Homeodomain-independent activity of the *fushi tarazu* polypeptide in *Drosophila* embryos

V. Danial Fitzpatrick*, Anthony Percival-Smith*, C. James Ingles & Henry M. Krause

Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, Canada
* Present addresses: Department of Protein Chemistry, Genentech Inc., 460 Point Street, Bruno, South San Francisco, California 94080, USA (V.D.F.); Department of Zoology, University of Western Ontario, London, Ontario N6A 5C1, Canada (A.P.-S.)

THE *Drosophila* segmentation gene *fushi tarazu* (*ftz*) encodes a homeodomain-containing protein, ftz, that can act as a DNA-binding activator of transcription[1–5]. In the developing embryo, ftz is expressed in seven stripes[6] which correspond to the even-numbered parasegments[7]. These parasegments are missing in *ftz*⁻ embryos[8]. When ftz is expressed throughout blastoderm embryos under the control of a heat-shock promoter, the odd-numbered parasegments are lost[9]. This 'anti-*ftz*' phenotype has been attributed to autoactivation of the endogenous *ftz* gene by the ectopically expressed protein[10]. Here we show that the same phenotype is induced by ectopic expression of a ftz polypeptide containing a deletion in the homeodomain. Thus, ftz can alter gene expression without binding directly to DNA.

To delineate functional domains in the ftz protein, we generated a series of *ftz*-deletion constructs which could be expressed

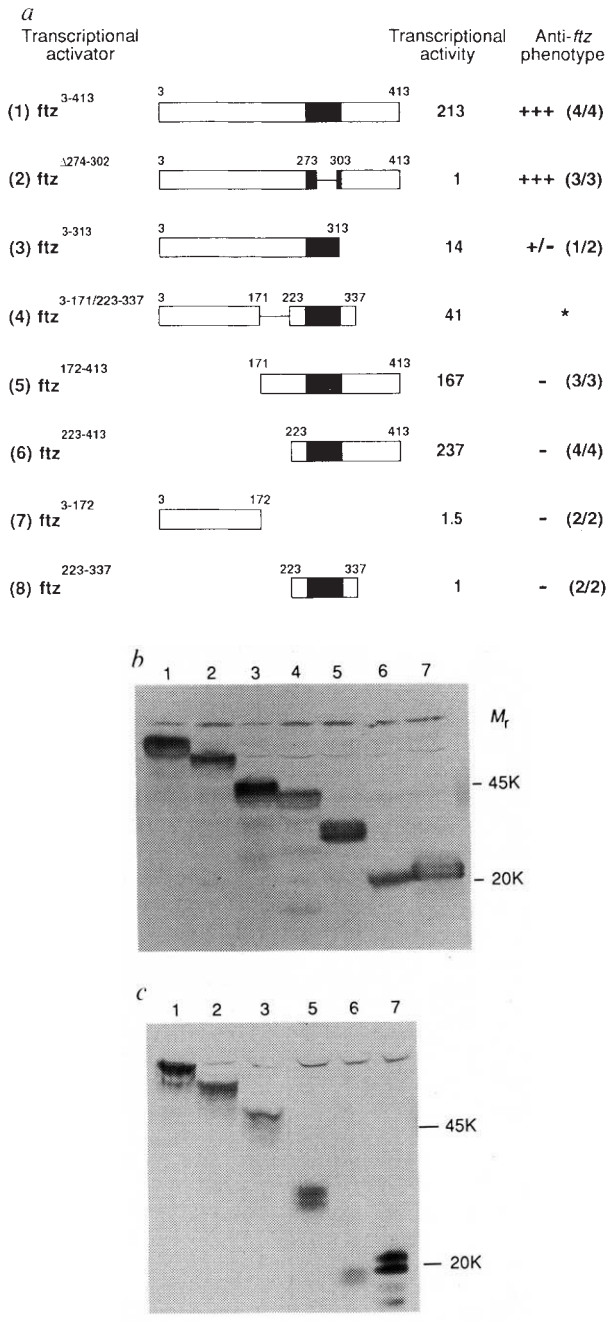

FIG. 1 *a*, Activities of ftz derivatives in tissue-culture cells and *Drosophila* embryos. To determine transcriptional activity of the ftz deletion derivatives, Schneider cells were co-transfected with the *ftz* expression constructs indicated and the ftz-dependent CAT reporter plasmid pD33NP6-CAT (ref. 1). Values given for transcriptional activity show CAT activities from cotransfected cell extracts relative to the CAT activity of extracts from cells transfected with pD33NP6-CAT alone. The same ftz derivatives were also ectopically expressed in *Drosophila* embryos and larval cuticles scored for an anti-*ftz* phenotype. The number of independent transformant lines obtained, as well as the number of transformant lines that produced the phenotype are indicated; +++ indicates the ability to induce a strong anti-*ftz* phenotype. Negative symbols indicate no phenotypic activity. Of the two ftz^{3-313} transformant lines, one yielded very weak anti-*ftz* phenotypes and the other yielded only wild-type cuticles. *Transformants were not obtained with this construct. *b*, *c*, Immunodetection of ftz polypeptides from tissue-culture cells (*b*) and third instar larvae (*c*). Protein extracts isolated from Schneider cells transfected with *ftz* expression constructs or from heat-shocked third instar larvae of *ftz* transformant lines, were analysed by western blotting using an anti-ftz polyclonal antibody[20]. Numbers above each lane correspond to the deletion constructs shown in *a*. The $ftz^{223-337}$ polypeptide, which does not resolve on 10 or 12% polyacrylamide gels, was detected on higher percentage gels at about the same levels of expression (not shown). Crossreactive non-ftz polypeptides indicate the equivalence of sample loading.

METHODS. The expression constructs used for Schneider cell transfections and P-element-mediated transformations were derivatives of the plasmids pPac[21] and pHT4[22] respectively. During construction, some additional non-ftz amino acids were introduced at the termini of the ftz polypeptides. Deletions ftz^{3-413}, $ftz^{\Delta 274-302}$, $ftz^{3-171/223-337}$, and ftz^{3-172} encode MDPEFIKEEKLTMRDP-$(T)^3$ftz at its N terminus; $ftz^{172-413}$ has MDPEFELGTRGSSR-$(V)^{171}$ftz at the N terminus; $ftz^{223-413}$ and $ftz^{223-337}$ encode MDPEFGACMPAGP-$(V)^{223}$ftz. Non-ftz amino acids at the C termini are: for $ftz^{3-171/223-337}$ and $ftz^{223-337}$, ftz$(E)^{337}$-GGILV; for ftz^{3-313} the C terminus is ftz$(K)^{313}$-NLYVYTCLCS. The non-ftz amino acids at the internal junction of $ftz^{3-171/223-337}$ are ftz $(V)^{171}$-PAGP-$(V)^{223}$ ftz. Each of the ftz deletion derivatives expressed from the pPac and pHT4 vectors were identical, with the exception of ftz^{3-313}, in which the N-terminal amino acids are MRDP-$(T)^3$ftz in pPac and MDPEFIKEEKLTMRDP-$(T)^3$ ftz in pHT4. The transfection protocol was essentially as described[21]. Transcriptional activity values were normalized for protein concentration. Correcting for transfection efficiency by incorporating β-galactosidase values did not significantly alter the results. Corrections for variations in ftz polypeptide concentrations were not made as there was a variable loss of epitopes in each of the ftz derivatives. Embryos collected for heat shock from P-element-transformed[23] fly lines at 2.75–3.25 h after egg laying were washed onto screens and heat-shocked for 10 min in Eppendorf tubes submerged in a 36.5 °C water bath. Devitilinized larvae were mounted in Hoyer's mountant[24]. Roughly 100 larvae from each transformant line were screened for cuticular phenotypes. Transformant lines with no anti-*ftz* activity were also phenotypically wild type after 15-min heat shocks. For western blots, protein extracts of Schneider cells transfected with 10 μg *ftz*-expressing plasmid were prepared by repeated freeze–thaw cycles of transfected cells which were resuspended in 100 μl 250 mM Tris, pH 8.0, 1.0 mM EDTA. Insoluble material was removed by centrifugation. Supernatants were electrophoresed in a 12% SDS–polyacrylamide gel. For protein extracts of transformant flies, third instar larvae were collected and heat-shocked at 36.5 °C for 1 h and homogenized in 2 × Laemmli sample buffer (5 μl × buffer per mg larvae) 30-min after the heat shock. The equivalent of 2 mg wet weight larvae was electrophoresed in a 10% polyacrylamide gel. Following transfer to nitrocellulose, ftz polypeptides were detected using anti-ftz polyclonal antibodies[20] and alkaline phosphatase-coupled secondary antibodies.

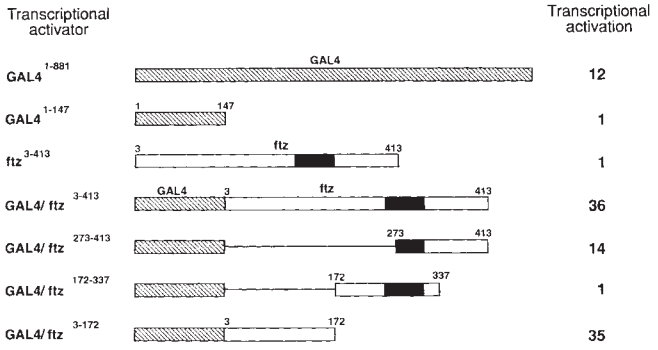

FIG. 2 Transcriptional activity of GAL4 and GAL4/ftz fusions assayed in *Drosophila* tissue culture cells. GAL4 and the GAL4/ftz chimaeric proteins were assayed for transcriptional activity in *Drosophila* Schneider cells by cotransfection of expression constructs with a GAL4-dependent CAT reporter. Values given for transcriptional activity show CAT activities from cotransfected cell extracts relative to the CAT activity of extracts of cells transfected with the CAT reporter costruct alone.

METHODS. Transfections and CAT assays were as described for Fig. 1 Expression plasmids for GAL4 or GAL4/ftz fusions were constructed by inserting sequences encoding either $GAL4^{1-881}$ or $GAL4^{1-147}$ into a unique *Bam*HI site of a pPac derivative. The $GAL4^{1-147}$ construct was used to make the GAL4/ftz fusions. The GAL4-dependent reporter was constructed by inserting a 600-base-pair *Sma*I/*Xho*I fragment containing the major GAL4 binding sites upstream of the GAL1 gene from pLR1Δ21 (ref. [25]) into the *Sal*1 site of pD-33CAT. The amino acids encoded at the GAL4/ftz junctions were: $GAL4(S)^{147}$-PEFIKEEKLTMRP-$(T)^{3}$ ftz for $GAL4/ftz^{3-413}$ and $GAL4/ftz^{3-172}$; $GAL4(S)^{147}$-PEFK-$(D)^{172}$ ftz for $GAL4/ftz^{172-337}$; $GAL4(S)^{147}$-PEFELGTRGSSRV-$(E)^{273}$ ftz for $GAL4/ftz^{273-413}$. The C terminus encoded by the $GAL4^{1-147}$ construct is $GAL4(S)^{174}$-PEF.

in cultured cells and developing embryos. The relative transcriptional activities of the deleted polypeptides were first measured in transfected cells (Fig. 1*a*). Plasmids expressing the various *ftz*-deletion constructs were cotransfected into *Drosophila* Schneider-2 cells together with a ftz-dependent chloramphenicol acetyltransferase (CAT), reporter plasmid. Each of the deletion derivatives was detected on a western blot (Fig. 1*b*). Not surprisingly, the relative CAT activities showed that the DNA-binding homeodomain was required but was not sufficient for activation of the ftz-dependent reporter gene (compare activities of ftz^{3-413}, $ftz^{\Delta 274-302}$ and $ftz^{223-337}$). Addition of N- or C-terminal sequences to the transcriptionally inactive homeodomain derivative restored transactivation potential. The presence of transactivation domains in the N and C termini of ftz was confirmed by fusing these domains to the DNA-binding domain of the yeast protein GAL4 and monitoring GAL4-dependent CAT reporter gene activity (Fig. 2). These data indicate that ftz, like other stimulatory transcription factors[11], contains modular DNA-binding and transcriptional-activation domains.

To determine the functional importance of these domains *in vivo*, we transformed heat-shock-inducible *ftz* deletion constructs into flies. Analysis by Southern (data not shown) and western blotting (Fig. 1*c*) confirmed that each of our transformants contained and expressed the correct constructs. Four (of four) full-length ftz transformant lines gave strong anti-*ftz* cuticular phenotypes characterized by the deletion of odd-numbered parasegments as previously described[9] (Figs 1*a*, 3*b*). Surprisingly, the only deletion derivative that generated a strong phenotype was the ftz polypeptide lacking a functional homeodomain. Three (of three) $ftz^{\Delta 274-302}$ transformant lines yielded cuticular phenotypes that were essentially identical to those generated by the full-length protein (Fig. 3*c*). Although many of the other ftz derivatives were transcriptionally active in cultured cells, all except ftz^{3-313} showed no activity in this assay

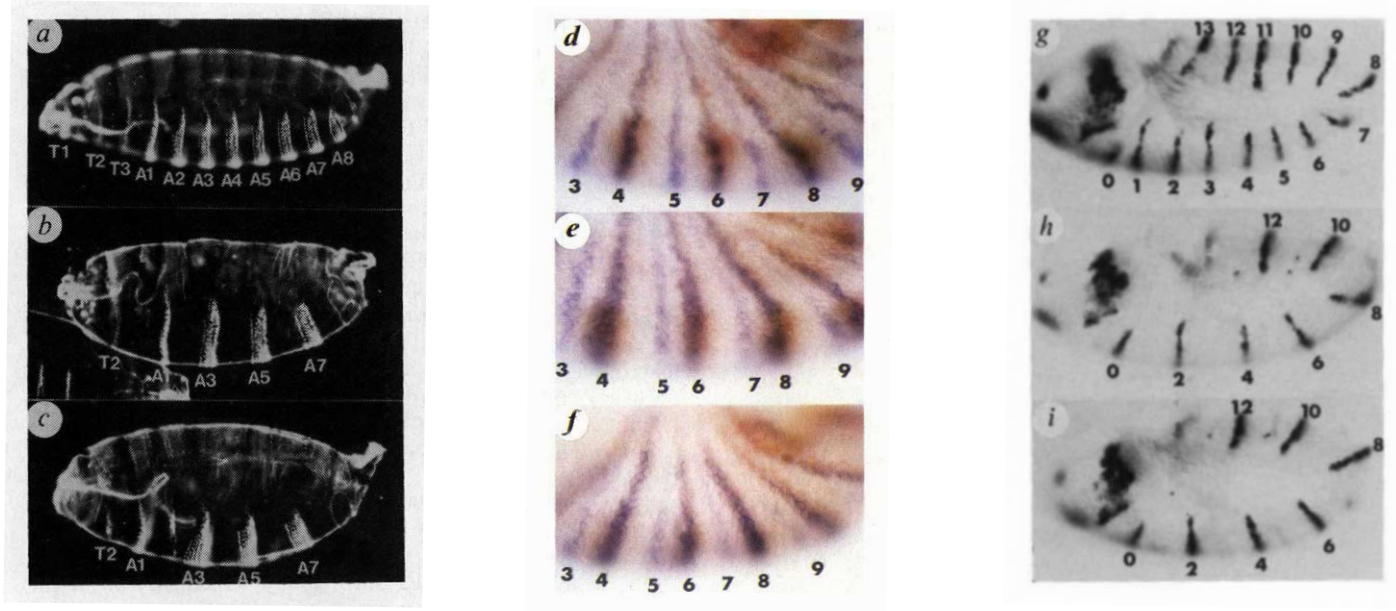

FIG. 3 Cuticular phenotypes and patterns of *en* and *wg* expression are similar in heat-shocked ftz^{3-413} and $ftz^{\Delta 274-302}$ embryos. Cuticular phenotypes (*a–c*), ftz and *en* expression (*d–f*), and *wg* expression (*g–i*) patterns are compared in heat-shocked wild type (*a, d, g*), ftz^{3-413} (*b, e, h*) and $ftz^{\Delta 274-302}$ (*c, f, i*) embryos. *a–c*, Dark-field photomicrographs for first instar larval cuticles. The wild-type cuticle (*a*) has been photographically reduced by ~30% relative to those in *b* and *c*. Photomicrographs *d–f* show embryos double-stained for *en* transcripts (blue) and ftz protein (brown) 45-min after heat shock. At this stage (3.5–4 h after egg laying), ftz stripes have normally resolved to stripes one or two cells wide which overlap completely with the even-numbered stripes of *en* (*d*). The anterior margins of ftz stripes in *e* and *f* are shifted anteriorly and coincide with the anterior margins of the widened *en* stripes. *g–i*, Embryos stained for *wg* transcripts 100 min after heat shock. Odd-numbered *wg* stripes were repressed in both ftz^{13-413} (*h*) and $ftz^{\Delta 274-302}$ (*i*) embryos.

METHODS. Cuticles were prepared as described in Fig. 1 legend. Whole-mount *in situ* hybridization followed ref. 26; double staining used antibody staining first [27], followed by *in situ* hybridization (A. Manoukian and H.M.K., unpublished).

(Fig. 1*a*). Thus, the ability to induce a strong anti-*ftz* phenotype required the regions of ftz encompassing both activation domains but did not require a functional homeodomain.

Ish-Horowicz *et al.*[10] have suggested that transient activation of the endogenous *ftz* gene by ectopic ftz leads to an anterior widening of the endogenous ftz stripes. This, in turn widens the even-numbered stripes of the segment polarity gene *engrailed* (*en*) and represses the odd-numbered stripes of the segment polarity gene *wingless* (*wg*). We tested whether our homeodomain-deleted ftz derivative also caused these changes (Fig. 3). Double-staining of ftz protein and *en* transcripts shows that the even-numbered *en* stripes were widened by both the full-length and homeodomain-deleted proteins, and that the anterior margins of the widened *en* stripes corresponded to the anterior margins of the widened ftz stripes (Fig. 3*e*, *f*). Both ftz derivatives also caused the repression of odd-numbered *wg* stripes (Fig. 3*h*, *i*). Thus, although the ftz homeodomain is required for DNA binding (H.M.K, unpublished results) and to rescue a *ftz* null phenotype (M. Mueller and W.J. Gehring, personal communication), it was not necessary to generate the anti-*ftz* phenotype. This result contrasts with previous ectopic expression studies in which the homeodomains encoded by the genes *Ultrabithorax*, *Antennapedia* and *Deformed* were shown to be crucial for the induction and determination of cuticular phenotypes[12-14].

The anti-*ftz* phenotype that we observe is probably initiated by protein–protein interactions alone. Such interactions may recruit ectopically expressed ftz into a protein–DNA complex that results in the activation of the endogenous *ftz* gene anterior to its normal domain of expression. Alternatively, ectopically expressed ftz might sequester or inactivate a factor that is a negative regulator of the *ftz* gene. There have been several reports of transcripts encoded by homeobox-containing genes in *Drosophila*[15], *Xenopus*[16,17] and mice[18] which are spliced so that their homeodomains are deleted or out of frame. In addition, a targeted deletion of the homeobox in the murine *En-2* gene was not lethal in homozygous $En\text{-}2^{hd}/En\text{-}2^{hd}$ mice[19]. Our results suggest that the proteins encoded by these genes could function in the absence of their DNA-binding homeodomains by means of protein–protein interactions. □

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Bipotential precursors of B cells and macrophages in murine fetal liver

Ana Cumano, Christopher J. Paige, Norman N. Iscove & Gerard Brady

The Ontario Cancer Institute, 500 Sherbourne Street, Toronto M4X 1K9, Canada

LYMPHOCYTES (B and T cells) derive continuously from the same multipotential stem cells that produce myeloid cells, including erythrocytes, granulocytes and macrophages[1,2]. Tri- and bipotential myeloid intermediates between the multipotential stem cells and later unipotential cells have been identified using clonal methods in culture. Although similar methods have detected committed pre-B cells in mouse fetal liver[3], earlier progenitors with additional non-B lineage options have not been demonstrated in normal tissues. We report the characterization and purification of fetal liver cells that generate clones containing both macrophages and B cells, identified biochemically and morphologically. The common origin of the two cell types was shown by culture of single precursor cells. Their dual potential and unrearranged immunoglobulin loci place the precursors before exclusive B-lineage commitment in the haematopoietic hierarchy. The availability of such cells in purified form will allow direct study of lineage choice in cells having both lymphoid and non-lymphoid options.

Haematopoietic precursors appear in mouse fetal liver at day 11 of gestation but mature B cells do not appear until day 17 (refs 4, 5). We have shown that 12-day fetal liver cells cultured with γ-irradiated marrow fibroblasts and interleukin 7 can generate clones of B cells that can be induced to differentiate terminally into immunoglobulin-secreting cells with appropriate B-cell mitogens[6-8]. The clonal precursors have unrearranged immunoglobulin loci and belong to the subpopulation, recognized by monoclonal antibody AA4.1 (ref. 9), that includes multipotential hematopoietic stem cells[10]. It therefore seemed possible that these early B-cell precursors might also be able to differentiate into other lineages.

In the present study, we purified the precursors extensively to allow examination of the clonal progeny of single isolated cells. Cells were successively panned on antibody-coated plates to separate them according to surface markers of stem cells (AA4.1 and Ly6A[11,12]), pre-B cells (B220) and macrophages (Mac-1). Cells retained on antibodies to B220 and Mac-1 (fraction IIa, Table 1) yielded clones in culture which contained either macrophages or B cells but not both. In contrast, limiting dilution cultures of the non-retained ($B220^-Mac^-$, fraction IIb) cells yielded culture wells containing B cells together with macrophage-like cells. When this fraction was further panned on plates coated with antibodies to Ly6A, the retained fraction (IIIb, $Ly6A^+$) contained over 50% of the total B-cell precursors in the original unfractionated fetal liver and only 0.1% of the starting cells.

The rest of the experiments focused on the $AA4.1^+B220^-Mac\text{-}1^-$ $Ly6A^+$ (fraction IIIb) population. The frequency of B-cell precursors was 1:3 to 1:7 in limiting dilution cultures with IL-7 and γ-irradiated S17 cells. Surprisingly, practically all of the wells containing small, round growing B-cell precursors also contained growing, plastic-adherent macrophage-like cells (Fig. 1). The frequency of mixed clones was independent of numbers of cells seeded down to 0.1 cells on average per well, strongly suggesting clonal origin of the mixed cultures. The high frequency of dual precursors made it possible to test rigorously for single cell origin by micromanipulation of single cells into culture wells under direct microscopic visualization. Of 70 single cells cultured alone, 13 formed dual B-lineage/adherent cell clones, and no wells contained B-lineage cells alone. These