Isolation of temperature-sensitive diphtheria toxins in yeast and their effects on *Drosophila* cells

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Summary

We have isolated temperature-sensitive diphtheria toxins (DT-A^{ts}) to develop a method that allows temporal impedement of cellular functions. Four DT-A^{ts} genes were isolated in a mutagenesis screen using the yeast, *Saccharomyces cerevisiae*. When expressed in yeast, these DT-A^{ts} arrest growth at 18°C but not at 30°C. Three DT-A^{ts} were subsequently tested in the R1-R6 photoreceptor cells of transgenic fruit flies, *Drosophila melanogaster*. The toxins show similar temperature dependence in both organisms, suggesting that they may

be useful in a wide range of non-homeothermic species. DNA sequence analysis revealed that three of the four DT-A^{ts} mutations are novel. Interestingly, the fourth DT-A^{ts} carries the same point mutation as the extensively characterized CRM197, an ADP ribosyltransferase-defective form of diphtheria toxin.

Key words: diphtheria toxin, inducible toxins, temperature-sensitivity, yeast, *Drosophila*.

Introduction

Laser-induced cell ablation experiments have proven to be useful in the study of cell-cell interactions in developing and adult organisms (Sulston, 1988). They allow us to examine the fate of specific cells, to determine cell lineages and to assess the role of cells in vivo. Unfortunately, ablation experiments are often technically difficult to perform and many cells of higher eukaryotes are not easily accessible. Therefore, Palmiter et al. (1987) initiated cell-lineage-specific ablation experiments in mice by introducing the gene for the fully active "A" subunit of the diphtheria toxin (DT-A) under the control of cell-specific regulatory sequences. This elegant approach to studying specific cell lineages has also been employed to define the role of specific cells in living mice (Behringer et al., 1988; Breitman et al., 1987; for review see Evans, 1989), and *Drosophila* (Kunes and Steller, 1991). However, the system is not generally applicable. First, constitutive expression of the toxin in essential cells is lethal, and precludes the establishment of stable lines for further study unless the toxins can be expressed conditionally (Kunes and Steller, 1991). Second, relatively few highly time- and tissue-specific regulatory sequences of genes have been characterized. Such sequences may be difficult to identify as many genes that seem to be expressed in specific tissues at a particular time of development are

often differentially expressed in other tissues or at other developmental stages (Bellen et al., 1989; Wilson et al., 1989; Grossniklaus et al., 1989). These regulatory sequences cannot be used for ablation experiments, although they are tissue specific at some stage of development. Third, temporal control of the expression of the toxin in a particular tissue or cell is dependent on the regulatory sequences that control the expression of the toxin. This temporal control cannot be easily manipulated. Thus, there are almost no cases in which one can govern time, duration and tissue-specificity of gene expression. To circumvent these problems, we have isolated temperature-sensitive variants of the diphtheria toxin in yeast.

Diphtheria toxin was chosen because its mechanism of action is understood (Collier, 1975), its sequence is known (Greenfield et al., 1983), its expression has been adapted for eukaryotic cells (Maxwell et al., 1987), and it is active in all eukaryotic cells tested (Pappenheimer et al., 1980) including yeast (Perentesis et al., 1988). The toxin's action is mediated by the inhibition of protein synthesis through ADP-ribosylation of elongation factor 2 (for review see Pappenheimer, 1977). In addition, recent but controversial evidence indicates that the toxin also acts as an endonuclease in vitro (Chang et al., 1989; Bruce et al., 1990; Nakamura and Wisnieski, 1990). A single molecule of the fully active toxin is sufficient to kill a mammalian cell

(Yamaizumi et al., 1978). The toxin has two subunits. The B subunit is required for receptor binding, whereas the A subunit used in this study is the toxic catalytic domain. Expression of DT-A inside cells usually leads to death without apparent damage to neighboring cells. Should any DT-A escape (e.g from death cells) the absence of DT-B precludes cell attachment and acid translocation.

Materials and methods

Cloning methods

pSE979 was constructed by means of a three-way ligation of an *Eco*R1-*Sma*1 GAL1 fragment derived from pSE278 (unpublished data), an *Eco*RV-*Not*1 DT-A fragment from pSE968 (unpublished data, this DT-A fragment was originally present in p2249-1, Maxwell et al., 1987) into the *Eco*R1-*Not*1 sites of pUN35 (Elledge and Davis, 1988). The restriction map of this and other vectors is shown in Fig. 1.

The pRXC4 (where X=M, R, I and N) plasmids were constructed using three-way ligations of the following fragments: a *Bam*H1-*Kpn*1 fragment from the *ninaE* promoter sequences from plasmid pDPS48 (a kind gift from Dean P.

Smith and Charles Zuker) and the *Kpn*1-*Xba*1 DT-A sequences (fully active (=D)) or temperature-sensitive toxins (=M, R, N, I) from pSE979 (D) or mutagenized pSE979 (M, R, I, N) into the *Bam*H1-*Xba*1 sites of pCaSpeR4 (Pirrotta, 1988). Only the most relevant restriction enzyme sites are shown in Fig. 1. The restriction enzyme sites that are shown in bold can be used to replace the *ninaE* promoter with regulatory sequences of other genes.

Sequencing strategies

To sequence the wild-type and mutant DT-A^{ts} clones, we subcloned the *Kpn*1-*Xba*1 fragments referred to in Cloning Methods into Bluescript KS and SK. Single-stranded DNA was sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with the automated fluorescence procedure (Applied Biosystems) as described by Smith et al. (1986). This allowed us to obtain one full-length sequence in all cases. To obtain the complete sequence on both strands, we used four internal primers to the DT-A. These additional sequences were obtained using the manual dideoxy chain-termination method on double-stranded DNA using ^{35}S as label.

Fly strains, culture and temperature shifts

y w fly strains were obtained from V. Pirrotta. Fly strains used

Restriction map of **pSE979** (7.2 kb). This plasmid is derived from the pUN35 shuttle vector and contains the fully active toxin (DT-A) (about 800 bp) under the control of the yeast GAL 1 promoter (about 800 bp)
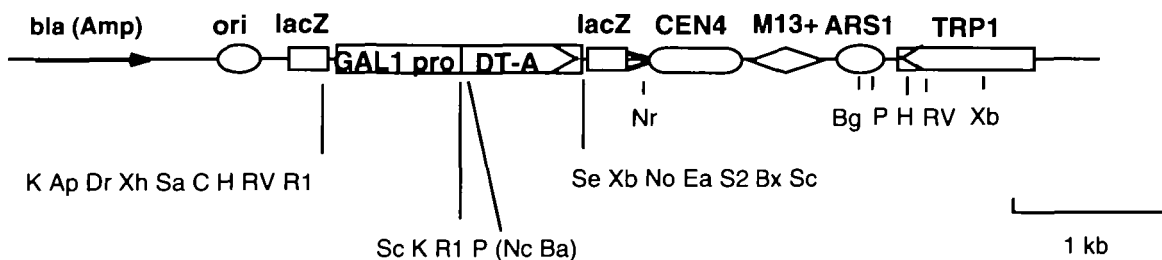
Restriction map of **pRXC4** (9.2 kb). This plasmid is derived from pCaSpeR4 and consists of the 5' and 3' ends of a P-element which are required for transformation into *Drosophila*, the *white* gene to allow identification of transformed flies, the *ninaE* promoter, which directs expression of foreign genes in R1-R6 cells, and the temperature-sensitive DT-Ats.
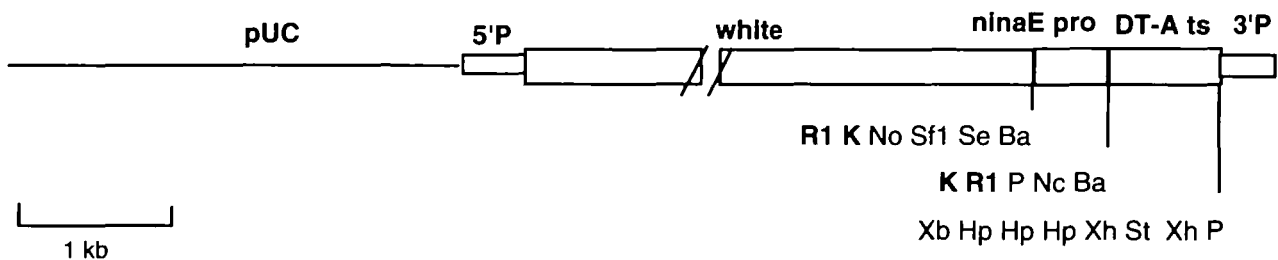

Fig. 1. Vectors used in this study. Abbreviations: Amp, Ampicillin; ori, origin of replication; *lacZ*, β-galactosidase gene; CEN4, yeast centromeric DNA; M13+, phage M13 origin of replication; ARS1, yeast autonomous replication sequence; TRP1, yeast tryptophan synthetase gene; *ninaE* pro., *ninaE* promoter; 5'P and 3'P, 5' and 3' ends of the *Drosophila* P-transposable element; Ap, *Apa*1; Ba, *Bam*H1; Bgl, *Bgl*1; Bg, *Bgl*II; Bx, *Bst*X1; C, *Cla*1; Dr, *Dra* II; Ea, *Eag*1; Hp, *Hpa*1; H, *Hind*III; K, *Kpn*1; Nc, *Nco*1; No, *Not*1; Nr, *Nar*1; P, *Pst*1; R1, *Eco*R1; RV, *Eco*RV; Sa, *Sal*1; Sc, *Sac*1; S2, *Sac*II; Se, *Spe*1; Sf, *Sfi*1; Sm, *Sma*1; Sp, *Sph*1; St, *Stu*1; Xb, *Xba*1; Xh, *Xho*1.
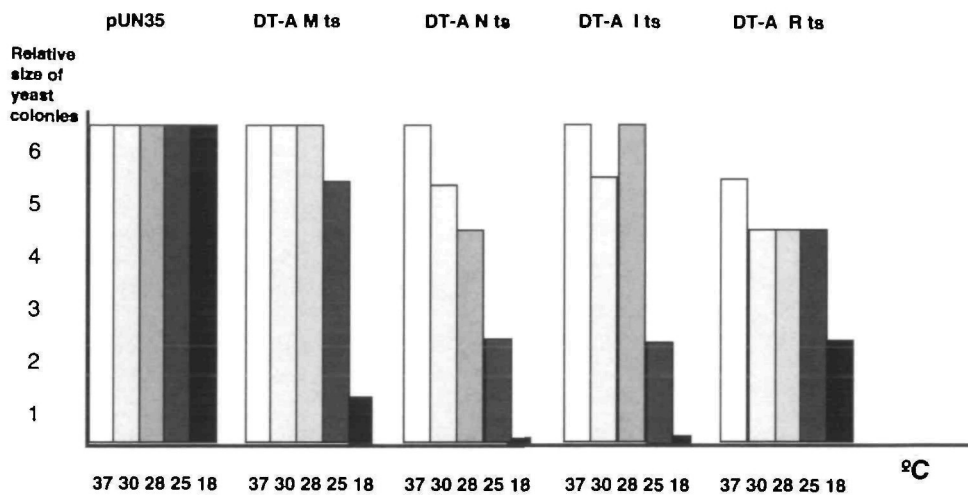
Fig. 2. Relative size of yeast colonies grown on galactose-containing media after several days at various temperatures.

to map the various insertions were obtained from Ed Grell. The *yw; Ki p^p P[delta 2-3]* strain was obtained from Y. Hiromi.

Flies were cultured on standard fly food supplemented with dry yeast at 16°C, 18°C, 25°C or 28°C in a water bath or a fly incubator. No substantial differences were observed between the two treatments. Unless stated otherwise, flies are grown for their entire life cycle at a specific temperature. Temperature shifts prior to pupariation (up or down) do not have any obvious effect on the morphology of the photoreceptor cells as long as the pupae and adults are kept at 25°C or 28°C. Temperature shifts are performed by transferring a vial containing mid- to late-stage pupae or 0-12 hour freshly emerged flies from an incubator to a water bath set at 16°C or 18°C for various lengths of time. The vials were subsequently returned to an incubator at 25°C or 28°C for 1-4 days.

Transformation and mapping of P-elements

Flies were transformed by injecting 300 ng/μl of the appropriate P-element vector (derivatives of pCaSpeR 4) into *yw* embryos according to standard protocols using the wings clipped vector as a helper (Rubin and Spradling, 1982). P-element insertions were assigned to chromosomes with standard crosses using *y w; CyO/Pm^{88k}* and *y w; TM6B, D^3/Ki H^{B55}* stocks, and many P-element insertions were cytologically mapped using digoxygenin-labeled probes.

We injected about four times as many embryos with the P[RDC4] constructs than for the other constructs. Two male P[RDC4] transformants were sterile but two females were fertile. Both original transformant strains established from these females are male sterile. In order to obtain additional transformants, we created transposant strains using the P[delta 2-3] methodology (Cooley et al., 1988; Robertson et al., 1988). A total of 9 fly strains carrying the P[RDC4] construct were established. Some of these fly strains are homozygous viable and male and female fertile. We generated at least five independent transformants for each construct.

Mosaic analysis

To create mosaic eyes, flies carrying P[RDC4] were crossed to *y w; Ki p^p P[delta 2-3]*. Progeny carrying both P[RDC4] and the *Ki p^p P[delta 2-3]* chromosome and exhibiting patches of red and unpigmented ommatidia were analyzed with the compound scope. Some heads were prepared for transmission electron microscopy (TEM) studies.

Determining the effects of DT-A expression in yeast cells

Single yeast colonies were grown in glucose media lacking tryptophan to select for pSE979 for two days. 10 μl of this culture was plated out on galactose media lacking tryptophan. The plates were inspected daily and the colony sizes were compared with those of cells containing the positive control (pUN35). The data shown in Fig. 2 correspond to a visual estimate of the size of the colonies on a scale of 6 at day three for plates kept at 37°C, 30°C, 28°C and 25°C (0=no growth, 6=size of yeast cell colonies carrying the pUN35 plasmid). The data reported for cells grown at 18°C were scored on day 6.

Histology

Heads of flies were fixed in 100 mM sodium phosphate (pH 7.0), 5 mM EDTA, 3% formaldehyde and 2.5% glutaraldehyde. After washing the heads with ddH₂O, the heads were fixed in 2% osmium tetroxide. Heads were embedded in historesin or Spurr's and semi-thin sections (3 μm) and thin sections (0.08 μm) were prepared for light microscopy and TEM.

Results

To isolate temperature-sensitive DT-A (DT-A^{tsX}), DT-A (Maxwell et al., 1987) was placed under the control of the GAL1 promoter on a yeast centromeric vector, pUN35, that contained TRP1 as a selectable marker (Elledge and Davis, 1988). This plasmid, pSE979, was used to transform the diploid CRY 3 yeast cells. A diploid strain was chosen because there are five recessive genes in yeast that confer resistance to the toxin when mutant (Perentesis et al., 1988), leading to high background reversion frequencies in haploids. CRY 3 (pSE979) cells grow on glucose-containing medium but fail to form colonies on galactose-containing medium, unlike the CRY 3 (pUN35) cells harboring the vector without the DT-A. CRY 3 (pSE979) cells were mutagenized with ethylmethane sulfonate (EMS, survival rate about 50%), allowed to recover for two generations and plated on media lacking tryptophan and containing galactose as a carbon source at 37°C. Approximately 600 colonies grew at
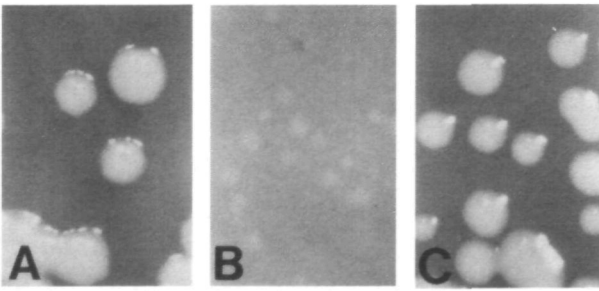
Fig. 3. Yeast colonies formed by CRY III strains carrying DT-A^{tsM}. (A) Colonies formed by a CRY III strain transformed with pSE979 containing the DT-A^{tsM}. Grown for 3 days at 30°C on galactose-containing medium (DT-A^{tsM} is expressed). (B) Same as in A but grown for 7 days at 18°C (DT-A^{tsM} is expressed and active). The photograph is overexposed to show what is growing on the plates. (C) Same as in B but transformed with pUN35 (no DT-A expressed).

37°C. 22 of the 600 colonies either failed to grow or grew very poorly at 18°C. It was shown that the temperature sensitivity was plasmid- dependent in at least four cases by retransforming yeast cells with the isolated mutant pSE979 plasmids. As illustrated in Fig. 2 and Fig. 3, the CRY 3 (pUN35) cells grew normally on selective medium containing galactose. In contrast, CRY 3 (pSE979) expressing wild-type DT-A did not grow on this medium at any temperature tested (not shown). CRY 3 expressing DT-A^{tsX} (X=M,N,I, and R) grew at elevated temperatures (37°C, 30°C and 28°C) but failed to grow or grew poorly at 18°C, but each independent ts-isolate displayed a characteristic temperature-dependence profile. As shown in Fig. 3, yeast cells containing the DT-A^{tsM} at 18°C form a very small translucent colony that can barely be seen. Yeast cells carrying DT-A^{tsX} are not always killed; some are only arrested or very severely retarded in their growth at 18°C. When the plated yeast cells expressing the DT-A$^{ts\ M,\ I\ and\ R}$ toxins are held at 18°C for 7 days and subsequently shifted to 25°C, few will resume growth and form colonies within 2-3 days. However, the cells harboring the DT-A^{tsN} require an extra day to recover. Hence, we conclude that cellular functions required for the growth of yeast are very severely affected at 18°C, but not at elevated temperatures.

To establish whether the DT-A^{ts} mutant variants isolated in yeast were also active and temperature sensitive in multicellular non-homeothermic eukaryotic organisms, they were introduced under the control of the ninaE promoter into the fruitfly. The ninaE gene is expressed abundantly in the R1-R6 photoreceptor cells (Mismer and Rubin, 1987) that are present in each of the ommatidia of the compound eye. Expression of the ninaE gene starts during the final stages of photoreceptor cell differentiation in mid pupae (48-60 hours after puparium), and continues in adult flies (Zuker et al. 1985). The gene is not known to be expressed at a detectable level in cells other than retinal cells and the larval photoreceptor organ (Mismer and Rubin, 1987; Pollock and Benzer, 1988). We surmised that it should

be possible to recover transformants that express the fully active DT-A as well as transformants that express the DT-A^{ts}, thereby allowing comparison of the defects caused by the different toxins in vivo. In addition, expression of DT-A in R1-R6 cells offers other advantages: first, transillumination of the head permits identification of the pseudopupil and rapid detection of defects in the organization of the photoreceptor cells and their rhabdomeres (Franceschini and Kirschfeld, 1971); second, the eight individual photoreceptor cells (R1-R8) can be easily identified on the basis of morphological features and position within each ommatidium in semi-thin histological sections; third, DT-A expression in the R1-R6 cells should not affect R7, R8 and other ommatidial cells if the DT-A toxins are cell autonomous.

We cloned the four DT-A^{ts} (M, N, I and R) genes as well as the wild-type DT-A gene under the control of the ninaE regulatory sequences (Mismer and Rubin, 1987) in the CaSpeR4 transformation vector (Pirotta, 1988). These plasmids named pRXC4 (R=ninaE promoter, X=DT-A M/N/I/R or D for the fully active toxin, C4=CaSpeR4), were injected into Drosophila embryos. Many transformant flies were recovered that contained P[RMC4], P[RRC4], P[RIC4] or P[RDC4]. No transformant flies were recovered that carried P[RNC4]. Flies of all these strains, except those that carry the fully active toxin, fail to show external morphological defects at any temperature. However, flies of the nine independent transformant lines that carry the fully active toxin, have rough eyes and abnormal ommatidia (compare Fig. 4A and B), and often lack bristles. Some strains lack many bristles whereas others lack one or two thoracic bristles only. Males of some of these strains are sterile, or substerile. However, we have been able to isolate and maintain healthy homozygous lines that carry the fully active toxin. Hence, we believe that the bristle defects and male sterility problems are due to ectopic expression of the fully active toxin. Since there is great variability for the bristle defects and fertility between the different strains it is very likely that these defects are the result of position effects (for review see Wilson et al., 1990).

To establish which cells and subcellular components were affected by DT-A, we prepared ultrathin sections for transmission electron microscopy (TEM). As shown in Fig. 4B, the morphology of the R1-R6 cells is severely altered in the P[RDC4] strains. When compared to normal R1-R6 cells (see Fig. 4A), the rhabdomeres of P[RDC4] flies are very small and abnormally shaped, the size of the R1-R6 cells is highly variable, they contain many fewer ribosomes, some cells contain many mitochondria, and the cytoplasm and nuclei stain less densely. In contrast, the R7 or R8 cells maintain essentially normal morphological features (see Fig. 4B), indicating that the DT-A acts in a cell autonomous fashion. Since the compounds used to reveal the cellular structures by TEM stain mostly proteins and nucleic acids and since the rhabdomeres are very small or absent, it is likely that protein synthesis is dramatically reduced or arrested and that
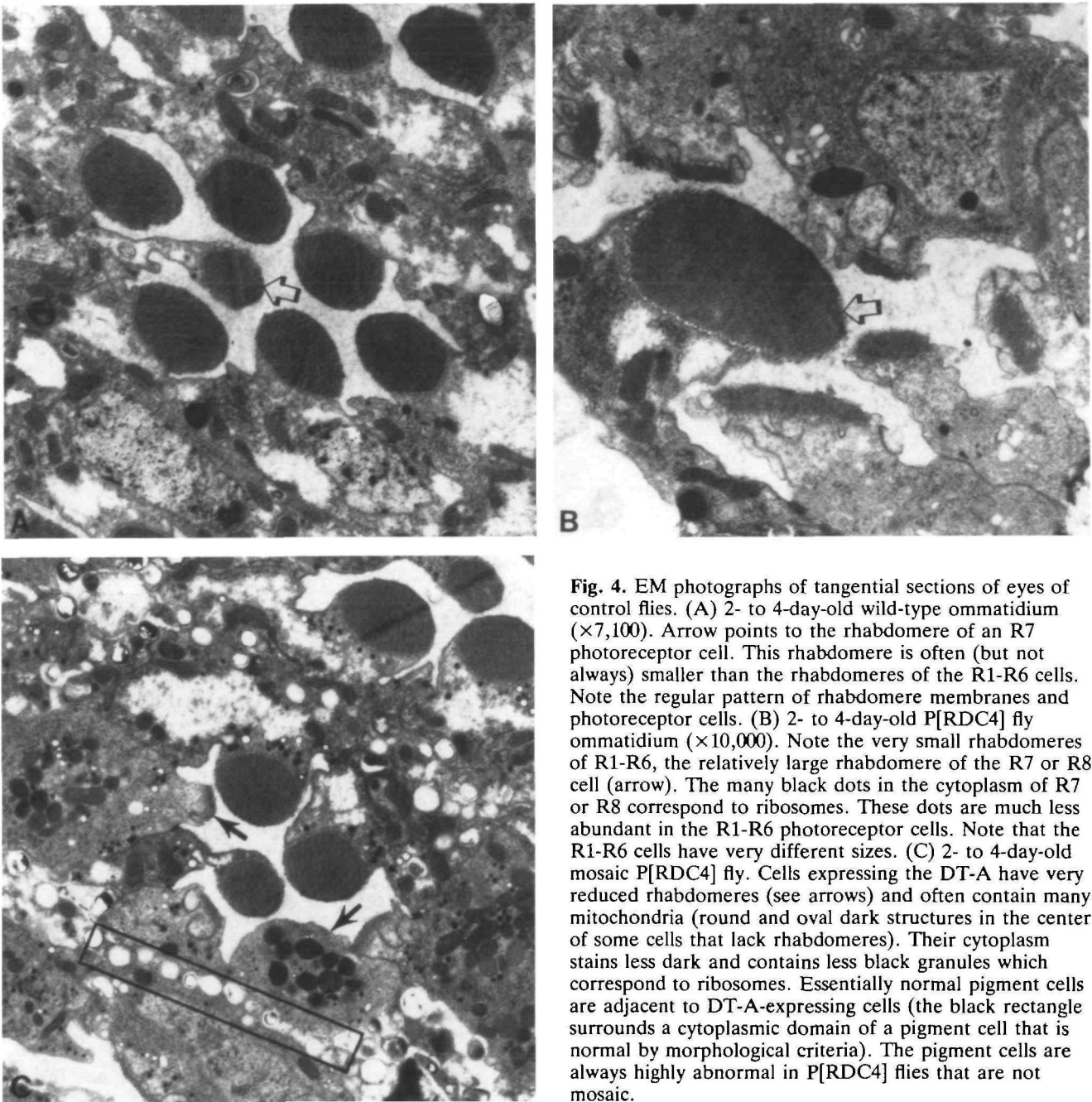
Fig. 4. EM photographs of tangential sections of eyes of control flies. (A) 2- to 4-day-old wild-type ommatidium (×7,100). Arrow points to the rhabdomere of an R7 photoreceptor cell. This rhabdomere is often (but not always) smaller than the rhabdomeres of the R1-R6 cells. Note the regular pattern of rhabdomere membranes and photoreceptor cells. (B) 2- to 4-day-old P[RDC4] fly ommatidium (×10,000). Note the very small rhabdomeres of R1-R6, the relatively large rhabdomere of the R7 or R8 cell (arrow). The many black dots in the cytoplasm of R7 or R8 correspond to ribosomes. These dots are much less abundant in the R1-R6 photoreceptor cells. Note that the R1-R6 cells have very different sizes. (C) 2- to 4-day-old mosaic P[RDC4] fly. Cells expressing the DT-A have very reduced rhabdomeres (see arrows) and often contain many mitochondria (round and oval dark structures in the center of some cells that lack rhabdomeres). Their cytoplasm stains less dark and contains less black granules which correspond to ribosomes. Essentially normal pigment cells are adjacent to DT-A-expressing cells (the black rectangle surrounds a cytoplasmic domain of a pigment cell that is normal by morphological criteria). The pigment cells are always highly abnormal in P[RDC4] flies that are not mosaic.

the intoxicated cells are non-functional. Other cells and structures of each ommatidium, like cornea and cone cells, seem not to be affected by the expression of DT-A. However, many pigment cells are absent or highly abnormal, indicating that DT-A is active in these cells. The partial lack and loss of pigment in many ommatidia can also be seen under the dissection microsope in some homozygous viable P[RDC4] flies. Hence, DT-A is either not cell autonomous for all eye cells or DT-A is expressed in certain pigment cells.

To determine if DT-A is indeed cell autonomous, P-transposase-mediated excisions of P[RDC4] were induced. In many mosaic eyes examined, some clusters of ommatidia were wild-type and white (DT-A and red

pigment absent because of the excision of P[RDC4]) whereas adjacent clusters of ommatidia were defective and red (DT-A and white+ genes of P[RDC4] expressed). TEM of these mosaic eyes showed that morphologically normal photoreceptor cells or pigment cells can be almost completely surrounded by cells that have the typical morphological defects of DT-A expressing cells (see Fig. 4C for a mosaic ommatidium). These observations indicate that the toxin acts in a cell autonomous fashion, and that the pigment cells express DT-A. Hence, the regulatory sequences of the *ninaE* gene most likely confer toxin expression to the pigment cells in our constructs. It is therefore possible that the *ninaE* gene is also expressed at low levels in sensitive
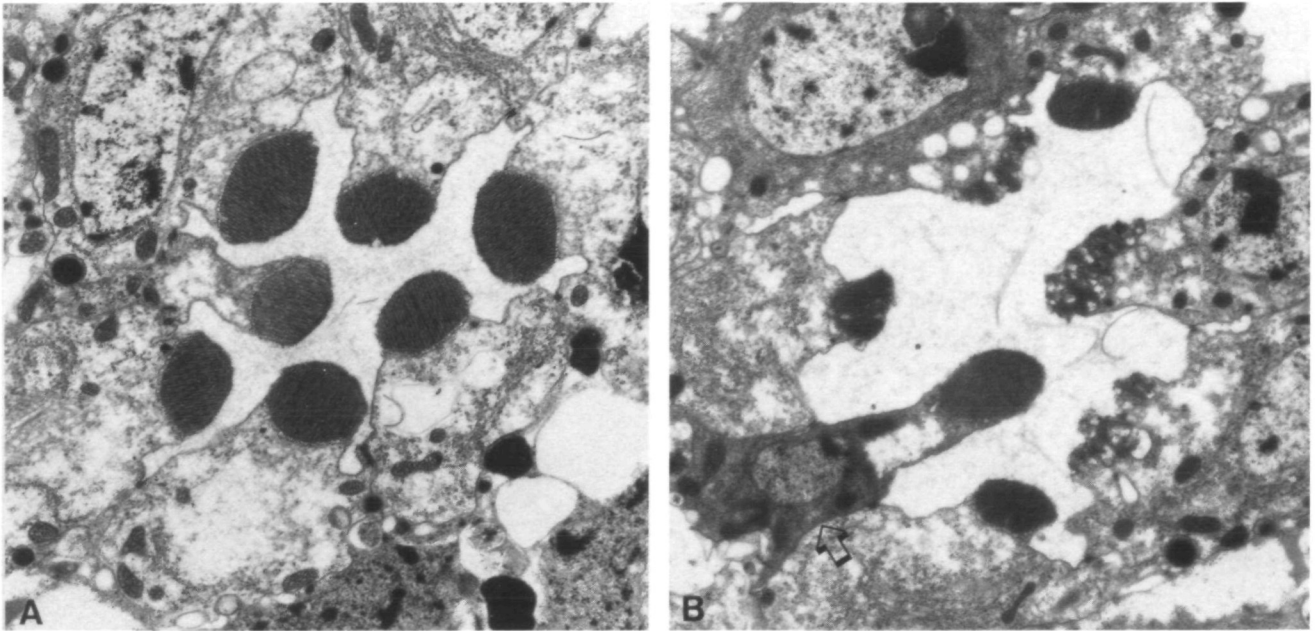
Fig. 5. EM photographs of tangential sections of eyes of flies that express DT-A^{tsM}. (A) 2- to 4-day-old P[RMC4]45A ommatidium (numbers after the brackets, like 45A, correspond to the cytological band where the P-element maps) of a fly maintained at 25°C. (B). 2- to 4-day-old ommatidium of a P[RMC4]45A fly maintained at 18°C. Compare the cell morphology of A and B. Note the reduced size of the rhabdomeres of the R1-R6 cells, the three totally abnormal rhabdomeres, the irregular shape and sizes of the R1-R6 cells, the abnormal spacing between the rhabdomeres, and the much darker cytoplasm of R7 or R8 (arrow).

pigment cells, and no evidence has been presented to the contrary in previous studies (Mismer and Rubin, 1987; Pollock and Benzer, 1988). Alternatively, the construct causes ectopic expression of the DT-A in pigment cells.

To date, we have established eight strains that carry single copies of the P[RMC4] (=ninaE-DT-A^{tsM})

construct. Flies carrying this construct show no roughened eye phenotype, bristle defects or male sterility. Transillumination of the eyes of flies reared at 25°C shows an essentially normal pseudopupil. However, transillumination of the eyes of flies of the same strains reared at 18°C shows an abnormal pseudopupil, indicating that the R1-R6 rhabdomeres of these flies are

Fig. 6. Phase-contrast microscopy photographs of tangential sections through the ommatidia of *Drosophila* eyes. The photographs show sections through 10-20 ommatidia of adult eyes (magnification=×2,000 or ×2,500). The rhabdomeres of the R7 and R8 photoreceptors are in the center of each ommatidium. Any section will only show a single central rhabdomere since the R7 and R8 rhabdomeres are only about half as long as R1-R6 rhabdomeres and are contiguous. In all cases, we analyzed sections of more than ten flies. (A) A 5-day-old *yw* fly. Arrow points to a rhabdomere of an R7 cell which is normally smaller than the rhabdomeres of R1-R6; (B) A one-day-old *yw* fly which carries a single copy of the P[RDC4]53A (wild-type DT-A) construct which was maintained at 25°C (insertion at 53A). The R1-R6 cells are almost not recognizable but they surround an essentially normal rhabdomere of the R7 or R8 cell (arrow, see also Fig. 4B); (C) A 10-day-old *yw* fly which carries a single copy of the P[RMC4]45A (DT-A^{tsM}) and which was maintained at 25°C. About 5% of the R1-R6 cells are abnormal and have reduced rhabdomeres and abnormal overall cell morphology (arrow); (D) A one-day-old *yw* P[RMC4]45A fly which developed at 18°C. Arrow at bottom points to an R7 or R8 rhabdomere which appears larger and rounder than the rhabdomeres of R1-R6. The cytoplasm of the R1-R6 cells is enlarged (top arrow); (E

and F) Flies kept in conditions similar as in C and D. However, this strain carries an insertion in chromosome arm 3L (=P[RMC4]3L); (G) a 5-day-old *yw* P[RMC4]3L fly (conditions see C.); (H) A 2-day-old P[RMC4]3L fly which was cold treated at 16°C for 6 hours on the first day. Note the relatively large size of the R7 or R8 rhabdomeres, the flattening on the cytoplasmic side of the rhabdomeres, and the enlarged cytoplasmic area; (I) A 10-day-old P[RMC4]45A which was kept at 25°C before eclosion and 18°C after eclosion. Note the round and centrally positioned rhabdomere in each ommatidium, and the virtual absence of rhabdomeres in other photoreceptor cells; (J) A 2- to 3-day-old *yw* fly which carries a single copy of the P[RRC4]3C-D (DT-A^{tsR}) and which was maintained at 28°C. Note that many rhabdomeres have different sizes and shapes (see arrows); (K) A 2- to 3-day-old *yw* P[RRC4]3C-D fly which was maintained at 18°C. Compare K with J. Many R1-R6 cells in K are severely affected; (L) A 2- to 3-day-old *yw* P[RRC4]3L fly which was maintained at 28°C. Very few cells have defective structures. Some of the R1-R6 cells have reduced and abnormal rhabdomeres (arrow). (M) A 2- to 3-day-old *yw* P[RRC4]3L fly maintained at 18°C. The R1-R6 cells in each ommatidium exhibit obvious morphological defects but the defects are significantly less severe than in the strain shown in K.

defective. We observe either no or minor defects in the overall organization and structure of the individual ommatidia or cells at 25°C (see Figs 5A, 6C, E and G). Approximately 5% of the R1-R6 cells in the P[RMC4] transformant strains exhibit an abnormal rhabdomere when the flies are maintained at 25°C. This indicates that DT-A^{tsM} activity is strongly attenuated at 25°C but that some residual activity may occasionally cause cell damage. This residual activity is not detrimental to more than 90% of the cells since 10-day-old flies (see

Fig. 6C, and E) have the same subtle defects as one-day-old flies (data not shown). In contrast, freshly emerged P[RMC4] flies that are reared at 18°C show severe morphological defects in the R1-R6 cells and the pigment cells, but have seemingly normal R7 and R8 cells (see Fig. 5B, 6D, and F). The morphological defects in R1-R6 cells are less severe than those observed in R1-R6 cells of flies of the same age that carry the fully active toxin (compare Figs 5B and 4B, and Fig. 6B with F and D). The rhabdomeres of the R1-
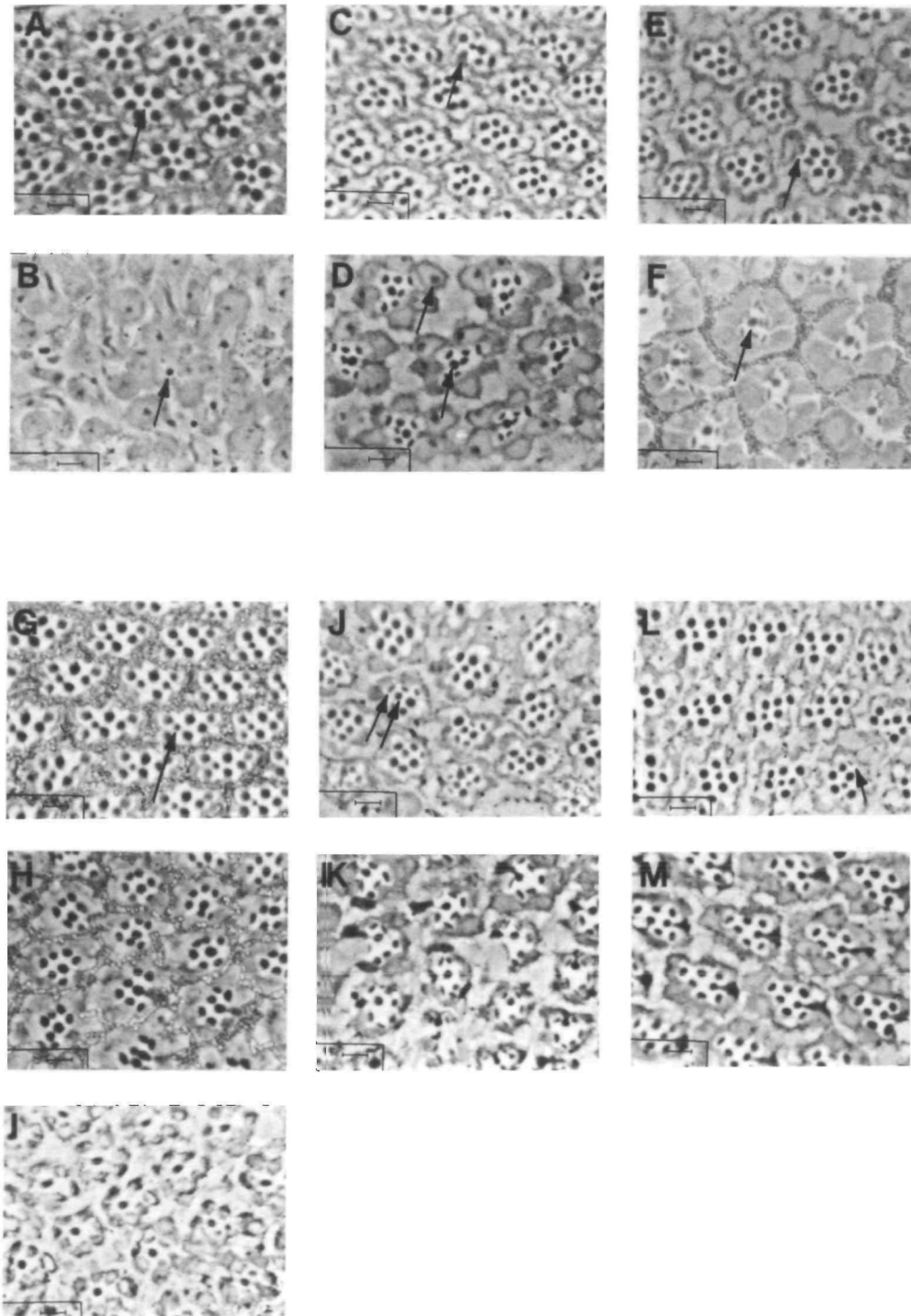
R6 cells are abnormal. Most rhabdomeres are significantly smaller than those of R7 or R8 (normally R7 and R8 rhabdomeres are smaller than those of R1-R6). In addition, many rhabdomeres contain vacuoles that disrupt the organization of the membranes of the rhabdomeres. Finally, as is observed in flies that carry P[RDC4], the pigment cells seem to be affected and many pigment granules are not included in cells, suggesting that the integrity of these cells is affected and that pigment cells may be more sensitive to the toxin than the photoreceptor cells. Hence, we conclude that DT-A^{tsM} is temperature-sensitive, attenuated and functional in both yeast and *Drosophila* cells. In addition, the temperature sensitivity in *Drosophila* is similar to that in yeast since the DT-A^{tsM} toxin shows no overt phenotype in yeast at 25°C but inhibits growth almost completely at 18°C.

To establish the minimal cold treatment necessary to induce obvious morphological defects in the R1-R6 cells of strains expressing DT-A^{tsM}, we exposed flies to different temperatures for various length of time. The minimal cold treatment that caused obvious morphological defects in differentiated R1-R6 cells was consistently obtained with a 6 hour cold treatment at 16°C of freshly emerged flies (see Fig. 6G, H and legend for description of the defects). Shorter cold treatments (3 hours) led to no obvious defects. In general, cold treatments after photoreceptor differentiation (adults) cause less severe defects than cold treatments during photoreceptor differentiation (mid to late pupae). However, if the cold treatment of adult flies that where raised at 25°C is maintained for 5 to 10 days, severe morphological defects can be induced (see Fig. 6I). Thus, altering the structural features of cells that are fully differentiated appears to be more difficult than altering those of differentiating cells.

Analysis of the six established strains that carry single P[RRC4] (=ninaE-DT-A^{tsR}) insertions revealed that significant differences exist among various P[RRC4] strains: some strains exhibited obvious defects at 28°C but the defects are much more pronounced at 18°C (compare Fig. 6J and K); others show very subtle or no defects in most ommatidia at 28°C and the defects at 18°C are not very severe (compare Fig. 6L and M). This indicates that DT-A^{tsR} is attenuated and that its effects are dependent on the position of the insertion in the fly genome. Similar observations were made for the DT-A^{tsI} although this toxin seems to be more active than DT-A^{tsR}. Since each P[RRC4] or P[RIC4] strain consistently exhibits a more severe phenotype at 18°C than at 28°C, DT-A^{tsR} ^{and} ^{I} are also temperature sensitive. Furthermore, as noted for DT-A^{tsM}, the temperature-sensitive profiles of DT-A^{tsR} ^{and} ^{I} in yeast and *Drosophila* are similar.

The four DT-A^{ts} genes and the wild-type DT-A gene were sequenced to characterize the molecular alterations underlying the temperature sensitivity (see Fig. 7). DT-A^{tsM} carries a point mutation resulting in a Gly^{53} → Glu^{53} substitution that has already been reported (Giannini et al., 1984). The corresponding mutant form of diphtheria toxin is known as CRM197.

ATG GAT CCT GAT GAT GTT GTT GAT TCT TCT AAA TCT TTT GTG ATG GAA 48
met asp pro asp asp val val asp ser ser lys ser phe val met glu 16
ATA
no met in DT-Ats

AAC TTT TCT TCG TAC CAC GGG ACT AAA CCT GGT TAT GTA GAT TCC ATT 96
asn phe ser ser tyr his gly thr lys pro gly tyr val asp ser ile 32
CAA AAA GGT ATA CAA AAG CCA AAA TCT GGT ACA CAA GGA AAT TAT GAC 144
gln lys gly ile gln lys pro lys ser gly thr gln gly asn tyr asp 48
TAA
*** in DT-Atsi

GAT GAT TGG AAA GGG TTT TAT AGT ACC GAC AAT AAA TAC GAC GCT GCG 192
asp asp trp lys gly phe tyr ser thr asp asn lys tyr asp ala ala 64
GAG
glu in DT-Atsm

GGA TAC TCT GTA GAT AAT GAA AAC CCG CTC TCT GGA AAA GCT GGA GGC 240
gly tyr ser val asp asn glu asn pro leu ser gly lys ala gly gly 80
GTG GTC AAA GTG ACG TAT CCA GGA CTG ACG AAG GTT CTC GCA CTA AAA 288
val val lys val thr tyr pro gly leu thr lys val leu ala leu lys 96
GTG GAT AAT GCC GAA ACT ATT AAG AAA GAG TTA GGT TTA AGT CTC ACT 336
val asp asn ala glu thr ile lys lys glu leu gly leu ser leu thr 112
GAA CCG TTG ATG GAG CAA CTC GGA ACG GAA GAG TTT ATC AAA AGG TTC 384
glu pro leu met gly gln val gly thr glu glu phe ile lys arg phe 128
GGT GAT GGT GCT TCG CGT GTA GTG CTC AGC CTT CCC TTC GCT GAG GGG 432
gly asp gly ala ser arg val val leu ser leu pro phe ala glu gly 144
AGT TCT AGC GTT GAA TAT ATT AAT AAC TGG GAA CAG GCG AAA GCG TTA 480
ser ser ser val glu tyr ile asn asn trp glu gln ala lys ala leu 160
TAG
*** in DT-Atsm

AGC GTA GAA CTT GAG ATT AAT TTT GAA ACC CGT GGA AAA CGT GGC CAA 528
ser val glu leu glu ile asn phe glu thr arg gly lys arg gly gln 176
GAT GCG ATG TAT GAG TAT ATG GCT CAA GCC TGT GCA GGA AAT CGT GTC 576
asp ala met tyr glu tyr met ala gln ala cys ala gly asn arg val 192
AGG CGA TCT CTT TGT GAA GGA ACC TTA CTT CTG TGG TGT GAC ATA ATT 624
arg arg ser leu cys glu gly thr leu leu leu trp cys asp ile ile 208
GGA CAA ACT ACC TAC AGA GAT TTA AAG CTC TAA   657
gly gln thr thr tyr arg asp leu lys leu ***   218

Fig. 7. Sequence comparisons of the wild-type and temperature-sensitive diphtheria toxins. The eukaryotic expression construct of Maxwell et al. (1987) encodes a protein that differs from the wild-type diphtheria toxin at the N-terminal end as it lacks the signal peptide. In addition, our sequence data revealed that this construct encodes a toxin A subunit that bears 23 additional amino acids at the carboxy terminus not found in the wild-type toxin (underlined).

The three other ts-forms of DT-A that we have isolated have not previously been reported or engineered (Collier, 1990).

Discussion

In this work, we describe the isolation of four temperature-sensitive diphtheria toxins (DT-A^{ts}) in yeast that are active at 18°C and have reduced activity or are inactive at 25°C to 37°C. Three of these DT-A^{ts} as well as the fully active DT-A were transformed under the control of the regulatory sequences of the *Drosophila ninaE* gene into the fruitfly. This is to our knowledge the first report in which viable and healthy transformants in *Drosophila* have been obtained that carry fully active or attenuated toxins (for previous attempts see Kunes and Steller, 1991). The activities of DT-A^{tsM} and ^{R} are clearly attenuated at all temperatures, with DT-A^{tsR} activity being most dependent on the transgene position in the *Drosophila* genome. The

activity of DT-A^{tsI} is the least attenuated as several strains show very severe defects in the R1-R6 at 18°C (not shown). The DT-A^{tsM} toxin may be the most useful toxin as it causes very few or no defects at 25°C and 28°C (data not shown), and seems to act relatively fast at 16°C since a 6 hour cold treatment has an effect on the morphology of the photoreceptor cells in which it is expressed.

Preliminary experiments show that the DT-A$^{tsM \text{ and } R}$ can easily be integrated in the fly genome under the control of other regulatory sequences such as the yeast regulatory sequences (UAS) that respond only to the yeast transcriptional activator GAL4. Hence, these toxins may become useful tools to express the DT-A^{ts} in tissues other than the photoreceptor cells using a binary system (Ornitz et al., 1991) with on the one hand, enhancer detector strains (Bellen et al., 1989; Wilson et al., 1989) that express the GAL4 gene instead of the β-galactosidase gene, and, on the other hand, the strains that carry the UAS-DT-A^{ts}. A. Brand and N. Perrimon have developed such a binary system in *Drosophila* (personal communication.).

Analysis of the sequence of the mutant toxins has revealed a number of important observations that should help resolve controversial issues with respect to the action of the DT-A itself. Indeed, DT-A^{tsM} was found to carry the same point mutation as CRM197. CRM197 has no cytotoxic effects (Uchida et al., 1973), exhibits structural and biochemical properties that distinguish it from the wild-type toxin (Bruce et al., 1990; Hu and Holmes, 1987; Bigio et al., 1987; Mekada and Uchida, 1985; Papini et al., 1987; Collins and Collier, 1985), does not cause ADP ribosylation of elongation factor-2 in vitro between 14°C and 37°C (B. J. Wisnieski, personal communication), and is much more sensitive to intracellular proteases (Yamaizumi et al., 1982). Interestingly, CRM197 shows high endonuclease activity in vitro (Bruce et al., 1990) between 14°C and 37°C (B. J. Wisnieski, personal communication). It is possible that the endonuclease activity is responsible for its temperature-dependent action. Alternatively, CRM197 is active in vivo due to a thermal refolding, which is not observed in vitro.

In DT-A^{tsR,} a C → T transition introduces a translational stop at amino acid 155. This may produce a readthrough and/or a truncated protein that is attenuated and temperature sensitive. DT-A^{tsN} contains a G → A transition in the initiation codon. Peabody (1989) has shown that translation initiation can occur at non-AUG triplets, like AUA, but in that case methionine would still be the initiating amino acid. As DT-A^{tsN} is temperature sensitive in yeast, it is likely that the initiation of translation occurs elsewhere. In DT- A^{tsI} a stop codon replaces gln^{37}. Such a short peptide is almost certainly inactive. It is more likely that a rare readthrough replaces gln^{37} with another amino acid to yield a low-abundance temperature-sensitive protein (Firoozan et al., 1991). The DT-A and DT-A^{tsX} transformed yeast and *Drosophila* strains should now permit study of the action of these different mutant toxins in living eukaryotic organisms.

In summary, we have isolated and partially characterized the first functional temperature-sensitive forms of diphtheria toxin. These DT-A^{ts} will aid research on how the structure of the DT-A domain affects toxicity in living eukaryotic cells and shed some new light on the action of CRM197. Because the DT-A^{ts} are active in very different cell types in many different organisms, they should enable us to arrest or severely impede cell growth and affect morphogenesis in a broad spectrum of organisms, including some plants, provided that suitable expression systems are available. Since the affected cells may maintain contacts with other cells for extended periods of time, such studies may answer different developmental questions than those answered by classical cell ablation experiments. Finally, the use of yeast as a host for the isolation of conditional mutations in heterologous genes may prove to be a general method for creation of conditional alleles of genes from other eukaryotic organisms less amenable to molecular genetic manipulations.

References

Behringer, R. R., Mathews, L. S., Palmiter, R. D. and Brinster, R. L. (1988). Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes and Dev.* 2, 453-461.

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila. Genes and Dev.* 3, 1288-1300.

Bigio, M., Rossi, R., Nucci, D., Antoni, G., Rappuoli, R. and Rattl, G. (1987). conformational changes in diphtheria toxoids. Analysis with monoclonal antibodies. *FEBS Lett.* 218, 271-276.

Breitman, M. L., Clapoff, S., Rossant, J., Tsui, L. C., Glode, L. M., Maxwell, I. H. and Bernstein, A. (1987). Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. *Science* 238, 1563-1565.

Bruce, C., Baldwin, R. L., Lessnick, S. L. and Wisnieski, B. J. (1990). Diphtheria toxin and its ADP-ribosyltransferase-defective homologue cRM197 possess deoxyribonuclease activity. *Proc. Natn. Acad. Sci. USA* 87, 2995-2998.

Chang, M., Baldwin, R. L., Bruce, B. and Wisnieski, B. J. (1989). Second cytotoxic pathway of diphtheria toxin suggested by nuclease activity. *Science* 246, 1165-1168.

Collier, R. J. (1975). Diphtheria toxin: mode of action and structure. *Bacteriol. Rev.* 39, 54-85.

Collier, R. J. (1990). Diphtheria toxin: structure and function of a cytocidal protein. In *ADP-ribosylating Toxins and G-proteins* (ed. J. Moss and M. Vaughan), Washington D.C: ASM

Collins, C. M. and Collier, R. J. (1985). Circular dichroism of diphtheria toxin, Pseudomonas aeruginosa exotoxin A, and various derivatives. *Biochem. Biophysica Acta* 828, 138-143.

Cooley, L., Kelly, R. and Spradling, A. (1988). Insertional mutagenesis of the Drosophila genome with single P-elements. Science 239, 1121-1128.

Elledge, S. J. and Davis, R. W. (1988). A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in Saccharomyces cervisiae. Gene 70, 303-312.

Evans, G. A. (1989). Dissecting mouse development with toxigenics. Genes and Dev. 3, 259-63.

Firoozan, M., Grant, C. M., Duarte, J. A. B. and Tuite, M. F. (1991). Quantitation of readthrough of termination codons in yeast using a novel gene fusion assay. Yeast 7, 173-183.

Franchesini, N. and Kirschfeld, K. (1971). Etude optique in vivo des elements photorecepteurs dans l'oeil compose de Drosophila. Kybernetik 8, 1-13.

Giannini, G. R., Rappuoli, R. and Ratti, G. (1984). The amino sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. Nucleic. Acids. Res. 12, 4063-4069.

Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G., Collier, R. J. and Kaplan, D. A. (1983). Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage B. Proc. Natn. Acad. Sci. USA 80, 6853-6857.

Grossniklaus, U., Bellen, H. J., Wilson, C. and Gehring, W. (1989). P-element-mediated enhancer detection applied to the study of oogenesis in Drosophila. Development 107, 189-200.

Hu, V. W. and Holmes, R. K. (1987). Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behavior. Biochem. Biophys. Acta. 902, 24-30.

Kunes, S. and Steller, H. (1991). Ablation of Drosophila photoreceptor cells by conditional expression of a toxin gene. Genes and Dev. 5, 970-983.

Maxwell, F., Maxwell, I. H. and Glode, L. M. (1987). Cloning, sequence determination, and expression in transfected cells of the coding sequence for the tox 176 attenuated diphtheria toxin A chain. Mol. Cell Biol. 7, 1576-1579.

Mekada, E. and Uchida, T. (1985). Binding properties of the Diphtheria toxin to cells are altered by mutation in the fragment A domain. J. Biol. Chem. 260, 12148-12153.

Mismer, D. and Rubin, G. M. (1987). Analysis of the promoter of the ninaE opsin gene in Drosophila melanogaster. Genetics 116, 565-578.

Nakamura, L. T. and Wisnieski, B. J. (1990). Characterization of the Deoxyribonuclease activity of diphtheria toxin. J. Biol. Chem. 265, 5237-5241.

Ornitz, D. M., Moreadith, R. W. and Leder, P. (1991). Binary system for regulating transgene expression in mice: targetting int-2 gene expression with yeast GAL4/UAS control elements. Proc. Natl. Acad. Sci. USA, 88, 698-702.

Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H. and Brinster, R. L. (1987). Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. Cell 50, 435-43.

Papini, E., Colonna, R., Schiavo, G., Cusinato, F., Tomasi, M., Rappuoli, R. and Montecucco, C. (1987). Diphtheria toxin and its mutant CRM197 differ in their interaction with lipids. FEBS letter 215, 73-78.

Pappenheimer, A. M., Jr (1977). Diphtheria toxin. Annu. Rev. Biochem. 46, 69-94.

Pappenheimer, A. M., Jr, Dunlop, P. C., Adolph, K. C. and Bodley, J. W. (1983). Occurrence of Diphthamide in Archaebacteria. J. Bacteriol. 153, 1342-1347.

Peabody, D. S. (1989). Translation initiation at non-AUG triplets in mammalian cells. J. Biol. Chem. 264, 5031-5035.

Perentesis, J., Genbauffe, F. S., Veldman, S. A., Galeotti, C. L., Livingston, D. M., Bodley, J. W. and Murphy, J. R. (1988). Expression of diphtheria toxin fragment A and hormone-toxin fusion proteins in toxin resitant yeast mutants. Proc. Natn. Acad. Sci., USA 85, 8386-8390.

Pirrotta, V. (1988). Vectors for P-element mediated transformation in Drosophila. In Vectors. A Survey of Molecular Cloning Vectors and their Uses. (ed. R. L. Rodriguez, and D. T. Denhardt) pp. 437-456. Boston: Butterworths.

Pollock, J. A. and Benzer, S. (1988). Transcript localization of four opsin genes in the three visual organs of Drosophila; RH2 is ocellus specific. Nature 333, 779-782.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schiltz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable source of P-element transposase in Drosophila melanogaster. Genetics 118, 461-470.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science 218, 348-353.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. and Hood, L. E. (1986). Fluorescence detection in automated DNA sequence analysis. Nature 321, 674-679.

Sulston, J. (1988). Cell lineage. In The Nematode Caenorhabditis elegans. (ed. W. Wood). CSH Laboratory press.

Uchida, T., Pappenheimer, A. M. and Harper, A. A. (1973). Diphtheria toxin and related proteins. II. Kinetic studies on intoxication of HeLa cells by diphtheria toxin and related proteins. J. Biol. Chem. 248, 3845-3850.

Wilson, C., Bellen, H. and Gehring, W. (1990). Position effects on eukaryotic gene expression. Annu. Rev. Cell Biol. 6, 679-714.

Wilson, C., Pearson, R. K., Bellen, H. J., O'Kane, C. J., Grossniklaus, U. and Gehring, W. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in Drosophila. Genes and Dev. 3, 1301-13.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 15, 245-250.

Zuker, C. S., Cowman, A. F. and Rubin, G. M. (1985). Isolation and structure of a rhodopsin gene from D. melanogaster. Cell 40, 851-858.