.

Stocks: The origins and phenotypes of *torpedo* mutations analyzed in this work, with the exception of top^{4A}, are described in CLIFFORD and SCHÜPBACH (1989). The top^{4A} allele was isolated in an X-ray mutagenesis by J. PRICE (PRICE *et al.* 1989); it is a weak zygotic embryonic lethal mutation that shows abnormally severe wing vein defects in combination with the viable mutation top^{1}.

G. STRUHL kindly provided a chromosome containing the visible mutations *straw*, *pawn* and *cinnabar*, which was used for clonal analysis. The $M(2)c^{33a}$ mutation was obtained from the Mid-America Drosophila Stock Center. See LINDSLEY and ZIMM (1992) for descriptions of the marker mutations used in this work.

Reclassification of *torpedo* alleles: The top^{JE1} and top^{JE13} mutations were originally placed in class IV because they exhibit unexpectedly mild wing vein defects in *trans* to viable alleles. Further analysis indicates that the weak wing venation abnormalities observed for these alleles are due to dominant suppression by a *plexus* (*px*) mutation present on the top^{JE1} and top^{JE13} chromosomes. The *px* mutation, which produces excess wing veins, was previously shown by DÍAZ-BENJUMEA and GARCÍA-BELLIDO (1990) to enhance ectopic wing vein production by the gain-of-function *torpedo* mutation Elp^{1}. Removal of *px* from the *dp cn a* top^{JE1} *px sp* chromosome by recombination leads to a more severe wing vein phenotype in *trans* to top^{1}. While 62% of L4 veins ($n = 84$) and 96% of anterior crossveins ($n = 84$) from *cn* top^{1} *bw/dp cn a* top^{JE1} *bw* animals were defective, 39% of L4 veins ($n = 56$) and 55% of anterior crossveins ($n = 56$) from *cn* top^{1} *bw/dp cn a* top^{JE1} *px sp* flies were gapped or missing.

Complementation analysis: Crosses were performed at room temperature (~22°). Four to six top^{x}/CyO or $top^{x}/SM1$ females were mated to an equal number of top^{y}/CyO or $top^{y}/SM1$ males. For embryonic lethal heteroallelic combinations, embryos were collected on apple juice agar plates as described in WIESCHAUS and NÜSSLEIN-VOLHARD (1986). The *trans*-heterozygous phenotype, in most cases, is based on the examination of 50–150 dead embryos. For postembryonic lethal heteroallelic combinations, matings were performed in vials. Mutant viability was calculated from the number of eclosing top^{x}/CyO (or $SM1$), top^{y}/CyO (or $SM1$) and top^{x}/top^{y} adults.

Mosaic analysis: To allow identification of *torpedo* and wild-type clones, we constructed mutant and control chromosomes marked with *straw*, *pawn*, *cinnabar* and *brown*. The markers *stw* and *pwn*, which produce yellowish and truncated bristles, respectively, allow identification of clones in the cuticle (GARCÍA-BELLIDO and DAPENA 1974). In a *cn* background, eye tissue that also is genotypically *bw* is completely unpigmented.

To maximize clone size, $Minute^{+}$ marked cells were induced in a $M(2)c^{33a}/+$ background. The dominant mutation $M(2)c^{33a}$ reduces the mitotic rate of cells; therefore cells within clones enjoy a growth advantage in the imaginal disc (FERRUS 1975; MORATA and RIPOLL 1975).

stw pwn cn bw sp, *stw pwn cn* top^{2C82} *bw sp* and *stw pwn cn* top^{2L65} *bw sp* mosaics were produced by irradiating 48 ± 12-hr-old progeny of *stw pwn cn* top^{x} *bw (sp)/CyO* females mated to *pr cn* $M(2)c^{33a}/CyO$ males in a Torrex 150D X-ray machine (12 min at 145 kv and 5 mA, using an aluminum filter). Irradiated animals completed development at 25°. *stw pwn cn bw*, *stw pwn cn* top^{EC20} *bw* and *stw pwn cn* top^{CO} *bw* mosaic animals were generated by γ-irradiation (1800 rad from a cobalt source at 36–60 hr of development). These irradiated animals completed development at 22°.

stw pwn cn top^{x} *bw (sp)/pr cn* $M(2)c^{33a}$ flies were examined for eye clones under a dissecting microscope at 25×, then stored in 70% ethanol prior to mounting. Wings, heads and bodies were dissected apart, cleared with 10% NaOH and mounted in Faure's medium as described in WIESCHAUS and NÜSSLEIN-VOLHARD (1986).

Isolation of genomic DNA: Total genomic DNA was prepared from embryos, larvae, pupae and adults essentially as described in BENDER *et al.* (1983).

Polymerase chain reaction (PCR): Fragments of the *torpedo* gene were amplified by the polymerase chain reaction (SAIKI *et al.* 1988) using the primers shown in Figure 2. DNA isolated from five embryos or 1/10 of an adult provided sufficient template for a typical PCR amplification. PCR buffer contained 50 mM KCl, 10 mM Tris (pH 8.3), 0.01% gelatin and either 1 mM or 2 mM $MgCl_2$. Amplification conditions for each genomic fragment are shown in Table 1.

Denaturing gradient gel electrophoresis (DGGE): Twenty prospective mutant alterations described in this work were identified and molecularly mapped through a DGGE (MYERS *et al.* 1985) protocol developed by M. GRAY. Fragments of the

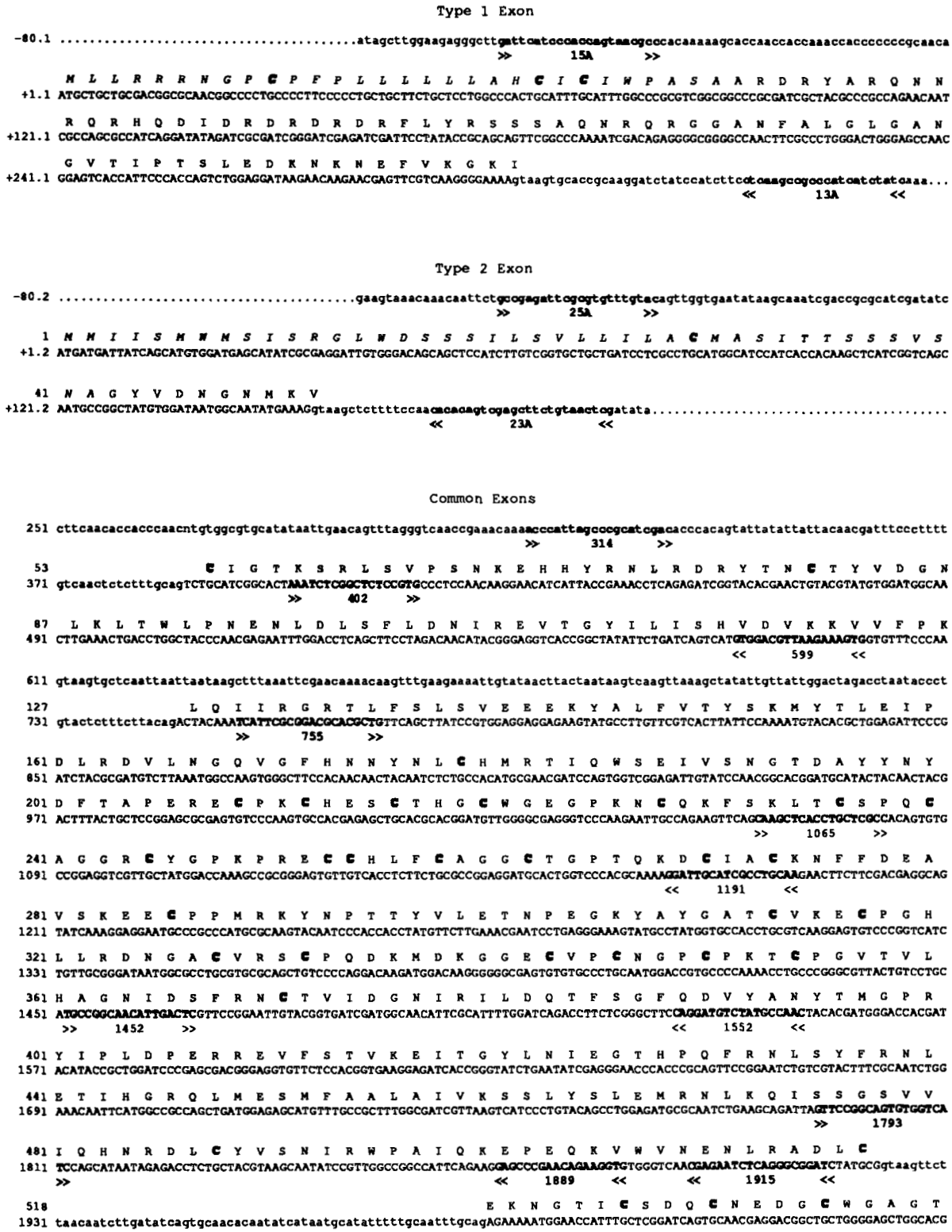


FIGURE 2.—The revised *torpedo* genomic sequence. The protein sequence is shown above the nucleotide sequence. Amino acid 1 of Torpedo corresponds to the initiation methionine for the type 2 receptor; residues specific to the type 1 receptor are not numbered. Signal pepide residues are italicized, cysteines in the extracellular domain are outlined, transmembrane domain residues are underlined and the tyrosine kinase domain is in bold italics. Nucleotide coordinates of the *torpedo* gene are based on LIVNEH *et al.* (1985). Coding sequences are in upper case; noncoding and intron sequences are in lower case. Primer sequences are boldfaced, and the direction of the arrows indicates whether the primer corresponds to the coding or noncoding strand of the gene. Names of amplification primers are boldfaced; names of sequencing primers are in regular type. Three nucleotides absent from the published *torpedo* sequence are underlined.

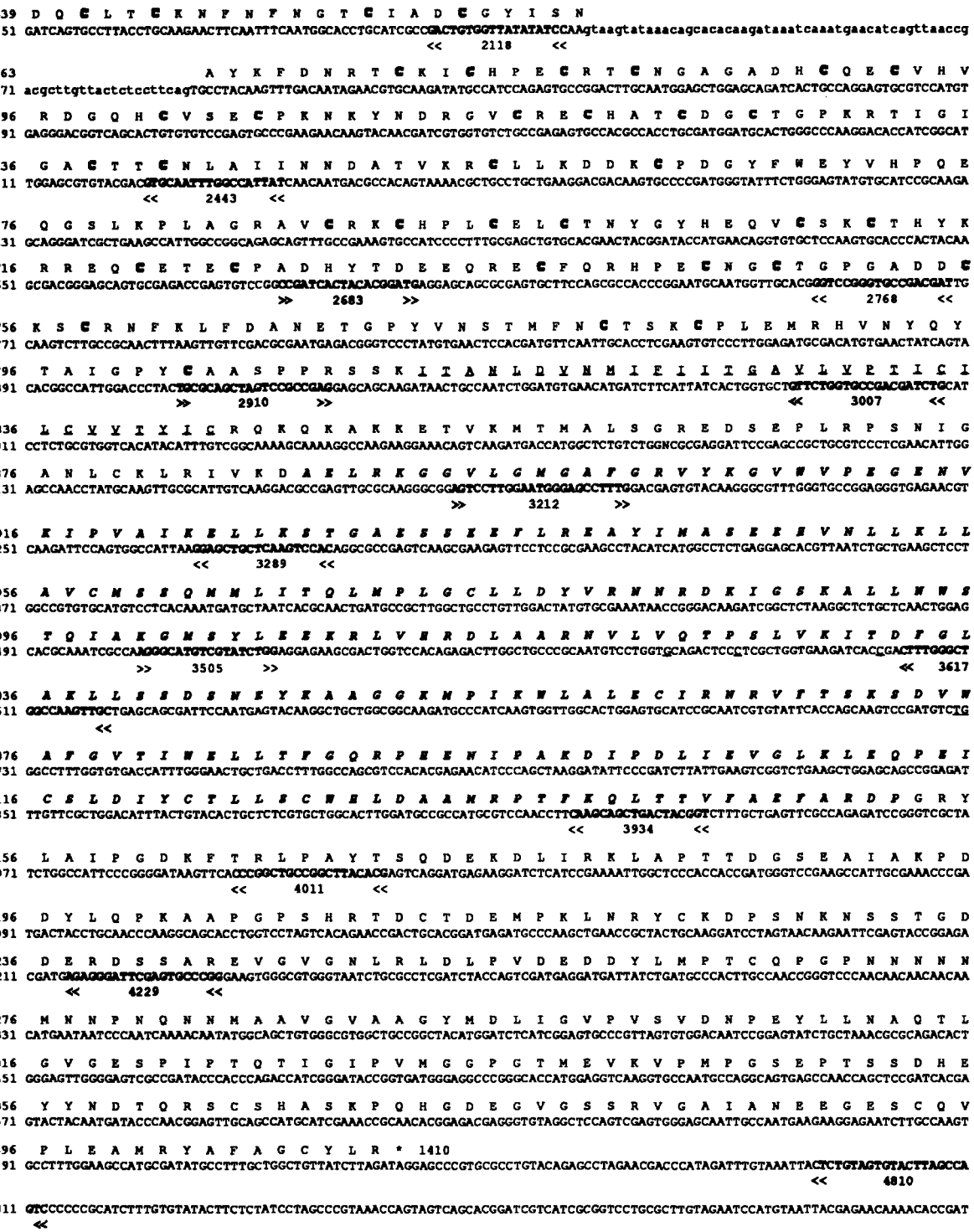


FIGURE 2.—*Continued*

torpedo gene were amplified from genomic DNA by PCR. After phenol/chloroform and chloroform extraction, followed by ethanol precipitation, 1/10 of the product of the PCR amplification was cut with a restriction enzyme(s) to yield fragments approximately 200–800 base pairs in length. Digested mutant and control DNA samples were electrophoresed in polyacrylamide gels containing a gradient of the denaturants urea and formamide for 20 hr at 60° and 70 V, then visualized by ethidium bromide staining. For most mutants characterized, amplified fragments comprising 80% or more of the coding region were examined (Figure 3).

A physical mismatch within a melting domain of a duplex DNA molecule destabilizes the domain, thereby lowering its melting temperature. This property was exploited to increase the sensitivity of the electrophoretic screen. DNA fragments produced from heterozygous (mutant/balancer or mutant/

TABLE 1

PCR amplification conditions for genomic fragments

Primers	$[Mg^{2+}]$ (mM)	Duration (sec)		
		Denaturation	Annealing	Synthesis
15A-13A	1	30 (94°)	60 (55°)	90 (72°)
25A-23A	2	30 (94°)	60 (60°)	90 (72°)
314-1889	1	30 (94°)	60 (60°)	150 (72°)
314-1915	1	30 (94°)	60 (60°)	150 (72°)
755-1889	1	30 (94°)	60 (55°)	150 (72°)
1793-3007	1	30 (94°)	60 (60°)	120 (72°)
2683-4229	1	30 (94°)	90 (55°)	150 (72°)
2910-3617	2	30 (95°)	60 (60°)	90 (72°)
3212-4229	1	30 (94°)	90 (55°)	150 (72°)
3212-4810	1	30 (94°)	60 (55°)	150 (72°)

parental) template DNA, half of which were heteroduplex molecules, were analyzed by DGGE.

Once a band showing aberrant electrophoretic mobility was detected, it was often possible to determine which product of the restriction digest contained the mutant lesion through double restriction digests and DGGE. In most cases, we could map prospective mutant lesions to genomic fragments less than 600 base pairs in length. Following its localization, each of these molecular lesions was identified by sequencing the fragment of the gene to which it maps.

None of the genomic fragments amplified from the top^{1}, top^{CJ4A}, top^{EE38} and top^{SH2} chromosomes showed abnormal mobility in denaturing gradient gels. Molecular lesions present on these mutant chromosomes were identified by sequencing fragments of *torpedo* encoding the extracellular domain of the receptor.

Sequencing: Mutant lesions were identified by sequencing single-stranded DNA produced from genomic DNA via the polymerase chain reaction. Double-stranded fragments were amplified from total genomic DNA as described above. After purification with the Magic PCR Preps kit (Promega), 1/10 of the double-stranded product was reamplified with a single internal or end primer. Single-stranded product from the second round of PCR amplification was extracted once with phenol/chloroform, then washed four times with 500 μl of distilled H_2O on a Microcon-100 filter (Amicon). One-fourth of the purified single-stranded DNA was sequenced using the Sequenase dideoxy kit (U.S. Biochemical Corp.).

Each mutation was verified by sequencing genomic fragments amplified from at least two independently prepared DNA samples. These samples were derived from homozygous, as well as heterozygous (mutant/parental or mutant/balancer) mutant animals.

Because we did not sequence the entire *torpedo* gene on each mutant chromosome, we cannot rule out the possibility that some *torpedo* alleles contain multiple molecular lesions. Nevertheless, we believe that the relevant DNA alteration was identified in most cases (see DISCUSSION).

Revised *torpedo* sequence: The genomic sequence of *torpedo* shown in Figure 2 is a revised version of the DER sequence submitted to GenBank (LIVNEH *et al.* 1985; SCHEJTER and SHILO 1989). Nucleotides 1–632 of the published sequence, which encode the first common exon and its flanking sequences, have been replaced by nucleotides 251–632 (SCHEJTER 1989). The identities of several ambiguous codons in the cytoplasmic portion of the molecule also have been resolved. Finally, three nucleotides (underlined in Figure 2) have been added to the region of the gene encoding the tyrosine kinase domain of the receptor. This last revision changes the identity of eight amino acids in subdomains VI and VII of HANKS *et al.* (1988) and adds a residue to subdomain VI; the updated protein sequence (. . . DLAARNVLVQTPSLVKITDFG . . .) more closely matches those of other members of the EGF-R subfamily of tyrosine kinases.

RESULTS

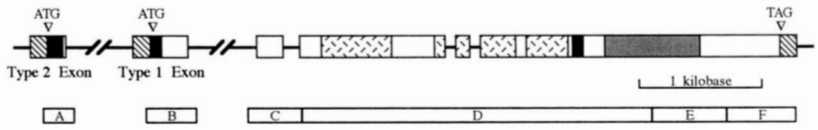
***torpedo* alleles show qualitative phenotypic differences:** Genetic analysis has shown that many mutations in the *torpedo* gene differentially affect the development of tissues that require receptor activity. In a previous study (CLIFFORD and SCHÜPBACH 1989) we used interallelic complementation analysis to assay the degree to which a collection of loss-of-function alleles affect each developmental process. The mutations examined displayed qualitative as well as quantitative phenotypic differences in the affected tissues. From this work, we concluded that *torpedo* encodes several differentially, but not independently, mutable activities. On the basis of their complementation behavior, we have divided the 24 *torpedo* alleles examined in this study into four functional categories (Table 2). These categories largely correspond to the four phenotypic classes defined in our earlier genetic studies.

The 12 class I lesions appear to disrupt all gene activities in a uniform manner. Alleles of this category range in severity from the partial loss-of-function, adult viable mutation top^{1} to the complete loss-of-function, embryonic lethal allele top^{1K35}. All homozygous lethal class I mutations enhance the female sterile and adult morphological defects of top^{1} and rarely survive in combination with the semiviable allele top^{CA}.

The class II mutations, in contrast, are embryonic lethal lesions that fully or partially complement the developmental defects of top^{1} and top^{CA}. These four alleles also substantially complement the postembryonic lethality of the pupal lethal lesion top^{EC20}. The class IIA alleles top^{101}, top^{2C824A} and top^{2L65} more fully complement top^{1}, top^{CA} and top^{EC20} than does the class IIB allele top^{2X51}, which was previously designated a class IV mutation.

The complementation behavior of the class III and IV *torpedo* mutations is consistent with their differentially affecting a subset of tissue-specific gene functions. The class III lesions top^{SH2} and top^{38} enhance the oogenesis defects of viable alleles to a lesser extent than do class I mutations of equivalent severity. The six class IV mutations show more severe or less severe adult morphological defects in combination with top^{1} than do class I alleles exhibiting an equivalent homozygous phenotype. In *trans* to a viable allele, top^{EA} and top^{EB} show unusually severe bristle defects, top^{4A} unexpectedly severe wing vein abnormalities, top^{CA} and top^{EE38} unusually weak eye defects and top^{ED16} abnormally severe eye defects.

Further genetic analysis has lead us to reclassify a second *torpedo* mutation examined in this study. The class I allele top^{JE1} was originally placed in class IV because it exhibits mild wing vein defects in *trans* to viable alleles. We now have evidence that the weak wing venation ab-



Allele	Genomic Regions Examined By DGGE					
	A	B	C	D	E	F
1	+	+	+	+	+	+
SH2	-	+	+	+	+	-
JE1	+	+	+	+	+	+
CA	-	+	+	+	+	+
CJ	+	+	+	+	+	+
EA	+	+	-	+	+	-
EB	+	+	-	+	-	-
EC20	+	+	+	+	+	+
ED16	+	+	-	+	+	-
ED26	+	+	-	+	+	-
EE38	+	+	+	+	+	+
EE39	+	+	+	+	+	+
38	+	+	+	+	+	+
101	+	+	-	+	+	-
4A	+	+	-	+	+	-
1F26	-	+	-	+	+	-
1K35	-	+	-	+	+	-
1P02	-	+	-	+	+	-
2C82	-	+	-	+	+	-
2G31	-	+	-	+	+	-
2L65	-	+	-	+	+	-
2W74	-	+	-	+	+	-
2X51	-	+	+	+	+	+
3B92	-	+	+	+	+	+

FIGURE 3.—Strategy for detecting *torpedo* mutations by DGGE. A schematic diagram of the *torpedo* gene is shown above the table. Exons are represented as boxes; nontranscribed sequences and introns are represented as lines. Black boxes represent signal sequences and the transmembrane domain; crosshatched boxes represent cysteine-rich repeats in the extracellular domain; a shaded box represents the tyrosine kinase domain. Diagonally hatched boxes represent 5′- and 3′-untranslated regions. The lettered boxes below the diagram of the locus indicate regions of the gene examined by DGGE. +, fragment was examined; −, fragment was not examined.

normalities observed for this alleles result from dominant suppression by the *plexus* mutation present on the mutant chromosome rather than an intrinsic property of the top^{JE1} allele itself (see MATERIALS AND METHODS).

Interallelic complementation: We have extended our previous genetic analysis of *torpedo* by performing pairwise complementation tests between 20 of the alleles molecularly characterized in this study. Examples of positive complementation, in which the phenotype of the *trans*-heterozygote is less severe than that of either homozygote, and instances of weak negative complementation, in which the heterozygous phenotype is more severe than anticipated, were seen (Figure 4). Some of these genetic interactions were also described by RAZ *et al.* (1991). The observed pattern of interallelic complementation argues that the physical association of receptors is essential for signal transduction by *torpedo* (see DISCUSSION).

Most instances of positive complementation involve four embryonic lethal mutations: the class IIA alleles top^{101}, top^{2C82} and top^{2L65}, and the class IIB allele top^{2X51} (Figure 4). All four mutations survive in *trans* to the postembryonic class I lethal allele top^{EC20}–10 to 50% of the *trans*-heterozygotes survive to adulthood, while the remainder die as pupae. The class IIA alleles are semiviable in combination with the class IV pupal lethal mutations top^{EB} and top^{ED16}. Class IIA mutations, in addition, partially suppress the embryonic lethal phenotypes of the class I alleles top^{1F26}, top^{2W74}, top^{3B92} and top^{JE1}. For example, while head morphogenesis and/or germband retraction abnormalities are seen in roughly 80% of top^{1F26} homozygotes and 60% of top^{2W74} homozygotes, we observe these defects in less than 5% and 30% of top^{1F26}/top^{2L65} and top^{2W74}/top^{2L65} heterozygotes, respectively (Figure 4; data not shown). Likewise, more than 90% of animals homozygous for top^{2L65}, top^{3B92} or top^{JE1} show a severe embryonic lethal phenotype, but more than 80% of top^{2L65}/top^{3B92} and top^{2L65}/top^{3B92} *trans*-heterozygotes display a significantly milder phenotype (Figures 4 and 5, E–G; data not shown). Positive complementation between top^{2W74} and the class IIA alleles top^{2C82} and top^{2L65} was also seen by RAZ *et al.* (1991). Further, all three Class IIA lesions partially complement the severe class IIB mutation top^{2X51} (Figure 4). The moderate Class IIA mutation top^{2C82} and the severe class IIA allele top^{2L65} show weak embryonic lethal phenotypes in *trans* to top^{2X51} (see also RAZ *et al.* 1991). Some top^{101}/top^{2X51} heterozygotes even survive to adulthood.

TABLE 2

Molecular alterations associated with *torpedo* mutations

Allele	Homozygous phenotype[a]	Genomic position	Nucleotide alteration	Amino acid alteration	Mutagen	Proposed structure[b]
Class I						
1	V	406	TCT to TTT	Ser^{58} to Phe	EMS	lig^- kin^+ ter^+
CJ	V	406	TCT to TTT	Ser^{58} to Phe	EMS	lig^- kin^+ ter^+
JE1	S	824	TCC to TTC	Ser^{152} to Phe	EMS	lig^- kin^+ ter^+
2G31	M/S	926	TGG to TAG	Trp^{186} to Amber	EMS	lig^0 kin^0 ter^0
1K35	S	1168	CAA to TAA	Gln^{267} to Ochre	EMS	lig^0 kin^0 ter^0
3B92	S	1318	TGT to AGT	Cys^{317} to Ser	EMS	lig^- kin^+ ter^+
EC20	P	1513	GAT to AAT	Asp^{382} to Asn	EMS	lig^- kin^+ ter^+
ED26	S	1755–1763	ΔCTGTACAGC	ΔLeu^{463} to Ser^{465}	EMS	lig^- kin^+ ter^+
EE39	M/S	1864	CAG to TAG	Gln^{499} to Amber	EMS	lig^0 kin^0 ter^0
1P02	S	3731	TGG to TGA	Trp^{1075} to Opal	EMS	lig^+ kin^0 ter^0
2W74	W^{ts}	3763	ACC to ATC	Thr^{1086} to Ile	EMS	lig^+ kin^- ter^+
1F26	W^{ts}	3844	CCG to CTG	Pro^{1113} to Leu	EMS	lig^+ kin^- ter^+
Class IIA						
101	W	3666	CCC to TCC	Pro^{1054} to Ser	EMS	lig^+ kin^- ter^+
2C82	M	3825	GGT to AGT	Gly^{1107} to Ser	EMS	lig^+ kin^- ter^+
2L65	S	3856	TCG to TTG	Ser^{1117} to Leu	EMS	lig^+ kin^- ter^+
Class IIB						
2X51	S	3989	GAT to GAGA	+1 frameshift	EMS	lig^+ kin^+ ter^0
Class III						
SH2	W^{ts}	839	CTG to CAG	Leu^{157} to Gln	ENU	lig^- kin^+ ter^+
38	M/S	1657	CAG to TAG	Gln^{430} to Amber	EMS	lig^0 kin^0 ter^0
Class IV						
EB	P	931	GAG to AAG	Glu^{188} to Lys	EMS	lig^- kin^+ ter^+
EE38	V	1099	CGT to TGT	Arg^{244} to Cys	EMS	lig^- kin^+ ter^+
CA	V	1336	CGG to TGG	Arg^{323} to Trp	EMS	lig^- kin^+ ter^+
ED16	P	2039	GGA to AGA	Gly^{535} to Arg	EMS	lig^- kin^+ ter^+
4A	W	2469	TGC to AGC	Cys^{662} to Ser	γ-Ray	lig^- kin^+ ter^+
EA	P/W	2611	GGA to GAA	Gly^{702} to Glu	EMS	lig^- kin^+ ter^+

[a] Phenotypes at room temperature. V, adult viable; P, pupal lethal; W, weak embryonic lethal; M, moderate embryonic lethal; S, severe embryonic lethal; ts, temperature-sensitive allele.

[b] Abbreviations: lig, extracellular domain; kin, tyrosine kinase domain; ter, carboxyl-terminal domain; +, wild type; −, mutant; 0, deleted.

Striking positive complementation is observed between certain *trans*-heterozygous combinations of class IIA mutations. The weak embryonic lethal mutation top^{101} fully complements the moderate embryonic lethal mutation top^{2C82} and the severe embryonic lethal mutation top^{2L65}: top^{101}/top^{2C82} and top^{101}/top^{2L65} animals are completely viable, morphologically normal and fertile (Figure 4, data not shown).

Certain class I and class IV postembryonic lethal alleles also exhibit positive genetic interactions with one another. The degree of complementation between these mutations, however, is less complete than that occurring between class IIA mutations. For example, top^{EC20}/top^{ED16} *trans*-heterozygotes usually survive to adulthood, but exhibit eye, wing and bristle abnormalities (Figure 4, data not shown).

We also observe examples of negative complementation. The pupal lethal class IV alleles top^{EB} and top^{EA}, for example, survive in combination with the class IV mutation top^{CA} less frequently than do the class I embryonic lethal alleles top^{1F26} and top^{2W74} (Figure 4; CLIFFORD and SCHÜPBACH 1989). The class IV mutation top^{4A} appears to show weak negative complementation in combination with a variety of alleles (Figure 4). In their genetic analysis, RAZ *et al.* (1991) observed negative complementation between the moderate embryonic lethal mutation flb^{JE3} (not examined in this study) and top^{1P02}, top^{3B41}, top^{3C87} and top^{JE1}.

We also observe differences in the complementation behavior of alleles showing similar homozygous phenotypes. The top^{4A}, top^{1F26} and top^{2W74} show similar weak embryonic lethal phenotypes at room temperature. Yet, in combination with top^{2L65}, top^{2X51} or top^{JE1}, the phenotype of top^{4A} is enhanced so that roughly 25% of the heterozygotes show a moderate or severe embryonic lethal phenotype. In contrast, fewer than 5% of animals heterozygous for top^{1F26} or top^{2W74} and top^{2L65}, top^{2X51} or top^{JE1} show these phenotypes. In fact, the embryonic lethal phenotypes of top^{1F26} and top^{2W74} are weakly *suppressed* by top^{2L65} (Figure 4, data not shown).

Class IIA alleles do not disrupt tissue-specific gene functions: Since the class IIA mutations fully complement the pupal lethality of top^{CA} and top^{EC20}, we proposed that they might preferentially disrupt a function of the *torpedo* gene required specifically for embryogenesis (CLIFFORD and SCHÜPBACH 1989). Under this hypothesis, the viability of animals *trans*-heterozygous for a class IIA allele and a pupal lethal mutation would result from the pupal lethal allele supplying normal gene function during embryogenesis and the class IIA allele supplying gene function during postembryonic development. If this were the case, we would expect class IIA lesions to

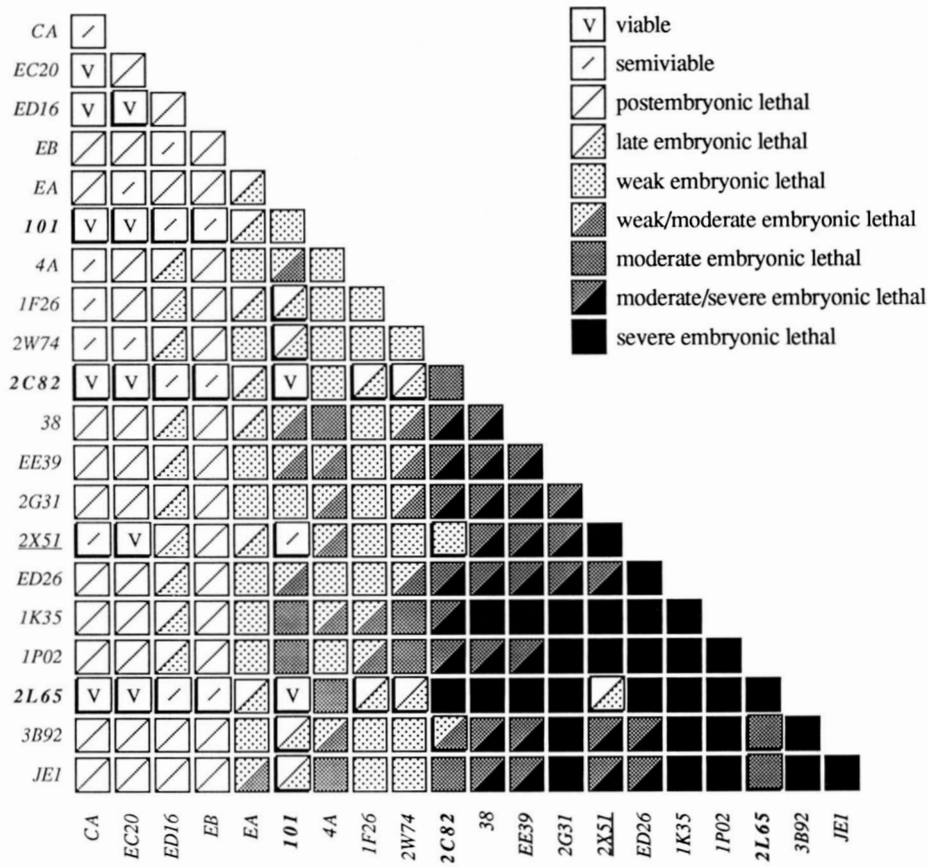


FIGURE 4.—Interallelic complementation between *torpedo* alleles. See MATERIALS AND METHODS for details of the crosses. Homozygous mutant phenotypes are indicated along the diagonal. Animals of viable mutant genotypes show at least 50% survival to adulthood relative to their $top^{x}/+$ sibs. Animals of semiviable mutant genotypes exhibit 10–50% survival to adulthood, with the balance dying during pupal development. Embryonic lethal phenotypes are shown in Figure 5. Examples positive complementation between *torpedo* alleles are highlighted. The names of the class IIA alleles are boldfaced and that of the class IIB allele is underlined.

complement the postembryonic lethality of all pupal lethal *torpedo* alleles and to show mild adult morphological abnormalities in combination with these mutations. Further, if the class IIA alleles primarily affect receptor function in the embryo, cells homozygous for these alleles should develop more-or-less normally as long as homozygosity is induced after embryogenesis.

To address this possibility we examined the phenotypes of the class IIA alleles top^{101}, top^{2C82} and top^{2L65} in combination with the pupal lethal alleles top^{EB} and top^{ED16}, which are more severe loss-of-function mutations than either top^{CA} or top^{EC20}. All three alleles show reduced viability in *trans* to top^{EB} and top^{ED16} (Figure 4); furthermore, heterozygous animals that survive to adulthood exhibit severe eye, wing and bristle defects (data not shown). These results argue that top^{101}, top^{2C82} and top^{2L65} disrupt the development of imaginal tissue.

In addition, we have shown that the class IIA mutations top^{2C82} and top^{2L65} strongly disrupt the development of imaginal tissues using genetic mosaic techniques (see MATERIALS AND METHODS). Clones of cells homozygous for top^{2C82} and top^{2L65} were recovered in the compound eye at a lower frequency than wild-type or top^{EC20} clones. Also, the eyes of some irradiated top^{2C82} and top^{2L65} heterozygous flies contained small scars similar to those seen in irradiated animals heterozygous for the amorphic class I allele top^{CO}. These scars are likely to result from the abnormal development of cells in a mutant clone (Table 3, data not shown). Likewise, top^{2C82} and top^{2L65} clones in the leg–like top^{CO} clones–were recovered less frequently than genetically wild-type clones (Table 3). Mutant leg clones were also smaller than wild type. While most wild-type clones in the leg consisted of at least 50 bristles, top^{2C82} and top^{2L65} mosaic legs contained fewer than 10 marked bristles (data not shown). Mosaic analysis therefore indicates that top^{2C82} and top^{2L65} impair the development of imaginal disc cells and behave as severe loss-of-function alleles in the imaginal discs.

These experiments show that the class IIA alleles top^{101}, top^{2C82} and top^{2L65} do not preferentially disrupt embryogenesis. Instead, the receptors encoded by these alleles are not fully functional in any of the various tissues examined. Therefore, the extensive complementation of pupal lethal alleles displayed by these class IIA mutations may result from their products interacting with other mutant receptors to transduce signals. Complementation between class IIA and pupal lethal alleles appears to occur in embryonic as well as in imaginal tissues. top^{ED16}, a pupal lethal allele complemented by the class IIA mutations, must be defective for a gene function required for embryonic development, since animals *trans*-heterozygous for top^{ED16} and any of a number of embryonic lethal mutations die during embryogenesis (Figure 4).

Sequence analysis of *torpedo* alleles: Genetic analysis reveals qualitative differences between *torpedo* alleles

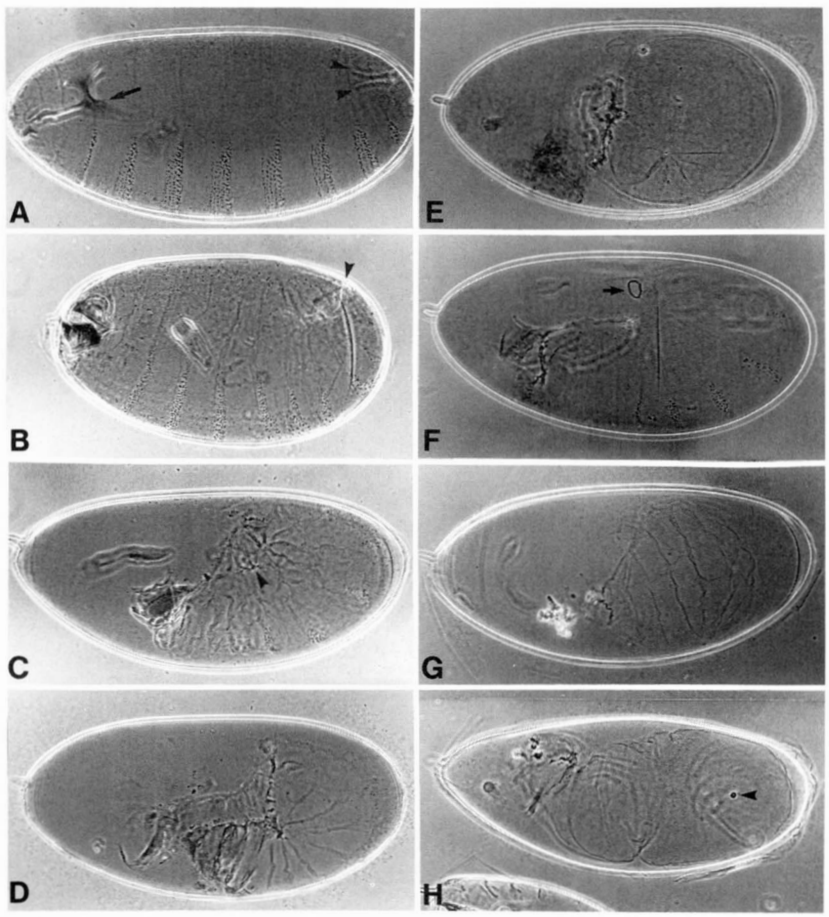


FIGURE 5.—Cuticular phenotypes of embryonic lethal *torpedo* mutants. Phase contrast photomicrographs of embryonic cuticles fixed inside the vitelline membrane. Anterior is to the left; dorsal is up. (A) Wild-type embryonic cuticular pattern. The head skeleton (arrow) and filzkorper (arrowheads) are below the plane of focus. Rows of denticles lie on the ventral surface of the embryo. (B) top^{2W74}/top^{2W74} animal showing a weak embryonic lethal phenotype. The head skeleton is collapsed, and the denticles are reduced in size. Because the animal has not fully retracted its germband, the filzkorper (arrowheads) are located more dorsally and anteriorly than in wild type. (C) top^{101}/top^{1K35} *trans*-heterozygote showing a moderate embryonic lethal phenotype. Most anterior cuticle and head skeleton structures are missing. The embryo has not undergone germband shortening; the posterior end of the animal, with rudimentary filzkorper (arrowheads), lies behind the head. The number and size of the denticles are reduced. (D) top^{JE1}/top^{JE1} animal exhibiting a severe embryonic lethal phenotype. Cephalic structures are absent. Germband shortening has not occurred. The animal lacks denticles and most ventral cuticle. (E) top^{2L65} homozygote showing a severe embryonic lethal phenotype. (F) top^{2L65}/top^{3B92} *trans*-heterozygote. This animal exhibits a weak to moderate embryonic lethal phenotype characterized by the production of denticle-bearing ventral cuticle and partial germband retraction (necrotic tissue surrounding a hole in the posterior cuticle is marked by an arrow). (G) top^{3B92} homozygote showing a severe embryonic lethal phenotype. (H) top^{38} homozygote showing a weak embryonic lethal phenotype. This animal suffers head skeleton defects and lacks denticles, but secretes considerably more cuticle and undergoes more complete germband retraction than an animal showing a severe embryonic lethal phenotype. An arrowhead marks a spot of filzkorper material.

and a complex pattern of interallelic complementation. To gain insight into the mechanistic basis of *torpedo*'s genetic complexity we have performed a molecular analysis of mutant alleles. Nucleotide alterations associated with 24 loss-of-function mutations in the *torpedo* gene, which represent each of the four phenotypic classes, have been identified (see MATERIALS AND METHODS). Five mutations described below, top^{1F26}, top^{2C82}, top^{2L65}, top^{2W74} and top^{2X51}, were also characterized by RAZ *et al.* (1991).

Nonsense mutations: Five alleles characterized in this study are nonsense mutations (Table 2, Figure 6). The predicted proteins encoded by top^{1K35}, top^{2G31}, top^{38} and top^{EE39} terminate within the extracellular domain of the receptor; these putative secreted molecules lack the bulk of the ligand binding domain, as well as the entire transmembrane, tyrosine kinase and carboxyl-terminal domains. The top^{1P02} product, on the other hand, is truncated within the tyrosine kinase domain of the receptor, resulting in the deletion of kinase subdomains X and XI of HANKS *et al.* (1988) and the carboxyl-terminal tail of the protein.

While most embryos homozygous for each of the nonsense mutations show a severe zygotic lethal phenotype, a fraction of top^{1P02}, top^{2G31} and top^{EE39} animals–10, 15 and 11%, respectively–exhibit a somewhat weaker

TABLE 3

Recovery of *top*^{2C82} and *top*^{2L65} clones in the eye and leg

Genotype	Eyes	Clones	Scars	Frequency of eye clones	Frequency of eye scars	Legs	Clones	Frequency of leg clones
stw pwn cn bw sp	116	12	0	0.103	0.000	120	14	0.117
stw pwn cn top^{2C82} *bw sp*	94	1	4	0.011	0.042	118	1	0.008
stw pwn cn top^{2L65} *bw sp*	204	3	2	0.015	0.010	119	1	0.008
stw pwn cn bw	408	23	0	0.056	0.000	468	32	0.068
stw pwn cn top^{EC20} *bw*	356	15	1	0.042	0.003	479	33	0.069
stw pwn cn top^{CO} *bw*	374	0	10	0.000	0.027	713	8	0.011

terminal phenotype (Table 4). However, even embryos homozygous for the deletion allele *top*^{18A} occasionally exhibit a moderate lethal phenotype (Table 4). It therefore seems likely that genetic background affects the final cuticle morphology of the mutant embryo. It is also possible that the *top*^{1P02}, *top*^{2G31} and *top*^{EE39} nonsense mutations are leaky.

Genetic background variation does not appear to be sufficient to explain the phenotype of the nonsense mutation *top*^{38}. Animals homozygous for this exceptional allele are 2-fold more likely than embryos homozygous for any other nonsense mutation, and 10-fold more likely than null embryos, to exhibit a moderate phenotype. Ten percent of *top*^{38}/*top*^{38} embryos, in fact, exhibit an embryonic lethal phenotype characterized by the production of essentially wild-type amounts of ventral cuticle (Table 4, Figure 5H). The partial loss-of-function character of *top*^{38} may be explained in several ways. One possibility is suppression by a second-site lesion that is linked to *top*^{38}. Alternatively, the hypomorphic behavior of *top*^{38} could result from readthrough of its amber codon or from translational reinitiation at a downstream methionine codon.

Deletion mutation: Nine contiguous nucleotides are deleted from the *top*^{ED26} chromosome, resulting in the loss of three amino acids located in subdomain III of the ligand binding domain of the protein (Table 2, Figure 6). Given the amorphic phenotype of *top*^{ED26} (Table 4), the three residues eliminated by this mutation appear to be essential for receptor function. Biochemical studies of the human EGF receptor implicate subdomain III of the molecule in receptor-ligand interactions (Lax *et al.* 1989, 1991; Wu *et al.* 1990). The portion of the ligand binding domain deleted by the *top*^{ED26} lesion is poorly conserved among receptor tyrosine kinases of the EGF-R subfamily (Figure 7G); such variability might be expected of a region of the molecule that is specialized for interaction with a specific ligand or set of ligands. It is possible, however, that the Top^{ED26} receptor is defective in receptor-receptor interactions or stability.

Frameshift mutation: In the *top*^{2X51} chromosome, a thymidine residue at genomic position 3989 is replaced by guanidine and adenine (Table 2, see also Raz *et al.* 1991). The protein encoded by this frameshift allele lacks most of its carboxyl-terminal tail (Figure 6), which includes tyrosine residues thought to mediate receptor-substrate interactions (reviewed in Schlessinger and Ullrich 1992). The carboxyl terminus of Torpedo is essential for receptor activity, as *top*^{2X51} homozygotes show a severe loss-of-function phenotype (Table 4). Despite this defect, Top^{2X51} does appear to be able to form functional heterodimers with other mutant receptors (Figure 4). For a discussion of this phenomenon, see Raz *et al.* (1991).

Missense mutations: Nucleotide alterations associated with 17 *torpedo* mutations lead to amino acid substitutions. Three of these amino acid replacements severely disrupt receptor activity, while the remainder result in a partial reduction of protein function.

Extracellular domain mutants: Twelve missense mutations alter residues lying within the ligand binding domain of the Torpedo protein. Five of these affect amino acids located in the relatively cysteine-poor subdomains I and III, which, in the case of the human EGF receptor, contain determinants of ligand binding (Lax *et al.* 1989, 1991). The viable *top*^{1} and *top*^{CJ} chromosomes, which were isolated independently but show similar phenotypes, contain the same amino acid alteration. The two alleles were induced on different parental chromosomes that can be distinguished by their characteristic sequence polymorphisms (Figure 8). In both cases, a non-conserved serine residue in subdomain I is changed to phenylalanine (Table 2, Figures 6, 7A and 8). The nucleotide alteration associated with the severe lesion *top*^{JE1} results in a Ser to Phe substitution in a highly variable region of extracellular subdomain I (Table 2, Figures 6 and 7B). Chemical cross-linking studies indicate that the homologous region of HER is in close proximity to the ligand (Woltjer *et al.* 1992). The T to A substitution associated with the weak embryonic lethal allele *top*^{SH2} leads to the replacement of a Leu residue five amino acids carboxyl-terminal to the serine affected by the *top*^{JE1} mutation with Gln. This portion of the molecule may form an amphipathic α-helix, as all receptors of the subfamily show a conserved spacing of hydrophobic residues separated by hydrophilic amino acids (Ala/Val/Met-*X*-*X*-Leu/Val-*X*-Leu/Met/Ile-*X*-*X*-Leu-*X*-*X*-Ile/Val). The second hydrophobic residue of this sequence is altered in the Top^{SH2} protein (Table 2, Figures 6 and 7B).

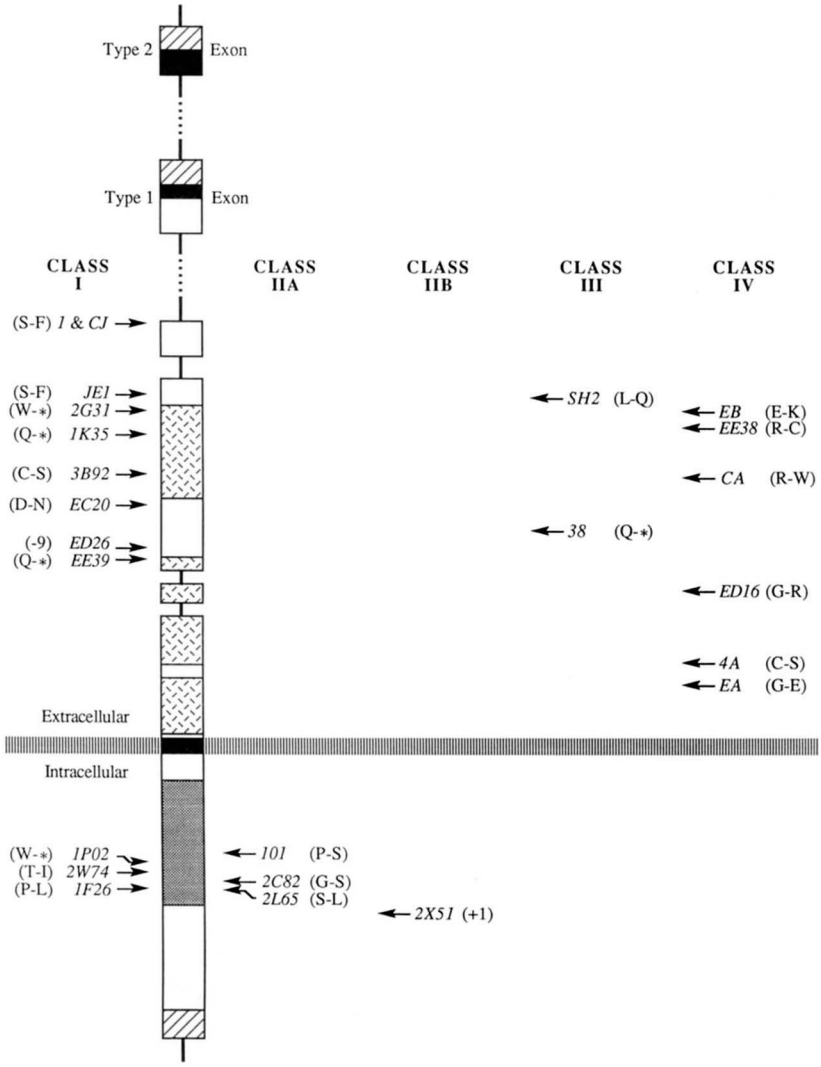


FIGURE 6.—Distribution of *torpedo* alleles by phenotypic class. Schematic diagram of the *torpedo* gene flanked by alleles from the five phenotypic categories. Exons are represented as boxes; nontranscribed sequences and introns are represented as lines. Signal sequences and the transmembrane domain are black boxes; cysteine-rich repeats in the extracellular domain are crosshatched boxes; the tyrosine kinase domain is a shaded box. The 5′- and 3′-untranslated regions are are represented by diagonally hatched boxes.

TABLE 4

Phenotypic distributions of select null, nonsense and missense alleles

Allele	Total embryos	Hatched embryos	Unhatched embryos[a]						Percent mutant	Moderate + weak / Total mutant
			Severe	Moderate	Weak	Normal	Other	Lost		
18A[b]	467	214	123	2	0	113	13	2	26.8	0.016
38[b]	331	150	51	19	10	85	3	13	24.2	**0.363**
ED26[b]	595	403	151	9	0	18	12	2	26.9	0.056
EE39[b]	446	293	89	8	3	39	7	7	22.4	0.110
1K35[c]	574	408	134	0	0	25	4	3	23.3	0.000
1P02[c]	679	451	151	15	1	43	13	3	24.6	0.096
2G31[c]	353	216	78	13	1	39	2	4	26.0	0.152
2L65[c]	651	211	147	11	2	212	34	34	24.6	0.081
2X51[c]	568	378	107	5	0	63	15	0	19.7	0.045
3B92[c]	665	442	179	5	0	12	22	5	27.6	0.027
JE1[d]	523	344	126	3	2	23	22	3	25.0	0.038

[a] The severe, moderate and weak phenotypes are shown in Figure 5.
[b] Induced on a *b pr cn sca* chromosome.
[c] Induced on a *cn bw sp* chromosome.
[d] Induced on a *dp cn a px sp* chromosome.

The nucleotide alteration associated with the partial loss-of-function, pupal lethal mutation top^{EC20} produces an Asp to Asn substitution in subdomain III of the ligand binding domain (Table 2, Figures 6 and 7F). Although the affected residue is not conserved among members of the EGF-R subfamily, most proteins in the subfamily do possess at least one negatively charged amino acid between the conserved Gly and Phe residues (Figure 7F).

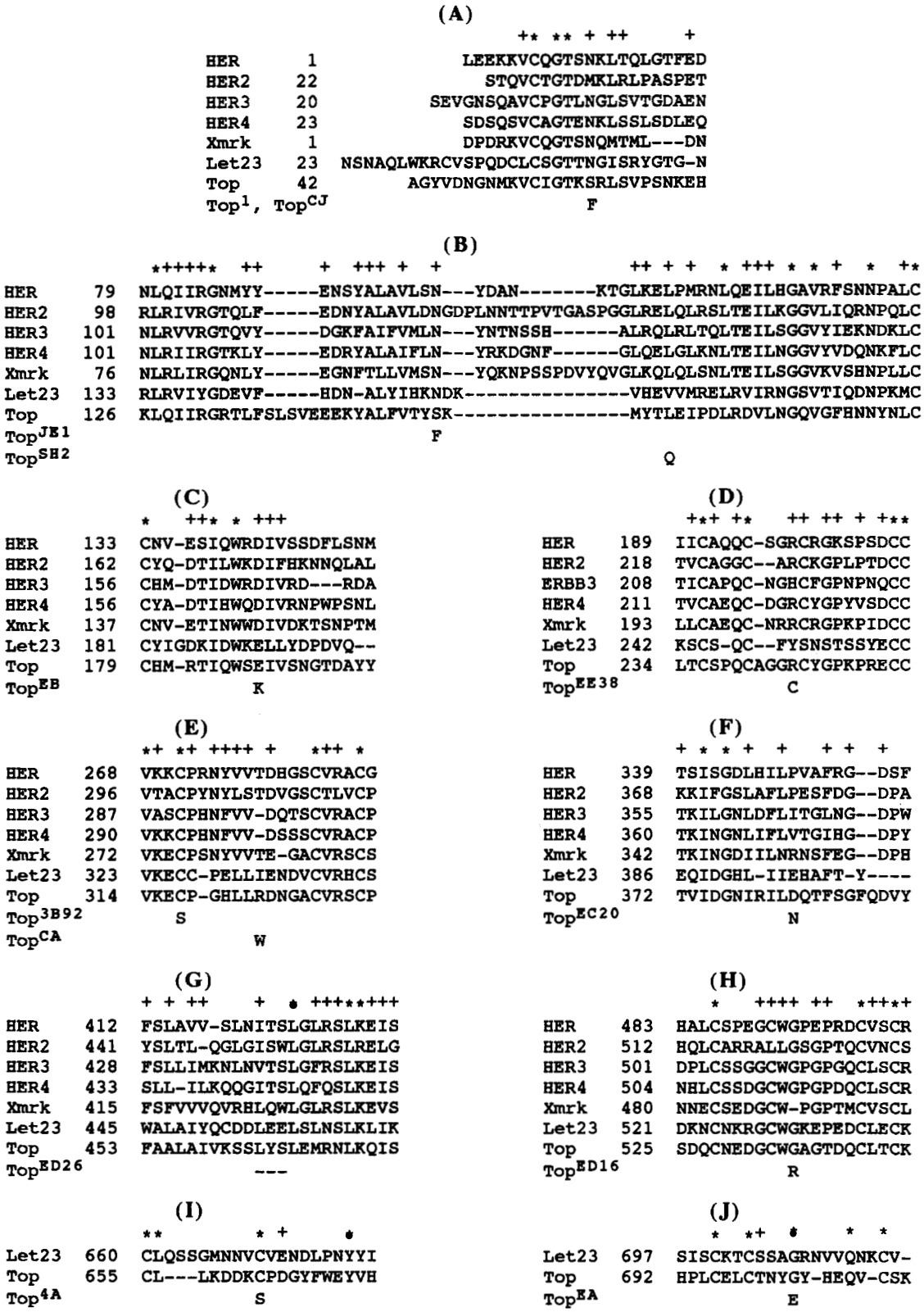


FIGURE 7.—Conservation of extracellular domain amino acids affected by missense and deletion mutations. Aligned protein sequences for the human epidermal growth factor receptor (HER) (ULLRICH *et al.* 1984), $p185^{erbB2}$ (HER2) (COUSSENS *et al.* 1985), $p160^{erbB3}$ (HER3) (KRAUS *et al.* 1989) and $p180^{erbB4}$ (HER4) (PLOWMAN *et al.* 1993) receptor tyrosine kinases, the melanoma-associated receptor tyrosine kinase of Xiphophorus (Xmrk) (WITTBRODT *et al.* 1989), the *let-23* receptor tyrosine kinase of *Caenorhabditis elegans* (Let23) (AROIAN *et al.* 1990) and the *torpedo* receptor tyrosine kinase of *D. melanogaster* (Top) (LIVNEH *et al.* 1985; this work). *, invariant residue; +, amino acid conserved among at least five proteins. Conservative amino acids: D/E; F/Y; I/L/M/V; H/K/R; N/Q; S/T. Torpedo residues altered by missense and deletion lesions are shown below the aligned sequences.

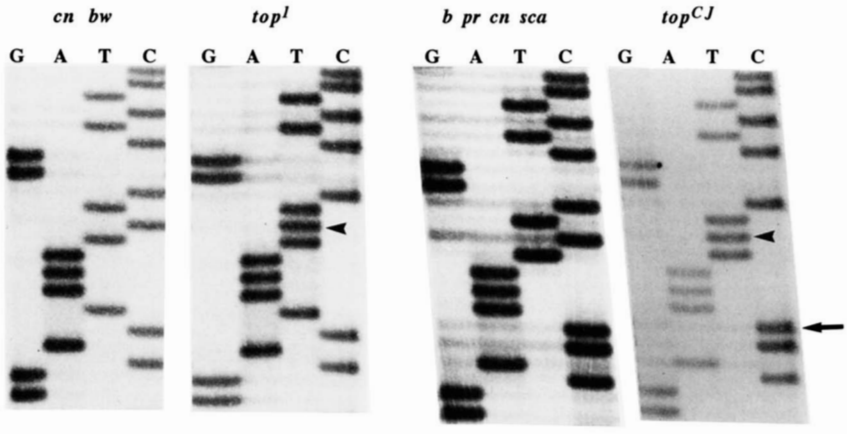


FIGURE 8.—Nucleotide alterations of top^{1} and top^{CJ}. Autoradiographs of sequencing gels showing the nucleotide sequences of top^{1} and top^{CJ} and the wild-type chromosomes, *cn bw* and *b pr cn sca*, on which they were induced. The identical C to T transition at nucleotide 406 of both mutant *torpedo* alleles is indicated by an arrowhead. The silent DNA polymorphism at nucleotide 401 that distinguishes the *cn bw* and *b pr cn sca* chromosomes is indicated by an arrow.

Seven mutations affect residues within the cysteine-rich repeats of the protein. In the human EGF receptor, these subdomains (II and IV) appear to serve as structural elements of the ligand binding domain (LAX *et al.* 1989, 1991). The two most severe mutations in this group change cysteine residues to serine. The product of top^{3B92}, a severe embryonic lethal allele, lacks a cysteine residue in subdomain II; the receptor encoded by top^{4A}, a weak embryonic lethal allele, lacks a cysteine located in subdomain IVB of the ligand binding domain (Table 2, Figures 6 and 7, E and I). Nucleotide alterations associated with the weakest mutations in this group, top^{EE38} (viable), top^{CA} (semiviable), top^{EA} (late embryonic lethal), top^{EB} (pupal lethal) and top^{ED16} (pupal lethal), alter non-cysteine residues in subdomains II and IV. In the Top^{CA} and Top^{EB} proteins, charged residues are changed to hydrophobic or oppositely charged amino acids (Table 2, Figures 6 and 7, C and E); in Top^{EE38}, a charged residue is replaced by cysteine (Table 2, Figures 6 and 7D); in Top^{EA} and Top^{ED16}, charged amino acids substitute for glycines (Table 2, Figures 6 and 7, H and J). Charged residues, which can form salt bridges, and glycines, which provide flexibility to the polypeptide chain, may play important roles in maintaining protein structure. The relative severity of mutations in the cysteine-rich motifs is consistent with the idea that disulfide bonding plays a central role, and ionic interactions a more peripheral role, in maintaining the structure of the extracellular domain.

Tyrosine kinase domain mutants: Nucleotide changes associated with five missense mutations lead to the alteration of residues in the kinase domain of Torpedo. The recent determination of the crystal structure of the catalytic subunit of murine cAMP-dependent protein kinase has revealed that the catalytic core domain of a protein kinase is composed of two lobes. Amino-terminal residues of the kinase domain form the smaller lobe, which is comprised of two β-sheets and two α-helixes; carboxyl-terminal residues of the domain form the larger lobe, which contains two β-sheets and seven α helixes (KNIGHTON *et al.* 1991). All of the kinase domain *torpedo* mutations affect residues located within the large lobe of the domain, between β-strand 9 and α-helix H (Figure 9), a portion of the kinase domain that contacts substrate molecules.

The nucleotide alteration associated with the weak embryonic lethal allele top^{101} mutation produces a conservative Pro to Ser change in the loop connecting β-strand 9 to α-helix F of the kinase domain (Table 2, Figures 6 and 9). This amino acid lies in a portion of the tyrosine kinase domain proposed to act as a determinant of protein kinase specificity. In this region of the kinase domain,the consensus sequence for protein-tyrosine kinases is *X*-Pro-Ile/Val-Lys/Arg-Trp-Thr/Met-Ala-Pro-Glu, while that for serine/threonine kinases is Gly-Thr/Ser-*X*-*X*-Tyr/Phe-*X*-Ala-Pro-Glu (HANKS *et al.* 1988). The Let-23 protein-tyrosine kinase, however, possesses Ala, rather than Pro, at this position (AROIAN *et al.* 1990).

In the Top^{2W74} protein, Ile replaces Thr^{1086}, a highly conserved residue situated at the carboxyl-terminal end of helix F (Table 2, Figures 6 and 9). This amino acid change generates a contiguous stretch of four hydrophobic residues with fairly extensive side chains (Leu-Leu-Ile-Phe); such a sequence may not assume a stable α-helical conformation.

The remaining three kinase domain mutants map to the loop connecting helix G and helix H. In the product of the moderate embryonic lethal mutation top^{2C82}, the Gly residue just carboxyl-terminal to helix G is changed to Ser. In the Top^{1F26} receptor, $proline^{1113}$, a residue in the loop connecting helixes G and H, is replaced by the nonconservative amino acid leucine. The product of the severe zygotic embryonic lethal allele top^{2L65} suffers a Ser to Leu substitution at the residue abutting, or within, the amino terminus of helix H (Table 2, Figs. 6 and 9). The serine altered in Top^{2L65} is less highly conserved than the residues affected by other kinase domain *torpedo* mutations, as alanine, proline, serine and threonine can be found at this position in tyrosine kinases (HANKS *et al.* 1988).

Temperature-sensitive mutations: Three *torpedo* missense mutations, top^{SH2}, top^{1F26} and top^{2W74}, are temperature-sensitive. Animals homozygous for these alleles show a severe embryonic lethal phenotype at 29° and a

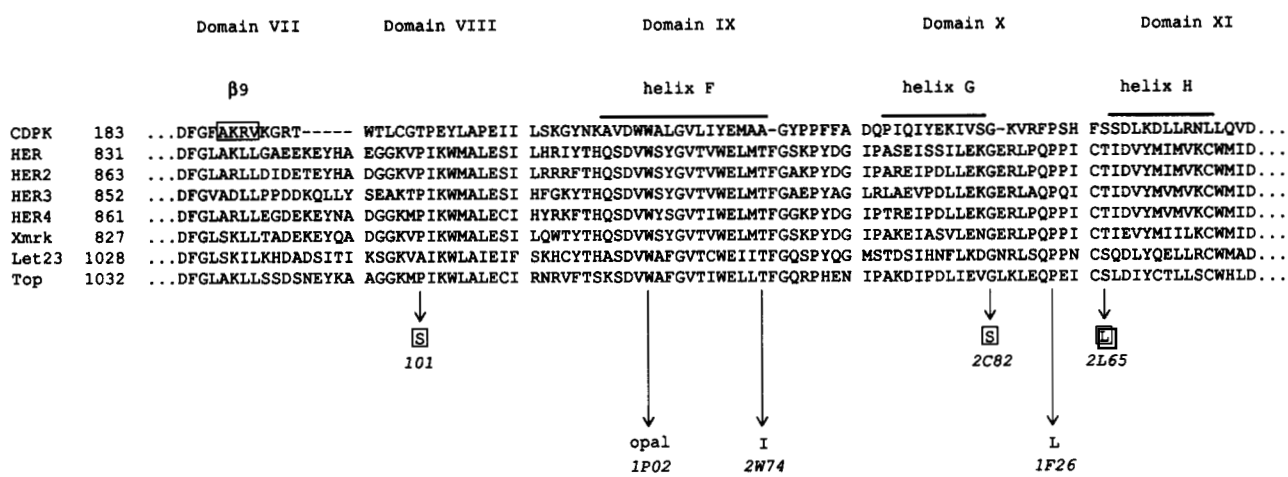


FIGURE 9.—Conservation of kinase domain amino acids affected by missense and nonsense mutations. Aligned protein sequences for the human, Xiphophorus, Caenorhabditis and Drosophila members of the EGF receptor subfamily of tyrosine kinases. See Figure 7 for references. The domains of HANKS *et al.* (1988), as well as structural features determined from the crystal structure of the murine cAMP-dependent protein kinase (CDPK) (KNIGHTON *et al.* 1991), are indicated. Helix, α-helix; β, β-sheet. Torpedo residues altered by missense and nonsense mutations are shown below the aligned sequences. Class I mutations are in plain text; class II mutations are boxed.

weak embryonic lethal phenotype at 18° (NÜSSLEIN-VOLHARD *et al.* 1984; CLIFFORD and SCHÜPBACH 1989, 1992; RAZ and SHILO 1992).

The nucleotide change associated with the top^{SH2} mutation alters a leucine in subdomain I of the ligand binding domain to the polar residue glutamine. The affected amino acid may lie within an amphipathic α-helix (see above).

In the product of the top^{2W74} allele, a polar amino acid in helix F of the kinase domain is replaced by a hydrophobic residue. The conditional allele sev^{AE2} contains a homologous alteration which changes the peptide sequence Ile-Leu-Thr-Leu to Ile-Leu-Ile-Leu; MULLINS and RUBIN (1991) provide evidence that this mutant Sevenless protein is temperature-sensitive for activity, rather than synthesis. This alteration could produce a temperature-sensitive product in a number of ways. Perhaps the bulky, relatively inflexible side chain of isoleucine produces steric hindrance resulting in the destabilization of the α-helix. This instability might worsen with increasing temperature. Another possibility is that the isoleucine residue participates in hydrophobic interactions with neighboring nonpolar amino acids. The strength of these novel interactions may be greater at higher temperatures.

The nucleotide alteration in the top^{1F26} chromosome leads to the substitution of Leu for Pro in the random coil linking helixes G and H of the tyrosine kinase domain. There appears to be a strict requirement for a proline within this portion of the kinase domain, as every protein kinase in the database of HANKS *et al.* (1988) possesses at least one proline within two residues of Pro^{1113}. Loss of this amino acid could increase the flexibility of the loop, a defect that might become more extreme at higher temperatures.

Correlation between amino acid changes and mutant phenotypes: We observe a striking nonrandom distribution of lesions within the gene. Mutations producing a general disruption of gene functions (class I) are scattered throughout the coding region of *torpedo*, while those affecting a subset of gene functions (classes II, III and IV) are clustered within specific functional domains of the Torpedo protein (Figure 6).

Class I mutations appear to reduce the activity of the Torpedo protein in all tissues. A variety of biochemical defects (ligand binding, receptor dimerization, tyrosine kinase activity, substrate interaction) could lead to a general disruption of receptor activity. Likewise, altering residues at many locations in the protein could lead to defects in stability, post-translational processing or transport to the membrane. Consistent with this expectation, we find that some class I lesions alter amino acids located in the extracellular domains while others affect amino acids lying in the cytoplasmic domain of Torpedo.

The class IIA mutations analyzed in this study, top^{101}, top^{2C82} and top^{2L65}, may disrupt a subset of receptor activities required in every tissue (see DISCUSSION). The nucleotide changes associated with these alleles alter amino acids lying in the portion of the tyrosine kinase domain that physically interacts with substrates; thus it is possible that the Top^{101}, Top^{2C82} and Top^{2L65} proteins are unable to phosphorylate one or more ubiquitous substrates. Receptor autophosphorylation sites, as well as cellular proteins, could serve as these substrates. The class IIB allele top^{2X51} encodes a receptor lacking a carboxyl-terminal domain. While this molecule should possess kinase activity, it should not be able to physically associate with SH2 domain-containing cellular proteins.

The product of the class III allele top^{SH2} carries an amino acid substitution in subdomain I of the ligand

binding portion of the receptor. Chemical cross-linking studies with the human epidermal growth factor receptor show that bound EGF lies in close physical proximity to this portion of the molecule, which suggests the intriguing possibility that the top^{SH2} missense mutation alters a region of Torpedo involved in ligand binding. The class III mutation top^{38}, on the other hand, is a nonsense mutation located in the extracellular domain of the protein that exhibits a partial loss-of-function phenotype (Table 4). The weak female sterile phenotype of this mutation in *trans* to top^{1} might indicate that the mechanism by which this mutation is suppressed operates more efficiently in the ovary than in other tissues.

All class IV alleles are missense mutations that produce amino acid substitutions in the ligand binding domain of the receptor (Figure 6). top^{CA}, top^{EA}, top^{EB}, top^{ED16} and top^{EE38} differentially affect the development of adult ectodermal structures, the eye, wing veins and bristles. The molecular nature of these mutations suggests the possibility that their products may be preferentially impaired in binding tissue-specific ligands. As top^{4A} shows a slightly antimorphic phenotype in the wing (data not shown), its product may not dimerize properly.

DISCUSSION

Mutations in the *torpedo* gene show a complex pattern of interallelic complementation. Based on genetic studies, we have divided the alleles into four phenotypic classes. Molecular analysis reveals that mutations of certain classes cluster within particular subdomains of the receptor. Class II mutations, embryonic lethal lesions that completely or partially complement a variety of embryonic and pupal lethal *torpedo* alleles, map to the portion of the gene encoding the intracellular domain of the Torpedo protein. The class IV mutations differentially affect the development of specific adult tissues, such as the eye, wing veins or bristles. These tissue-preferential alleles alter single amino acids in the extracellular domain of the receptor. Likewise, the class III mutation top^{SH2}, which differentially affects oogenesis, changes a residue in the extracellular domain of Torpedo.

Do the identified molecular alterations correspond to the mutant lesions?: Given the large coding region of *torpedo* (~4.4 kb), we chose to search for mutant lesions by DGGE rather than by sequencing the entire gene. For this reason, we cannot rule out the possibility that some mutant chromosomes contain molecular lesions in addition to the ones identified. Nonetheless, we believe that the relevant DNA alterations have been identified in most cases for the following reasons. First, only a single PCR fragment amplified from each mutant chromosome showed a mobility shift in the DGGE assay (80% or more of the *torpedo* coding region was typically examined) (Figure 3). Second, nucleotide alterations associated with the *torpedo* alleles are consistent with their mode of generation. Molecular characterization of a large collection of lesions induced at the *rosy* locus by ethyl methanesulfate (EMS) or ethylnitrosurea (ENU) (summarized in LINDSLEY and ZIMM 1992) has shown that these alkylating agents usually generate G/C to A/T substitutions. Nineteen of twenty-three nucleotide alterations detected in chromosomes carrying EMS- or ENU-induced *torpedo* mutations are G to A transitions (Table 2). Third, studies in which EMS-induced mutations in the *sevenless* (MULLINS and RUBIN 1991), *Toll* (SCHNEIDER *et al.* 1991) and *dorsal* (ISODA *et al.* 1992) loci were identified by sequencing the entire coding region of the gene indicate that double hits are infrequent. Twenty-seven of 29 alleles characterized in these studies possess single molecular alterations.

In addition, the biochemical properties of certain mutant gene products are consistent with their proposed molecular structure. The receptors encoded by top^{JE1} and top^{3B92}, which are predicted to carry missense alterations in their extracellular domains, possess kinase activity (SCHEJTER and SHILO 1989). The Top^{1K35}, Top^{1P02}, Top^{2G31} and Top^{2X51} proteins, which are predicted to lack autophosphorylation sites, are kinase$^-$ in both *in vivo* and *in vitro* assays.

Further, the locations of the top^{1} and top^{CA} mutant lesions are confirmed by genetics. A wild-type chromosome produced by a recombination event between the top^{1} and top^{CA} mutations (J. PRICE and T. SCHÜPBACH, unpublished) carries a sequence polymorphism specific to top^{CA} at nucleotide 401 and a polymorphism specific to top^{1} at nucleotide 1545 (data not shown).

Alleles that differentially affect the development of specific tissues encode receptors with altered extracellular domains: Eight alleles examined in this study show tissue-preferential effects. These mutations show complementation behavior consistent with the idea that they peferentially disrupt a subset of *torpedo*-dependent developmental processes (see above). Seven of these alleles encode receptors carrying amino acid substitutions in their extracellular domains (Table 2). Several mechanisms can account for the genetic behavior of these mutations. One possibility is that the amino acid alterations resulting from the class III and IV missense mutations destabilize the Torpedo protein in a tissue-preferential fashion.

A second is that alterations in the extracellular domain of Torpedo differentially affect the development of certain tissues by disrupting different aspects of receptor dimerization. If the kinetics of signal transduction differ among tissues, mutations decreasing the rate of dimer formation could preferentially disrupt the development of one cell type, while those disrupting the maintenance of stable dimers could preferentially disrupt the development of another cell type.

A third possibility is that amino acid substitution in the extracellular domain of the receptor preferentially disrupt the binding of tissue specific ligands. Indeed, Torpedo does appear to be activated by tissue-specific

ligands. *gurken*, a gene that exclusively regulates Drosophila oogenesis, is required in germ cells and acts upstream of *torpedo* in the signalling pathway (SCHÜPBACH 1987). *gurken* encodes a molecule showing similarity to transforming growth factor-α (TGF-α) (NEUMAN-SILBERBERG and SCHÜPBACH 1993), a ligand for EGF-R (DERYNCK *et al.* 1984), and thus may represent a germline-specific ligand for Torpedo. The product of the *spitz* gene of Drosophila, which also encodes a TGF-α homolog (RUTLEDGE *et al.* 1992), may represent another ligand for Torpedo. Animals that lack zygotic *spitz* expression die during embryogenesis and show a subset of the defects seen in severe *torpedo* mutants (MAYER and NÜSSLEIN-VOLHARD 1988; RAZ and SHILO 1992; RUTLEDGE *et al.* 1992). The three mechanisms are not mutually exclusive.

Torpedo may phosphorylate multiple substrates: The most dramatic examples of complementation between mutant receptors involve the products of the class IIA embryonic lethal *torpedo* alleles: top^{101}/top^{2C82} and top^{101}/top^{2L65} *trans*-heterozygotes are indistinguishable from genetically wild-type animals (Figure 4). Since the molecular lesions associated with these three alleles alter codons coding for amino acids in a region of the kinase domain that is likely to contact receptor substrates (KNIGHTON *et al.* 1991), we favor the idea that these mutant receptors are impaired in their ability to interact with substrate molecules. Because top^{101}, top^{2C82} and top^{2L65} disrupt the development of every tissue tested, we propose that their products are defective in interacting with ubiquitous cellular substrates (either cytosolic proteins or specific tyrosine residues in the carboxyl-terminal domain of Torpedo itself). Complementation between Top^{101} and Top^{2L65}, for example, would occur because the two mutant receptors fail to interact with different sets of substrates. The lack of complementation between Top^{2C82} and Top^{2L65}, on the other hand, would result from the inability of both mutant receptors to interact with one or more common substrates.

The class I proteins Top^{1F26} and Top^{2W74} partially complement Top^{101}, Top^{2C82} and Top^{2L65} (Figure 4). Since the residual activity possessed by these class I proteins allows only weak complementation of Top^{101}, Top^{2C82} and Top^{2L65}, we favor the idea that the amino acid alterations produced by top^{1F26} and top^{2W74} impair receptor interactions with all substrates.

Other mechanisms can account for the complementation occurring between alleles that encode receptors with altered kinase domains. One alternative is that complementation reflects the mutual stabilization of mutant receptors. Another is that receptor-receptor, receptor-substrate or even receptor-ligand interactions that are defective in homodimers are stable in heterodimers. Further studies are required to determine which of these possibilities is correct.

Signal transduction by *torpedo* may require the physical interaction of receptors: Biochemical studies of the human EGF receptor suggest that receptor oligomerization–stimulated by ligand binding–is essential for signal transduction by this molecule (Figure 1; reviewed in ULLRICH and SCHLESSINGER 1990). The extracellular domain of EGF-R plays a dual role in receptor dimerization. This portion of the molecule contains not only determinants of ligand binding (LAX *et al.* 1989, 1991; WU *et al.* 1990; WOLTJER *et al.* 1992) but also determinants mediating the physical association of receptors (SPIVAK-KROIZMAN *et al.* 1992).

The pattern of complementation between *torpedo* alleles is consistent with the the idea that signal transduction by Torpedo depends upon the physical association of receptors. In several cases partial complementation is observed between a Torpedo molecule with an altered extracellular domain but normal tyrosine kinase and carboxyl-terminal domains (abbreviated as $lig^- kin^+ ter^+$ in Table 2) and a Torpedo protein with an altered tyrosine kinase domain but normal extracellular and carboxyl-terminal domains (abbreviated as $lig^+ kin^- ter^+$ in Table 2). Top^{EB} and Top^{2L65} represent one such pair of mutant receptors. A simple interpretation of this complementation is that the mutant receptors form a functional heterodimer. In this model, formation of the heterodimer would be mediated by the intact extracellular domain of the $lig^+ kin^- ter^+$ receptor and activation of the receptor complex would be mediated by the intact tyrosine kinase domain of the $lig^- kin^+ ter^+$ receptor.

If the biochemical defect of a kin^- receptor could be complemented simply by the expression of a kin^+ molecule in the same cell, then all Torpedo proteins possessing an intact tyrosine kinase domain should complement a given kinase-impaired receptor equally well. This is not the case (Figure 4). Instead we find that the capacity of a kinase impaired molecule, such as Top^{2L65}, to form an active signal transduction complex with a $lig^- kin^+ ter^+$ Torpedo protein is roughly inversely proportional to the severity of the lesion in the extracellular domain, such that receptors with the most severely affected extracellular domains (as judged by homozygous mutant phenotypes) show the weakest complementation with $lig^+ kin^- ter^+$ proteins. This finding is consistent with the idea that intermolecular complementation depends upon the physical association of mutation receptors and is mediated through the extracellular domain of the molecule.

The pattern of interallelic complementation argues that the extracellular domain of Torpedo plays a critical role in mediating complementation between mutant receptors. This requirement is illustrated by the positive complementation behavior of the class II alleles, whose products appear to possess normal ligand binding and carboxyl-terminal domains and an altered tyrosine kinase domain. While the $lig^+ kin^- ter^+$ product of the severe class IIA allele top^{2L65} fully complements the $lig^+ kin^- ter^+$ molecule encoded by the weak embryonic le-

thal class IIA mutation top^{101}, it only partially complements the pupal lethal alleles top^{EB} and top^{ED16}, which encode lig$^-$ kin$^+$ ter$^+$ receptors (Figure 4). Likewise, top^{2L65} is complemented more fully by the severe embryonic lethal mutation top^{2X51} (lig$^+$ kin$^+$ ter^0 product) than by the severe embryonic lethal alleles top^{3B92} and top^{JE1} (lig$^-$ kin$^+$ ter$^+$ products) (Figure 4).

Further evidence that the ligand binding domain of Torpedo is required for complementation between mutant receptors is provided by the genetic behavior of top^{4A}, top^{1F26} and top^{2W74}. top^{4A} encodes a lig$^-$ kin$^+$ ter$^+$ receptor, while top^{1F26} and top^{2W74} encode lig$^+$ kin$^-$ ter$^+$ products; all three alleles, at room temperature, show similar homozygous embryonic lethal phenotypes. However, top^{4A} generally shows more severe phenotypes in combination with top^{2L65}, top^{2X51} and top^{JE1} than do top^{1F26} and top^{2W74} (Figure 4). In an analogous fashion, the semiviable mutation top^{CA} (lig$^-$ kin$^+$ ter$^+$ product) partially complements top^{1F26} and top^{2W74} but fails to complement the pupal lethal allele top^{EB} (lig$^-$ kin$^+$ ter$^+$ product) (Figure 4).

Alleles whose products have altered extracellular domains generally complement other mutant alleles less completely than do equally (or, in some cases, more) severe alleles encoding receptors with altered kinase or carboxyl-terminal domains. These observations argue that the extracellular domain of Torpedo is necessary for interallelic complementation. Full complementation occurs between certain lig$^+$ receptors and partial complementation between lig$^+$ and lig$^-$ receptors.

Alternatively, the complementation behavior of the class II mutations may reflect the increased cell surface expression or stability of their protein products relative to those encoded by other classes of *torpedo* alleles. If they are expressed at higher levels than receptors encoded by the class I, III and IV alleles, the products of the class II alleles would have a greater probability of interacting with–thereby stabilizing or forming functional heterodimers with–other mutant Torpedo molecules.

Does signal transduction require ligand binding by both receptors?: top^{3B92} and top^{JE1}, which encode proteins with altered ligand-binding domains (Table 2), behave as total loss-of-function mutations when homozygous or in *trans* to chromosomal deficiencies. However, since these alleles partially complement *torpedo* mutations encoding receptors with altered kinase domains (Table 2, Figure 4), the Top^{3B92} and Top^{JE1} proteins apparently can form some functional heterodimers.

Missense mutations within the extracellular domain of Torpedo could abolish receptor function, yet still allow intermolecular complementation, in a number of ways. One possibility is that the amino acid alterations produced by top^{3B92} and top^{JE1} only affect protein stability. If this were the case, complementation would reflect stabilization of the Top^{3B92} and Top^{JE1} proteins through the co-expression of another species of mutant receptor.

Another possible mechanism for the inactivation of Top^{3B92} and Top^{JE1} is that they bind ligand and initiate dimer formation normally, but do not form stable receptor complexes. The formation of short lived heterodimers between these molecules and kinase-impaired Torpedo proteins might be sufficient for weak signal transduction.

A third possibility is that these mutant Torpedo proteins are defective in ligand binding and, consequently, in the initiation of dimer formation. The stability of dimers containing these proteins, however, might be normal. Biochemical studies suggest that receptor dimerization and signal transduction may require ligand binding only by one member of the pair. Epidermal growth factor stimulates the dimerization of EGF-R and HER2 *in vivo* (Quian *et al.* 1992; Spivak-Kroizman *et al.* 1992), as well as the aggregation of purified EGF-R and HER2 extracellular domains in solution (Spivak-Kroizman *et al.* 1992). Neither HER2 nor its purified extracellular domain possesses EGF binding activity. When coexpressed with kinase$^-$ EGF-R, HER2 protein autophosphorylates and associates with PLC-γ in response to EGF stimulation (Spivak-Kroizman *et al.* 1992). In an analogous manner, the Top^{3B92} and Top^{JE1} proteins might be completely defective in ligand binding, yet still be able to associate with and be activated by a ligand-bound Torpedo molecule with a defective kinase domain.

Conclusions: We have molecularly characterized 24 loss-of-function mutations at the *torpedo* locus of Drosophila, which encodes a receptor tyrosine kinase of the EGF receptor subfamily. Genetic interactions between 21 of these alleles were examined (this work; Clifford and Schüpbach 1989). Based on the correlation observed between the genetic behavior and amino acid alterations of these mutations, we draw the following conclusions: (i) Torpedo may be activated by tissue-specific ligands, (ii) Torpedo may phosphorylate multiple substrates within the same tissue, (iii) signal transduction by *torpedo* appears to require the physical interaction of receptors, (iv) the extracellular domain of Torpedo plays a critical role in mediating receptor-receptor interactions. Our results complement and extend the previous work of Raz *et al.* (1991), and are consistent with models of receptor tyrosine kinase function derived from tissue culture studies.

Using a functional (genetic) assay, we have identified residues in the extracellular and intracellular domains of Torpedo that are essential for signal transduction. Since a number of the affected amino acids are conserved between the the fly and vertebrate receptors, it should be possible to investigate the biochemical consequences of these mutant alterations on receptor function by engineering them into the human epidermal growth factor receptor.

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