The torso response element binds GAGA and NTF-1/Elf-1, and regulates tailless by relief of repression

Gwo-Jen Liaw,1 Karen M. Rudolph,1 Jian-Dong Huang,2 Todd Dubnicoff,2 Albert J. Courey,2,3 and Judith A. Lengyel1,3,4

1Department of Molecular, Cell and Developmental Biology, 2Department of Chemistry and Biochemistry, and 3Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90095 USA

Modulation of transcription factor activity leading to changes in cell behavior (e.g., differentiation versus proliferation) is one of the critical outcomes of receptor tyrosine kinase (RTK) stimulation. In the early Drosophila embryo, activation of the torso (tor) RTK at the poles of the embryo activates a phosphorylation cascade that leads to the spatially specific transcription of the tailless (tll) gene. Our analysis of the tor response element (tor-RE} in the tll promoter indicates that the key activity modulated by the tor RTK pathway is a repressor present throughout the embryo. We have mapped the tor-RE to an 11-bp sequence; using this sequence as the basis for protein purification, we have determined that the proteins GAGA and NTF-1 (also known as Elf-1, product of the grainyhead gene} bind to the tot-RE. We demonstrate that NTF-1 can be phosphorylated by MAPK (mitogen-activated protein kinase), and that tll expression is expanded in embryos lacking maternal NTF-1 activity; these results make NTF-1 a likely target for modulation by the tor RTK pathway in vivo. The data presented here support a model in which activation of the tor RTK at the poles of the embryos leads to inactivation of the repressor and therefore, to transcriptional activation (by activators present throughout the embryo) of the tll gene at the poles of the embryo.

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the posterior of the embryo, however, *tll* and *hkb* are regulated only by the the activated tor RTK, and the biological effect of tor RTK activation can be explained entirely by the transcriptional activation of these two genes, both of which encode transcription factors (Strecker et al. 1986; Pignoni et al. 1990, 1992; Weigel et al. 1990, Brönnner et al. 1994).

The terminal system is one of the genetically best-characterized RTK systems. The roles in this pathway of the *Drosophila* Ras, Raf-1, MAPKK, and MAPK (ERKA) have been established by demonstrating interactions between mutations in the genes encoding these proteins with both loss-of-function and gain-of-function *tor* mutations (for review, see Perrimon 1993). These *tor* mutations are invaluable reagents for evaluating the in vivo function of putative tor response elements in genes regulated by the pathway (Liaw and Lengyel 1992; Liaw et al. 1993).

Because the diversity of effects of activated RTKs might be attributable in part to the modulation of distinct constellations of transcription factors, identification of additional RTK-targetable transcription factors is of prime importance for our understanding of ligand-dependent alteration of cell function. Like the other components of the tor RTK-activated pathway, the transcription factors regulating *tll* transcription are almost certainly provided maternally (for review, see Perrimon 1993) and therefore, difficult to identify directly by genetic techniques. As an alternative biochemical approach, we have taken a first step toward identifying such transcription factors by mapping tor response elements (*tor*-REs), to which these proteins are predicted to bind, in the promoter of the target gene *tll*. Here, we report data supporting a model in which an 11-bp *tor*-RE mediates transcriptional repression (rather than activation) of the *tll* gene. Where the tor kinase pathway is activated at both poles of the embryo, this repression is lifted and transcriptional activation is brought about by activators most likely present throughout the embryo. As it is present in embryos, which can be obtained in large quantity, the tor system is biochemically accessible. We have purified two proteins from *Drosophila* embryo extracts, GAGA and NTF-1, that bind to the *tor*-RE; we discuss the possible in vivo roles of these proteins in regulating *tll* expression.

**Results**

**A tor responsive minimal *tll* regulatory module**

We initiated mapping of the *tor*-RE within a “minimal regulatory region” that drives expression in two polar caps in response to *tor* activation. This region was identified on the basis of previous work in which we used promoter–reporter fusion constructs to show that the regulation of *tll* expression by the activated terminal system is mediated by synergistically interacting proximal and distal regulatory regions (Liaw and Lengyel 1992). Within the proximal regulatory region (shown in Fig. 1) we identified a 121-bp fragment (region D1,2, composed of regions P, Q, and R in Fig. 1) that mediates regulation of expression by the tor RTK pathway (Liaw and Lengyel 1992, Liaw et al. 1993). Although a construct [GS] containing region PQR drives spatially correct expression, the level of *lacZ* expression is very low and can only be detected by in situ hybridization to the *lacZ* mRNA (Liaw et al. 1993). Addition of the 60 bp of region O gives a regulatory fragment [region O–R, inserted into construct G11] that drives expression in the characteristic early *tll* pattern [i.e., in two symmetrical caps at the poles of the embryo (Fig. 1)], the higher level of this expression is sufficient for further dissection analysis (see below). Region O–R does not drive expression of the later appearing anterior stripe [that responds to *bicoid* and *dorsal* as well as *tor* regulation], elements responsible for this stripe have been mapped to other portions of the *tll* regulatory region (Liaw and Lengyel 1992). Region O–R does have the critical feature of mediating response to the *tor* gene; it does not drive expression at the poles of the embryo [terminal expression] in *tor* loss-of-function embryos (*torKR*), and it drives ectopic uniform expression in *tor* gain-of-function embryos (*torKR*AB) (data not shown). On the basis of the above characteristics, we define the 181-bp O–R region as a minimal regulatory region responding to tor RTK activation. Analysis of deletion mutants of this minimal regulatory region reveals that it contains elements that mediate activation throughout the embryo, as well as elements mediating spatially specific repression.

**Activator elements**

In addition to region O [mentioned above], regions P and R1 [see Fig. 3, below] also contribute to the overall level of *tll* expression. When these regions are deleted or substituted, the overall level of expression is diminished throughout the embryo (constructs G11 and G16 in Fig. 1; construct G26 in Fig. 3). We presume that there are ubiquitous activator proteins that bind to sites within these regions and activate *tll* expression. Further analysis of these regions and of the proteins binding to them will be presented elsewhere (G.-J. Liaw, A. Gu, A.J. Courey, and J.A. Lengyel, in prep.).

**Spatially specific repression**

The spatially restricted *lacZ* terminal expression driven by construct G11 is brought about not by a spatially specific activation element, but rather by relief of repression. This is deduced by comparing expression driven by construct G11 [region OPQR, expression restricted to the poles of the embryo] to that driven by construct G14 [region OPQ, uniform expression] (Fig. 1B,C). Other construct pairs possessing and lacking region R [i.e., cf. G21 and G17 [OPR vs. OP] and cf. G16 with G18 [OQR vs. OQ]] give similar results [Fig. 1]. The deletion of the R region results in uniform expression that resembles the expression pattern of the *tll* gene in *tor* gain-of-function embryos (Steigrimsson et al. 1991). The most reasonable interpretation of these data is that an element in the
57-bp R region mediates repression of transcription in the central domain of the embryo so that expression, driven by activators present throughout the embryo (see below), is limited to the poles.

We localized two other weak repression elements, one in the M region and one in the Q region. The presence of a weak repression element in the M region is revealed by a comparison of the expression driven by constructs G31 and G32, addition of the M region leads to some repression in the center of the embryo (Fig. 1H, I). Because embryos carrying either G14 (possessing Q) or G17 (lacking Q) show uniform expression, it appears that the Q region is not sufficient to repress central expression on its own. The presence of a weak repression element in the Q region is suggested, however, by the observations that (1) the expression domain driven by construct G21 (OPR) is slightly expanded relative to that driven by construct G11 (OPQR) (Fig. 1B, G), and (2) the expression level driven by construct G19 (OR) is stronger than that driven by construct G16 (OQR) (Fig. 1D, F).

The repressor elements can repress a heterologous promoter

The above data strongly suggest that the region M–R contains negative regulatory elements that repress lacZ expression in the central domain of the embryo. To further test this idea, we placed region M–R, oligomerized
fourfold, upstream of a DNA fragment containing a portion of the Krüppel (Kr) promoter followed by a hsp70 basal promoter element and the lacZ gene (Hoch et al. 1990). The Kr–hsp70 promoter alone drives a strong central band of expression [Fig. 2A]. When four copies of the M–R region are inserted upstream of the Kr–hsp70 promoter, the central Kr-promoter-driven stripe becomes both narrower and significantly weaker (Fig. 2B). In effecting repression from a position 2.5 kb from the Kr promoter and thus affect the ability of the adjacent M–R repeats to drive terminal expression. If this latter interpretation is correct, then Tailless-binding elements must also function as silencer elements.

**Identification of the tor-RE**

Because removal of the 57-bp R region results in ectopic expression of till throughout the embryo, a pattern similar to till expression in tor gain-of-function embryos (Steingrimsson et al. 1991), the mechanism for regulation of till expression by the tor RTK pathway is most likely to be relief of transcriptional repression. The repressor element mapped to the R region would then fulfill the definition of the tor-RE. Candidate sequences within the R region for the tor-RE are a 26-bp sequence protected from DNase I digestion by nuclear extract from early embryos (footprint D) and an overlapping 28-bp sequence (box III) conserved between *Drosophila melanogaster* and *D. virilis*, species that diverged at least 40 million years ago [see Fig. 1, Throckmorton 1975; Liaw et al. 1993].

To localize precisely the tor-RE, the R region in construct G11 was mutagenized by linker scanning with a 14-bp sequence, dividing up the R region into four portions: R1, R2, R3, and R4 [see constructs G22–G26 in Fig. 3A]. Analysis of the expression pattern driven by these various mutagenized constructs in transformant embryos showed that the only mutation that had an effect on the spatially localized pattern of expression is that in construct G25 (Fig. 3C). The crucial result is that alteration of the R2 region results in strong derepression. The fact that the R2 region is located in the center of the D footprint, in the portion that overlaps with box III, supports the idea that the R2 region plays an important regulatory role.

Within the *D. melanogaster* and *D. virilis* till regulatory regions, we identified five additional sequences that display close similarity to the sequence found in the R2 region. A consensus, TGCTCAATGAA, derived from these six sequences, is shown in Figure 4B. On the basis of the results of the mutagenesis described above, as well as additional experiments to be described below, we define the six sequences that share this 11-bp consensus as tor-REs. Two tor-REs are found in the proximal *D. melanogaster* till regulatory region, one within footprint D [and analyzed by mutagenesis as described above], and another within region M, which [also as described above] contains a weak repression element (see Fig. 1). Two tor-REs are found in the distal till regulatory region, in a

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**Figure 2.** Negative regulatory elements in region M–R repress a heterologous activator. lacZ mRNA distributions in embryos are revealed by in situ hybridization. (A) Expression driven by the Kr–hsp70/lacZ construct. The shaded box indicates 2.5 kb of the Kr promoter that drives strong expression in the middle of embryo [Hoch et al. 1990]. (B) When the fourfold oligomerized M–R region of the till proximal promoter (Fig. 1A), indicated by four rightward-pointing arrows, is added to the 5' end of the Kr promoter, expression in the middle of the embryo is reduced. The embryo shown in B represents the intermediate level of repression seen with the indicated construct; of six transformant lines analyzed, two showed a stronger repression, three an equal repression, and one a weaker repression. (C) Expression driven by the oligomerized region M–R alone.
630-bp fragment (Fig. 4; Dm distal a and Dm distal b) that, like the PQR region, also drives terminal-specific expression [K.M. Rudolph, G.-]. Liaw, A.J. Courey, and J.A. Lengyel, in prep]. Like one of the tor-REs in the proximal promoter, one of the tor-REs in the distal promoter is also in the center of a sequence (footprint F) protected from DNase I by embryonic nuclear extract (Fig. 4D). Two additional tor-REs are found in the proximal and distal D. virilis till presumptive regulatory regions (Fig. 4; Dv proximal and Dv distal).

The tor-RE in the distal regulatory region was tested in experiments similar to those used to test the footprinted tor-RE in the proximal regulatory region. A 150-bp region containing box III sequence (which is protected by the F footprint) was oligomerized and placed upstream of the lacZ gene; this construct gives expression at the embryonic termini (Fig. 5B; construct K12). When the tor-RE in the K12 construct was replaced with random nucleotide sequence (construct K13), repression was lost in the middle of embryo (Fig. 5C). Although terminal expression of till is not normally regulated by the dorsoventral system (Pignoni et al. 1992), the exclusively ventral expression driven by constructs K12 and K13 is most likely attributable to activation by the Twist protein (see Fig. 5 legend).

In summary, the experiments shown in Figures 3 and 5 show that the two tor-REs that can be footprinted, one in the proximal and one in the distal till regulatory region, can be mutated independently to cause the terminal expression domains to expand, resulting in uniform expression along the anterior/posterior axis. These results support the notion that these sequence elements act as tor-REs.

To test the function of the proximal, footprinted tor-RE in more detail, three mutant constructs with base substitutions in the 11-bp consensus sequence were generated (see Fig. 3A). Embryos carrying construct G29, with four base changes placed symmetrically in the center of the tor-RE, show an almost uniform expression (see Fig. 3F). Embryos transformed with the constructs G27 and G30, with only two base changes each, show expanded polar expression (i.e., less central repression).
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Figure 4. Consensus sequences in the distal and proximal tll promoters of both D. melanogaster and D. virilis. Box III sequences (below line) are found at several positions in the proximal and distal regulatory regions of both D. melanogaster (A) and D. virilis (this study, Liaw et al. 1993, G.-J. Liaw, data not shown). The positions of footprints of embryonic nuclear extract (from Liaw et al. 1993, and D, below) are indicated by solid boxes above the line (A). Alignment of the box III-related sequences reveals an 11-bp consensus sequence (boxed face type letters) (B), on the basis of experiments described in the text, this consensus is referred to as the tor-RE consensus sequence. The significance of the 6-bp core sequence CTCAAT (arrows) found in some of the tor-REs is also discussed in the text. The tor-RE footprinting activity by using probes containing the CTCAAT core sequence are also seen in footprint C (C). One of the tor-REs in the distal promoter region is also protected from DNase I digestion (indicated by box F in A) with embryonic nuclear extract (D). A distal promoter DNA fragment (−2926 to −2298) of the core sequence (indicated by arrows in Fig. 4B), was 5′ labeled at the −2926 position and used as probe. The probe was sequenced using the Maxam and Gilbert sequencing reactions C + Y (lane 1) and G + A (lane 2), or incubated with no embryonic nuclear extract (lane 3), 5 μl of extract (lane 4) or 10 μl of extract (lane 5). Similar experiments show that the tor-RE in the D. virilis proximal promoter is also protected from DNase I digestion by embryonic nuclear extract (data not shown).

These results implicate specific nucleotides within the 11-bp consensus sequence as being essential for the correct function of the tor-RE.

We identified a 6-bp core sequence, CTCAAT, within the tor-REs (see Fig. 4B). Only four of the six tor-REs (i.e., those containing a perfect match to the CTCAAT core sequence [indicated by arrows in Fig. 4B]), were protected from DNase I digestion by embryonic crude nuclear extract (Figs. 3A and 4D). In addition to the footprinted tor-REs, footprint C also contains three imperfect repeats of the CTCAAT core sequence. These results, summarized in Figure 4, suggest that the core sequence CTCAAT is crucial for protein binding to the tor-RE in vitro.

There are reasons to believe that the entire 11-bp tor-RE consensus sequence, not just the core sequence CTCAAT contained within it, constitutes the tor-RE. When footprint C, which contains three repeats of the CTCAAT core sequence, is deleted from the minimal promoter, there is only a minor loss of transcriptional repression (see Fig. 1). In addition, making a change outside of the core sequence (GA to CT) does have an effect on repression (Fig. 3, construct G30).

In conclusion, our results show that the gene activation mediated by the tll promoter in response to activation of the tor RTK occurs by relief of repression. Within small regulatory regions that drive terminal expression of tll, we have identified several elements that share an 11-bp consensus sequence, are footprinted by embryonic nuclear extract, and when mutated, cause relief of repression. These elements satisfy the definition of the tor-RE. The 6-bp core sequence, CTCAAT, may constitute a necessary, but not sufficient portion of the tor-RE.

**Purification and identification of two candidate tor-RE-binding proteins**

As described above (see Fig. 4) and in previous work (Liaw et al. 1993), there is an activity in crude embryonic nuclear extract that footprints the tor-RE; this activity appears equally concentrated in both 0- to 4- and 0- to 12-hr extracts (G.-J. Liaw, data not shown). The first step in the purification of the tor-RE-binding protein (tor-REB) was the application of nuclear extract from 0- to 12-hr embryos to heparin-Sepharose. Fractions eluting at 0.2 M, 0.3 M, and 0.5 M KCl were assayed for specific tor-RE footprinting activity by using probes containing the wild-type tor-RE (construct G11, Fig. 1A) and the mutated tor-RE (construct G29, Fig. 3A). Based on the ability to footprint the wild type, but not the mutant...
tor-RE, two distinct activities were identified that separated in the initial heparin-Sepharose chromatography. Subsequently, each activity was purified separately; we refer to these two proteins in what follows as tor-REB$_a$ and tor-REB$_b$.

**tor-REB$_a$ is the GAGA protein**

One tor-RE footprinting activity was identified in the material eluting from heparin-Sepharose at 0.5 M KCl. As indicated in the flowchart in Figure 6A, this material was further purified by CM Sepharose column chromatography, and finally DNA affinity chromatography using the tor-RE sequence (see Materials and methods). Analysis of the protein bound by the final DNA-affinity column by SDS–gel electrophoresis revealed the major component to be a polypeptide with an apparent molecular mass of 65 kD [Fig. 6B]. This protein is recognized by an anti-GAGA antibody in a Western blot [Fig. 6B]. Furthermore, both the tor-REB$_a$ and the purified bacterially expressed GAGA protein [see Materials and methods] give identical footprints on the tor-RE [Fig. 7A]. We conclude that the tor-REB$_a$ is the GAGA protein.

**tor-REB$_b$ is the DRE-binding protein, which is NTF-1 (Elf-1)**

A second tor-RE protein eluted from heparin–Sepharose at 0.3 M KCl. This eluate also contains activity binding to the dpp repression element [DRE], the subject of another investigation [Huang et al. 1995]. tor-REB$_b$ was found to copurify with the DRE-binding protein. Using the protocol developed for purification of the DRE-binding protein [except the final DNA affinity chromatography, for which the tor-RE was used [see Materials and methods]] we purified a second tor-RE protein. Because the DRE-binding protein has been shown to be identical to the Drosophila transcription factor NTF-1 [also known as Elf-1] [Huang et al. 1995], we compared footprinting by NTF-1 and the purified protein we called tor-REB$_b$. Bacterially expressed GST–NTF-1 and tor-REB$_b$ give identical footprints on the tor-RE [Fig. 7B]. The tor-REB$_b$ [NTF-1 purified from embryos] shows a reduced ability to footprint the mutated tor-RE (i.e., it is unable to bind to the region where the DNA sequence is altered, although it still binds to the flanking regions) [Fig. 7B]. We conclude that the tor-REB$_b$ is NTF-1.

**NTF-1 is a substrate for MAPK (ERKA)**

Because the tor-RE is the cis-regulatory element responding to activation of the tor RTK pathway, the tor-REBs are candidate substrates of the MAPK activated by the terminal system, ERKA. Bacterially expressed ERKA is not active in vitro [W. Biggs and L. Zipursky, pers. comm.]; therefore, we used the homologous mammalian protein, bacterially expressed ERK2 [Haystead et al. 1992]. Bacterially expressed GAGA and NTF-1 were tested as potential substrates by incubation with [$\gamma$-32P]ATP and activated ERK2. Figure 8 shows that NTF-1 [but not GAGA] is phosphorylated by ERK2. The molar ratio of 32P incorporated into NTF-1, relative to that incorporated into myelin basic protein [which is phosphorylated on one threonine, Erickson et al. 1990], indicates that there is also one phosphate incorporated per NTF-1 molecule.

**Decreased NTF-1/Elf-1 activity results in expansion of tll expression**

The availability of mutants in Drosophila can often provide the opportunity to test the in vivo function of a protein identified biochemically. This is, in principle, possible for NTF-1 and the GAGA protein, as mutations

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**Figure 5.** A tor-RE in the distal tll promoter. (A) Schematic drawing of the D. melanogaster distal region [top line, see Fig. 3A]. Footprint F is shown as a black box, with the tor-RE [box III] shown beneath [restriction sites: Av/ Aval, Al/ AluI]. Construct K12 contains four copies of the wild-type distal region [see Fig. 3]. Construct K13 contains four copies of the distal region with the 16-bp centered around the tor-RE replaced by the random sequence GACTGCAGACTGTCAG {which contains an internal PstI site} using site-directed mutagenesis. For construct K13, the asterisk indicates the mutagenized tot-RE [box III] element. To the right of the constructs are cartoons of lacZ mRNA expression pattern, as seen at the early cellular blastoderm stage, summarized from the expression seen in whole embryos. (B,C) Embryos at the cellular blastoderm stage carrying the constructs K12 and K13, respectively. The ventral expression of the K12 and K13 constructs is most likely attributable to activation by the Twist protein for the following reasons: the constructs drive no expression in embryos lacking the Dorsal protein (K.M. Rudolph, data not shown), which is required to activate expression of the twist gene on the ventral side of the embryo, within the -2770 to -2620 fragment of the distal region there are no Dorsal-binding sites but there are four E-box containing sites that can be footprinted by Twist protein [G.-J. Liaw, data not shown].
in the genes encoding these proteins have been identified. NTF-1 is encoded by the gene *grainyhead* (*grh*); loss-of-function *grh* mutations result in embryonic lethality with no obvious terminal defects [Bray and Kafatos 1991]. The GAGA protein is encoded by the *Trithorax-like* (*Trl*) gene; loss-of-function *Trl* mutations are not embryonic lethal, but do result in lethality during the third larval instar [Farkas et al. 1994]. Because the known components of the terminal system upstream of *tll* and *hkb* are provided maternally [Perrimon 1993], and as *tll* transcription is activated very early in embryogenesis (i.e., by nuclear cycle 9, Pignoni et al. 1992), it is likely that any NTF-1 or GAGA protein involved in regulating initial expression of *tll* is provided for by maternal transcription. Analysis of ovaries and early embryos indicates that mRNAs for both proteins are indeed part of the maternal dowry [Soeller et al. 1993; Huang et al. 1995]. Because null alleles of *grh* and *Trl* are zygotically lethal, the effect of these mutations on early *tll* expression can best be tested by generating germ-line clones in heterozygous females. If either of these proteins plays a required role in repression of *tll*, one would predict an expansion of *tll* expression in embryos from germ-line clones lacking expression of the particular protein.

Germ-line clones can be generated by X-irradiation of females carrying a dominant female sterile mutation, or by use of the recently developed *ovo* FL-P-FRT system [Perrimon and Gans 1983; Chou et al. 1993]. Using three different *Trl* alleles and both the FLP-FRT system and X-irradiation, however, we were unable to obtain an increase above background in the number of eggs laid by heterozygous *Trl/ovo* females [G.-J. Liaw; data not shown]. Therefore, it is likely that *Trl* may be required for early events of oogenesis. For the weak allele *Trl*13C, homozygous escaper females can be obtained; embryos from these females show an approximately twofold decrease in level of *tll* expression, but no expansion in the *tll* expression domains [G.-J. Liaw; data not shown]. Thus, at present, genetic experiments do not provide evidence supporting a requirement for GAGA in the regulation of *tll* expression.

Our ability to generate germ-line clones in heterozygous *grh*/+ females allows a more definitive conclusion to be reached about the role of NTF-1 in *tll* regulation. Although it was not possible to use the FRT-FLP system [because the insertion site of *P(ovo)0* on the available FRT *ovo* chromosome is a lethal allele of *grh*], we were able to generate homozygous *grh* clones by X-irradiation of females of the genotype *grhB37/Fs(2)D*. A portion of the embryos from these *grhB37* germ-line clones showed the predicted expansion of *tll* expression: There was a range in the size of the posterior cap of *tll* expression, from a domain covering 0%--20% egg length (no expansion, some of these embryos may have come from escaper *grh/CyO* females) to a domain covering 0% to ~40% egg length [Fig. 9A,B]. In no case was *tll* expression expanded to cover the entire embryo, as is seen in *tor*-dominant gain-of-function alleles [Steingrimsson et al. 1991]. Interestingly, this expansion often appeared as a set of stripes rather than being continuous [Fig. 9C]; thus, there may be repressors of *tll* that are not distributed uniformly throughout the embryo.

In the above experiments, we were not able to distinguish between embryos lacking *grh* only maternally, or embryos lacking *grh* both maternally and zygotically. That the embryos showing expansion of *tll* expression indeed come from *grh* germ-line clones is indicated by two pieces of evidence: (1) expansion is not seen in embryos from unirradiated *grh/Fs(2)D* females crossed to...
pression. Although we were unable to obtain germ-line clones using the grh<sup>B32</sup> allele, embryos from an inter se grh<sup>B32</sup>+/+ cross showed weak expansion of <i>tll</i> expression, always seen as two additional stripes rather than as a continuous domain (Fig. 9D). Thus, grh<sup>B32</sup> may be a stronger allele than grh<sup>B37</sup>, indicated by its apparent maternal effect as a heterozygote and the fact that germ-line clones of the former allele were not obtained.

In conclusion, these genetic experiments are consistent with a required role for NTF-1 in the repression of <i>tll</i>.

**Discussion**

<i>tll</i> regulation occurs by relief of repression

Our in vivo promoter dissection has revealed that transcriptional activation of <i>tll</i> by the activated <i>tor</i> RTK pathway occurs by relief of repression. An 11-bp element, the tor-RE, is critical for this repression as mutation of this element in the context of either the proximal or distal <i>tll</i> regulatory module results in expanded or, in some cases, uniform reporter gene expression. The finding that <i>tll</i> regulation involves relief from repression suggests the following model for how localized activation of the terminal system results in spatially specific transcription. In the central region of the embryo where the terminal system is not activated, repres-

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**Figure 7.** Comparison of the DNA-binding specificity between tor-REB<sub>a</sub> and GAGA, and between tor-REB<sub>b</sub> and NTF-1. The footprinting probe was either the wild-type tor-RE or a mutated version of the tor-RE that eliminates repression (see Fig. 3A). The binding to each of these probes by purified bacterially expressed GAGA (GAGA) is compared to that of purified tor-REB<sub>a</sub> in A, whereas the binding of purified bacterially expressed GST-NTF-1 (NTF-1) is compared to that of purified tor-REB<sub>b</sub> in B. Solid boxes in A and hatched boxes in B indicate the sites of footprints; asterisks indicate the location of substituted bases in the mutated tor-RE [the upper footprints in both A and B are to non-tor-RE sites]. An alignment over the tor-RE of the GAGA footprint, the NTF-1 footprint, and the previously described footprint D [Liaw et al. 1993] is shown in C. At a low concentration, GAGA footprints only the left half of the D footprint [solid line], at higher concentrations, the GAGA footprint increases [shaded line]. Similarly, NTF-1 at a low concentration footprint the right side of the D footprint [boxed hatching], at higher concentrations, the NTF-1 footprint increases [unboxed hatching]. Lane 1 in A is a sequencing ladder, lanes 2–8 in A and 1–6 in B contain wild-type probe, and lanes 7–9 in B contain mutant tor-RE. In A, 1 μl [lanes 3, 6, 10], 2 μl [lanes 4, 7, 11], and 5 μl [lanes 5, 8, 12] of solutions of GAGA and tor-REB<sub>b</sub> at 1 and 0.03 mg/ml, respectively, were used. In B, 1 μl [lane 2] and 5 μl [lane 3] of GST–NTF-1 at 0.1 mg/ml, and 2 μl [lanes 5, 8] and 10 μl [lanes 6, 9] of the tor-REB<sub>a</sub> at 0.005 mg/ml [estimated], were used. "-" [lanes 2.9 in A, and lanes 1, 4, 7 in B] indicates no protein added to the reaction mixture.

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**Figure 8.** Phosphorylation of NTF-1 by MAPK. Activated MAPK was prepared as described in Materials and methods, and incubated with GST–NTF-1 [N], GAGA [GA], either one of the control proteins GST [G] or myelin basic protein [My], or with no protein [C]. The reaction products were electrophoresed on an SDS–acrylamide gel and detected by silver staining (A); an autoradiograph of this gel is shown in B. M [lane 1] contains protein size markers. The activated MAPK [P<sup>42</sup>] and My are indicated with arrows. Except for GAGA [1 μg], 0.1 μg of each protein was added to the phosphorylation reaction. As determined by scanning of the gel, the ratio of dpm in the My doublet band to the dpm in the GST–NTF-1 band was 11:1. The molar ratio of My to GST–NTF-1 was 9:1 [because equal masses of My and GST–NTF-1 were loaded on the gel], and the relative molecular masses of these two proteins are 18.1 and 167 kD, respectively. Because MAPK phosphorylates one site per My molecule [Erikson et al. 1990], these numbers suggest that under the reaction conditions used, MAPK also phosphorylates roughly one site per GST–NTF-1.

<sup>grh/CyO</sup> males, and [2] embryos from an inter se cross of <i>grh</i><sup>B37</sup>+/+ do not show significant expansion of <i>tll</i> expression.
or proteins that interact with the tor-RE and perhaps with other DNA regulatory elements serve to silence the tll promoter. At the poles of the embryo, one or more of the proteins required for tll repression is modified and therefore, inactivated by the terminal system; this allows terminal-specific tll transcriptional activation by globally distributed activating factors. A prediction of this model is that one or more factors interacting with critical tll cis-regulatory elements [e.g., the tor-RE] will be found to be substrates of the terminal system phosphorylation cascade.

Various lines of evidence suggest that the tor-RE may silence transcription in an active manner rather than by the exclusion of activator proteins from overlapping binding sites. First, a region of the tll promoter containing the tor-RE is able to reduce the activity of the Kr enhancer at a distance. Second, deletion of the tor-RE from either the proximal or the distal regulatory module results in an expansion of the spatial domain in which transcription occurs, but does not result in a reduction in the level of transcription. If the tor-RE contained overlapping repressor- and activator-binding sites, then we would expect deletion of the tor-RE to result in a reduction in the overall level of expression.

Multiple regulatory factors may contribute to tll repression

Chromatographic fractionation of Drosophila embryo extracts allowed purification of two proteins that bind specifically to the tor-RE: GAGA and NTF-1. Because GAGA binds to the sequence TGAG [Kerrigan et al. 1991], it is not surprising that it also binds to the tor-RE, which contains TGAG on the opposite strand of the core sequence CTCAAT. The fact that mutations that render the tor-RE inactive also reduce or eliminate binding of GAGA suggests that this protein could contribute to tll repression. Although we have not been able to generate embryos completely lacking GAGA to test this idea in vivo, we note that, as GAGA is present in the freshly laid egg [Soeller et al. 1993], there is at least a potential for GAGA to play a role in tll expression.

Although there is no obvious motif shared between the previously reported NTF-1-binding site [Bray et al. 1989; Dynlacht et al. 1989] and the tor-RE that would explain the binding of NTF-1 to both of these sequences, a number of considerations suggest that NTF-1 plays a role in regulation of tll expression. First, as found for GAGA, mutations in the tor-RE that eliminate its repression function also reduce or eliminate NTF-1 binding. Second, NTF-1 mRNA is synthesized during oogenesis and appears to be transferred to the developing oocyte [Huang et al. 1995]; therefore, it is likely that NTF-1 is present in the early embryo when transcription is activated during the syncytial blastoderm stage. Third, NTF-1 is phosphorylated in vitro by the mammalian MAPK homolog of ERK1, an essential protein kinase component of the terminal signaling cascade [Brummer et al. 1994b]. Fourth, elimination of NTF-1 activity from early embryos by the generation of germ-line clones results in an expansion of tll expression. We conclude that NTF-1 plays an essential role in the repression of tll; it seems likely that its phosphorylation, as a result of tor RTK activation, diminishes its repressor activity.

Although it appears that NTF-1 (and possibly also GAGA) contributes to the repression of tll, previous studies on NTF-1 and GAGA have focused on their roles as transcriptional activators. In vitro, NTF-1 binds to specific cis-regulatory elements in the Dopa decarboxylase, Ultra-bithorax, and fushi tarazu promoters [Bray et al. 1989; Dynlacht et al. 1989] and acts as a transcriptional activator [Dynlacht et al. 1989, Attardi and Tjian 1993]. GAGA binds in vitro to many regulatory regions, including those of various pattern formation, heat shock, and histone genes [for review, see Soeller et al. 1993], both biochemical and genetic studies suggest that GAGA, by modulating chromatin structure, allows access to the promoter of the basal transcriptional machinery and other activating transcription factors [Kerrigan et al. 1991; Lu et al. 1993; Tsukiyama et al. 1994; Farkas et al. 1994; O'Brien et al. 1995]. Because a number of transcription factors have now been shown to be capable, in a context-dependent manner, of acting either as activators or repressors [for review, see by Roberts and Green 1995], it is reasonable to imagine that this might also be possible for NTF-1 and GAGA.
Several pieces of evidence suggest that there are proteins additional to GAGA and NTF-1 that might be involved in regulation of \( \text{tll} \). First, the expansion of \( \text{tll} \) expression in NTF-1-deficient embryos does not lead to uniform expression of \( \text{tll} \) throughout the embryo, as is seen in \( \text{tor}^D \) embryos (Steingrimsson et al. 1991), indicating there must be factors present in the early embryo, in addition to NTF-1, that also repress \( \text{tll} \) expression. Second, the multimerized 11-bp tor-RE only weakly (if at all) represses expression of the heterologous promoter \( \text{dpp} \) [data not shown], suggesting that DNA sequences additional to the tor-RE (and hence the proteins that bind to them) are required for a high level of repression. Third, in our analysis of the minimal \( \text{tll} \) regulatory region, deletion of the region covered by footprint \( C \), which is adjacent to the tor-RE, results in a slight expansion of the spatial expression pattern, suggesting that a protein binding to this element might be involved in repression. Fourth, the observation that loss-of-function \( \text{groucho} \) germ-line clones result in embryos with terminal defects (Paroush et al. 1994) suggests that maternally encoded Groucho protein might play a role in \( \text{tll} \) regulation. Although Groucho does not bind to DNA (Hartley et al. 1988), it might bind to other proteins regulating \( \text{tll} \). Thus, there may be a complex of proteins regulating \( \text{tll} \) that responds to the activation of the tor RTK. Consistent with this notion, multiprotein complexes have been implicated in transcriptional activation and repression in both \( \text{Drosophila} \) and yeast; in addition, the GAGA protein belongs to the Trithorax group of proteins that is thought to be involved in a possible activator complex in \( \text{Drosophila} \) (Orlando and Paro 1995).

The sevenless (sev) RTK pathway in the \( \text{Drosophila} \) eye imaginal disc shares many components of the tor RTK pathway, in particular Drk, Sos, Ras1, Raf, and ERKA proteins (for review, see Dickson and Hafen 1994), raising the question of whether any transcription factor targets of these pathways might also be shared. Two \( \text{ets} \) domain transcription factors, Pointed-P2 and Yan, are targets of the sev pathway and are phosphorylated by ERKA; this phosphorylation converts Pointed-P2 to a transcriptional activator, and inactivates the transcriptional repression function of Yan (Brunner et al. 1994a; O’Neill et al. 1994). Also, the \( \text{Drosophila} \) lun homolog \( \text{DJun} \) has been shown to be a target of the sev pathway (Bohmann et al. 1994). Neither \( \text{ets} \)-nor API-binding sites, however, are found in either of the \( \text{tll} \) minimal regulatory modules, nor does bacterially produced Yan footprint within the proximal regulatory module [G.-J. Liaw, unpubl.].

**Multiple tor-REs and graded terminal system activity**

The multiple tor-REs in the proximal and distal \( \text{tll} \) promoter might play a role in setting the sharp borders of \( \text{tll} \) expression. Graded terminal system activity presumably leads to the graded modification and consequent inactivation of the repressor proteins that bind to the tor-RE (Casanova and Struhl 1989; Pignoni et al. 1992; Sprenger and Nüsslein-Volhard 1992). Graded repressor inactivation in the terminal patterning system would then be analogous to the Bicoid morphogen gradient in the anterior patterning system and the morphogen gradient of nuclear localized Dorsal in the dorsal/ventral patterning system. Just as multiple binding sites for Bicoid and Dorsal in the \( \text{hunchback} \) and the \( \text{twist} \) regulatory regions, respectively, appear to be necessary to set sharp, correctly placed borders of expression of these zygotic genes (for review, see Courey and Huang 1995), multiple tor-REs might similarly help to create distinct, properly positioned borders of \( \text{tll} \) transcription.

**Materials and methods**

**Construction of \( \text{tll} \) promoter-reporter fusion genes and germ-line transformation**

Various subfragments of the proximal and distal \( \text{tll} \) promoter regions [shown in Fig. 1A, with the exception of construct G21; K.M. Rudolph, G.-J. Liaw, A.J. Courey, and J.A. Lengyel, in prep.] were generated by means of the polymerase chain reaction or by synthesis of the DNA oligonucleotides using Gene Assembler Plus [Pharmacia]. The DNA fragments for the \( \text{G} \) series of constructs were inserted into the \( \text{PstI} \) site of the vector pPAP2, which is a derivative of pBluescript SK(+) [Stratagene]. This vector contains two Avai sites just flanking the \( \text{PstI} \) site. These two Avai sites, 5'-CTCGGG-3', were used to multimerize DNA fragments in tandem repeats [Liaw et al. 1993; Liaw 1994]. For each construct, multiple copies of the distal region [see Fig. 3A] were inserted into the Avai site of the vector pEA5, which is also a derivative of pBluescript SK(+) [Liaw 1994]. For each construct, plasmids were selected that carried four copies of the DNA fragment in question. Constructs G21–G27 and G29 (Fig. 4A) were generated by oligonucleotide-directed mutagenesis [Ausubel et al. 1994] using a tetramer of the construct G11 as template. Construct G28 was generated by inserting the fourfold oligomerized region M–R [excised from construct G2] into the NotI site of pKr672 [Hoch et al. 1990] [see the legend to Fig. 5].

DNA sequences of all constructs were then confirmed by DNA sequencing [Sanger et al. 1977]. The fourfold multimers were inserted into the multicloning site of the \( \text{P-element} \) vector PwHZ128, a derivative of PwHZ16 [Liaw et al. 1993]. These P-element constructs were injected into \( \text{w}1118 \) embryos as described [Rubin and Spradling 1982; Spradling and Rubin 1982]. For each construct, at least six independent lines were established.

**Characterization of promoter-driven \( \text{lacZ} \) expression and endogenous \( \text{tll} \) expression**

Expression of \( \beta \)-galactosidase in whole embryos was assayed by staining with X-gal [Raghavan et al. 1986]. Expression of mRNA of the \( \text{lacZ} \) gene and of the endogenous \( \text{tll} \) gene was monitored by in situ hybridization [Tautz and Pfeifle 1989] using random-primed digoxigenin-labeled [Boehringer Mannheim Biochemicals] \( \text{lacZ} \) DNA and a \( \text{tll} \) cDNA [Pignoni et al. 1990]. Embryonic stages were determined according to Campos-Ortega and Hartenstein (1985).
Purification of tor-REBs

Nuclear extracts from 0- to 12-hr embryos (~750 grams) were prepared as described (Soeller et al. 1986) and fractionated on a 50-ml heparin-Sepharose column. Fractions containing tor-REB, footprinting activity were eluted with 0.5 mM KC1 in HEMG-H [25 mM HEPES [pH 7.6], 0.1 mM EDTA, 12 mM MgCl2, 10% glycerol, and 1 mM dithiothreitol]. These fractions were pooled, diluted to 0.1 mM KC1 in HEMG (like HEMG-H except the concentration of MgCl2 is 2.5 mM) and applied to a 20-ml CM-Sepharose column. The column was eluted with 100 ml of a linear 0.1–0.5 mM KC1 gradient in HEMG. Fractions containing tor-REB footprinting activity (eluting between 0.18 and 0.24 mM KC1) were pooled, supplemented with Nonidet-P40 (0.1%), poly[d(C-A)], and purified by DNA affinity chromatography as described by Kadonaga and Tjian (1986). Two complementary oligonucleotides based on the DNA sequence of footprint F (5’-GATCCGAAGAGTT-TACTCAATGCTCAATGAATTCGGAGCGGCGGCGGCAA-3’, Fig. 4D) were used to prepare the DNA affinity resin. The columns were equilibrated with 0.2 mM KC1 HEMGN and the tor-REB was eluted at 0.4 mM KC1.

The procedure for the purification of the second tor-REB (tor-REBb) was identical to that described by Huang et al. (this issue) for purification of the DRE, with the exception that the DNA affinity column used was the tor-RE (the DNA sequence of footprint D, 5’-GATCGCTGCGTTCCTTGCTCAATGAATTTT-TCGCAATGGCAGCCTG-3’ instead of the DRE). The supernatant, divided into four parts, was applied to four 1-ml DNA affinity columns as described by Kadonaga and Tjian (1986). The columns were equilibrated with 0.25 mM KC1 HEMGN and the tor-REBb was eluted at 0.4 mM KC1.

The purplication of tor-REB footprinting activity [eluting between 0.18 and 0.24 mM KC1] were pooled, supplemented with Nonidet-P40 (0.1%), poly[d(C-A)], and purified by DNA affinity chromatography (Molecular Dynamics Inc.).

Purification of GAGA and GST–NTF-1

GAGA protein was purified from 0- to 12-hr embryonic nuclear extract by a simplification of the procedure of Biggin et al. (1988). Nuclear extract was loaded onto a 90 ml of SP-Sepharose column. Fractions containing GAGA footprinting activity, eluting at 0.35 mM KC1 HEMG, were pooled, supplemented with NP-40 and poly[d(A-T)], and purified by DNA affinity chromatography as described by Biggin et al. (1988).

GAGA was also purified from GAGA-expressing Escherichia coli BL21DE31SlyS3 containing the plasmid pARGAGA a generous gift of W. Soeller, Pfizer, Inc. Geno, CT). purification was as described by Lu et al. (1993), using LB medium and HEMG buffer instead of NZCYM medium and HEG buffer.

GST–NTF-1 was purified from expressing E. coli DH5α. NTF-1 cDNA [from R. Tjian] (Dynlacht et al. 1989) was cloned into the expression vector pGEX-2T (Pharmacia). The GST–NTF-1 fusion protein was purified using glutathione-agarose (Sigma) chromatography (Smith and Johnson 1988). To remove degradation products, the affinity-purified protein was concentrated to 0.5 ml, dialyzed against 0.1 mM KC1 HEMGN overnight and then chromatographed on a 50-ml Sephacyr S-300 (Pharmacia) column in 0.1 mM KC1 HEMGN. The first 2 ml of included volume from this column was 75% intact GST–NTF-1.

Protein concentrations were determined by the Bradford assay (Bio-Rad, Inc).

Western blotting using anti-GAGA antibodies

Proteins were loaded onto duplicate 9% polyacrylamide–SDS gels. Proteins in one gel were detected by silver staining (Ausbel et al. 1994), whereas proteins in the other gel were blotted to a nitrocellulose filter using a NovaBlot apparatus (model LKB 2117/250, Pharmacia). Incubation with antibody and detection of proteins on the nitrocellulose filter followed the Immunoblot Kit (Bio-Rad, Inc) protocol. The rat polyclonal anti-GAGA antibody (α519) was generously provided by C. Benyajati (University of Rochester, NY). Molecular mass markers were from Sigma Chemical Co. and Gibco; the sizes shown are the apparent molecular masses given by the supplier.

Phosphorylation of the NTF-1 protein by MAPK

Rat ERK2 (p42ppA) and constitutively active rabbit MAPK was generous gifts of T. Haystead (University of Virginia, Charlottesville). Phosphorylation was carried out as described by Haystead et al. (1992). ERK2 was activated by MAPKK in activation buffer (50 mM β-glycerolphosphate [pH 7.2], 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM DTT, 7.5 mM MgCl2, and 300 μM ATP) for 90 min at 30°C. Activated MAPK was dialyzed against activation buffer without Mg2+ and ATP. Aliquots (10 μl of activated MAPK were then incubated with either various proteins in activation buffer (with 100 μM [γ-32P]ATP rather than 300 μM ATP). Myelin basic protein was obtained from Sigma Chemical Co. After electrophoresis of the reaction products on a 10% polyacrylamide–SDS gel, phosphorylated proteins were detected by autoradiography. Incorporation of 32P into various bands was quantitated with a PhosphorImager (Molecular Dynamics Inc.).

Fly stocks and germ-line clones

Two genetic strains (gh) mutant lines, dp grh832 cn bw/CyO and dp grh832 cn bw/CyO, were obtained from S. Bray (University of Cambridge, UK). The dominant female sterile mutation Fsl2/D, which like grh maps to the right arm of the second chromosome (Schüpbach 1982), was obtained from T. Schüpbach, this chromosome was balanced with S1 Sp1 Ms(2)M1 bw2 CyO (obtained from the Bloomington stock center). Germ-line clones were generated by X-irradiation of first instar larvae from dp grh cn bw/CyO females crossed with Fsl2/D CyO males (Perrimon and Gans 1983; adult female progeny potentially carrying germ-line clones (i.e., dp grh cn bw/Fsl2/D) were mated in groups of 10 with grh/CyO males. Groups of females producing eggs with normal chorionic appendages (indicating the presence of homozygous grh germ-line clones, as germ-line cells carrying Fsl2/D produce eggs with abnormal or no dorsal appendages (Schüpbach 1982)) were used for egg collections.

The alleles Trr13c, Trrp2, and Trrp85 were obtained from F. Karch (Université de Genève, Switzerland). Trrp2 and Trrp85 were placed over the ovo31 FRT chromosome (obtained from the Bloomington stock center) and recombination induced with the heat shock FLP according to the protocol of Chou et al. (1993) but no clones were obtained. We also sought to generate germ-line clones by X-irradiation of Trr13c/ovo31 as described above, but again, no such clones were obtained.

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