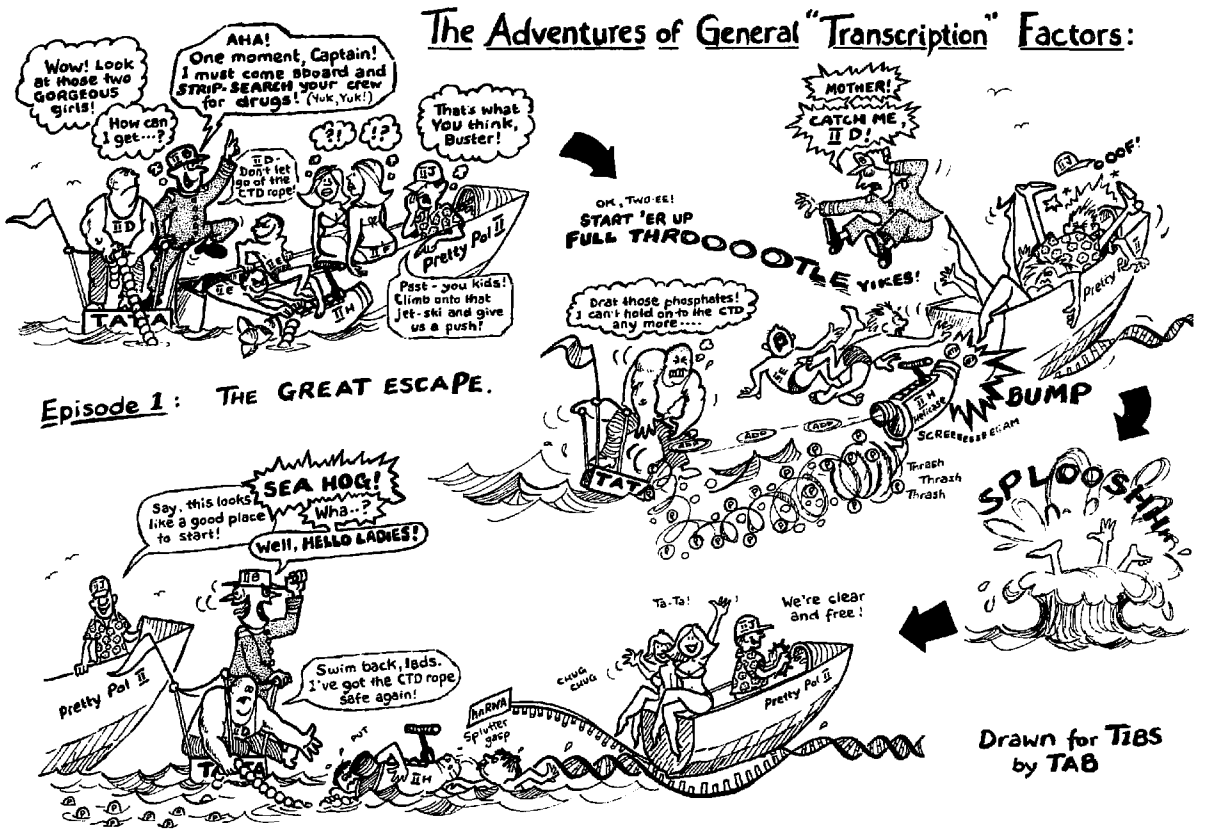

Signal transduction in the early *Drosophila* embryo: when genetics meets biochemistry

Norbert Perrimon and Claude Desplan

An elegant combination of genetic and biochemical approaches has been used to investigate a variety of signal transduction pathways in developmental processes. Here, we describe the 'terminal' signaling system in the *Drosophila* embryo, which is responsible for pattern formation in the polar regions of the embryo. This pathway involves a membrane-bound receptor tyrosine kinase (RTK) that is similar to other *Drosophila* RTKs, such as sevenless, and the mammalian RTKs, such as the epidermal growth factor or platelet-derived growth factor receptors.

IN THE DEVELOPMENT of *Drosophila*, many maternal and zygotic genes are involved in embryonic patterning and the establishment of the body plan[1]. These maternal genes are required in the mother for the proper development of the embryo. Transcripts from these genes are synthesized by the mother and their products are used by the early embryo for setting up the spatial expression patterns of the early zygotic genes. These genes, in turn, control a cascade of gene expression leading to the segmented embryo.

A series of maternal and zygotic mutations has been isolated that leads specifically to loss of structures in the terminal regions (anterior and posterior) of the embryo (see Table I for specific references). These mutations exhibit similar phenotypes, suggesting that they operate in a common developmental pathway. For instance, loss-of-function mutations of one of the maternal terminal genes, *torso* (*tor*), produce progeny that have lost all terminal structures. This phenotype is similar to that seen in embryos where the zygotic gene *tailless* (*tll*) is absent. Furthermore, the enlarged terminal domains seen in embryos from mothers carrying a gain-of-function *tor* mutation can be suppressed by the removal of zygotic *tll* function[2], suggesting that maternal *tor* controls the zygotic transcriptional activation of *tll*[3]. This type of genetic analysis has determined the order in which the 'terminal genes' act. The *tor* gene encodes a receptor tyrosine kinase (RTK) that is

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Table I. Terminal class genes

Gene	Requirement	Protein	Ref(s)
torso (*tor*)	Maternal	Receptor tyrosine kinase	5, 43
corkscrew (*csw*)	Maternal and zygotic	Nonreceptor protein tyrosine phosphatase	31
Son of sevenless (*Sos*)	Maternal and zygotic	Guanine nucleotide exchange factor	15, 16, 19
ras1	Maternal and zygotic	Small GTP-binding protein	15, 16, 19
D-raf	Maternal and zygotic	Serine/threonine kinase	17, 24, 25
D-sor-1	Maternal and zygotic	Tyrosine/threonine kinase	27
trunk (*trk*)	Maternal	Not determined	45
torsolike (*tsl*)	Maternal	Secreted ligand?	11
fs(1)polehole	Maternal	Not determined	44
fs(1)Nasrat	Maternal	Not determined	44
tailless (*tll*)	Zygotic	Transcription factor	3
huckebein (*hkb*)	Zygotic	Transcription factor	7

present throughout the membrane of the embryo[4,5]. In normal development, it is activated by an extracellular ligand at both poles of the embryo. Mutants that do not express *tor* do not develop terminal structures, while *tor* gain-of-function mutants have a constitutively active receptor throughout the embryo, leading to generalized expression of *tll*[4,6].

Two of the primary targets of the tor RTK are the products of the zygotic genes *tll* and *huckebein* (*hkb*), both of which code for transcription factors. The product of *tll* is a member of the nuclear receptor superfamily[3], and *hkb* encodes an Sp1/egr-like zinc-finger protein[7]. Both genes are expressed in two distinct domains at the embryo termini[3,8].

Activation of tor

A genetic approach to investigating the order of function of terminal genes has been crucial to understanding the mechanism underlying tor activation. Although the ligand that activates tor only at the embryo's poles has not yet been identified, several maternal genes have been implicated in the local production of the ligand (Fig. 1)[9]. One of these, *torsolike* (*tsl*), is required in a subset of the somatic follicle cells, located at the poles of the egg chambers, suggesting that cell communication between the follicle-cell epithelium and the oocyte is involved in defining the terminal regions[10]. This led to the hypothesis that the follicle cells secrete the ligand for tor, or provide an activity required to specify the site of interactions between tor and its ligand near the poles of the embryo (Fig. 1)[10]. The recent molecular characterization of *tsl* supports such a model[11,12]: *tsl* is expressed in polar follicle cells located at both ends of the oocyte, and encodes a putative secreted protein with a signal sequence but no transmembrane domain. Three other genes, *trunk* (*trk*), *fs(1)Nasrat* and *fs(1)polehole*, have been shown to act upstream of *tor*[2]. Further characterization of these genes will probably lead to insights into the process by which tor becomes activated locally.

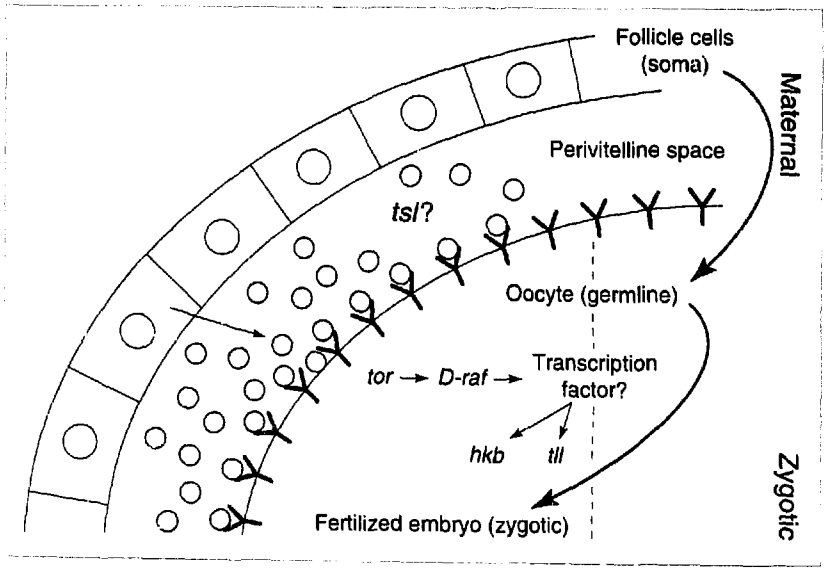

Figure 1

Maternal and zygotic genes involved in determination of the terminal regions of the *Drosophila* embryo. A signal from the follicle cells surrounding the oocyte (which is of somatic origin), is sent at the poles of the embryo. It activates a signal transduction cascade whose products, encoded by maternal genes, are stored in the egg. Activation of the cascade leads to the transcriptional activation of the zygotic effectors in the developing embryo. See Table I for abbreviations.

The tor pathway: parallels with *Drosophila* eye development

The products of the *ras* and *raf* oncogenes have been implicated in the control of cell proliferation as downstream effectors of RTKs[13,14]. Mutations in these general factors are likely to disrupt many pathways and be associated with multiple and complex phenotypes, resulting in death of the embryo[15–17]. Although the pleiotropic function of these molecules has made their genetic analysis difficult, the sophisticated classical and molecular genetic techniques of *Drosophila* have circumvented this problem[16–19]. Many of the genes involved in the Ras pathway were first identified in *Drosophila* because of their involvement in the induction of the R7 photoreceptor cell in the eye by its neighbor, the R8 photoreceptor[20], which involves activation of the sevenless (sev) RTK.

Since both tor and sev encode RTKs, this might suggest that members of the Ras pathway would be involved as maternal components of the terminal determination pathway. The function of Ras and the Sos guanine nucleotide exchange factor were indeed shown to be critical for terminal development: disruption of Ras activity leads to a terminal phenotype, while constitutively active Ras rescues the phenotype of *tor* loss-of-function mutations[15]. As might be expected, the genes involved in the Ras pathway act downstream of tor, but upstream of the gene encoding the *Drosophila* homolog of the Raf serine/threonine (Ser/Thr) kinase D-Raf (Fig. 2 and see below)[17]. The parallel between tor and sev has been emphasized further by the observation that

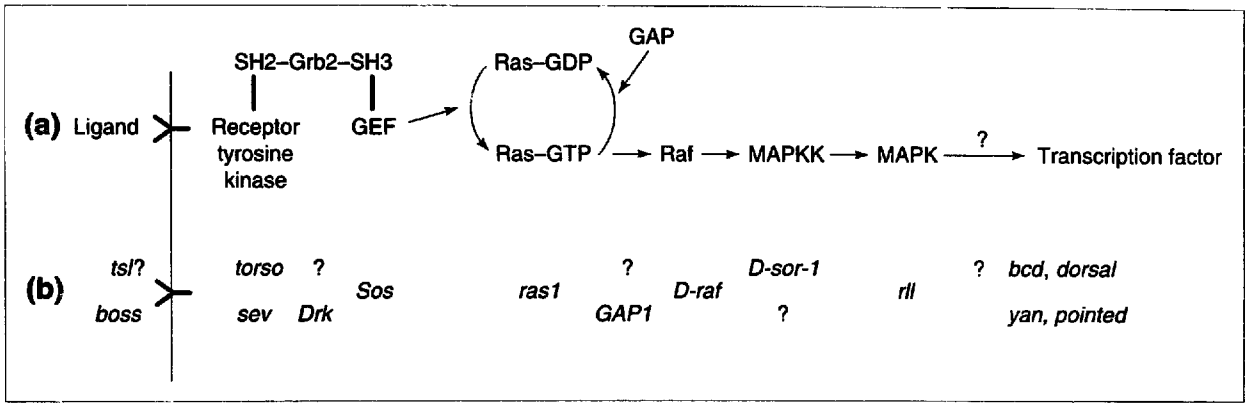

Figure 2

Cascade of activation from receptor tyrosine kinases. **(a)** The proposed direct interactions between proteins. **(b)** The genetic cascade leading to terminal differentiation (top) or R7 photoreceptor-cell induction (bottom). The genes involved in both pathways are positioned centrally. GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; boss, bride of sevenless; see Table I for other abbreviations.

constitutively active tor can induce R7 development in a *sev* mutant[21].

From RTK to Ras and Raf

After ligand binding, RTKs dimerize and autophosphorylate on tyrosine residues, which are recognized by Src-homology 2 (SH2) domains present on several adaptor molecules such as Grb2 in mammalian cells and Sem-5 in *Caenorhabditis elegans*. These adaptor molecules also contain SH3 domains, which bind proteins containing proline-rich motifs. Recently, a *Drosophila* homolog of these proteins, Drk[22,23], has been shown to interact both with the tyrosine-phosphorylated cytoplasmic domain of sev, through its SH2 domain, and with Sos, through its SH3 domains[22,23]. Interestingly, Drk is encoded by a gene whose loss-of-function mutations enhance the *sev* phenotype[16], consistent with a positive role in mediating the signal from the receptor. Therefore, it appears that sev acts on the Ras cycle by activating Sos via Drk.

D-Raf is another member of the terminal signal transduction cascade[15,17,24]. Mutations in *D-raf* exhibit a zygotic lethal phenotype, but specific disruption of *D-raf* in the germline leads to a maternal phenotype similar to that of *torso*[25]. Maternal null *D-raf* alleles completely suppress the dominant phenotype of a gain-of-function *tor* mutation[17], as well as the phenotype of embryos carrying activated Ras (see below)[15]. These observations place *D-raf* downstream of *tor* and *ras1* (Fig. 2) in this signaling pathway. Raf function has also been shown to act downstream of Ras in the sev pathway[26].

Downstream of Raf

Two suppressor screens have been used to isolate putative targets of Raf. They have identified dominant mutations in a gene encoding a protein that is very similar to mitogen-activated protein kinase (MAPK) kinase (MAPKK or MEK) called *D-mek* or *D-sor-1* (Refs 27, 28). Several genetic experiments place *D-mek* downstream of *tor* and *D-raf*, but upstream of the putative transcription factors modified by the cascade of kinases (Fig. 2)[27].

A *Drosophila* homolog of MAPK has been identified and shown to be encoded by the gene *rolled* (*rll*). Analysis of *rll* mutations has demonstrated that it is a component of the sev signaling pathway[29,30]. Interestingly, mutations that render the rll protein constitutively active result in a similar phenotype to gain-of-function *tor* mutations, suggesting that *rll* operates downstream of *D-mek* in the *tor* signaling cascade[30] to activate *tll* and *hkb*.

The role of phosphatases

A screen for maternal terminal phenotypes among a collection of *Drosophila* zygotic lethal mutations has led to the identification of the *corkscrew* (*csw*) gene, which encodes a soluble protein tyrosine phosphatase with two SH2 domains[31]. Mutants that do not express *csw* have only a partial terminal phenotype, and genetic epistasis indicates that *csw* acts downstream of *tor*. Therefore, it cannot be responsible for inactivating tor by interacting with its phosphotyrosines. Rather, csw appears to act as a positive factor in the tor signaling pathway, and might be involved in recycling tor, or in removing a negative regulatory phosphate[31], as do some other phosphatases (see Ref. 32 for a review).

A possible role for csw in the tor cascade has been provided by recent biochemical studies on Syp (also known as PTP1D, SH-PTP2, PTP2C and Shc), which encodes a mammalian homolog of csw (Ref. 31; L. Perkins, unpublished). Tyr1009 in the platelet-derived growth factor (PDGF) receptor has been shown to be required for binding of Grb2. However, the association of Grb2 with the receptor is not direct but is instead mediated by Syp[33]. A possible function of csw is to attach Drk/Grb2 to the RTK and thus promote Ras activation. This model is consistent with the observation that *csw* operates upstream of *ras1* (Ref. 33). Further biochemical characterization of the binding sites of csw and Drk to tor will clearly be necessary to test this model.

Transcriptional effectors of the terminal system

The gene(s) encoding the transcription factor(s) whose activity is modified by the tor kinase cascade has not yet been identified. This hypothetical factor (gene Y, Fig. 1)[9] is predicted to activate the expression of both *tll* and *hkb* in the terminal regions, in response to tor signaling. Transcriptional activation of the zygotic target genes is likely to be achieved by either (1) the activation by MAPK of a transcription factor that controls *tll* and *hkb* expression positively; or (2) the phosphorylation and inhibition by MAPK of a constitutively active repressor, resulting in localized derepression of the terminal genes at the poles. Although such a factor has not

been isolated in the terminal pathway, two Ets proteins, yan and pointed, appear to be involved in the sev pathway downstream of MAPK[34].

However, there is another known maternal transcription factor whose activity is regulated by the terminal cascade of kinases. In the anterior part of the embryo, the morphogen bicoid (bcd) controls head and thorax development. The bcd protein activates effector genes distinct from those of the terminal pathway[9]. However, tor activity represses transcription of the bcd target genes at the anterior pole[35]. This repression correlates with phosphorylation of bcd and depends on *D-raf* but not on either *tll* or *hkb*. Thus, tor downregulates the activity of bcd, possibly by phosphorylation of its transcriptional activation domain by a Ser/Thr kinase acting downstream of MEK (Fig. 1)[35]. This output of the terminal system is distinct from that leading to the modification of the product of gene Y and to the zygotic expression of *tll* and *hkb* (Fig. 2). Therefore, by using a new target in the anterior part of the embryo (bcd), the same biochemical pathway leads to different effects in the anterior versus posterior terminal regions.

In addition to the anterior system, the maternal dorsal–ventral system also appears to be regulated by the terminal system[36]. The repressive activity of the dorsal protein appears to be prevented by the activity of the tor pathway, representing a third output of this system.

Finding new genes in terminal development

As has been outlined above, many different transcriptional events may be switched on by the same signaling pathway. For example, *D-raf* and *D-mek*, in addition to their roles in tor signaling, have been shown to operate in the sev and epidermal growth factor (EGF) signaling pathways. A possible explanation for this is that common pathways diverge at the level of the transcriptional effector: the products of gene Y and *bcd* are not present in the eye, while the transcriptional effectors of sev are not present in the early embryo. One approach to identifying genes involved downstream of tor signaling is to use germline mosaic techniques. A recombination system (called the FRT-OvoD1 system) has been developed, which engineers adult females carrying germline mutations that are normally lethal. These females produce eggs that lack the maternal function to be tested[37–40]. Such screens may allow the identification of additional terminal genes, such as gene Y (Fig. 1)[9].

Another powerful approach for isolating components of a signal transduction pathway is to isolate second-site suppressor or enhancer mutations that modify the phenotype associated with a specific component of the tor pathway. Doyle and Bishop[41] identified seven such mutations that suppress the phenotype of a weak *tor* gain-of-function allele, three of which correspond to *Sos*, *Ras1* and *Drk*. Using a similar approach, Tsuda *et al.*[27] and Lu *et al.*[28] isolated MAPKK among suppressors of a weak *D-raf* allele (see above and Ref. 42). Further isolation of genes by these screens will probably identify other important players in the tor signaling pathway.

'Biochemical genetics'

The early embryo is a syncytium, so it is possible to micro-inject purified or overexpressed proteins believed to act in tor signaling and to test their *in vivo* function in different mutant backgrounds. More importantly, this technique will help unravel the order of interaction between the proteins involved in signaling. For example, Lu *et al.*[15] have microinjected an extract from baculovirus overexpressing the constitutively active mammalian v-Ras protein into an early embryo lacking *tor* function. Activated v-Ras rescued the maternal phenotype of *torso*, demonstrating its involvement in the pathway and its location downstream of *tor*[15]. In addition, they also showed that activated v-Ras could not rescue the maternal phenotype of *D-raf*, demonstrating that Ras, as suspected from studies in mammalian cells, operates upstream of *D-raf* in *tor* signaling.

Genetics and biochemistry: complementary approaches

The field of signal transduction in mammalian cells has made wide use of oncogenes and of somatic cell genetics. The molecules identified by genetic studies in yeast, *C. elegans* and *Drosophila* are, to a large extent, identical to those identified in mammals. The most important contribution of the genetic systems has been their ability to describe the genetic epistasis between the molecules involved in a single pathway, i.e. to link the different members and place them within developmental pathways, and identify rate-limiting steps. This not only gives a functional significance to biochemical reactions, but also offers models to test biochemically. However, genetics has difficulties isolating redundant or pleiotropic functions (see above) and cannot lead to an understanding of biochemical mechanisms. Thus, it is only by combining the two approaches and studying parallels between genetic and biochemical systems that we will obtain a complete mechanistic and functional description of signal transduction and its role in development.

Acknowledgements

The authors thank the members of the Desplan lab for useful comments and suggestions during the writing of this manuscript.

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Regulation of CFTR channel gating

David C. Gadsby and Angus C. Nairn

The debilitating symptoms of cystic fibrosis stem from the reduced Cl^- permeability of epithelial cells owing to mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. In cells with normal CFTR channels, receptor-mediated activation of cyclic-AMP-dependent protein kinase causes phosphorylation of several serines in the regulatory domain of CFTR, permitting channel opening and closing via cycles involving ATP hydrolysis. Cellular phosphatases rapidly dephosphorylate the channels, inactivating them. Here we discuss recent advances in our understanding of this complex mechanism for regulating channel gating.

CYSTIC FIBROSIS (CF), the most common lethal genetic disease in caucasian populations[1], is caused by alterations in a single gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR)[2]. The gene alterations disrupt hormonally regulated epithelial Cl^- transport, and consequently water movement, in the airways, pancreas, intestines, sweat glands, and testes[3]. Thick dehydrated mucus and bacterial infection in the lungs of CF patients cause debilitation and death[1]. Some two thirds of the diagnosed CF cases are caused by a single mutation in the CFTR gene – a deletion of the three nucleic acids that code for Phe508 (Fig. 1), but hundreds of other disease-causing mutations have been identified[4]. The relationship between specific mutations and progression of the disease remains to be clarified.

The cystic fibrosis gene encodes a Cl^- channel

CFTR is a 169 kDa protein that shows substantial organizational (Fig. 1) and sequence homology to members of the family of ATP-binding cassette (ABC) transport proteins, also called traffic ATPases[5], that includes the mammalian P-glycoprotein responsible for multi-drug resistance, the yeast **a** mating factor exporter (STE6) and bacterial periplasmic transporters. All ABC transporters incorporate two nucleotide-binding domains (NBDs; Fig. 1), which are thought to hydrolyse ATP and drive the active transport[5]. However, CFTR also has a unique regulatory (R) domain containing multiple consensus sites for phosphorylation by cyclic-AMP-dependent protein kinase (PKA) and protein kinase C (PKC)[2].

Despite its homology with ABC transporters, and the possibility that CFTR might regulate the processing, membrane insertion, or function of other ion channels[6,7], an overwhelming body of evidence has proved that CFTR is a small-conductance (8–10 pS), ohmic Cl^- channel whose gating is regulated by PKA and by hydrolysable nucleoside triphosphate. Expression of exogenous CFTR endows a variety of cell types with channels indistinguishable from epithelial CFTR channels[8,9]. Point mutations in putative transmembrane α-helices M1 and M6 (Fig. 1) alter properties of CFTR channel pores[10–14], and purified CFTR protein incorporated into lipid bilayers generates small-ohmic-conductance Cl^- channels that are activated by PKA plus ATP[15].

Activity of CFTR Cl^- channels is regulated by both PKA phosphorylation and ATP hydrolysis

Although there can be no doubt that CFTR is a channel, we are only just beginning to understand the complex

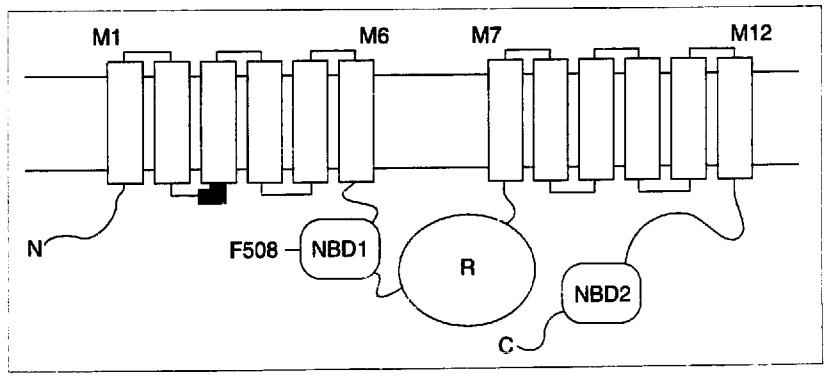

Figure 1
Topological model of CFTR[2], showing cytoplasmic nucleotide-binding domains (NBD1, NBD2), the regulatory (R-) domain, and predicted membrane-spanning α-helices (M1–M12). The thickened section of the M2–M3 cytoplasmic loop represents the peptide encoded by exon 5, believed to be spliced out of the cardiac isoform[54] of CFTR. F508 marks the site of the most common mutation in CF patients.

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