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Developmental Biology 278 (2005) 576–586

DEVELOPMENTAL BIOLOGY

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Genomes & Developmental Control

An endoderm-specific GATA factor gene, *dGATAe*, is required for the terminal differentiation of the *Drosophila* endoderm

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Received for publication 27 April 2004, revised 16 November 2004, accepted 16 November 2004
Available online 17 December 2004

Abstract

GATA factors play an essential role in endodermal specification in both protostomes and deuterostomes. In *Drosophila*, the GATA factor gene *serpent* (*srp*) is critical for differentiation of the endoderm. However, the expression of *srp* disappears around stage 11, which is much earlier than overt differentiation occurs in the midgut, an entirely endodermal organ. We have identified another endoderm-specific *Drosophila* GATA factor gene, *dGATAe*. Expression of *dGATAe* is first detected at stage 8 in the endoderm, and its expression continues in the endodermal midgut throughout the life cycle. *srp* is required for expression of *dGATAe*, and misexpression of *srp* resulted in ectopic *dGATAe* expression. Embryos that either lacked *dGATAe* or were injected with double-stranded RNA (dsRNA) corresponding to *dGATAe* failed to express marker genes that are characteristic of differentiated midgut. Conversely, overexpression of *dGATAe* induced ectopic expression of endodermal markers even in the absence of *srp* activity. Transfection of the *dGATAe* cDNA also induced endodermal markers in *Drosophila* S2 cells. These studies provide an outline of the genetic pathway that establishes the endoderm in *Drosophila*. This pathway is triggered by sequential signaling through the maternal *torso* gene, a terminal gap gene, *huckebein* (*hkb*), and finally, two GATA factor genes, *srp* and *dGATAe*.

Keywords: GATA factor; *dGATAe*; *srp*; Endoderm; *Drosophila*

Introduction

The endoderm gives rise to major parts of the gut tube of multicellular organisms. Regulatory mechanisms that establish the endoderm have recently received considerable attention with respect to the development of protostomes and deuterostomes. Certain important components of this genetic regulatory pathway/network have now been identified (for reviews, see Maduro and Rothman, 2002; Shivdasani, 2002). It remains unknown whether protostomes and deuterostomes share a common genetic mechanism of endoderm specification. However, GATA factor genes are expressed throughout endodermal development in both animal groups (Maduro and Rothman, 2002; Shivdasani, 2002). GATA factors have one or two characteristic zinc-finger motifs corresponding to $CXNCX_{17}CXNC$, and act as transcription factors that bind to a consensus DNA sequence WGATAR of specific target genes (Evans and Felsenfeld, 1989; Orkin, 1992; Tsai et al., 1989).

In vertebrates, GATA factor genes are classified into two groups, *GATA-1/-2/-3* and *GATA-4/-5/-6*. While *GATA-1*, *-2*, and *-3* are involved in hematopoiesis (Orkin and Zon, 1997), *GATA-4*, *-5*, and *-6* are essential for the development of endoderm-derived tissues, in that they activate endoderm-specific genes such as IFABP, gastric H^+/K^+-ATPase, HNF4, and albumin (Arceci et al., 1993; Bossard and Zaret, 1998; Fujikura et al., 2002; Gao et al., 1998; Koutsourakis et al., 1999; Laverriere et al., 1994; Maeda et al., 1996; Morrisey et al., 1998; Shivdasani, 2002). The GATA factor genes are also essential for

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doi:10.1016/j.ydbio.2004.11.021

endodermal development in the protostomes *Caenorhabditis elegans* and *Drosophila melanogaster* (Maduro and Rothman, 2002; Murakami et al., 1999). *C. elegans* has eleven GATA factor genes, and seven of these are known to be related to endodermal development, resulting in redundant regulatory pathways (Maduro and Rothman, 2002). *end-1* is the earliest GATA factor gene that is expressed specifically in the endoderm lineage. *end-1* mutant embryos fail to form the endoderm (Zhu et al., 1997), whereas overexpression of *end-1* can induce non-endodermal cells to switch to an endodermal fate (Zhu et al., 1998). *end-1* can also induce endoderm formation when it is expressed in *Xenopus* embryos (Shoichet et al., 2000), suggesting that the genetic mechanisms underlying endodermal development are at least partially shared between protostomes and deuterostomes.

In *C. elegans*, *end-1* activates another GATA gene, *elt-2*, and *end-1* expression ceases prior to overt differentiation of the endoderm. *elt-2* continues to be expressed in the gut throughout life, and activates genes that are required for various gut functions (Fukushige et al., 1998). In the protostome *D. melanogaster*, *serpent* (*srp*) is a GATA gene that is essential for the specification of endoderm (Rehorn et al., 1996; Reuter, 1994). *srp* is expressed in the anterior and posterior terminal regions of the blastoderm, which both give rise to the endoderm. In the *srp* mutant embryo, the prospective endodermal region differentiates into the ectodermal hindgut. In normal embryos, the prospective hindgut region abuts the prospective endoderm of the posterior terminal, and the hindgut is specified by the *Brachyury* ortholog, *brachyenteron* (*byn*) (Kispert et al., 1994; Murakami et al., 1995; Singer et al., 1996). The initial area in which *byn* is expressed in the cellular blastoderm includes the prospective posterior endoderm. However, *byn* expression in the posterior half of this region is soon repressed by *srp* and the region develops into the endoderm. In the *srp* embryo, *byn* expression expands to the prospective endodermal regions. Activation of *srp* depends on a zygotic gap gene, *huckebein* (*hkb*), which is triggered by maternal Torso activity at the anterior and posterior terminal regions of the fertilized egg, followed by activation of Ras signaling (Brönner and Jäckle, 1991; Brönner et al., 1994; Rehorn et al., 1996; Reuter, 1994). These events outline the genetic pathway that defines endodermal development in *Drosophila* to date, and represent one of the best examples of a genetic pathway that specifies organogenesis (Murakami et al., 1999).

Despite the significant progress that has been made in characterizing the pathway described above, another, as yet unknown, gene seems to be involved, since *srp* ceases to be expressed after stages 10–11 in the prospective endoderm, long before overt differentiation of midgut. Since several GATA factor genes are sequentially expressed during the development of the *C. elegans* endoderm, we expected to identify a novel GATA gene in *Drosophila*. Thus far, three GATA factor genes have been reported in *Drosophila*: *pannier* (*pnr*, also known as *dGATAa*), *srp* (also known as *dGATAb*), and *grain* (also known as *dGATAc*) (Abel et al., 1993; Lin et al., 1995; Ramain et al., 1993; Rehorn et al., 1996; Winick et al., 1993). By searching the *Drosophila* genome sequence, we found several sequences containing novel GATA factor motifs, and identified two GATA factor genes, *dGATAd* and *dGATAe*. While *dGATAd* is not expressed in the embryo, *dGATAe* is specifically expressed in the endoderm after stage 8, and it continues to be expressed in the endodermal midgut of larvae and adult flies. In this study, we analyzed the regulation and function of the *dGATAe*. We show that *dGATAe*, upon activation by *srp*, induces overt differentiation of the *Drosophila* endoderm. This finding has enabled us to delineate almost the entire genetic pathway of *Drosophila* endodermal development. This pathway is initiated by early maternal signals and results in the terminal differentiation of the midgut.

Materials and methods

Fly stocks

The following strains were used: OregonR; srp^{2} (amorphic); pnr^{VX6} (Heitzler et al., 1996); fkh^{XT6} (Weigel et al., 1989a,b); Kr^{1} (Liu and Jack, 1992); *Df(3R)sbd45* (a deficiency lacking *dGATAe* and *pnr*); UAS-*srp* (Hayes et al., 2001); UAS-*pnr* (Haenlin et al., 1997); *Ay*-GAL4, which is driven constitutively by Act5C promoter (Ito et al., 1997); *byn*-GAL4, which is specific to the hindgut primordium (Iwaki and Lengyel, 2002); G455.2-GAL4, which is specific to the hindgut primordium (San Martin and Bate, 2001); and PY258, an enhancer-trap line that marks the midgut (Murakami et al., 1994).

cDNA cloning, sequencing, and germ line transformation

In searching the *Drosophila* genome, we found two sequences that encode novel GATA-type zinc fingers, and identified the corresponding *Drosophila* EST clones (GH03570 and LD08432). The genes were sequenced and designated as *dGATAd* (accession No. AB055143) and *dGATAe* (accession No. AB054995, AB054996), respectively, in accordance with the nomenclature used in previous studies (Abel et al., 1993; Winick et al., 1993). While no embryonic *dGATAd* expression was detected, *dGATAe* was found to be expressed specifically in the endoderm, as detailed below. The EST clone of *dGATAe* (LD08432) has a premature stop codon immediately after the C-terminal finger, which most likely reflects a mutation of the clone, since sequences of the genomic and cDNA clones obtained from *Drosophila* cDNA libraries (Brown and Kafatos, 1988) did not have this premature stop codon. A cDNA clone that included the entire coding region of *dGATAe* was obtained from the cDNA libraries by PCR (accession No. AB124838) with the following primers, 5′-

AGGCGGCCGCCTAACGCTGGAAGAAACGCAAA-3′ and 5′-GGGGTACCCCCGTAGCTTAAGCCATAAGTC-3′, which included *Not*I and *Kpn*I adaptors. DNA sequencing of cDNA clones and EST clones were performed using a LI-COR model 4000 L. The CLUSTAL W program in the DNA Data Bank of Japan was used to perform multiple sequence alignments of GATA factor genes in *Drosophila*, *C. elegans*, and mouse (Thompson et al., 1994). A molecular phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

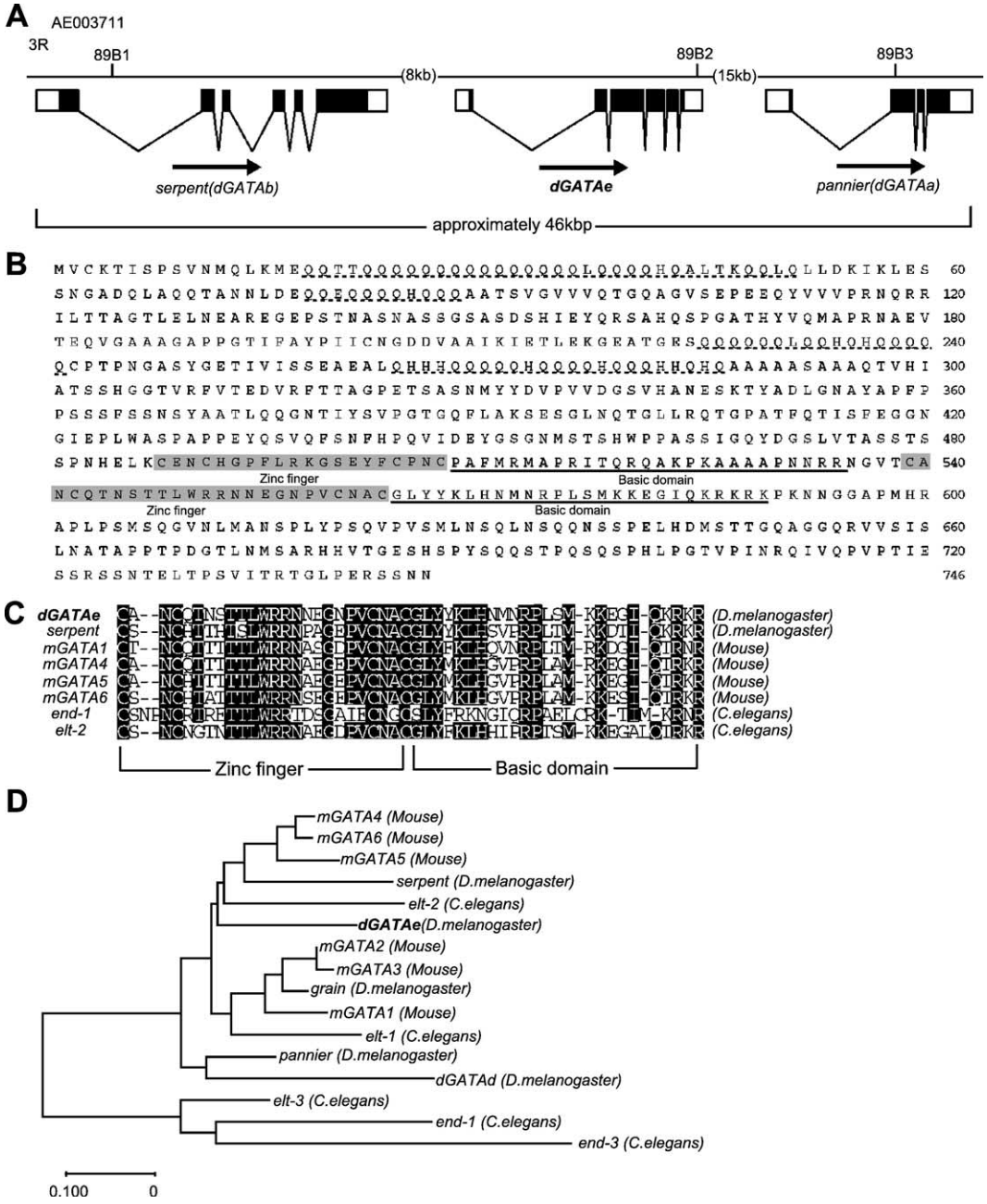

Fig. 1. Genomic structure, deduced amino acid sequence, and molecular phylogenetic tree of *dGATAe*. (A) Three GATA factor genes, *srp*, *dGATAe*, and *pnr* are clustered in a genomic region spanning about 46 kb at cytological map position 89A13-89B4 on the right arm of the 3rd chromosome. Codes on the bar indicate cytological map positions, and numbers in parentheses indicate the lengths of intergenic regions between the three genes. The structures of *pnr* and *srp* have been reported previously (Rehorn et al., 1996; Winick et al., 1993). (B) Deduced amino acid sequence of *dGATAe*. *dGATAe* encodes a predicted protein of 746 amino acids. GATA-type zinc finger motifs are marked with gray boxes, and the basic domain is underlined. The glutamine-rich domains are marked with dotted lines. (C) Alignment of the amino acid sequence of C-terminal finger and associated basic domain of *dGATAe* with those of known GATA factors of *Drosophila* (*srp*), *C. elegans* (*end-1* and *elt-2*), and mouse (*mGATA-1/4/5/6*). The alignment shows that these domains are highly homologous across species. (D) Molecular phylogenetic tree of the GATA factors. Amino acid sequences of the C-terminal finger and basic region were analyzed with the CLUSTAL W program (Thompson et al., 1994), and a molecular phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). *dGATAe* is relatively close to *mGATA-4/5/6*, *serpent*, and, *elt-2*, but distinct subgroupings are not recognized.

The *dGATAe* cDNA was subcloned into the *Not*I–*Kpn*I site of the pUAST vector, and germline transformation was carried out as described by Brand and Perrimon (1993). The resulting UAS-*dGATAe* strains were crossed with GAL4 driver strains and used for overexpression experiments.

In situ hybridization, immunostaining, and histochemical staining

Embryos were subjected to in situ hybridization using Dig-11-UTP-labeled RNA probes, as described previously (Takashima and Murakami, 2001). cDNAs or EST clones corresponding to the following genes were used as templates for Dig-RNA probes: *dGATAe*; *integrin* β_v (Yee and Hynes, 1993); *innexin7* (*inx7*) (Stebbings et al., 2002); *midgut expression 1* (*mex1*) (Schulz et al., 1991); *Race* (Tatei et al., 1995); *byn* (Murakami et al., 1995). Anti-Srp immunostaining was performed as previously described (Sam et al., 1996). Histochemical staining for β-galactosidase activity was performed as previously described (Murakami et al., 1994).

RT-PCR

Total RNA was prepared from the midgut and hindgut of third instar larvae and adult flies, and from S2 cells transfected with *dGATAe* cDNA. mRNA was purified from the total RNA samples with the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). First-strand cDNA was synthesized with a ReadyToGo kit (Amersham Pharmacia Biotech). PCR was performed using the first strand cDNA as a template and with specific primers for the following genes: *dGATAe*, 5′-AGGATGTACGCTTCACCACC-3′ and 5′-CTTAGGCTTCCGTTTGCGCT-3′; *integrin* β_v, 5′-CAGGTGGGATATCGTTGTCT-3′ and 5′-TCAGCACTTAGGATGTCCAC-3′; *inx7*, 5′-GACCAGAAGGAAACTGCCTA-3′ and 5′-GAATCAATCGTACAGACC-3′; *ras2* as an internal control, 5′-ACGAAGCAGTGCAACATCGAC-3′ and 5′-ATCCTGCTCGATGAAGGGACG-3′.

dsRNA-mediated genetic interference

To inactivate *dGATAe* mRNA in embryos, double-stranded RNA (dsRNA) encoding *dGATAe* was injected into fertilized eggs as described (Kennerdell and Carthew, 1998). Briefly, a fragment of *dGATAe* cDNA (nucleotides 1069–2339) was first amplified by PCR with primers containing T7 promoter–adapter sequences. Both sense and antisense RNA were then simultaneously synthesized with T7 RNA polymerase. The sense and antisense RNAs were annealed as described previously (Hughes and Kaufman, 2000). Annealed RNA products were then dissolved in ultrapure water at a concentration of 3 mg/ml and microinjected into fertilized eggs.

Transfection of S2 cells with dGATAe cDNA

The *dGATAe* cDNA was subcloned into the *Not*I–*Xba*I site of the pIZT/V5-HIS vector (Invitrogen). S2 cells were cultured in M3 INSECT medium (Sigma) with 10% fetal bovine serum, and the *dGATAe* cDNA was transfected into the cells using the calcium phosphate precipitation method. Two days after transfection, total RNA was isolated from the cells and then subjected to RT-PCR.

Results

dGATAe is a novel Drosophila GATA factor gene

While searching genomic databases in an attempt to identify novel *Drosophila* GATA transcription factor

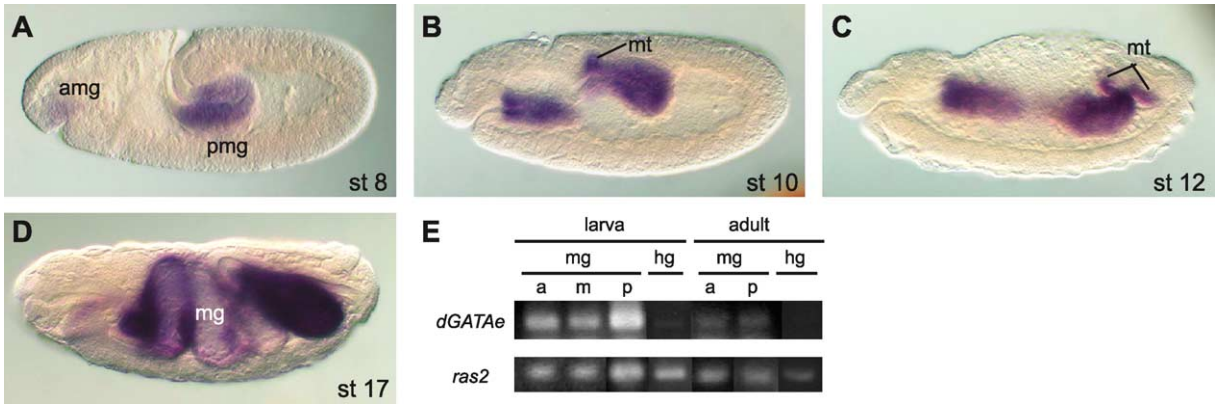

Fig. 2. Expression of *dGATAe* mRNA in wild-type embryos, and in the midgut of larvae and adult flies. Panels A through D show lateral views of wild-type embryos, with the anterior region to the left. (A) *dGATAe* mRNA is first detected in the invaginating anterior midgut (amg) and posterior midgut (pmg) at stage 8. (B) At stage 10, primordia of the Malpighian tubules (mt) also express *dGATAe*. (C, D) *dGATAe* expression in the endoderm continues throughout embryonic development, but expression in the Malpighian tubules becomes weak in later stages. (E) RT-PCR of *dGATAe* in the larval and adult midgut. Anterior (a), middle (m), and posterior (p) portions of the larval midgut (mg), as well as anterior (a) and posterior (p) halves of the adult midgut, express *dGATAe*. Hindgut (hg) was used as a negative control, and *ras2* primers were used for internal controls.

genes, we found two sequences containing novel GATA factor motifs. These cDNA sequences were cloned from *Drosophila* cDNA libraries and/or from EST clones. The two genes were named *dGATAd* and *dGATAe*, respectively, in accordance with the nomenclature adopted for other *Drosophila* GATA factor genes (Abel et al., 1993; Winick et al., 1993). Although embryonic *dGATAd* expression was not detected, *dGATAe* was expressed specifically in the endoderm, as described below. *dGATAe* is located at 89A13-B4, forming a cluster with two other known GATA factor genes, *pannier* (*pnr*, also known as *dGATAa*) and *srp* (also known as *dGATAb*) (Fig. 1A). The exon–intron structure of *dGATAe* was deduced by comparing the *dGATAe* cDNA sequence with the genomic sequence (AE003711). *dGATAe* encodes a predicted protein of 746 amino acids containing two GATA-type zinc finger motifs (Fig. 1B). The C-terminal finger, which is well conserved in various animals, is typical of the GATA family, corresponding to the $C\text{-}X_2\text{-}C\text{-}X_{17}\text{-}C\text{-}X_2\text{-}C$ motif, with a flanking basic domain. In contrast, the N-terminal finger, $C\text{-}X_2\text{-}C\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C$, is atypically short, although it is also has a flanking basic domain (Fig. 1B). The N-terminal region of *dGATAe* has several glutamine-rich domains (dotted lines in Fig. 1B), a feature also found in *pnr* and *srp*. Multiple alignment analysis of the amino acid sequence of the C-terminal finger and basic domain of *dGATAe* with those of other known GATA factors showed that *dGATAe* is closely related to certain endoderm-specific GATA factor genes, including *Drosophila srp*, *C. elegans elt-2*, and mouse *GATA-4/5/6*. However, distinct subgroupings could not be defined either between *Drosophila* and mouse or between *Drosophila* and *C. elegans* (Figs. 1C, D). Similar difficulties in classifying other known GATA genes into distinct subgroups have been previously reported (Lowry and Atchley, 2000).

dGATAe is expressed specifically in the endoderm throughout life

In situ hybridization on whole-mount embryos was used to determine the expression pattern of *dGATAe* during embryogenesis. *dGATAe* mRNA was first detected in the posterior endoderm at stages 7–8, and in the anterior endoderm at stage 8 (Fig. 2A). Malpighian tubule primordia also expressed *dGATAe* from stage 10 onwards (Fig. 2B). Expression of *dGATAe* in the endoderm, in the midgut (which is exclusively derived from the endoderm) and within Malpighian tubules continues throughout embryonic development (Figs. 2C, D). *dGATAe* expression was not detected in any embryonic tissues other than endoderm and Malpighian tubules.

We used RT-PCR to examine whether *dGATAe* expression in the endodermal midgut continues in post-embryonic stages. The midguts of third instar larvae were dissected into anterior, middle, and posterior segments, whereas adult fly midguts were dissected into anterior and posterior segments. Each of these midgut segments exhibited *dGATAe* mRNA expression (Fig. 2E), indicating that *dGATAe* is expressed in the midgut throughout life.

dGATAe is activated by srp

srp, another GATA factor gene, is required for endodermal development in *Drosophila*. *srp* is first expressed at the

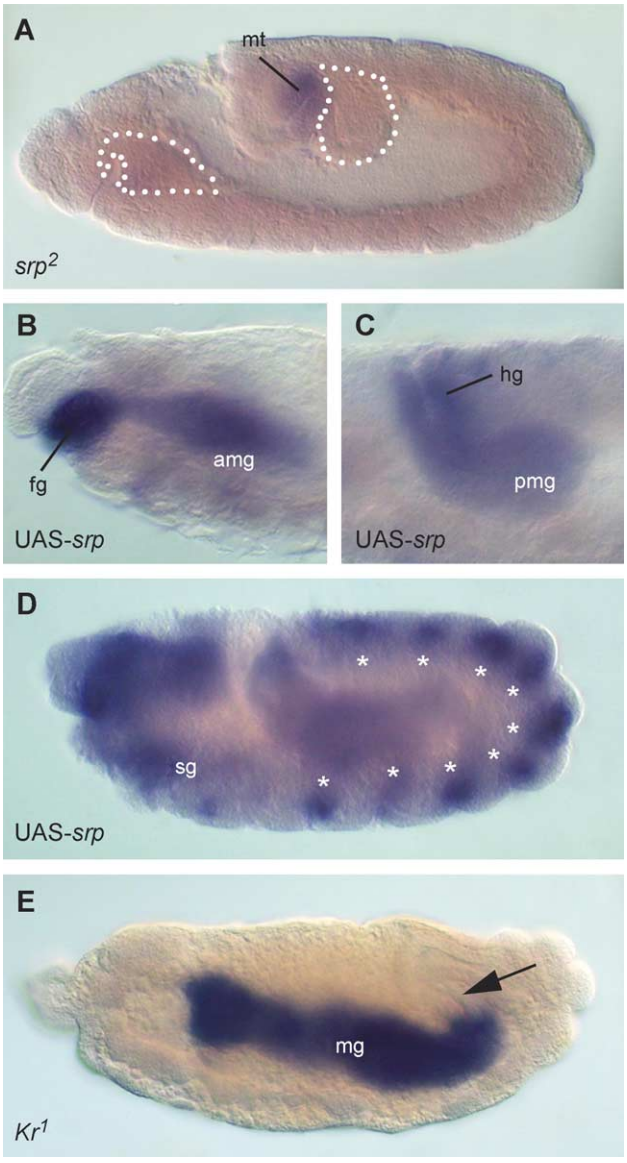

Fig. 3. *dGATAe* is activated by *srp*. (A) In the srp^2 mutant, *dGATAe* expression in the endoderm (indicated with dotted lines) disappears, but that of Malpighian tubules (mt) remains. (B, C) Ubiquitously misexpressed *srp* (*Ay*-GAL4 × UAS-*srp*) induces ectopic *dGATAe* expression in the foregut (B) and hindgut (C). amg, anterior midgut; pmg, posterior midgut; fg, foregut; hg, hindgut. (D) Ectopic expression of *dGATAe* was also induced in the ventral nerve chord (asterisks) and the salivary gland (sg). (E) In Kr^1 mutant, *dGATAe* expression in the prospective region of Malpighian tubules disappears (arrow).

cellular blastoderm stage in yolk cells, and is prospective regions of the endoderm, amnioserosa, and hemocyte primordium (Abel et al., 1993; Rehorn et al., 1996). Endodermal expression of *srp* disappears by stages 10–11(Rehorn et al., 1996). Embryos lacking *srp* activity fail to develop endoderm; instead, the prospective endodermal region develops into the ectodermal hindgut (Reuter, 1994). Since *srp* is expressed in the endoderm earlier than *dGATAe* is expressed, we examined whether *dGATAe* expression is activated by *srp*. Expression of *dGATAe* in the prospective endoderm region was abolished in the *srp* mutant (srp^2/srp^2) (Fig. 3A, dotted line), whereas *dGATAe* expression in the Malpighian tubules was not affected (Fig. 3A). Conversely, ubiquitously misexpressed *srp* caused strong ectopic expression of *dGATAe* in the foregut (Fig. 3B), and in the hindgut (Fig. 3C). Note that the foregut and hindgut

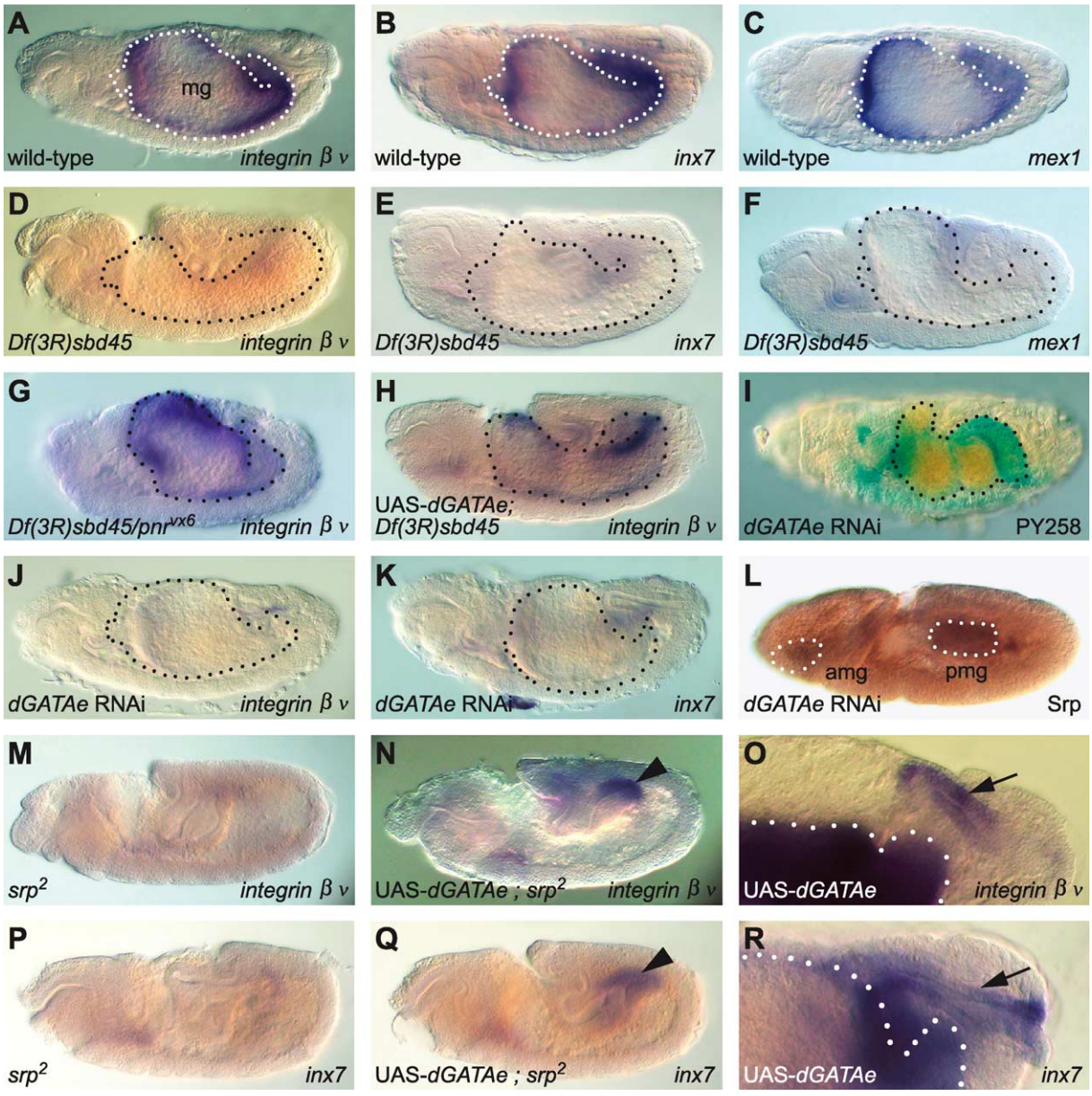

Fig. 4. *dGATAe* is required for the activation of differentiated midgut marker genes, *integrin* β_v, *inx7*, and *mex1*. All panels show lateral views. Dotted lines indicate the midgut. (A, B, C) Wild-type embryos at stage 15 express *integrin* β_v (A), *inx7* (B) and *mex1* (C) throughout the endodermal midgut. (D, E, F) Midgut of the *Df(3R)sbd45* homozygous embryo that lacks *dGATAe* locus fails to express *integrin* β_v (D), *inx7* (E), and *mex1*(F). (G) The *Df(3R)sbd45/pnr*^{vx6} heterozygous embryo normally expresses *integrin*β_v (H) Ubiquitous misexpression of *dGATAe* in *Df(3R)sbd45* embryos (*Ay*-GAL4/UAS-*dGATAe*; *Df(3R)sbd45*/*Df(3R)sbd45*) has rescued the expression of *integrin*β_v. (I, J, K, L) Midgut of the embryo injected with dsRNA of *dGATAe* shows normal gross morphology, with characteristic constrictions at stage 17 (I), but fails to express *integrin*β_v (J) and *inx7*(K). The dsRNA treatment does not affect the expression of Srp protein in the anterior (amg) and posterior midgut (pmg) at stage 8 (L). (M, P) The srp^2 embryos fail to express *integrin* β_v (M) and *inx7* (P). (N, Q) Ubiquitously misexpressed *dGATAe* in srp^2 embryos (*Ay*-GAL4/UAS-*dGATAe*; srp^2/srp^2) has rescued the expression of *integrin* β_v (N) and *inx7* (Q) in a portion of the prospective midgut region (arrowheads), though midgut morphology is still strongly affected. (O, R) Misexpression of *dGATAe* in the hindgut (with *byn*-GAL4 driver) strongly induces expression of *integrin* β_v (O) and *inx7* (R) in the hindgut (arrows).

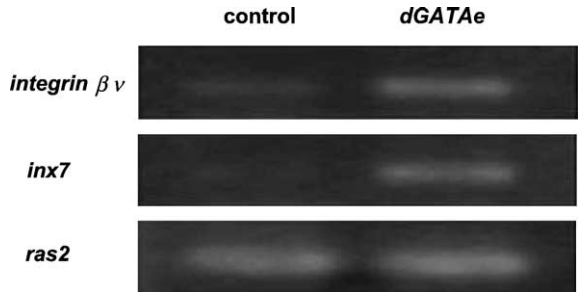

Fig. 5. RT-PCR detection of *integrin* β_v and *inx7* transcripts in S2 cells transfected with *dGATAe* cDNA. Transfection of *dGATAe* cDNA subcloned in the pIZT/V5-HIS vector (Invitrogen) induced expression of *integrin* β_v and *inx7* in S2 cells. *ras2* expression was used as internal controls of RT-PCR.

arise immediately anterior and posterior to the endoderm, respectively. This ectopic expression pattern was transient. During embryogenesis, *dGATAe* was also induced ectopically in the salivary gland and segmentally in the ventral nerve cord (Fig. 3D). These results strongly suggest that *srp* activates *dGATAe* in the endoderm. *fork head* (*fkh*) is expressed throughout the prospective gut, and the gut primordia degenerate during germband retraction in *fkh* mutants (Weigel et al., 1989a,b). However, *dGATAe* expression was not affected in the *fkh* mutant (fkh^{XT6}/fkh^{XT6}) (data not shown).

As shown in Fig. 3A, *dGATAe* expression in the Malpighian tubules was not affected in the *srp* mutant (Fig. 3A), indicating that this expression depends on some other gene. *Krüppel* (*Kr*) is known to be required for the development of Malpighian tubules (Liu and Jack, 1992), so we investigated whether the *dGATAe* expression in this organ depends on *Kr*. *dGATAe* expression was completely abolished in the prospective region of the Malpighian tubules of *Kr* mutant embryos (arrow in Fig. 3E. Compare with Fig. 2C), indicating that *Kr* is required for *dGATAe* expression in the Malpighian tubules.

dGATAe induces gene expression of differentiated midgut

The findings described above suggest that *dGATAe* is required for the later stages of endodermal development, which leads to terminal differentiation of the midgut. To test this, we examined the role of *dGATAe* in the expression of midgut-specific *integrin* β_v, and the midgut-specific gap junction gene, *inx7*. *integrin* β_v and *inx7* are both expressed throughout the endoderm from embryonic stage 11 onwards (Figs. 4A, B). RT-PCR analyses indicated that *integrin* β_v and *inx7* are expressed in the midgut of third instar larvae and adult flies (data not shown). Thus, these genes can serve as markers of the differentiated midgut.

The *Df(3R)sbd45* embryo lacks *dGATAe* and *pnr* loci, but, retains the *srp* locus (Heitzler et al., 1996; Jürgens et al., 1984). In contrast to the *srp* mutant, in which the prospective endodermal region develops into ectodermal tissues, including a portion of the hindgut (Reuter, 1994), the *Df(3R)sbd45* embryo formed apparently normal midgut primordium surrounding the yolk (see Figs. 4D, E), with constrictions that are characteristic of the late stages of normal midgut (data not shown). However, the midgut primordium failed to express *integrin* β_v (Fig. 4D) and *inx7* (Fig. 4E). Another midgut-specific gene, *midgut expression 1* (*mex1*) (Schulz et al., 1991) was also not expressed in this embryo (Figs. 4C, F). Since the *Df(3R)sbd45* strain also lacks the *pnr* locus, we tested the hypothesis that this phenotype is caused by the lack of *dGATAe*, but not by the lack of *pnr*. Both *integrin* β_v and *inx7* were normally expressed in both pnr^{VX6}/*Df(3R)sbd45* heterozygotes (Fig. 4G) and pnr^{VX6} (null) homozygotes (data not shown). Unlike the misexpression of *dGATAe* (see below), misexpressed *pnr* did not induce *integrin* β_v or *inx7* expression in the hindgut (data not shown). When *dGATAe* was ubiquitously misexpressed in the *Df(3R)sbd45* homozygotes, expression of *integrin* β_v was restored (Fig. 4H), although *inx7* expression was not restored under these conditions (data not shown). To further confirm that the loss of *integrin* β_v and *inx7* expression is due to the lack of *dGATAe*

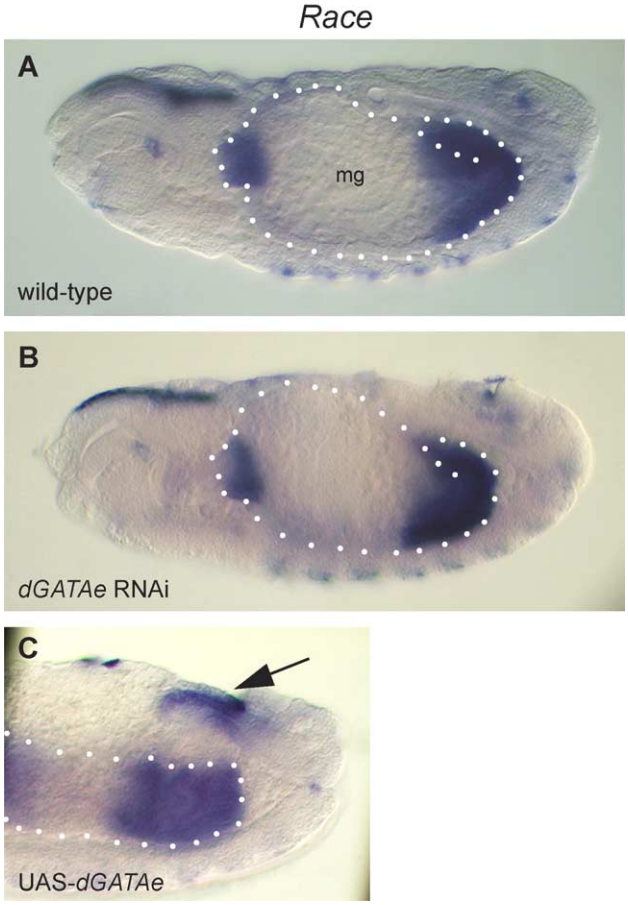

Fig. 6. *dGATAe* is not required for the expression of *Race*, an early endodermal marker. All panels show lateral views. White dotted lines indicate endodermal midgut. (A) A wild-type embryo at stage 15, where *Race* is expressed in the midgut. (B) dsRNA of *dGATAe* does not affect the *Race* expression in the midgut. (C) Misexpression of *dGATAe* in the hindgut induces ectopic expression of *Race* in the hindgut (arrow).

activity, we inactivated *dGATAe* transcripts with RNA interference (RNAi) using dsRNA (Kennerdell and Carthew, 1998). The gross morphology of the midgut primordium of dsRNA-injected embryos appeared to be normal, forming normal constrictions in late stages (Fig. 4I). However, embryos injected with dsRNA corresponding to *dGATAe* failed to express either of the *integrin* β_v and *inx7* (Figs. 4J, K). The loss of midgut markers in response to dsRNA treatment was not a secondary effect of the loss of *srp* activity, since Srp protein was detected at normal levels in embryos injected with dsRNA (Fig. 4L). When *dGATAe* was ubiquitously misexpressed in the *srp* mutant embryos, expression were restored (Figs. 4M, N, P, Q). In addition, misexpression of *dGATAe* in the hindgut of wild-type embryos strongly induced ectopic expression of *integrin* β_v and *inx7* (Figs. 4O, R). Misexpressed *pnr* did not induce these marker genes (data not shown). Furthermore, both *integrin* β_v and *inx7* were also induced in S2 cells transfected with *dGATAe* cDNA (Fig. 5). These results clearly demonstrate that *dGATAe* induces gene expression in the differentiated midgut.

dGATAe is not required for the expression of an early endodermal marker

Next, we examined whether the *dGATAe* plays a role in expression of an early marker gene in the endoderm. The *Race* gene is an early marker for endoderm and amnioserosa. *Race* expression begins in the invaginating endoderm slightly before *dGATAe* expression begins, and *Race* expression persists throughout embryogenesis (Tatei et al., 1995). *Race* expression in the endoderm was not affected in either *Df(3R)sbd45* embryos (data not shown) or in embryos treated with *dGATAe* dsRNA (Figs. 6A, B). These results show that *dGATAe* is not essential for the expression of *Race* during normal development. Nevertheless, misexpression of *dGATAe* in the present study induced ectopic expression of *Race* in a portion of the hindgut (Fig. 6C). Since *Race* is a target of *srp*, it is likely that misexpressed *dGATAe* activates the *srp* target gene because of the possible structural similarities between the protein products of *dGATAe* and *srp*.

dGATAe maintains endodermal identity by repressing byn in the endoderm

In the posterior terminal region of the blastoderm, prospective regions of the posterior endoderm and hindgut abut each other. *byn* is responsible for determination of the prospective hindgut (Kispert et al., 1994; Murakami et al., 1995; Singer et al., 1996). *byn* is expressed throughout the prospective posterior endoderm during early stage 5, but soon disappears in this region. It is the *srp* gene that represses *byn* in the prospective endoderm. Thus, the boundary between the endoderm and hindgut is established by the repressive activity of *srp* on *byn* (Murakami et al., 1999; Reuter, 1994). We next examined whether *dGATAe* also represses *byn*, as *dGATAe* continues to be expressed after endodermal *srp* expression ceases. Ectopic expression of *byn* in the prospective endodermal domain was observed in embryos that lack *dGATAe* (Fig. 7A), although the area of ectopic expression was much smaller than that observed in the *srp* mutant, in which ectopic expression of *byn* was observed throughout the entire prospective endoderm (Fig.

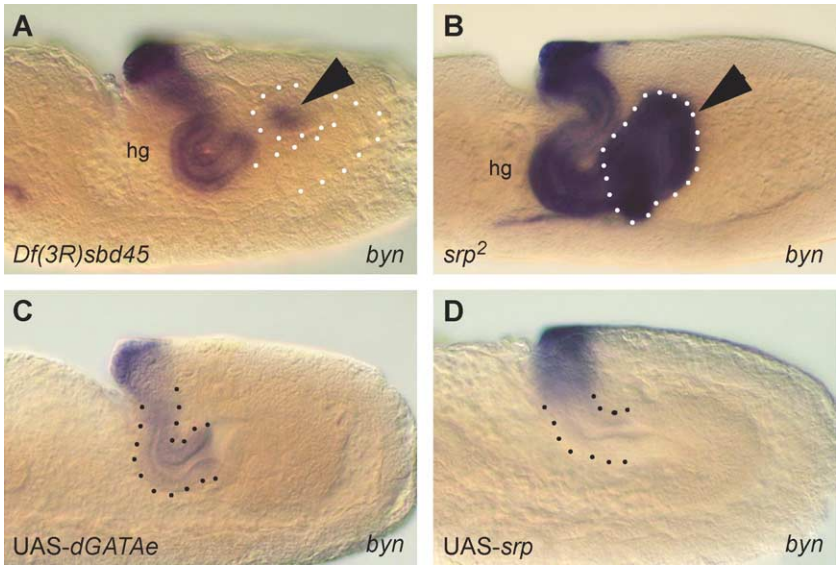

Fig. 7. *dGATAe*, as well as *srp*, has repressive activity on *byn*, thus, maintaining the endodermal identity. Embryos were hybridized with *byn* probe. White dotted lines indicate the prospective midgut region. (A) The *Df(3R)sbd45* embryo, which lacks *dGATAe*, shows ectopic expression of *byn* in a portion of the prospective midgut region (arrowhead). (B) In the srp^2 embryo, *byn* expression expands to the entire endodermal domain (dotted line). (C) Misexpression of *dGATAe* in the hindgut with G455.2-GAL4 strongly represses *byn* expression in the hindgut. Dotted lines indicate the expression domain of G455.2-GAL4 driver. (D) Misexpressed *srp* strongly represses *byn* in the hindgut.

7B). Moreover, when misexpressed in the prospective hindgut domain, both *dGATAe* and *srp* strongly repressed *byn* expression (Figs. 7C, D). Thus, *dGATAe* is required to maintain the endodermal identity that is initially established by *srp*.

Discussion

srp and dGATAe sequentially drive endodermal development

srp is the first GATA factor gene to be expressed within the *Drosophila* endoderm, and it is essential for its endodermal specification (Rehorn et al., 1996; Reuter, 1994). *srp* is expressed in the prospective endoderm in the cellular blastoderm stages, but its expression disappears by stages 10–11. In this study, we show that *srp* activates another GATA factor gene, *dGATAe*, and that *dGATAe* is required for expression of specific genes in the differentiated midgut. *dGATAe* induced the expression of late endodermal marker genes even in the absence of *srp* activity. Since *dGATAe* expression in the endodermal midgut persists throughout the embryonic, larval, and adult stages of *Drosophila*, it seems likely that *dGATAe* is also necessary for maintaining gene expression in the differentiated midgut. Inactivation of *dGATAe* transcripts with dsRNA did not cause any marked morphological defects (Fig. 4I), but most of these embryos failed to hatch (data not shown), suggesting that *dGATAe* is essential for differentiated midgut and for viability of the larva. It should be noted that the endodermal midgut is subdivided into four chambers (Bienz, 1994), and further arranged into 13 subdomains with distinct gene expression patterns (Murakami et al., 1994). Homeotic genes expressed in the visceral muscle were shown to cause subdivision of the midgut into the four chambers, but the mechanisms that generate the various subdomains are still unknown.

The sequential activation of *srp* and *dGATAe* during endodermal development in *Drosophila* is analogous to the gene regulatory cascade that occurs during endodermal development in *C. elegans*. The earliest endodermal GATA factor expressed in *C. elegans* is *end-1*, which is expressed in the endoderm. *end-1* then activates the subordinate GATA factor gene, *elt-2*, which activates late endodermal genes (Fukushige et al., 1998; Zhu et al., 1997). These results suggest that the genetic mechanism underlying endodermal development is at least partially conserved between *Drosophila* and *C. elegans*. However, the molecular phylogenetic relationship of these endoderm-specific GATA factor genes has not yet been established (see Fig. 1D).

GATA factors are also essential for endodermal development in vertebrates. In the *Xenopus* embryo, *GATA-4* and *GATA-5* are expressed in the prospective endoderm, and both genes can induce formation of the endoderm (Weber et al., 2000; Yasuo and Lemaire, 1999). *GATA-5* also plays an important role in endodermal development in zebrafish, where it function as an upstream regulator of *Sox17a*, which is essential for endodermal specification (Kikuchi et al., 2001; Reiter et al., 2001; reviewed in Shivdasani, 2002). In *Drosophila* and *C. elegans*, the GATA factor genes *dGATAe* and *elt-2*, respectively, continue to be expressed in the differentiated gut, as well as in the early stages of endodermal specification. In mammals, the GATA-4, and -6 proteins are expressed in the differentiated stomach and intestine, and bind to the gastric H^+/K^+-ATPase gene (Maeda et al., 1996). Taken together, these findings suggest that vertebrate GATA factor genes also function in endodermal tissues after terminal differentiation.

Gene regulatory pathways that establish the endodermal midgut and ectodermal hindgut in Drosophila

The *Drosophila* endoderm arises at the anterior and posterior terminal regions of the early blastoderm. Previous studies have revealed the gene regulatory pathway leading to endodermal development in some detail (for review, see Murakami et al., 1999). A Torso-like protein secreted by the

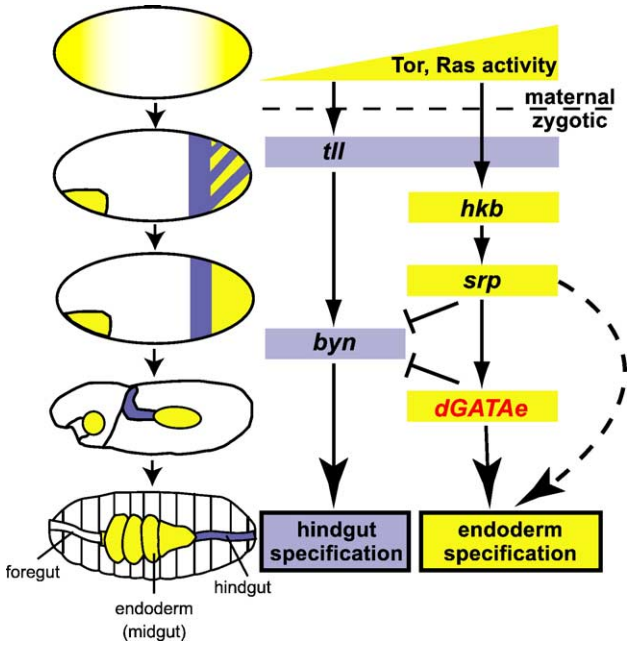

Fig. 8. Schematic model of gene regulatory pathways that specify the endodermal midgut and the ectodermal hindgut. Graded Ras signaling, which is caused by graded Tor activity, activates two gap genes, *tll* and *hkb*, depending on the signaling intensity. *hkb* activates *srp*, and *srp*, in turn, activates *dGATAe*. Both *srp* and *dGATAe* are required for the specification of the endoderm. After *srp* ceases to be expressed, *dGATAe* continues to be expressed in the endoderm, and activates genes of terminally differentiated midgut. On the other hand, *tll*, another target of the Ras signal, activates *byn*, which is responsible for specification of the hindgut, initially in the prospective regions of the hindgut and endoderm. A short time later, *byn* expression in the prospective endoderm is repressed by *srp*, and thereby, the boundary between the endoderm and the hindgut is established. *dGATAe* also has represses *byn* in the endoderm, which may contribute to maintaining endodermal identity.

follicle cells covering both the anterior and posterior terminal regions of the egg triggers activation of the receptor tyrosine kinase, Torso, resulting in a graded Ras signal that peaks at both terminals (Furriols et al., 1998; Martin et al., 1994; Savant-Bhonsale and Montell, 1993). A zygotic gap gene, *huckebein* (*hkb*) is activated by high levels of the Ras signal, and *hkb*, in turn, activates *srp* (Greenwood and Struhl, 1997; Rehorn et al., 1996). The present study revealed that *srp* activates another GATA factor gene, *dGATAe*, and the latter leads to terminal differentiation of the midgut (see Fig. 8). *dGATAe* may be necessary to maintain gene expression in the terminally differentiated midgut, since *dGATAe* expression persists in the midgut throughout life. In addition to playing essential roles in the development of the endoderm, *srp* and *dGATAe* also act to restrict the area of the adjacent hindgut. The ectodermal hindgut is specified by a *Brachyury* ortholog, *brachyenteron* (*byn*) (Kispert et al., 1994; Murakami et al., 1995; Singer et al., 1996). The gene regulatory pathway leading to the activation of *byn* is closely linked with that of *srp*. Ras signaling in the posterior terminal region of the fertilized egg activates another gap gene, *tailless* (*tll*) (Greenwood and Struhl, 1997; Pignoni et al., 1990). *tll* is required for the activation of *byn*. The *byn*-positive domain in the early cellular blastoderm stages includes the prospective endoderm domain, but the expression in the prospective endoderm soon disappears in response to the repressive activity of *srp* (see Fig. 8). *byn* expression in the hindgut persists throughout life, as does *dGATAe* expression in the midgut. These rather simple regulatory pathways lead to the activation of *dGATAe* and *byn*, and consequently, to the terminal differentiation of the midgut and hindgut (Fig. 8). It should be noted that these pathways not only delineate the process of endodermal development in *Drosophila*, but also highlight conserved genetic components underlying the endodermal development of multicellular animals.

Acknowledgments

We thank Dr. D. Hoshizaki for providing UAS-*srp* strain and Srp antibody and Dr. M. Haenlin for providing UAS-*pnr* strain and Dr. T. Hamaguchi for his valuable advice on midgut marker genes. This research was supported in part by Grant-in-Aids from the Ministry of Education, Science, Sports, Culture and Technology, Japan and JSPS to R.M. (16027236 and 1657017).

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