Requirement of the *Drosophila raf* homologue for *torso* function

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IN *Drosophila* the correct formation of the most anterior and posterior regions of the larva, acron and telson is dependent on the maternally expressed terminal class of genes[1]. In their absence, the anterior head skeleton is truncated and all the structures posterior to the abdominal segment seven are not formed[1–6]. The protein predicted to be encoded by one of these genes, *torso* (*tor*), seems to be a transmembrane protein with an extracytoplasmic domain acting as a receptor and a cytoplasmic domain containing tyrosine kinase activity[7]. Here we report that another member of the terminal-genes class, *l(1)polehole* (*l(1)ph*)[2,3,8], which is also zygotically expressed, is the *Drosophila* homologue of the v-*raf* oncogene and encodes a potential serine-and-threonine kinase. We also show that functional *l(1)ph* gene product is required for the expression of a gain-of-function *tor* mutant phenotype, indicating that *l(1)ph* acts downstream of *tor*. Together, these results support the idea that the induction of terminal development occurs through a signal transduction system, involving the local activation of the *tor*-encoded tyrosine kinase at the anterior and posterior egg poles, resulting in the phosphorylation of the *l(1)ph* gene product. In turn, downstream target proteins may be phosphorylated, ultimately leading to the regionalized expression of zygotic target genes. Such a process is in agreement with the finding that both *tor* and *l(1)ph* messenger RNAs are evenly distributed.

It has been shown previously that the gene encoding a potential serine-and-threonine kinase with homology to the v-*raf* oncogene[9–11] maps near the *l(1)ph* locus[12,13]. Molecular analysis of the DNA lesions associated with *l(1)ph* mutations indicates that the *Drosophila raf* homologue, *D-raf*, is in fact the *l(1)ph* gene (Fig. 1). Confirmation of the identity of *D-raf* and *l(1)ph* was obtained by P-element-mediated rescue experiments. A DNA fragment of 4.3 kilobases (kb) containing only the *D-raf* coding sequences rescued both the zygotic and maternal effects of *l(1)ph* mutations (Fig. 1*b*).

The *D-raf* transcripts are distributed homogeneously

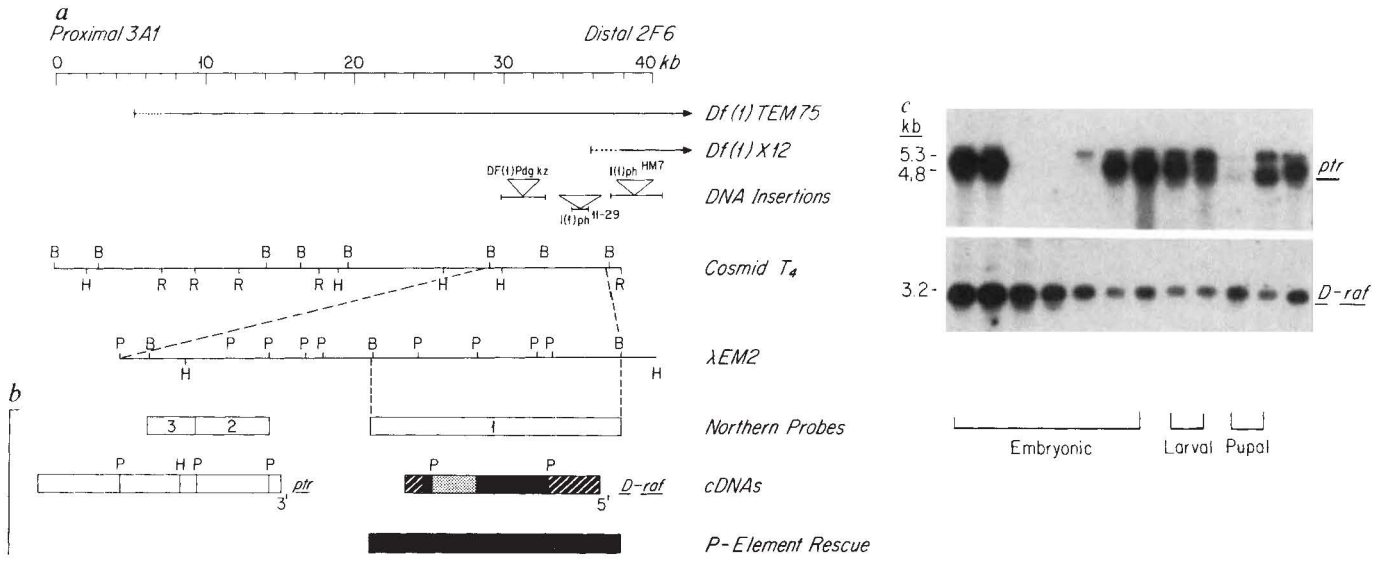

FIG. 1 The molecular organization of the 2F6-3A1 cytogenetic region containing the *l(1)ph* locus. The reference bar is graduated in kb with the position 0 defined by the proximal end of the T4 cosmid[23]. *a*, The *l(1)ph* gene lies between deficiency breakpoints *Df(1)TEM75* and *Df(1)X12*[8] which map to positions 6–8 and 36–38, respectively. The solid lines represent DNA sequences not removed by the deficiencies and the broken lines represent the maximum limits within which the deficiency breakpoints fall. *Df(1)TEM75* complements *l(1)ph* mutations, defining a proximal limit for the *l(1)ph* gene. The *Df(1)X12* chromosome does not complement *l(1)ph* mutations, indicating at least some of the *l(1)ph* gene lies proximal to the breakpoint at position 36–38. DNA polymorphisms in three different strains are identified as insertions in the *l(1)ph* region. Two mutant alleles of *l(1)ph*, *l(1)ph*^{HM7} and *l(1)ph*$^{11-29}$ (L.A., manuscript in preparation) have ~4-kb DNA insertions mapping to positions 38–41 and 35–36, respectively. The *Df(1)Pdg kz* chromosome has wild-type *l(1)ph* activity but contains a 7-kb DNA insertion at position 30–33. These DNA insertions are depicted at their approximate positions by the inverted triangles. The deficiency breakpoints and DNA insertions were mapped using standard methods as described previously[24]. *b*, Two non-overlapping transcription units lie within the genomic region containing the *l(1)ph* gene. The *D-raf* gene was localized to position 33–37 using a *Bam*H1 subclone (nothern-blot probe 1) of the genomic probe *D-raf1* (ref. 12). A second transcription unit called *ptr* (proximal to *raf*) was identified by northern-blot analysis using probes 2 and 3. No additional bona fide transcription units are encoded within the DNA spanning positions 0–40. Complementary DNAs homologous to *D-raf* and *ptr* transcription units were isolated from λgt11 (gift of K. Zinn and C. Goodman) and plasmid pNB50 (ref. 25) cDNA libraries, respectively. The 3.2-kb *D-raf* cDNA contains both 700 base pairs-5′ and 600 base pairs-3′ nontranslated regions (hatched boxes), and a coding region of 1.9 kb (shaded boxes) which includes the putative serine-threonine kinase domain (vertical striped box). The 4-kb insertion in *l(1)ph*$^{11-29}$ maps within the *D-raf* coding region, whereas the *l(1)ph*^{HM7} insertion maps 5′ to it. The complete structure of the 4.3 kb *ptr* cDNA has not been determined; but the *Df(1)Pdg kz* DNA insertion occurs within sequences encoding the *ptr* transcript. *Df(1)Pdg kz*/*Df(1)X12* females show only truncated *ptr* transcripts, have the normal 3.2 kb *D-raf* transcript, and are viable, fertile adults (data not shown). These data indicate that it is the *D-raf* rather than the *ptr* transcription unit which encodes the *l(1)ph* gene. A transformed strain, B-13-1 (ref. 13) containing a P-element construct carrying the wild-type *D-raf* gene was tested for complementation to *l(1)ph* mutations. All pleiotropic aspects of the mutant *l(1)ph* phenotype including the maternal effect, were rescued by the transformed DNA fragment *in trans* configuration (data not shown). *c*, Accumulation of *ptr* and *D-raf* transcripts during development. The *ptr* transcription unit encodes a 5.3 kb and a 4.3 kb RNA species and its expression is developmentally regulated. The 3.2 kb *D-raf* transcript is present at all stages of development. The time points for the embryonic period are given as hours after egg laying. These northerns blots re-probed with an actin 5C probe indicate that each lane contains an equivalent concentration of RNA (data not shown). Preparation of poly(A)$^+$ RNA and northern-blot analysis were performed as described previously[26].

throughout the oocyte and embryo at all stages (Fig. 2) and are not preferentially localized to the termini. Based on evidence presented below, it is likely that the maternal *D-raf*-encoded protein is also evenly distributed in the embryo. Similarly, there is no specific pattern to the localization of *tor* mRNA in the egg or embryo, and a uniform distribution of *tor* gene product is also predicted[7]. Thus the spatial restriction of both *D-raf* and *tor* activities, and hence the proper determination of terminal cell fates, does not rely on the spatial localization of the corresponding gene products, but could rather depend on their localized activation, possibly by one of the other terminal genes.

The finding that the proteins encoded by *D-raf* and *tor* contain domains with potential kinase activities indicates a possible mechanism by which such restricted activity of evenly distributed gene products occurs. In mammalian cells, the proto-oncogene homologue of v-*raf*, *c-raf-1* (refs 14, 15) encodes a protein which functions as a transducer of extracellular growth signals. Binding of growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) to receptors with tyrosine kinase activity causes the phosphorylation of *c-raf-1* product and the stimulation of its kinase activity[16]. Activated *c-raf-1* protein in turn phosphorylates other proteins, which may include itself and transcription factors, to initiate the programme for cell division. The proteins encoded by *tor* and *D-raf* could function in an analogous signal transduction pathway. The *tor* gene product could be the receptor for a spatially localized 'terminal' signal potentially secreted by the follicle cells during oogenesis. Binding of the localized ligand to the extracellular receptor domain of the *tor* gene product would activate the kinase domain, which would then locally phosphorylate the ubiquitous *D-raf* gene product. Thus, local activation of the *D-raf* gene product would lead to the induction of a programme of zygotic gene expression specific for the termini of the embryo.

We tested genetically for the interactions between *tor* and *D-raf* which such a model predicts. Loss-of-function alleles of *tor* lead to the loss of terminal structures identical to those described for null *l(1)ph* mutations. The maternal effect of some *tor* alleles, however, produce an opposite, gain-of-function embryonic phenotype[17,18]. Females homozygous for the *tor* gain-of function allele tor^{RL3} produce at 25 °C embryos that lack thoracic and abdominal structures but show differentiation of the terminal anlagen. Loss of abdominal segments is correlated with the suppression of early gap and segmentation gene expression, indicating that tor^{RL3} could encode a protein which is ectopically active in the central domain of tor^{RL3}-derived embryos[17,18].

If the *D-raf* gene product is required to transfer the activated *tor* signal to other downstream terminal genes, then the absence of *D-raf* should suppress the tor^{RL3} gain-of-function phenotype. To test whether this occurs, embryos derived from eggs lacking maternal *D-raf* and containing tor^{RL3} activities were examined. Two classes of embryos were observed with equal frequency after 24 hours of development at either 18 °C or 25 °C. Class 1 embryos showed the terminal class defect, with anterior and posterior regions of the embryos failing to develop. These embryos most probably represent the progeny lacking maternal *D-raf* activity. Class 2 embryos suffered massive cell death and developed little cuticle. These embryos are equivalent to those lacking both maternal and zygotic *D-raf* activity. No embryos exhibited the tor^{RL3} phenotype. Thus, functional *D-raf* gene product is required for expression of the gain-of-function tor^{RL3} phenotype. Loss of *D-raf* gene product blocks the transfer of the *tor* terminal signal in the central domain and at the termini of tor^{RL3}-derived embryos. This result also indicates that *D-raf* gene product is present and can be activated in the central domain of tor^{RL3} and wild-type embryos, which is consistent with the ubiquitous presence of *D-raf* transcripts (Fig. 2).

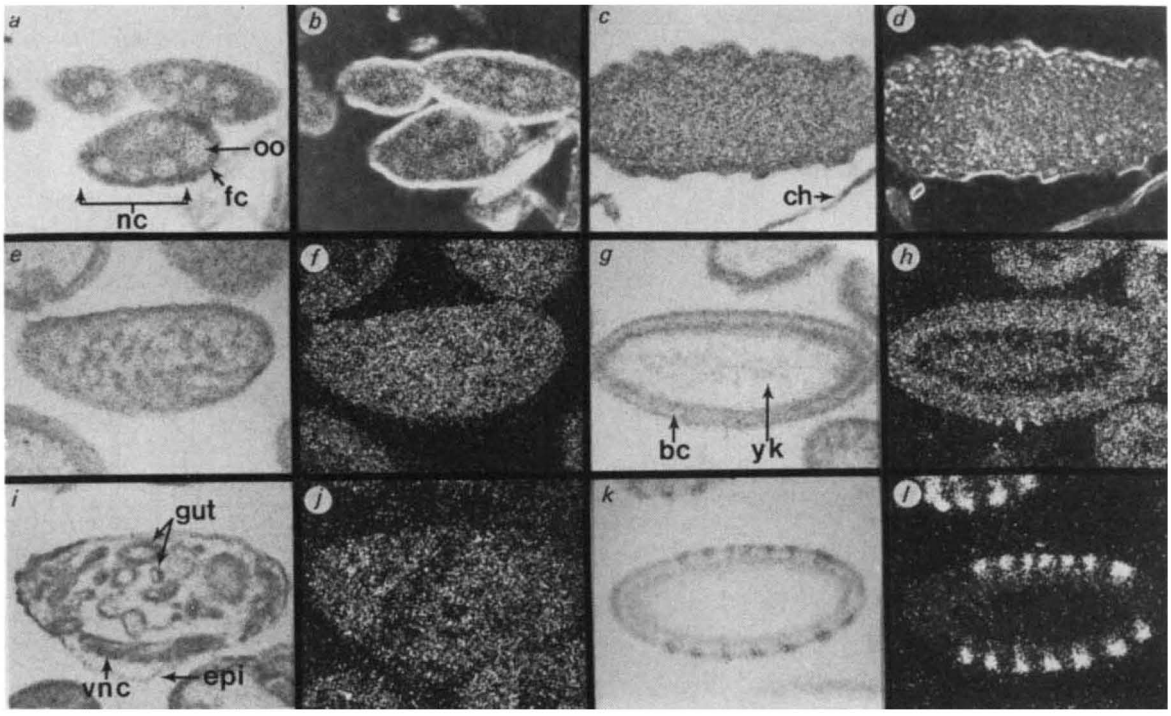

FIG. 2 Accumulation of *D-raf* transcripts during oogenesis and embryogenesis. *a*, *b*, An equal distribution of *D-raf* transcripts is observed for germ-line-derived nurse cells, oocyte and somatic follicle cells at stages 5–9 of oogenesis. *c*, *d*, A mature stage-14 oocyte shows no spatial localization of maternal *D-raf* RNAs. *e*, *f*, Accumulation of *D-raf* RNA in a syncitial blasoderm staged embryo 2 h after fertilization. *g*, *h*, At 3 h, *D-raf* RNA is present in the peripheral blastoderm cells and in the internal yolk region. *i*, *j*, At later stages of embryogenesis (16 h) *D-raf* transcripts are present in endodermal, mesodermal and ectodermal tissues. *k*, *l*, Control blastoderm stage embryo showing localization of *fushi tarazu* (*ftz*) transcripts[27]. Preparation of tissue sections and hybridization conditions using ^{35}S-labelled *D-raf* and *ftz* probes were as described previously[26]. Abbreviations: bc, blastoderm cells; ch, chorion; epi, epidermis; fc, follicle cells; nc, nurse cell complex; oo, oocyte; vnc, ventral nerve cord; yk, yolk.

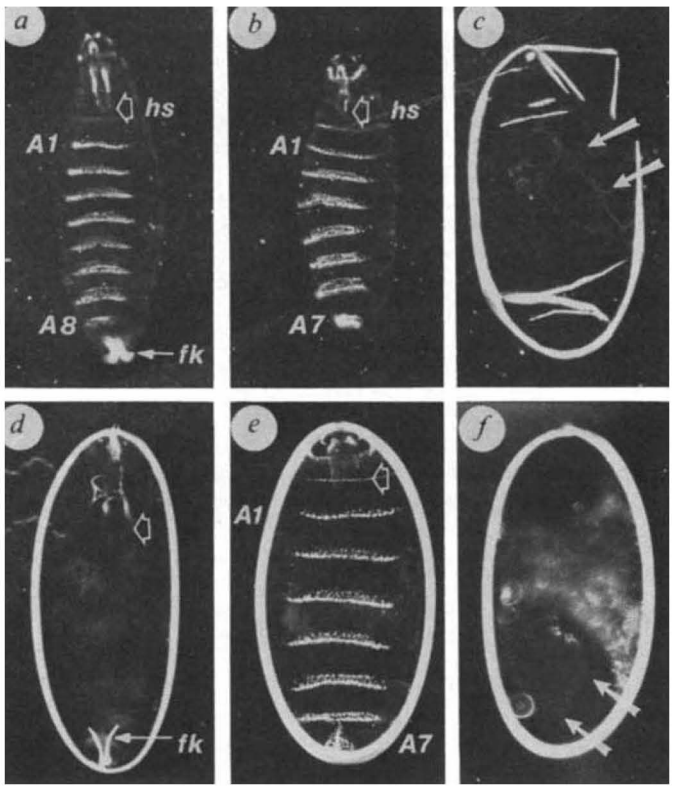

FIG. 3 The phenotype of embryos derived from double mutant *l(1)ph* and tor^{RL3} female germ cells. Dark field photographs of cuticular preparations of wild-type embryos (*a*) and of embryos derived from; homozygous *l(1)ph* female germ cells (*b, c*); homozygous tor^{RL3} females (*d*); homozygous *l(1)ph*;tor^{RL3} female germ cells (*e, f*). In *b* and *e*, embryos show the terminal class phenotype: a truncated head skeleton and the lack of structures posterior to abdominal segment seven. These embryos developed without maternal *D-raf* activity. In *c* and *f*, embryos show the *l(1)ph* 'null' phenotype; little cuticle is formed. These embryos developed without maternal and zygotic *D-raf* activity. *d*, The tor^{RL3} gain-of-function phenotye; at 19 °C embryos form head and tail structures without thoracic or abdominal segments. The $l(1)ph^{EA75}$ allele used to generate female germ cells lacking *D-raf* activity as previously described[28]. All females were crossed to Oregon R wild-type males. Abbreviations: fk, filzkorper; hs, head skeleton.

Suppression of the gain-of-function tor^{RL3} phenotype is also observed in embryos derived from eggs containing only half the wild-type amount of *D-raf* gene product. At 18 °C, the proportion of embryos that hatched was greater for these embryos than for those with normal levels of *D-raf* activity (Table 1). The maternal effect of tor^{RL3} is cold sensitive[18]; at 25 °C, embryos show complete loss of abdominal segmentation, whereas at 18 °C a few embryos (1%) have normal segmentation and hatch. This weak gain-of-function phenotype of the tor^{RL3} allele at 18 °C is indicative of a low-level of ectopic *tor* activity. But when the maternal contribution of functional *D-raf* gene product was reduced by half there was a 10-fold increase (11%) in the number of embryos that survive to hatching. The enhanced survival can be attributed to the reduced levels of maternal *D-raf* activity, and, thus, to an indirect reduction of ectopic tor^{RL3} activity. When the availability of *D-raf* gene product is reduced, the efficiency of signal transfer may also decrease, thereby restoring normal embryonic development. At the termini, where the activity of tor^{RL3} is at least normal, a 50% reduction of *D-raf* product does not adversely affect the development of head or tail structures.

These results support the idea that induction of terminal development acts through a signal transduction system involving a phosphorylation cascade. Spatial restriction of activity is achieved in this pathway by the localized activation of evenly distributed gene products. Two genes likely to participate in this cascade are *tor* and *l(1)ph* (*D-raf*). We have shown here that *D-raf* acts downstream of the *tor* gene product to transfer the maternal terminal signal. Our data is consistent with the model proposed above and by Sprenger *et al.*[7]: activation of *tor*-encoded tyrosine kinase in a spatially restricted manner results in the phosphorylation of *D-raf* gene product, which in turn phosphorylates serines and threonines of downstream target proteins. One such target could be the protein encoded by the *tailless* (*tll*) gene[19]; phosphorylation by *D-raf* gene product of maternally expressed Tll protein[18] could cause its activation, leading directly or indirectly to zygotic expression of *tll*. Other downstream terminal genes, including *fork head*[20] and *spalt*[21] could play a part in mediating the terminal class signal transmitted by *D-raf*.

In contrast to the other maternal terminal class genes, *l(1)ph* gene activity is also required at later embryonic and larval stages of development for the maintenance of cell viability[3] and for the proliferation of somatic cells[2], respectively. Thus the *l(1)ph* gene product acts in several different developmental pathways; only its maternal role in the terminal class pathway has been addressed here. □

TABLE 1 Partial suppression of the tor^{RL3}

Maternal genotype	*T* (°C)	*N*	No. of embryos hatched	Hatching (%)
A. tor^{RL3}/tor^{RL3}	18	1,920	20	1
	19	2,812	0	0
	20	1,882	0	0
B. *l(1)ph/FM3* tor^{RL3}/tor^{RL3}	18	1,906	220	11.5
	19	2,788	110	3.9
	20	1,320	3	0.22
C. ±/*FM3*; tor^{RL3}/tor^{RL3}	18	1,950	30	1.5
	19	1,478	11	0.7
	20	1,520	0	0

The frequency of embryonic hatching was assayed 48 h after egg laying; females were mated to Oregon R wild-type males. *A*, Eggs produced by tor^{RL3}/tor^{RL3} females collected from the original stock obtained from T. Schupbach. For *B* and *C* the following stock was made: $l(1)ph/FM3/Dp(1;Y)w^{+303};tor^{RL3}/CyO$. From this stock *l(1)ph/FM3*; tor^{RL3}/tor^{RL3} females were collected and assayed in *B*. For the females assayed in *C*, *l(1)ph/FM3*; tor^{RL3}/CyO females were mated to tor^{RL3}/CyO males and the ±/*FM3*; tor^{RL3}/tor^{RL3} females collected. In this experiment, surviving larvae were phenotypically normal and some developed into adults. The unhatched embryos had segmentation defects which were more extreme at 20 °C than at 18 °C. A null allele, $l(1)ph^{EA75}$, was used in this study. Descriptions of balancer chromosomes, *FM3* and *CyO* in ref. 22.

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Substantial increase of protein stability by multiple disulphide bonds

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DISULPHIDE bonds can significantly stabilize the native structures of proteins[1–3]. The effect is presumed to be due mainly to a decrease in the configurational chain entropy of the unfolded polypeptide[4–7]. In phage T4 lysozyme, a disulphide-free enzyme, engineered disulphide mutants that crosslink residues 3–97, 9–164 and 21–142 are significantly more stable than the wild-type protein[8–11]. To investigate the effect of multiple-disulphide bonds on protein stability, mutants were constructed in which two or three stabilizing disulphide bridges were combined in the same protein. Reversible thermal denaturation shows that the increase in melting temperature resulting from the individual disulphide bonds is approximately additive. The triple-disulphide variant unfolds at a temperature 23.4 °C higher than wild-type lysozyme. The results demonstrate that a combination of disulphide bonds, each of which contributes to stability, can achieve substantial overall improvement in the stability of a protein.

Unpaired cysteine residue(s) in a protein containing a disulphide bond(s) can lead to oligomerization through thiol/disulphide interchange[12]. Previously described single-disulphide mutants (designated as 3C–54T, 9C–164C-wt* and 21C–142C-wt*, see Fig. 1) were, therefore, constructed in an otherwise cysteine-free pseudo wild-type lysozyme (wt*)[11,13]. For the same reason the two double-disulphide mutants D3–97/9–164 and D9–164/21–142 and the triple disulphide mutant T3–97/9–164/21–142 were also designed and constructed to have no unpaired cysteines. Figure 1 shows the locations of the disulphide bonds and ancillary mutations introduced into T4 lysozyme. Each of the mutant genes was expressed in *Escherichia coli* and the proteins purified to homogeneity. Immediately after purification, the mutant enzymes were found to be a mixture of oxidized (crosslinked) and reduced (noncrosslinked) forms, as judged by ion-exchange high-performance liquid chromatography (HPLC)[11], reversed-phase HPLC[8,13] and titration of protein thiols with Ellman's reagent (data not shown). After exposure of proteins to air under mild alkaline conditions (pH 8.0) for several days, however, the mutant proteins were all converted to the oxidized forms (Table 1). Nonreducing SDS-PAGE showed that the double- and triple-disulphide mutants migrate faster than the disulphide-free wild-type lysozyme, whereas all the multiple-disulphide mutants had mobility identical with wild type in the presence of reducing agent (data not shown). This observation indicates that the crosslinked proteins have a more compact structure in the denatured state.

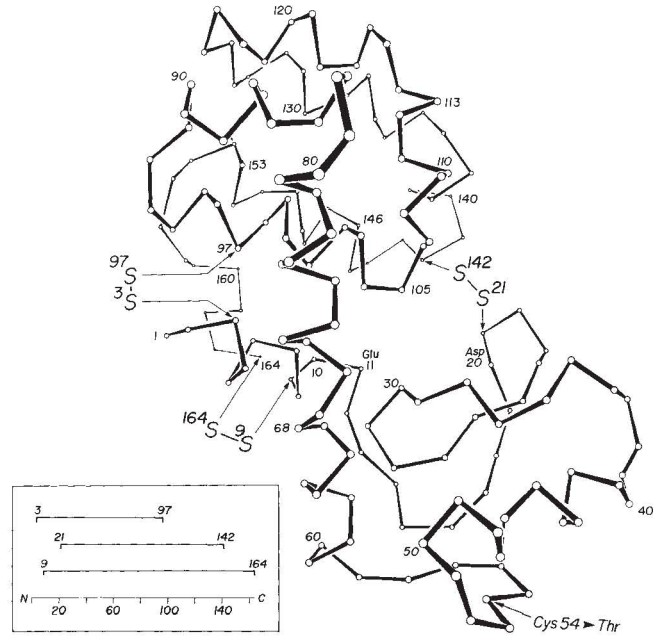

FIG. 1 Backbone of T4 lysozyme showing the locations of the three engineered disulphide bridges. The insert illustrates the loops formed by these bridges. Identification and generation of the mutant lysozymes is as follows:

Variant	Amino-acid replacements 3	9	21	54	97	142	164	No. of cysteines	No. of disulphide bonds
wt	Ile	Ile	Thr	Cys	Cys	Thr	Leu	2	0
wt*				Thr	Ala			0	0
3C–54T	Cys			Thr				2	1
9C–164C-wt*		Cys		Thr	Ala		Cys	2	1
21C–142C-wt*			Cys	Thr	Ala	Cys		2	1
D3–97/9–164	Cys	Cys		Thr			Cys	4	2
D9–164/21–142		Cys	Cys	Thr	Ala	Cys	Cys	4	2
T3–97/9–164/21–142	Cys	Cys	Cys	Thr		Cys	Cys	6	3

Recombinant DNA techniques used in the construction of the disulphide mutants were essentially as described[17]. Mutagenic oligonucleotides (21–23 mer) were synthesized using a model 380B DNA synthesizer (Applied Biosystems) and purified by a C18 Sep-Pak cartridge (Millipore). The single-stranded DNA template for site-directed mutagenesis was an M13mp18 derivative containing the T4 lysozyme gene on a 630 base-pair *Bam*HI–*Hin*dIII fragment. The mutagenesis was performed according to Kunkel *et al.*[18] using *E. coli* strain CJ236 (*dut1, ung1, thi1, relA1*/pCJ105 (Cm^r)). After the repair synthesis of DNA *E. coli* JM101 (ref. 19) was transformed and the mutants identified either by DNA sequencing[20] or plaque hybridization with the [^{32}P]-labelled mutagenic prime[21]. The mutations were verified by sequencing the entire T4 lysozyme gene. The mutated T4 lysozyme gene on M13 was digested with *Bam*HI and *Hin*dIII, and cloned into the expression plasmid pHSe5 that contains *tac* and *lac*UV5 promoters, $lacI^q$ gene as well as *trp* terminator[22]. *E. coli* RR1 (ref. 17) was then transformed by the recombinant pHSe5 and the mutant protein was overproduced by addition of isopropyl-β-thiogalactoside. The mutant proteins were purified to homogeneity by CM-Sepharose and SP-Sephadex (Pharmacia) chromatography as described[22].

The activity of the D3–97/9–164 mutant (Table 1) is indistinguishable from that of wild-type enzyme in both the oxidized and reduced forms. The result is consistent with the observation that the corresponding single-disulphide mutants have essentially the same activities as wt* in both oxidized and reduced forms[11]. In addition it indicates that virtually all the D3–97/9–164 molecules have the correct pairing of the disulphide bridges as any mispairing would presumably lead to a loss of activity. The wt* lysozyme loses enzymatic activity at ~55 °C at pH 7.4, whereas the D3–97/9–164 mutant, which is more thermostable than wt* by 15.7 °C at pH 2.0 (see below), retains activity up to

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