Different levels of Ras activity can specify distinct transcriptional and morphological consequences in early *Drosophila* embryos

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**SUMMARY**

The terminal portions of the *Drosophila* body pattern are specified by the localized activity of the receptor tyrosine kinase Torso (Tor) at each pole of the early embryo. Tor activity elicits the transcription of two 'gap' genes, *tailless* (*tll*) and *huckebein* (*hkb*), in overlapping but distinct domains by stimulating the Ras signal transduction pathway. Here, we show that quantitative variations in the level of Ras activity can specify qualitatively distinct transcriptional and morphological responses. Low levels of Ras activity at the posterior pole direct *tll* but not *hkb* transcription; higher levels drive transcription of both genes. Correspondingly, low levels of Ras activity specify a limited subset of posterior terminal structures, whereas higher levels specify a larger subset. However, we also show that the response to Ras activity is not uniform along the body. Instead, levels of Ras activity which suffice to drive *tll* and *hkb* transcription at the posterior pole fail to drive their expression in more central portions of the body, apparently due to repression by other gap gene products. We conclude that *tll* and *hkb* transcription, as well as the terminal structures, are specified by two inputs: a gradient of Ras activity which emanates from the pole, and the opposing influence of more centrally deployed gap genes which repress the response to Ras.

Key words: *Drosophila*, body pattern, gradients, Ras, terminalia

**INTRODUCTION**

Receptor tyrosine kinases are involved in transducing a variety of extracellular signals that elicit diverse cellular responses. Yet, transduction of these signals generally depends on a common intracellular pathway involving the small G-protein Ras, and the serine-threonine kinases Raf, MEK and MAPK (Marshall, 1994; McCormick, 1994; Marshall, 1995). Moreover, activity of this pathway alone is often sufficient to transduce the signal and lead to the expected cellular response (Perrimon, 1994; Kayne and Sternberg, 1995; Marshall, 1995). Thus, a central question is how activation of the Ras pathway can elicit so many distinct outputs, either in different cell types or through the exposure of equivalent cells to different kinds or amounts of ligand.

Specificity could be achieved in several ways. For example, cells of different type might express different constellations of transcription factors and hence be predisposed to respond to Ras activity in different ways (Brunner et al., 1994; Cowley et al., 1994). Alternatively, cells of the same type might express more than one receptor tyrosine kinase and the activation of each of these receptors could produce a different output by engaging additional signal transduction pathways (Kazlauskas, 1994). Here, we consider a third way, namely that Ras activity may be continuously varied like a rheostat in response to different levels of a ligand and in this way provide a series of thresholds that elicit distinct outputs. Such a mechanism is of particular interest in developing tissues because some receptor tyrosine kinases are thought to mediate the patterned responses of cells to polarized or graded distributions of a given ligand (Casanova and Struhl, 1989; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993; Katz et al., 1995; Schweitzer et al., 1995).

One instance of such a patterning phenomenon is the control of terminal body patterning in *Drosophila* embryos, which depends on the receptor tyrosine kinase Torso (Tor) (Schüpbach and Wieschaus, 1986; Nüsslein-Volhard et al., 1987; Klingler et al., 1988; Casanova and Struhl, 1989; Sprenger et al., 1989). Although Tor is expressed uniformly along the surface of the early embryo, it is activated only at the poles, apparently in response to the localized activity of an extracellular ligand, possibly the protein Trunk (Casanova and Struhl, 1989; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993; Casanova et al., 1995). Activation of the Tor receptor then initiates a localized cascade of sequential Ras, Raf, MEK and MAPK activity within the embryo leading to the zygotic expression of two genes, *tailless* (*tll*) and *huckebein* (*hkb*), in overlapping but distinct domains at the poles (Pignoni et al., 1992; Lu et al., 1993a; Tsuda et al., 1993; Brunner et al., 1994; Casanova et al., 1994; reviewed by Lu et al., 1993b; Duffy and Perrimon, 1994). At least at the posterior pole, most if not all aspects of terminal patterning appear to be specified by the Tll and Hkb proteins which function as transcription factors to regulate the expression of a number of other target genes.
genes (Pignoni et al., 1990; Weigel et al., 1990; Brönné and Jäckle, 1991). Experiments in which the level of Tor activity is controlled by temperature, rather than by the distribution of its ligand, have shown that high levels of Tor activity specify the more terminal structures (such as the anal pad and anal tuft at the anterior end), whereas lower levels specify less terminal structures (such as derivatives of abdominal segments A7 and A8; Casanova and Struhl, 1989). Thus, the localized activity of the Tor receptor might organize \textit{tll} and \textit{hkb} expression and thereby specify terminal body pattern by generating a Ras activity gradient (Casanova and Struhl, 1989; Sprenger et al., 1989; Furriols et al., 1996).

In this paper, we demonstrate that different levels of constitutive Ras activity can elicit distinct molecular outputs – the transcription of either \textit{tll} alone, or \textit{tll} and \textit{hkb} together – as well as the formation of distinct portions of terminal body pattern. However, we also provide evidence that the response to a given level of Ras activity is not absolute but depends on cellular context, in particular on the presence or absence of other gap gene products. These findings indicate that cell pattern can be organized by mechanisms which depend on variations in both the level of Ras activity and in the responsiveness of cells to this activity.

**MATERIALS AND METHODS**

**Composition of transgenes for ubiquitous expression of RasV12 in early embryos**

To generate early embryos in which the constitutively active RasV12 protein is expressed ubiquitously, we constructed two transgenes in which the RasV12 coding sequence was placed under the control of the promoter of either the ribosomal protein 49 (rp49) gene (O’Connell and Rosbash, 1984; Kongsuwan et al., 1985) or the Tubulin\textsubscript{t1} (\textit{Tub1}) gene (Theurkauf et al., 1986). The rp49 promoter fragment is an approx. 2 kb segment of DNA beginning at a \textit{Pst} site at the 5’ end and extending to the A\textsc{TG} at the 3’ end which has been mutated to GG\textsc{TAC}C (a \textit{Kpn1} site; D. Kalderon, personal communication). The \textit{Tub1} fragment has been described previously (Basler and Struhl, 1994). The rp49 and \textit{Tub1} promoters direct low or moderate levels of transcription respectively, in most cells, as determined by examining the expression of \textit{rp49-lacZ} and \textit{Tub1-lacZ} transgenes (G.S., unpublished findings). Because even low level constitutive expression of the RasV12 coding sequence might be expected to be lethal, these transgenes were rendered conditional by inserting a Flp-out cassette between the promoter and coding sequence to terminate transcription (as in Struhl and Basler, 1993).

\textit{rp49>\textit{w}'>rasV12} transgene

A genomic fragment containing the \textit{rp49} promoter was placed upstream of the \textit{rasV12} coding sequence (Fortini et al., 1992) followed by the 3’ UTR of the \textit{hsp70} gene (Struhl and Basler, 1993), and the resulting \textit{rp49-rasV12-hsp70} 3’ UTR gene inserted into a derivative of the \textit{Carnegie} 20 transformation vector (Rubin and Spradling, 1982) lacking the \textit{y\textsc{+}} rescuing marker. A \textit{\textit{w}'>}\ Flp-out cassette was then constructed by introducing the \textit{\textit{w}'} minigene derived from the \textit{Casper} transformation vector (Pirrotta, 1988) between two minimal FRTs in the \textit{J33} plasmid (Struhl and Basler, 1993) and the cassette inserted between the \textit{rp49} and \textit{rasV12} sequences to create the final \textit{rp49>\textit{w}'>\textit{rasV12}} transgene.

\textit{Tub1>\textit{w}'>\textit{rasV12}}

A similar strategy was followed to that described for the \textit{rp49>\textit{w}'>\textit{rasV12}} transgene except that a genomic fragment containing the \textit{Tub1} promoter (Basler and Struhl, 1994) was used in place of the \textit{rp49} promoter fragment.

**Genetics**

**Generating 1X and 2X \textit{rp49-rasV12} embryos**

A balanced stock of the following genotype was generated by standard crosses: \textit{y w}\textit{; tor\textsubscript{EF} hsp70-flp.2}\textsc{CyO}; \textit{rp49>\textit{w}'>rasV12}/TM2. Late larvae and early pupae from this stock were heat shocked at 37°C for 60 minutes to excise the \textit{\textit{w}'>}\ Flp-out cassette from most of the resident transgenes (Struhl and Basler, 1993) and adult \textit{tor\textsubscript{EF}tor\textsubscript{EF} females carrying either one or two copies of the transgene were selected based on the presence or absence of the TM2 balancer chromosome. These females were crossed to wild-type males and allowed to lay eggs for 3–4 days before embryos were collected for analysis (to ensure that the mutant embryos derive exclusively from mutant female germ cells, rather than from mosaic nurse cell/oocyte complexes).

**Generating 1X \textit{rp49-rasV12} bcd osk tsl embryos**

Females of the genotype \textit{y w hsp70-flp.1; bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691}/TM2} were crossed to males of the genotype \textit{y w; rp49>\textit{w}'>rasV12; bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691}} to generate \textit{y w y w hsp70-flp.1; \textit{rp49>\textit{w}'>rasV12}; bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691}/bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691} females which were treated as described above. To obtain \textit{bcd osk} or \textit{bcd osk tsl} females carrying a single copy of the \textit{Tub1>rasV12} transgene, \textit{y w hsp70-flp.1; bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691}} \textit{Tub1>\textit{w}'>rasV12}/TM2 females were crossed to either \textit{bcd\textsuperscript{E1} osk\textsuperscript{166} or bcd\textsuperscript{E1} osk\textsuperscript{166} tsl\textsuperscript{E691}} males.

**In situ analysis**

RNA in situ were performed as described by Jiang et al. (1991) with the following modifications. For double labelling experiments, one RNA probe was labelled with dioxigenenin-conjugated UTP while the other probe was labelled with fluorescein-conjugated UTP. Both probes were hybridized at the same time and then detected sequentially using blue and red alkaline phosphate substrate reactions (as described by Strahle et al., 1994). For single labelling experiments, probes were labelled with dioxigenenin-conjugated UTP. Plasmids containing \textit{tll} (Pignoni et al., 1990) and \textit{hkb} (Brönné and Jäckle, 1991) cDNAs were a gift from J. Casanova (Casanova et al., 1994). Genomic PCR, using the primers CGGAATTCGATGCAGCATCCAGGG and CGGGCGCTAAGCTATCC was performed to obtain a 483 bp DNA fragment from the 3’ region of the \textit{byn} transcript (Kispert et al., 1994; Singer et al., 1996). This fragment was subcloned into bluescript (Stratagene) and used for the production of RNA probes.

**Cuticular analysis**

Embryos were allowed to develop for 24 hours and then mounted in a 1:1 mixture of Hoyer’s mountant and lactic acid (Struhl, 1984).

**RESULTS**

**Molecular and morphological responses to localized Tor activity at the posterior pole**

The Tor receptor, as well as Ras, Raf, MEK and MAPK, are required for the normal patterns of transcription of the gap genes \textit{tll} and \textit{hkb} at each end of the early embryo. The products of these gap genes then, in turn, specify the pattern of the most anterior and most posterior portions of the body (see Introduction; Fig. 1). At the posterior pole, the requirement for Tor and the Ras/Raf signal transduction pathway is absolute – neither \textit{tll} nor \textit{hkb} are expressed in their absence, and no terminal structures form. In contrast, both \textit{tll} and \textit{hkb} are expressed at the anterior pole in the absence of terminal...
signaling, albeit in abbreviated domains, under the control of the anterior determinant Bicoid (Pignoni et al., 1992; Liaw and Lengyel, 1993). In the present study, we are concerned with how localized Tor activity organizes different patterns of gene expression and cuticular differentiation; hence, to simplify the analysis, we focus on these responses in the posterior half of the body.

As shown previously (Pignoni et al., 1990; Brönner and Jäckle, 1991) and in Fig. 1, hkb and tll are expressed in overlapping domains which extend approximately 8% and 15% egg’s length (EL) from the posterior pole in syncytial blastoderm embryos (late stage 4 and early stage 5; staging as in Campos-Ortega and Hartenstein (1985)). The combined activities of tll and hkb then specify the more complex patterns of expression of subordinate transcription factors such as forchhead, hunchback, AbdominalB (AbdB), cad, and brachybere (byn) (Casanova, 1990; Weigel et al., 1990; Kispert et al., 1994; Singer et al., 1996; reviewed by Jürgens and Hartenstein, 1993). For example, tll activates byn transcription, while hkb represses it, resulting in a stripe of byn expression (Kispert et al., 1994; see Fig. 1). All of these gene functions appear to play significant roles in directing the formation of the posterior terminalia, including the ventral denticle belt of the eighth abdominal segment (A8), the posterior spiracles, the anal pad and tuft, and internal structures such as the hindgut, posterior midgut, and Malpighian tubules (Casanova, 1990; Jürgens and Hartenstein, 1993).

**Different levels of Ras activity specify distinct transcriptional responses.**

To test whether different levels of Ras activity can suffice to specify distinct transcriptional responses at the posterior pole, we sought to generate embryos that lack Tor, but express different levels of a constitutively active form of Ras, RasV12 (Trahey and McCormick, 1987). This was accomplished by creating females with the following genetic properties. First, they were homozygous for a null allele of the tor gene: embryos developing from such mutant females lack Tor-dependent activity of endogenous Ras. Second, they carried one or two copies of a Flp-out transgene, rp49>W+>rasV12, composed of the promoter from the ubiquitously expressed ribosomal protein 49 (rp49) gene (O’Connell and Rosbash, 1984), a W+ Flp-out cassette which blocks transcription (>W+>, and the rasV12 coding sequence (Materials and Methods). Finally, they carried an hsp70-flp transgene in which the coding sequence for the yeast recombinase Flp is placed under the control of the Drosophila hsp70 heat shock promoter. Late third instar larvae and early pupae of this genotype were heat shocked to catalyze the excision of the Flp-out cassette from most of the resident rp49>W+>rasV12 transgenes (see Materials and Methods). We refer to embryos derived from such tor- female germ cells as 1X rp49>rasV12 and 2X rp49>rasV12 embryos depending on whether the females carried one or two copies of the transgene. By the same convention, we refer to embryos derived simply from tor or wild-type females as 0X rp49>rasV12 or ras+ respectively.

As shown in Fig. 1, neither tll nor hkb transcripts are expressed posteriorly in 0X rp49>rasV12 embryos. However tll is expressed in a narrow domain at the posterior of 1X rp49>rasV12 embryos and in a broader domain in 2X rp49>rasV12 embryos. We also found that tll appears to be expressed at higher level at the posterior pole of 2X rp49>rasV12 embryos compared to the posterior pole of 1X rp49>rasV12 embryos (data not shown). In contrast, we failed to detect posterior hkb expression in 1X rp49>rasV12 embryos, but could readily detect hkb transcription at the posterior pole of 2X rp49>rasV12 embryos. hkb is expressed at the posterior pole of these 2X rp49>rasV12 embryos in a domain somewhat narrower, and at a level somewhat lower, than that seen in ras+ embryos.

We draw three conclusions from these results. First, the different levels of constitutive RasV12 activity in 1X and 2X rp49>rasV12 embryos appear to distinguish between two qualitatively distinct transcriptional outputs: tll alone, and tll plus hkb. Second, the level of RasV12 activity is also related quantitatively to target gene transcription, as the higher level of RasV12 activity in 2X rp49>rasV12 embryos appears to generate a higher level of posterior tll expression than that generated in 1X rp49>rasV12 embryos. Third, even though we would anticipate that RasV12 is active uniformly throughout these embryos, we find that tll and hkb are only transcribed in tightly restricted domains at the poles, with the boundary of the tll domain depending on the different levels of RasV12 activity generated in 1X and 2X rp49>rasV12 embryos. These last results suggest the existence of a local differential in the ability of nuclei to respond to a constant level of RasV12 activity.

We also examined the transcription of the byn gene, in 1X and 2X rp49>rasV12 embryos (Fig. 1). In 1X rp49>rasV12 embryos we observe that byn transcripts are expressed in a small cap (approx. 9% EL; egg length), whereas the domain of expression in 2X rp49>rasV12 embryos is significantly broader (approx. 15% EL). We note that byn expression extends back to the posterior end of 2X rp49>rasV12 embryos, unlike in ras+ embryos in which byn expression is normally repressed at the posterior pole. Because both the activation and repression of terminal byn expression are known to depend, respectively, on tll and hkb, we surmise that higher levels of Ras activity are required at the posterior of wild-type (ras+) embryos to drive sufficiently high levels of Hkb expression to repress byn expression. These results reinforce those obtained for tll and hkb transcription and further suggest that both these genes are regulated quantitatively as well as qualitatively by the level of Ras activity.

**Different levels of Ras activity specify distinct terminal structures.**

We next examined the effects of different levels of Ras activity on the differentiation of terminal structures. As shown in Fig. 1, 1X rp49>rasV12 embryos show a modest restoration of posterior terminal structures which are absent in the 0X rp49>rasV12 embryos. In particular, these embryos form the least terminal of the posterior terminal structures: the eighth abdominal denticle band and the posterior spiracles. The extent of restoration is considerably greater in 2X rp49>rasV12 embryos: these form additional terminal structures such as the anal tuft and anal pads. As observed for the patterns of tll and hkb transcription in 2X rp49>rasV12 embryos, these structures appear in the normal spatial order, and hence the external cuticular pattern of these embryos is similar to that of wild-type (ras+) embryos. Thus, we conclude that different levels of Ras can elicit the formation of distinct subsets of terminal
pattern elements, just as they distinguish between different transcriptional outputs.

**Different responses to a constant level of Ras activity**

As noted above, the localized expression of *tll* and *hkb* at the posterior poles of *IX* and 2X *rp49 rasV12* embryos indicates that nuclei located at different positions along the anteroposterior axis of late syncytial embryos do not respond in the same way to a constant level of RasV12. The gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*), and *giant* (*gt*) are expressed in overlapping central domains of the body, organized in large part by the anterior and posterior determinants Bicoid (*Bcd*) and Oskar (*Osk*) (reviewed by St. Johnston and Nüsslein-Volhard, 1992). All four of these gap genes encode transcription factors, and at least some are capable of acting as repressors (reviewed by Gray and Levine, 1996). Hence, it is possible that the localized expression of one or more of these factors blocks the response of nuclei in more central portions of the body to Ras activity.

To test this possibility, we have examined the consequences of generating uniform RasV12 activity in embryos lacking the anterior and posterior determinant systems as well as Tor receptor activity. This was accomplished by creating females that (i) were homozygous for recessive loss-of-function mutations of the *bcd*, *osk*, and *torso-like* (*tsl*) genes (the *tsl* gene is required during oogenesis for the activity of the Tor receptor during embryogenesis; Stevens et al., 1990; Savant-Bhonsale and Montell, 1993; Martin et al., 1994), (ii) carried the *hsp70-flp* transgene and (iii) carried one copy of either the *rp49 w+ rasV12* transgene or a *Tubalinat >w+ rasV12* transgene which has a stronger ubiquitous promoter derived from the *Tubulinat* (*Tub*) gene (Materials and Methods).

In the absence of exogenous RasV12 activity, embryos derived from *bcd* osk* tsl* females express *Kr* uniformly and at high level, but lack detectable *hb*, *kni*, *gt*, *tll* and *hkb* transcription (Struhl et al., 1992; data not shown). These embryos also express moderate levels of Hb protein derived from ubiquitous, maternally derived *hb* transcripts. When *bcd* osk* tsl* females carrying a single copy of either the *rp49 w+ rasV12* or *Tubalinat >w+ rasV12* transgene were heat shocked as late larvae or early pupae, they gave rise to embryos that expressed *tll* (Fig. 2B and data not shown). Thus, all of the nuclei in these embryos appear to respond similarly to low to moderate levels of constitutive RasV12 activity by transcribing *tll*, but not *hkb*.

As shown in Fig. 1G, doubling the dosage of the *rp49 rasV12* transgene in *tor* females generates embryos that have sufficient RasV12 activity to direct *hkb* expression at the posterior pole, and a similar result is obtained using a single copy of the *Tubalinat rasV12* transgene in place of two copies of the *rp49 rasV12* transgene (Fig. 2A). Nevertheless, the same level of constitutive RasV12 activity is not sufficient to drive

![Fig. 1. Qualitatively distinct responses in embryos containing different levels of Ras activity.](Image 199x255 to 559x550)
of the Tub\(\alpha T > ras^{V12}\) transgene. These embryos retain wild-type tsl function and hence give rise to embryos in which ubiquitous Ras\(^{V12}\) activity is supplemented at the poles by normal levels of endogenous Ras activity. As shown in Fig. 2C, they showed localized transcription of hkb at both poles, presumably in response to normal, localized activity of endogenous Ras. Thus, we infer that higher levels of Ras activity generated under these conditions can suffice to drive hkb transcription.

Finally, it is informative to compare the patterns of \(tll\) and hkb transcription at the posterior poles of embryos derived from \(bcd^{-}\-osk^{-}\) females (Fig. 2D) with those derived from \(tor^{-}\) females which carry one copy of the Tub\(\alpha T > ras^{V12}\) transgene (Fig. 2A). In the former, these patterns depend solely on the normal, localized activity of the Tor receptor, and hence on the polarized activity of wild-type Ras. In the latter, the only Ras activity is that of the uniformly expressed Ras\(^{V12}\) protein; consequently, the restricted expression of \(tll\) and hkb presumably reflects the ability of the remaining gap gene products to repress Ras-dependent transcription of \(tll\) and hkb. Nevertheless, each input can generate a correctly ordered pattern of \(tll\) and hkb expression, suggesting that they function in a cooperative fashion to organize \(tll\) and hkb expression during normal development.

### DISCUSSION

During early embryogenesis, terminal patterning is organized by a mechanism involving local activation of a transmembrane receptor tyrosine kinase, Tor, and transduction by the Ras signal transduction pathway (reviewed by Perrimon, 1993). Here, we show that different levels of a constitutively activated form of Ras, Ras\(^{V12}\), are able to generate different transcriptional responses and specify the formation of distinct structures at the posterior end of the body. Low levels of Ras\(^{V12}\) suffice only to direct \(tll\) transcription and the formation of posterior structures such as the A8 ventral denticle band and the posterior spiracles, whereas higher levels induce both \(tll\) and hkb transcription and specify additional, more terminal structures such as the anal pads and anal tuft. Thus, our results establish that quantitative variation in the level of the Ras activity can lead to qualitatively distinct outcomes, and hence suggest that localized activation of the Tor receptor organizes terminal body pattern by creating a gradient of activity of the Ras signal transduction pathway. Our results also draw attention to the presence of another significant influence on terminal body pattern, namely the localized deployment of other gap gene products in more central portions of the body. At least one of these gap gene products appears to dampen or block the responsiveness to activated Ras, counteracting the influence of the Ras activity gradient emanating from the pole.

### Generation and interpretation of a Ras activity gradient

The receptor Tor is thought to be activated by an extracellular ligand that is generated locally at each pole and then adsorbed and sequestered by binding to the receptor (Stevens et al., 1990; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993). Diffusion of the ligand before binding might suffice to generate a graded distribution of activated Tor and hence of activated Ras. It is also possible that formation of the

### Fig. 2. Opposing roles of Ras and centrally expressed gap genes in controlling \(tll\) and hkb transcription. The diagrams adjacent to each embryo represent, in simplified form, early gap gene expression and Ras activity in the embryo. In embryos derived from \(tor^{-}\) females carrying one copy of the Tub\(\alpha T > ras^{V12}\) transgene (A), as in embryos derived from \(tor^{-}\) females carrying two copies of the \(rp49 > ras^{V12}\) transgene (Fig. 1G-I), moderate levels of uniform Ras\(^{V12}\) activity induce \(tll\) and hkb expression at the poles. In embryos derived from \(bcd^{-}\-osk^{-}\-tsl^{-}\) females (B), Hb and Kr are expressed uniformly and Kni and Gt expression are repressed by Hb (Struhl et al., 1992); under these conditions, moderate levels of uniform Ras\(^{V12}\) activity derived from the Tub\(\alpha T > ras^{V12}\) transgene drive \(tll\), but not hkb, transcription throughout the embryo. hkb expression is, however, restored at the posterior pole of embryos derived from \(bcd^{-}\-osk^{-}\) females carrying one copy of the wild-type tsl allele and one copy of the Tub\(\alpha T > ras^{V12}\) transgene (C), presumably due to high levels of endogenous Ras activity generated by the Tor receptor. As shown in (D), this endogenous Ras activity is sufficient to specify essentially normal domains of expression of both \(tll\) and hkb in embryos derived from \(bcd^{-}\-osk^{-}\) females that lack Ras\(^{V12}\) activity, even though the remaining gap gene products are initially either absent or uniformly expressed.

hkb transcription in embryos lacking all three determinant systems, even at the posterior pole (Fig. 2B). We note that the particular constellation of gap gene proteins uniformly expressed in embryos from \(bcd^{-}\-osk^{-}\-tsl^{-}\) females (High Kr, moderate Hb, no Kni and no Gt; Struhl et al., 1992) is not found at the posterior pole of embryos derived from either wild-type or \(tor^{-}\) females. Thus, the abnormal presence of Hb and/or Kr throughout these embryos might be responsible for their failure to express hkb in response to moderate Ras\(^{V12}\) activity.

To ask whether a higher level of Ras activity can elicit hkb transcription in embryos lacking both the anterior and posterior determinant systems, we examined \(tll\) and hkb transcription in embryos derived from \(bcd^{-}\-osk^{-}\) females carrying a single copy
Ras activity gradient depends at least in part on subsequent movement of activated Tor, Ras or other downstream signaling components within the syncytial embryo.

The most likely intracellular targets of the Ras transduction pathway are factors which regulate \textit{tll} and \textit{hkb} transcription. In the case of \textit{tll}, analysis of \textit{cis}-acting regulatory sequences upstream of the promoter suggests that activity of the Ras pathway normally activates \textit{tll} transcription by antagonizing the action of a ubiquitously expressed transcriptional repressor which binds to these sequences (Liaw et al., 1995). One candidate for this repressor is the product of \textit{grainyhead} gene, a homologue of the vertebrate \textit{NFT-1} gene. \textit{grainyhead} function is apparently required to prevent general transcription of the endogenous \textit{tll} gene (Liaw et al., 1995). Moreover, MAPK is capable of phosphorylating \textit{NFT-1} in vitro (Liaw et al., 1995). Hence, a Ras activity gradient might generate a graded distribution of phosphorylated Grainyhead protein, relieving repression of \textit{tll} transcription when a sufficient fraction of the protein is phosphorylated and hence inactivated.

A similar mechanism could also apply to the localized transcription of \textit{hkb}. For example, if Grainyhead were to act directly as a repressor of both \textit{tll} and \textit{hkb}, different levels of the active form of the protein might suffice to repress each gene, and hence, different levels of Ras activity would be required to release them from repression. Alternatively, other transcription factors that have a different sensitivity to the Ras signal transduction pathway may be responsible for regulating \textit{hkb} transcription.

Whatever the mechanism, our findings indicate that the \textit{tll} and \textit{hkb} genes respond in qualitatively distinct ways to different levels of Ras activity and suggest that a relatively small difference in Ras activity – that resulting from a two-fold increase in the maternal dose of the \textit{rp49>rasV12} transgene – can suffice to distinguish between the ‘off’ and ‘on’ states of transcription. Thus, the threshold sensitivity of \textit{tll} and \textit{hkb} to differences in Ras activity may be similar to that exhibited by other gap genes in response to the Bicoid and Hunchback gradient morphogens (Struhl et al., 1989, 1992).

Although most, or all, aspects of posterior terminal pattern are governed by the actions of \textit{Tll} and \textit{Hkb} protein, the resulting patterns of gene expression and cuticular differentiation are complex, involving overlapping patterns of transcription of many target genes such as \textit{byn}, \textit{fkh}, \textit{caudal} and \textit{abdB}, and the formation of diverse morphological structures. Previous studies (Strecker et al., 1988; Casanova, 1990; Diaz et al., 1996) have provided evidence that this complexity arises in part from the formation of local gradients of \textit{Tll} and \textit{Hkb} expression within the domains in which the two genes are transcribed. We have observed that the levels of both \textit{tll} and \textit{hkb} transcription appear to be sensitive to the level of Ras activity. For example, \textit{tll} is expressed at higher level at the posterior end of 2X \textit{rp49>rasV12} embryos compared to 1X \textit{rp49>rasV12} embryos, and the level of posterior \textit{hkb} transcription appears similarly dependent when 2X \textit{rp49>rasV12} and \textit{ras+} embryos are compared. As illustrated by the transcription of \textit{byn} in these embryos, quantitative differences in \textit{tll} and \textit{hkb} transcription appear to correlate with corresponding differences in the transcription of further downstream target genes. Thus, the activity gradient of Ras may be translated into local gradients of \textit{tll} and \textit{hkb} which in turn have instructive roles in organizing subordinate gene expression and differentiation.

A complicating factor in interpreting the organizing influence of the Ras activity gradient is the role played by other gap genes, which appear to modulate the response to Ras activity. Our results establish that nuclei at different positions along the anteroposterior axis are predisposed to respond in distinct ways to the same level of constitutive \textit{rasV12} activity. Moreover, they indicate that this predisposition results from the localized activities of one or more of the remaining gap genes in more central portions of the body. In the posterior half of the body, the gap proteins Hunchback (Hb), Krüppel (Kr), Knirps (Kni) and Giant (Gt) are expressed in an ordered series of overlapping domains, and these are organized principally in response to the anterior and posterior determinants Bicoid (Bcd) and Osk (Osk). Hence, irrespective of the activity of the terminal determinant system, nuclei at different positions from the posterior pole will be exposed to different constellations of these proteins, all of which are DNA binding transcription factors. As shown in Figure 2, localized expression of these ‘central’ gap gene proteins can suffice to organize overlapping posterior domains of \textit{tll} and \textit{hkb} transcription in 1X \textit{Tubat1>rasV12} embryos in which all nuclei are exposed to the same level of Ras activity.

Thus, nuclei in the posterior half of the body are subject to two, independent inputs which control the expression of \textit{tll} and \textit{hkb}: a Ras activity gradient spreading from the posterior pole which drives their expression, and an opposing gradient of one or more central gap gene proteins which repress their expression. Under the appropriate circumstances, either input can generate a correctly ordered pattern of \textit{tll} and \textit{hkb} transcription. In the wild-type condition, the two inputs may function in a mutually reinforcing fashion to generate the normal patterns of transcription of both target genes. This situation is analogous to the opposing roles of Bcd and Osk in organizing the expression of the central gap genes themselves (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Wharton and Struhl, 1991; Struhl et al., 1992), and may reflect a common strategy used in pattern formation. As is the case for Bcd and Osk, the opposing activities of the Ras gradient and the central gap genes also appear to be mutually exclusive. Conditions which force high levels of ectopic gap gene expression at the posterior pole block terminal development, probably through the repression of \textit{tll} and \textit{hkb} transcription (Gaul and Jäckle, 1989; Struhl, 1989a). Conversely, conditions which force high levels of ectopic Tor or Ras activity in the central portion of the body can block transcription of the central gap genes (Klingler et al., 1988; Casanova and Struhl, 1989; Steingrimsson et al., 1991). A similar phenomenon of mutual exclusion and collaboration between opposing signals is observed in the developing adult legs, where Wg and Dpp restrict each other’s expression defining the dorso-ventral axis, but operate in conjunction to establish the proximo-distal axis (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996).

A rheostat function for Ras: implications for other systems

As noted in the Introduction, receptor tyrosine kinases are capable of transducing a variety of signals through the Ras pathway, in some cases generating distinct outputs to different ligands or to different concentrations of the same ligand (reviewed by Marshall, 1995). Our present demonstration that
different levels of a constitutively active form of Ras can elicit distinct transcriptional outputs indicates one mechanism by which this specificity can be achieved. In essence, Ras can function as a rheostat in which quantitative variations in activity provide one or more thresholds that differentially regulate gene expression. This mechanism may be particularly important in developmental contexts in which the graded or localized distribution of ligand appears to organize cell pattern. In addition to terminal body patterning, receptor tyrosine kinases have been implicated in a number of patterning phenomena of this kind in Drosophila, including organization of the follicular epithelium during oogenesis and control of dorso-ventral epidermal pattern in the embryo (Clifford and Schüpbach, 1989; Price et al., 1989; Brand and Perrimon, 1994; Schweitzer et al., 1995). A rheostat mechanism might also be involved in situations in which cells of the same type can respond in distinct ways to different ligands, each received by a different receptor tyrosine kinase (Marshall, 1995). A possible example of this phenomenon in Drosophila is the R7 cell in the Drosophila eye, which requires the activity of both Sevenless and the EGF receptor during development (Campos-Ortega et al., 1979; Tomlinson and Ready, 1987; Xu and Rubin, 1993; Freeman, 1996).

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