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Repression of *Drosophila* pair-rule segmentation genes by ectopic expression of *tramtrack*

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SUMMARY

The *tramtrack* (*ttk*) protein has been proposed as a maternally provided repressor of the *fushi tarazu* (*ftz*) gene in *Drosophila* embryos at the preblastoderm stage. Consistent with this hypothesis, we have detected by immunohistochemistry the presence of *ttk* protein in preblastoderm embryos. This is followed by a complete decay upon formation of the cellular blastoderm when *ftz* striped expression is at its peak. In addition, the highly complex pattern of zygotic *ttk* expression suggests specific functions for *ttk* late in development that are separate from the regulation of *ftz*. We have produced *ttk* protein ectopically in blastoderm-stage embryos transformed with a *heat shock-ttk* construct. Ectopic *ttk* caused complete or near-complete repression of the endogenous *ftz* gene, as well as significant repression of the pair-rule genes *even skipped*, *odd skipped*, *hairy* and *runt*. These findings suggest that specific repression by *ttk* (or by undiscovered repressors) may be more than an isolated phenomenon during the rapid cleavage divisions, a period when the need for genetic repression has not been generally anticipated.

Key words: *tramtrack*, *Drosophila*, pair-rule gene, segmentation gene, ectopic expression, repressor

INTRODUCTION

The establishment of the segmental body pattern of *Drosophila* is governed by a hierarchy of maternal and zygotic genes. The zygotically expressed segmentation genes have been classified into three categories according to their temporal and spatial expression patterns. The gap genes are expressed as broad domains along the antero-posterior axis of the embryo. The pair-rule genes are expressed with double-segment periodicity in the early embryos and the segment polarity genes are expressed in a part of every segment of the developing embryo (for reviews see Akam, 1987; Nusslein-Volhard et al., 1987; Scott and Carroll, 1987; Ingham, 1988).

The *fushi tarazu* (*ftz*) gene is a member of the pair-rule class of segmentation genes. Embryos homozygous for the *ftz* mutation are missing the even-numbered parasegments and die late in embryogenesis (Wakimoto and Kaufman, 1981). *Ftz* RNA is first detected weakly throughout the embryo during nuclear cycle 10 (Hafen et al., 1984; Weir and Kornberg, 1985). During the later division cycles, expression becomes restricted to 15%-65% egg length (0% is the posterior pole). By late nuclear cycle 14 (the cellular blastoderm stage), *ftz* RNA and protein expression evolves into 7 evenly spaced stripes encircling the embryo (Carroll and Scott, 1985; Krause et al., 1988; Karr and Kornberg, 1989). These stripes of *ftz* expression correspond approximately to the regions that are missing in a *ftz* mutant embryo (Wakimoto and Kaufman, 1981). *Ftz* is expressed in specific cells of every segment of the developing nervous system at 5-12 hours of embryogenesis (Carroll and Scott, 1985; Doe et al., 1988), and in a restricted section of the developing hindgut around 12-15 hours of development (Krause et al., 1988).

The expression of *ftz* is largely controlled at the level of transcription (Hiromi et al., 1985; Edgar et al., 1986). Three *cis*-acting elements have been mapped by promoter fusion analysis (Hiromi et al., 1985): the zebra element (740 bp) confers a weak striped pattern of expression in the early embryonic mesoderm; the upstream element (6.1 kb to 3.4 kb upstream of the translational start) has enhancer-like properties that ensure high levels of *ftz* expression in the ectoderm and mesoderm, and the neurogenic element (2.45 kb upstream from the translational start) is necessary for *ftz* expression in the developing nervous system.

A number of *trans*-regulators of *ftz* have been identified by genetic studies. The pair-rule segmentation gene *hairy* plays a critical role in repressing *ftz* in the interstripe regions (Carroll and Scott, 1986; Howard and Ingham, 1986; Frasch and Levine, 1987; Hiromi and Gehring, 1987; Ish-Horowicz and Pinchin, 1987; Carroll et al., 1988; Hooper et al., 1989). Although it has been shown that repression by *hairy* is mediated through the zebra element, it remains to be established whether *hairy* protein binds DNA directly, or acts indirectly through the association with another factor. In addition, *ftz* protein positively autoregulates *ftz*

expression through the binding of its homeodomain to multiple sites in the upstream element (Pick et al., 1990; Schier and Gehring, 1992).

Biochemical approaches have led to the identification of many protein binding sites within the regulatory sequences of *ftz* (Harrison and Travers, 1988, 1990; Dearolf et al. 1989a, 1989b; Ueda et al., 1990; Pick et al., 1990; Brown et al., 1991; Topol et al., 1991). A number of the DNA-binding factors that interact with these sequences have now been identified, including the gap gene *caudal* and two previously unidentified genes *FTZ-F1* and *FTZ-F2* / *tramtrack*. The gap gene *caudal* acts as an activator of *ftz* in the posterior region of the embryo (Dearolf et al., 1989b). *FTZ-F1* is a new member of the steroid hormone receptor superfamily and is implicated in the overall activation of *ftz*, particularly in stripes 1, 2, 3 and 6 (Ueda et al., 1990; Lavorgna et al., 1991). *Tramtrack* (*ttk*) / *FTZ-F2* has been identified as a zinc finger protein that is capable of binding to several sites in the zebra element and to sites in the enhancer element of *ftz* (Harrison and Travers, 1988, 1990; Brown et al., 1991). The *ttk* protein has also been shown to bind in vitro to the pair-rule gene *even skipped* (*eve*) (Read et al., 1990; Jiang et al., 1991; Read and Manley, 1992a,b).

In a previous study, we have proposed that *ttk* / *FTZ-F2* (hereafter referred to as *ttk*) functions as a negative regulator of *ftz*, since point mutations that eliminate *ttk* binding to the zebra element cause derepression of *ftz-lacZ* constructs in transformed embryos (Brown et al., 1991). In addition to abberant expression in the germband-extended-stage embryos, global derepression of *ftz* was observed in preblastoderm-stage embryos, as early as the third nuclear division cycle. With the exception of the detection of *engrailed* protein in pre-cycle 9 embryos (Karr et al. 1989), transcriptional activity at such an early stage of embryogenesis has not been observed. The earliest transcription by RNA polymerase II from the zygotic genome is known to occur around nuclear division cycles 9 or 10 (Lamb and Laird, 1976; McKnight and Miller, 1976; Anderson and Lengyel, 1979; Edgar and Schubiger 1986). It is widely assumed that preblastoderm-stage embryos are transcriptionally silent due to the absence of specific activators or to the inactivity of the general transcriptional machinery. Our findings with the mutated *ftz-lacZ* constructs suggest that, in the case of *ftz*, there is specific repression by *ttk* in pre-cycle 10 embryos.

In this paper, we have examined the distribution of *ttk* protein during embryogenesis and the effects of ectopic *ttk* expression on the expression of *ftz* and on subsequent embryonic development. Our results provide further evidence in support of the hypothesis that *ttk* acts as a direct, maternal repressor of *ftz*, and perhaps of other pair-rule segmentation genes.

MATERIALS AND METHODS

Cloning of the full-length ttk gene into the Studier vector system

An *Nde*I site was engineered at the transcription start site of the *ttk* gene by site-directed mutagenesis. The full-length clone was then isolated as a 2.5kb *Nde*I-*Bam*HI fragment (the *Bam*HI restriction was a partial digest since there is a *Bam*HI site near the 3′ end of the gene), and cloned into *Nde*I-*Bam*HI restricted JC10 (Clos et al., 1990), a derivative of the pET3C vector (Studier and Moffatt, 1986). The resulting plasmid (pJLBG), has *ttk* under the control of the T7 promoter.

Expression of the full-length clone and extraction of the *ttk* protein

400 ml Luria-Bertani broth with 100 mg/ml ampicillin was inoculated with a fresh transformant colony of pJLBG in BL21(DE3) and grown with shaking at 37°C until the culture reached OD_{600nm} 0.75. The expression of *ttk* was induced by adding IPTG to 0.4 mM and shaking at 37°C for 1 hour. Cells were harvested by spinning 10 minutes at 6000 ***g***, resuspended in 4 ml lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.75 mg aprotinin, 5 mg/ml leupeptin, 2 mg/ml pepstatin, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 0.5 mM DTT) and frozen at −70°C.

The cell suspension was subjected to three rapid freeze-thaw cycles, sonicated with 6×30 second bursts at 100 mW and incubated for 5 minutes at 37°C with 2 mg/ml deoxycholic acid. DNAase I was added to a final concentration of 10 mg/ml and incubated 30 minutes at 25°C. The lysate was centrifuged for 10 minutes at 6000 ***g***, at 4°C. The supernatant was discarded and the pelleted inclusion bodies were resuspended in a Dounce homogeniser in 9 ml lysis buffer containing 0.5% Triton X-100, 10 mM EDTA, incubated at room temperature for 10 minutes, and centrifuged for 10 minutes at 6000 ***g*** at 4°C. The resuspension and centrifugation steps were repeated twice and the final pellet was resuspended in 4 ml Laemmli gel loading buffer (50 mM Tris-HCl pH 6.8, 2 mM EDTA, 1% SDS, 1% mercaptoethanol, 8% glycerol, 0.025% bromphenol blue). The sample was divided into 750 ml aliquots and either frozen at −70°C, or loaded on an SDS gel for electrophoretic purification of the protein.

Purification of *ttk* and preparation of *ttk* antibodies

750 μl aliquots of *ttk* protein in loading buffer were electrophoresed on 7.5% SDS gels. A small vertical slice was excised and stained with Coomassie Blue to locate *ttk* protein. The slice was realigned with the remainder of the gel. The gel region containing *ttk* was excised and homogenised with 4 ml gel running buffer. The slurry was incubated at 25°C for 48 hours and passed over glass wool to remove residual acrylamide. The protein was precipitated by adding KCl to 1 M, and incubating for 20 minutes on ice, and pelleted by centrifugation in a microfuge for 10 minutes. The pellet was dissolved in 500 ml 10 mM Tris pH 8.0, 0.1% SDS and dialysed for 4 hours against the same buffer. Protein concentration was measured with the Bradford assay and purity was assayed by applying an aliquot to an SDS gel and staining with Coomassie Blue after electrophoresis.

Polyclonal antibodies against *ttk* were prepared by subcutaneously injecting rats with 50 μg of purified *ttk* protein followed by two injections of 50 μg each at 3 week intervals, (Hazleton Laboratories).

Western analysis

SDS-PAGE (7.5% polyacrylamide) was performed according to standard procedures. Proteins were electrophoretically transferred to nitrocellulose membrane in 50 mM Tris, 380 mM glycine, 0.1% (w/v) SDS, 20% methanol. Membranes were processed with primary antibody (1:250 dilution rat polyclonal anti-*ttk*, pre-absorbed overnight at 1:5 dilution against fixed embryos), followed by secondary antibody (1:500 dilution alkaline phosphatase-conjugated goat anti-rat IgG). The alkaline phosphatase II kit (Vector Laboratories) was used for color development according to the manufacturer's instructions.

Nuclear extracts used were as described in Brown et al. (1991). Bacterial extracts were prepared as described above from BL21(DE3) cells carrying pJLBG or from XL1 Blue (Strategene) cells carrying plasmids with ttk p69 (pHD) or p88 (λ409) fused to β-galactosidase at the aminoterminus (Read and Manley, 1992a). The p69 and p88 proteins were induced with IPTG.

Gel mobility shift assay in the presence of antibodies against *ttk*

2 μl of nuclear embryonic extract (see Brown et al., 1991) was incubated in a total volume of 10 μl containing 20 fmoles labelled DNA-binding site, 10 μg tRNA, 4 μg poly(dI-dC).poly(dI-dC), 1 μl 5% BSA, 3.5 μl PBS and 1 μl of antibody or prebleed serum (diluted in PBS). Samples were incubated for 30 minutes at 25°C, supplemented with 2 μl gel loading buffer (2.5% Ficoll 400, 0.5× TBE and tracking dyes) and electrophoresed on a 1% agarose, 0.5× TBE gel.

Construction of the *hs/ttk* transformed flies

pLB20, a plasmid containing the full-length *ttk* cDNA in pBluescript with an *Nde*I site engineered at the transcription start site, was cut with *Nde*I and filled in with Klenow. The cDNA insert was excised with *Xba*I. The *Nde*I-*Xba*I fragment was ligated to *Sac*II (cut and filled in)/*Xba*I cut pCaSpeRhs vector (gift of C. Thummel and V. Pirotta), thereby putting the *ttk* cDNA under the control of the heat-shock promoter. The filling in reaction had some exonuclease activity which nibbled the fragment ends but which left the ATG codon and the downstream sequences intact and fortuitously maintained the necessary translation start signals. The resulting plasmid is named p*hs-ttk* and the organisation is illustrated in Fig. 3A. Our initial experience with a *heat shock-ttk* construct with 252 nt of 5′ untranslated leader sequence showed that these sequences interfered with the translation of the p69 polypeptide (unpublished observations).

P element-mediated germline transformations were performed as described in Rubin and Spradling (1982). The host strain, *Df(1)w67c2,y* was injected with 300 μg/ml p*hs-ttk* and 150 μg/ml p*d2-3wc* helper DNA. Transformed G_1 progeny were selected on the basis of partial rescue of the w^- phenotype. Homozygous flies were generated by crossing with the appropriate balancer strains; two homozygous lines were obtained. In the transformant line 4C, the p*hs-ttk* construct is inserted on chromosome 2 and, in line 4B, on chromosome 3.

Embryo collection, heat shock and embryo staining

For antibody staining and in situ hybridization, untransformed embryos and *hs-ttk* embryos were processed in parallel. The embryos were collected at 25°C for 45 minutes on grape juice agar plates, washed, dried, transferred onto a coverslip and covered with Voltaleff 3S halocarbon oil to clear the chorion. Embryos were allowed to develop (at 25°C) until most of the embryos were in late syncytial blastoderm or cellular blastoderm formation. Unfertilised eggs and postblastoderm stages were discarded before heat shock. Embryos were heat shocked at 36°C for 15 minutes in a humidified chamber on a constant temperature block. After heat shocking, the embryos were allowed to recover for 30 minutes at 25°C and were then rinsed with heptane to remove the halocarbon oil and dechorionated in 50% Chlorox.

Fixation, devitellinisation and antibody staining of embryos was as described in DiNardo and O'Farrell (1987) using the Vectastain Elite ABC kit. The polyclonal *ttk* serum and the anti-*ftz* antibody (a gift from H. Krause), were used at a concentration of 1:500 after preabsorbing at a 1:5 dilution against Oregon R embryos. The color reaction was developed using the peroxidase substrate kit DAB (Vector Laboratories). Embryos were mounted in Permount (Fisher Biotech.) and examined using Nomarski optics.

In situ hybridisation to detect mRNA was based on the method of Tautze and Pfeifle (1989) with modifications according to Rick Garber (personal communication). After fixation and devitellinisation, the embryos were transferred to EtOH and were stored (for up to 6 months) at −20°C. For staining, the embryos were rinsed in 50% EtOH, 50% xylene and soaked in 100% xylene for several hours to reduce the background; they were then rinsed again with 50% EtOH, 50% xylene followed by several rinses in 100% EtOH (rinses are for 5 minutes each unless otherwise stated). The embryos were then rinsed in MeOH, followed by 50% MeOH, 50% PBT (PBS [130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4] with 0.1% Tween 20) plus 5% formaldehyde and fixed for 20 minutes in PBT plus 5% formaldehyde. After fixing, the embryos were rinsed three times in PBT (2 minute rinses) and then digested with 50 μg/ml proteinase K (Boehringer Mannheim) for 4 minutes at 37°C. Digestion was terminated by rinsing 2×2 minutes with 2 mg/ml glycine in PBT followed by two rinses in PBT. The embryos were postfixed for 20 minutes in PBT plus 5% formaldehyde. The fixative was removed and the embryos were washed 5×5 minutes with PBT. The embryos were then rinsed in 50% PBT, 50% hybridization solution (50% deionised formamide, 5× SSC, 100 μg/ml sonicated, boiled salmon sperm DNA, 100 μg/ml tRNA, 50 μg/ml heparin, 0.1% Tween 20) and incubated in 100% hybridization solution at 48°C for 2 hours. Hybridisation was at 48°C overnight in 100 μl of hybridization solution containing 0.1 μg/ml heat-denatured DNA probe.

DNA probes were prepared from gel-purified restriction fragments of pair-rule gene cDNA clones. The *ftz* probe was 1.7 kb *Eco*RI fragment (gift of A. Laughon and M. Scott); the *even skipped* probe was a 1.1 kb *Hin*dIII-*Eco*RI fragment (gift of M. Levine and M. Scott); the *runt* probe was a 2.2 kb *Bam*HI fragment (gift of A. Manoukian, H. Krause, and P. Gergen); the *hairy* probe was a 2.0 kb *Eco*RI fragment (gift of M. Horner and C. Rushlow); the *odd skipped* probe was 400 bp *Eco*RI fragment (gift of D. Coulter); and the *Kruppel* and *hunchback* probes were 2.1 kb *Pst*I/*Eco*RI and 2.3 kb *Xba* I fragments, respectively (obtained through D-H. Hwang). 100 ng of DNA fragment was incubated in a total volume of 15 μl with 100 μg random primers, $pd(N)_6$ (Pharmacia), denatured by boiling 5 minutes and quick chilled on ice. 2 μl of 10× Vogel buffer (1 M Pipes (pH 6.6), 50 mM $MgCl_2$, 0.6 mM β-mercaptoethanol), 2 μl deoxynucleotide mix containing digoxigenin-UTP (Genius kit, Boehringer Mannheim), and 5-10 units of Klenow enzyme were added. The labelling reaction was incubated overnight at 15°C and then for 4 hours at room temperature after the addition of a further 5-10 units of Klenow. The reaction was stopped by adding 0.5 M Na_2EDTA to 50 mM and incubating at 65°C for 10 minutes. The probe was precipitated at −70°C after the addition of 80 μl of H_2O, 50 μg tRNA, 5 M LiCl to 0.4 M and 300 μl ethanol.

After hybridization, excess probe was removed and the embryos were washed for 20 minutes at 48°C with hybridization solution, 50% hybridization solution/50% PBT, and 5 washes with PBT. The embryos were then incubated for 1 hour at room temperature with 1:2000 dilution of anti-digoxigenin alkaline-phosphate-conjugated antibodies (Genius kit) in PBT with 0.1% BSA (the antibodies were preabsorbed at a 1:5 dilution against an equal volume of embryos). The embryos were washed four times for 20 minutes with PBT and then rinsed 2× 20 minutes in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween 20. To the second wash (1 ml), 4.5 μl nitroblue tetrazolium (NTB) and 3.5 μl of X-phosphate (Genius kit) were added for the color reaction and were incubated for several hours in the dark until the color developed. The reaction was stopped by rinsing several times with PBT. The embryos were stored overnight at 4°C, mounted in Permount (Fisher Biotech) and examined using Nomarski optics.

Cuticle preparations

Embryos were collected and heat shocked as described above. After heat shock the embryos were returned to a humid chamber at 25°C for at least 24 hours to allow development to proceed. The number of hatched versus unhatched embryos was scored under the microscope for each class. The embryos were then rinsed with heptane to remove the halocarbon oil, dechorionated in 50% chlorox for 3 minutes, rinsed with embryo wash, dried, transferred to a microscope slide with a drop of 1:1 acetic acid/Hoyers reagent (as described in Wieschaus and Nusslein-Volhard, 1986) and incubated overnight at 65°C. The cuticle preparations were then examined by phase-contrast microscopy.

RESULTS

The *tramtrack* gene encodes two related proteins of predicted molecular masses 69×10^3 (p69) and 88×10^3 (p88), with different DNA-binding specificities generated through alternative splicing of related zinc-finger motifs (Read and Manley, 1992a). Complementary DNAs for *ttk* p69 have been previously cloned and shown to interact specifically with *ftz* regulatory elements (Harrison and Travers, 1988, 1990; Brown et al., 1991). We have raised rat polyclonal antibodies to full-length recombinant *ttk* p69 and used them to analyze the distribution of *ttk* protein during embryogenesis by immunohistochemical staining.

Antibodies to *ttk* p69 react with one major and three minor *ttk* protein-DNA complexes that are formed when embryo extracts are incubated with a segment of the *ftz* zebra element and analyzed by native gel electrophoresis (Fig. 1A). The putative *ttk* proteins (previously named *FTZ-F2 A-D* and defined by their specific binding to TGC_AGGAC_T sequences on the zebra element [Brown et al. 1991]) are all supershifted by treatment with immune serum but not with pre-immune serum. Hence, all four gel shift complexes are composed of multiple forms of *ttk* p69 (or a highly related antigen) that could result from covalent modification, proteolytic degradation or association as homomeric or heteromeric complexes.

The specificity of the antibodies to *ttk* p69 was determined by western blot analysis of embryonic nuclear extracts after separation on a SDS-polyacrylamide gel. The antibodies showed reaction to a major protein species of M_r 92×10^3, which corresponds to the anomalously migrating, full-length p69 protein expressed in *E. coli* (Fig. 1B; Read and Manley, 1992a). Anomalous migration of other developmental transcription factors on SDS gels, e.g. *ftz* (Krause et al. 1988) and *Kruppel* (Ollo and Maniatis, 1987) has been observed previously. Since full-length *ttk* p69 shares significant sequence identity with the *ttk* p88 isoform, we expected substantial cross-reaction of our antiserum with the latter isoform. However, the antiserum only faintly stained a band of M_r ~150×10^3 on the western blot (Fig. 1B), corresponding to the anomalous migration of the p88 isoform (Read and Manley, 1992a). The preferential staining of *ttk* p69 over p88 was observed with nuclear extracts prepared from embryos staged throughout embryonic development, and with the bacterially expressed *ttk* isoforms (Fig. 1C). Hence, the results indicate that the antibodies are primarily directed against epitopes unique to p69, the *ttk* isoform that has been implicated in the repression of *ftz*.

The endogenous distribution of *ttk* protein

We employed the antibodies to *ttk* p69 to determine the pattern of p69 expression at various stages throughout embryogenesis (Fig. 2). At the earliest stages observed (pre-stage 3 embryos; stages according to Campos-Ortega and Hartenstein, 1985) staining for *ttk* protein, presumably of maternal origin, can be detected throughout the embryo (Fig. 2A). This early appearance of *ttk* does not seem to accumulate exclusively in the cleavage nuclei. The general distribution of *ttk* persists through the syncytial blastoderm stage and staining for *ttk* is also observed in the pole cells (Fig. 2B). During formation of the cellular blastoderm, staining for *ttk* diminishes, and staining is completely absent by the completion of the cellular blastoderm (Fig. 2C), a period when *ftz* expression in stripes is at its height.

The zygotic expression of *ttk* is first evident around late embryonic stage 9-early stage 10, in the anterior midgut primordium (Fig. 2D). This is followed in stage 10 by the staining of isolated cells along the mesodermal surface of the yolk space (Fig. 2E). These cells may correspond to the precursors of macrophages, which have been identified morphologically by Campos-Ortega and Hartenstein (1985) in stage 11 embryos. Weak staining of the pole cells is also detectable at this stage. Subsequently, *ttk* expression is observed in the posterior midgut primordium and in patches of cells along the embryo that correspond to staining of the tracheal placodes. The salivary gland placode and the head region also show staining at this stage (Fig. 2F). By the time of germband retraction, the pattern becomes harder to discern. There is strong staining of the visceral mesoderm and weaker staining of the ectoderm (Fig. 2G). At full germband retraction, the staining is more general, although the staining is stronger in the visceral mesoderm and the ectoderm of the embryo. There is also weak staining of the amnioserosa (Fig. 2H).

The distribution of *ttk* protein that we have observed during embryogenesis is similar to the distribution of *ttk* RNA (Harrison and Travers, 1990; Read and Manley, 1992a,b; Brown and Wu; unpublished observations), although the appearance of *ttk* protein lags behind that of *ttk* RNA by a short interval (about 30 minutes). The presence of *ttk* protein in preblastoderm-stage embryos is consistent with its proposed role as a maternal repressor of *ftz*. The complex zygotic expression of *ttk* in late embryos suggests that *ttk* has multiple regulatory functions separate from the repression of *ftz* at specific stages throughout embryogenesis.

Ectopic expression of *ttk*

In an earlier study, we proposed that *ttk* functions as a negative regulator of *ftz*, since point mutations that eliminate *ttk* binding to the zebra element cause derepression of *ftz-lacZ* constructs in transformed embryos (Brown et al., 1991). To test this hypothesis, we generated flies carrying *ttk* under the control of the heat-shock promoter and examined the effects of ectopic expression of *ttk* on *ftz* expression. The full-length *ttk* p69 cDNA clone (with the entire 5′ leader sequence removed; see Methods) was inserted into the pCaSpeRhs vector as shown in Fig. 3A. The ability of the *ttk* construct lacking 5′ leader sequences to be translated was confirmed by in vitro transcription-

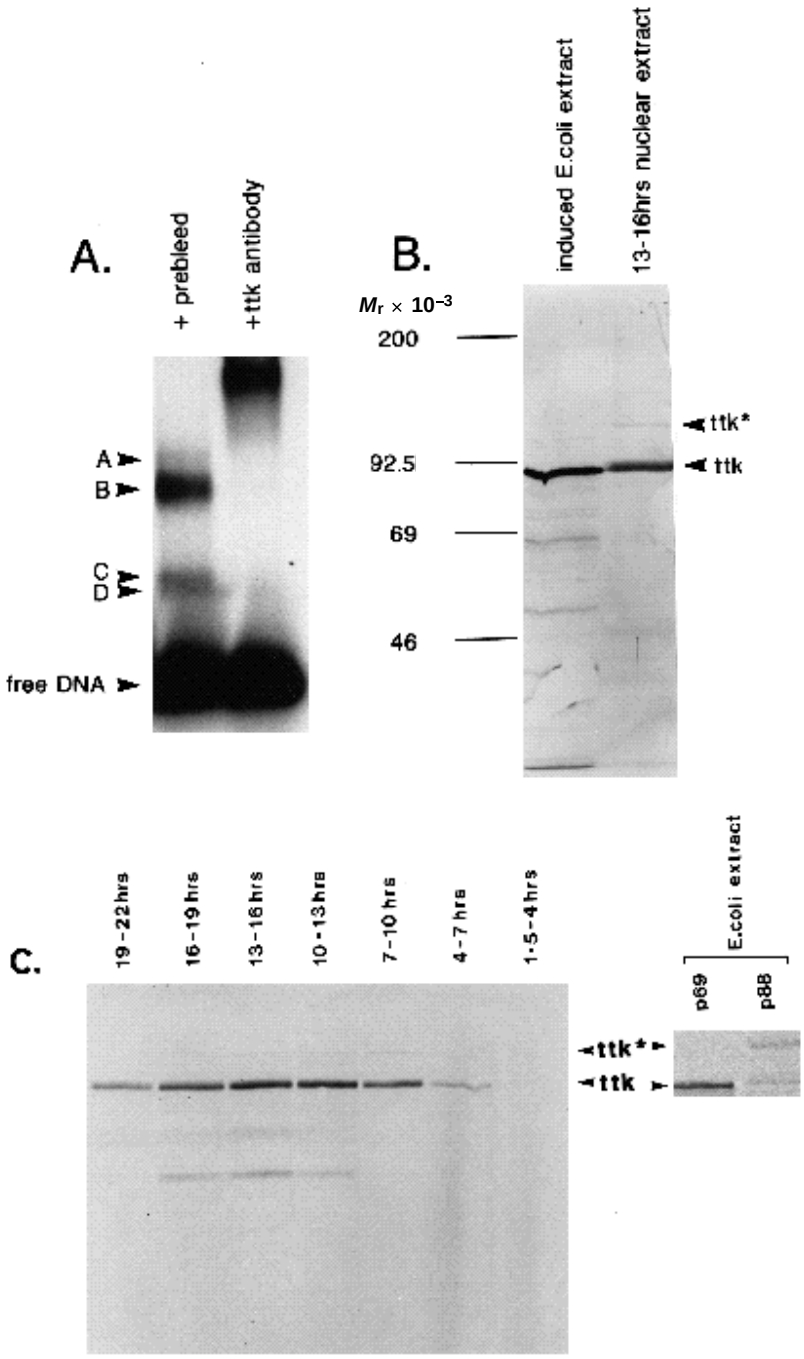

Fig. 1. Characterisation of the antibody to *ttk* p69. (A) Gel mobility shift assay. Autoradiogram of a 1% agarose gel showing a gel mobility shift assay of 10-13 hour embryo nuclear extract using a ^{32}P labelled *Ban*I-*Hpa* II fragment (position −131 to −200) of the *ftz* gene, with the inclusion of prebleed sera or the polyclonal antibody to p69. The 10-13 hour embryo extract contains no *FTZ-F1* activity and the *FTZ-F3* activity has been competed away by including 100-fold excess (100 ng) of the *FTZ F3* binding site (position −172 to −142 of the *ftz* zebra element; see Brown et al., 1991 for further details) (B) Western blot analysis. Gel lanes loaded with a bacterial extract containing recombinant *ttk* p69, or 13-16 hour embryo nuclear extract were probed with the antibody to *ttk* p69. *ttk** corresponds to weak staining of *ttk* p88. Prebleed sera recognises neither p69 nor p88 (data not shown). (C) Western blot analysis of nuclear extracts prepared from embryos staged at the indicated intervals, and bacterial extracts containing expressed *ttk* (p69) and *ttk** (p88). Equivalent amounts of bacterially expressed proteins were loaded on the gel lanes.

translation assays before proceeding with P element-mediated germ-line transformation. Two independent transformant lines were established as homozygous stocks.

Since blastoderm-stage embryos represent the earliest developmental period when the heat-shock response can be elicited, embryos were harvested and aged at 25ºC until the late syncytial blastoderm or early cellular blastoderm stages. Embryos were then heat shocked for 15 minutes at 36ºC and allowed to recover for 30 minutes at 25ºC before fixation and analysis by whole-mount in situ hybridisation or immunohistochemical staining. The lack of *ttk* protein expression at the cellular blastoderm stage of unshocked embryos carrying p*hs-ttk* is confirmed in Fig. 3B, panel (a). Upon heat shock, these embryos showed extensive *ttk* protein staining with clear nuclear localization essentially throughout the length of the embryo. In some cases, p*hs-ttk* expression seems to be slightly lower in the polar regions and is not evident in the pole cells (Fig. 3B, panel (b). The half-life of ectopic *ttk* protein was found to be approximately 90 minutes.

Embryos collected from flies lacking or carrying the p*hs-ttk* construct were heat shocked in parallel and stained for *ftz* RNA expression by in situ hybridization (Fig. 4). As shown in Fig. 4A,B, the normal expression of *ftz* in seven stripes is not affected by heat shock of embryos derived from the strain lacking p*hs-ttk*. However, heat-shock-

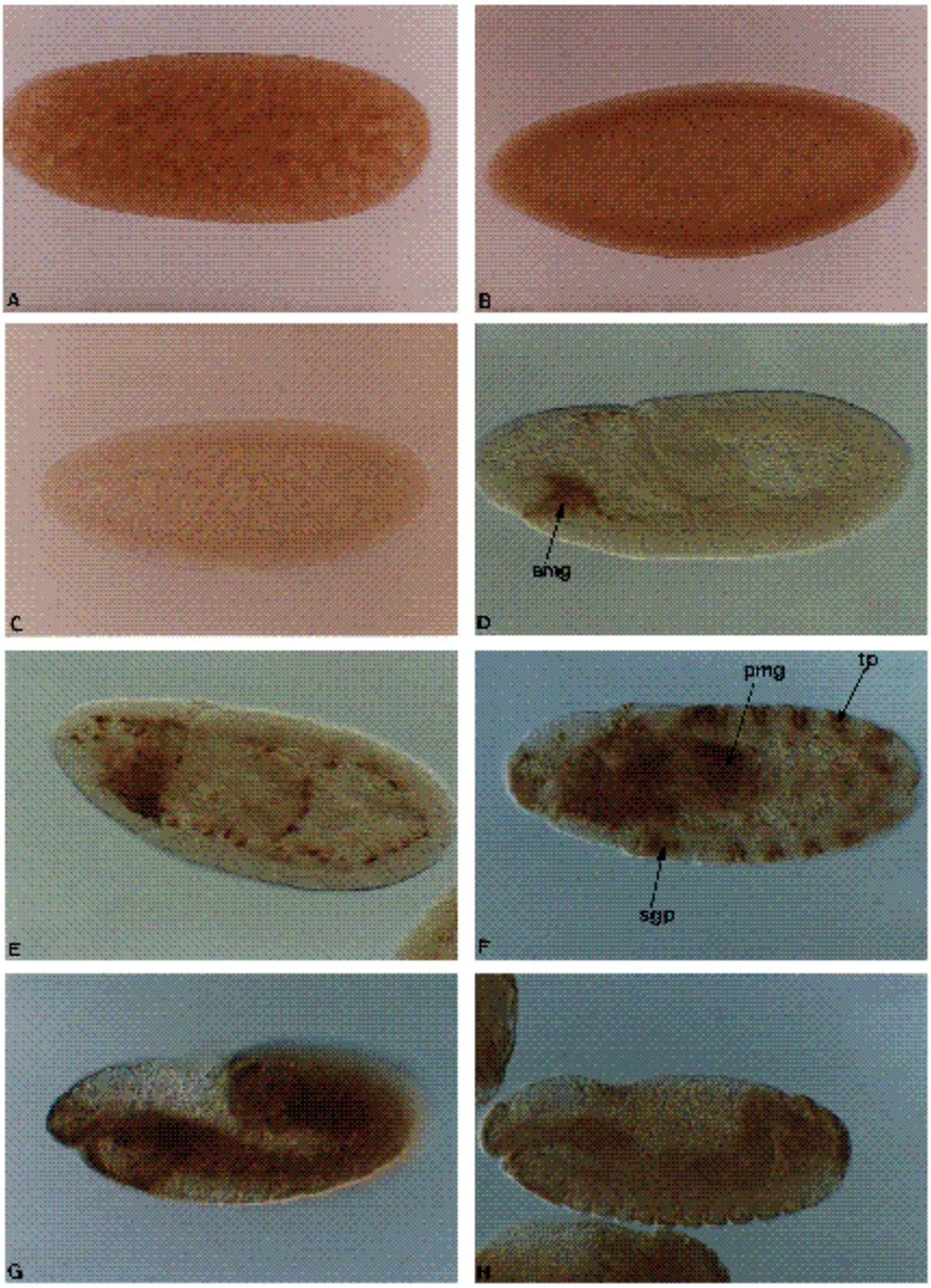

Fig. 2. *ttk* protein distribution during embryogenesis. (A) Pre-stage 3 embryo. (B) Syncytial blastoderm embryo. (C) Cellular blastoderm. (D) Late stage 9/early stage 10 embryo; amg, anterior midgut primordium. (E) Embryonic stage 10, in addition to the anterior midgut primordium, staining is now detected in isolated cells along the mesodermal surface of the yolk space. (F) Embryonic stage 11; pmg, posterior midgut primordium, sgp, salivary gland placode, tp, tracheal placodes. (G) Germband retraction stage. (H) Full germband retraction. No staining was observed with prebleed sera (data not shown).

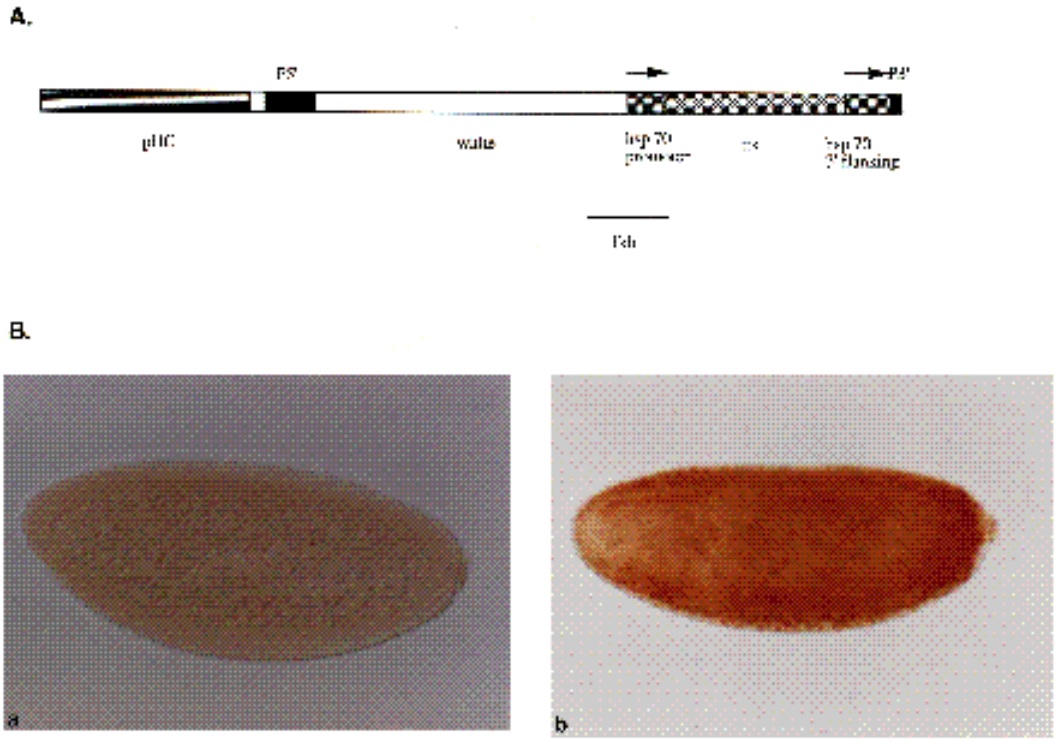

Fig. 3. Ectopic expression of *ttk* protein. (A) Structure of the p*hs-ttk* plasmid. The solid portion represents P-element sequences; the stipled portions, *hsp70* sequences (promoter and 3′ polyadenylation signals); the checked portion, the *ttk* cDNA and the open portion, *white* sequences. (B) Heat-shock-induced expression of *ttk*. Embryos stained with the antibody to *ttk* p69 are oriented anterior to the left. (a) an unshocked embryo at the cellular blastoderm stage, (b) a heat-shocked embryo at the same stage.

induced expression of *ttk* in embryos carrying p*hs-ttk* caused a drastic reduction in the expression of *ftz* in the entire population of shocked embryos. Out of a cohort of 150 embryos examined, 60% showed no detectable *ftz* RNA expression (Fig. 4D) and the remaining 40% showed only weak, residual staining in stripes 2,3 and 7 (see Fig. 4E). 100% of non-heat-shocked embryos carrying p*hs-ttk* showed the normal staining for *ftz* in 7 stripes (Fig. 4C). The above observations were similar for both transformant lines analyzed.

When heat-shocked embryos carrying p*hs-ttk* were stained for *ftz* protein expression by immunohistochemistry, the same trend was observed, although the appearance of *ftz* protein lagged slightly behind that of *ftz* RNA, so that most embryos had residual *ftz* expression in at least some of the seven stripes (Fig. 5). Nonetheless, the residual expression in the stripes was limited to small patches in embryos expressing p*hs-ttk* and in no case was the three- to four-cell width of normal *ftz* expression retained (compare Fig. 5E and F).

Phenotype of embryos after ectopic *ttk* expression

In order to analyze the phenotype of embryos after ectopic expression of *ttk*, embryos carrying p*hs-ttk* were collected and allowed to develop at 25°C to late syncytial blastoderm or early cellular blastoderm stages. The embryos were then heat shocked for 15 minutes at 36°C, returned to 25°C and permitted to develop for another 24 hours. The numbers of hatched larvae and unhatched larvae or embryos were scored by microscopic analysis. Typical results of such an experiment are shown in Table 1. No embryos carrying p*hs-*

Table 1. Percentages of hatched and unhatched embryos of non-heat-shocked (NHS) and heat-shocked (HS) *Df(1)w67c2,y* controls, or transformant lines carrying p*hs-ttk* (4B and 4C)

	Hatched embryos (%)	Unhatched but normal looking embryos (%)	Unhatched but abnormal looking embryos (%)	Number of embryos examined
Df(1)w67c2,y NHS	66	27	7	108
Df(1)w67c2,y HS	50	30	20	86
4B NHS	68	17	15	95
4B HS	0	0	100	97
4C NHS	71	20	9	94
4C HS	0	0	100	104

Unhatched embryos were scored as normal if they showed all the features diagnostic for stage 17 embryos (Campos-Ortega and Hartenstein, 1985). Abnormal embryos were scored as such if they did not correspond to any recognisable developmental stage.

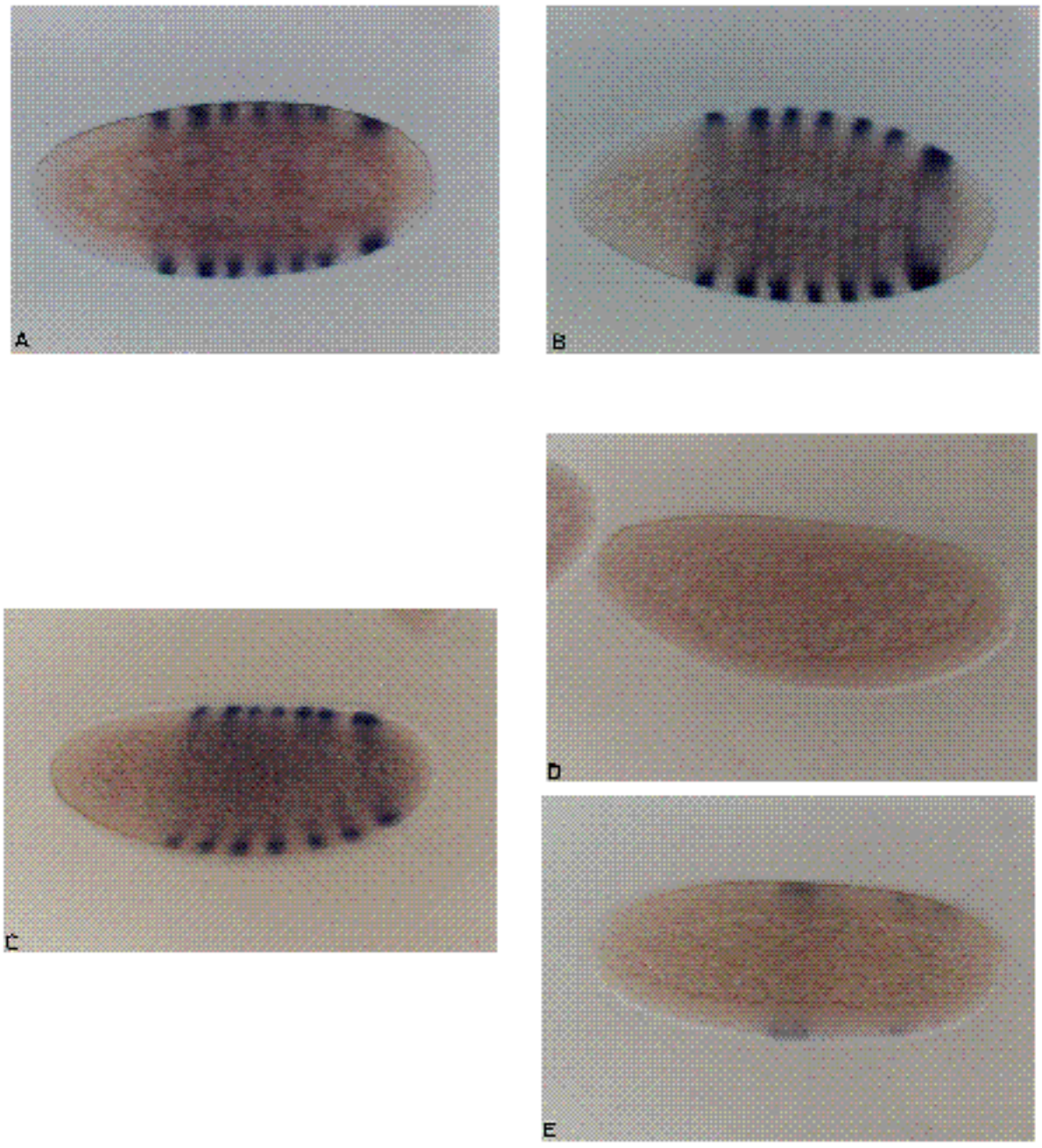

Fig. 4. Effect of ectopic *ttk* expression on the *ftz* RNA pattern, analyzed by whole-mount in situ hybridization. (A) An unshocked *Df(1)w67c2,y* embryo at the syncytial blastoderm stage; (B) a similar embryo subjected to heat shock; (C) an unshocked syncytial blastoderm embryo from the *Df(1)w,67c2,y* strain transformed with p*hs-ttk* (line 4C). (D, E) similarly staged line 4C embryos subjected to heat shock. Similar results were obtained with the second independent transformant line 4B.

ttk survived after the heat shock to hatch from the eggshell (100% lethality) while the lethality was 20-50% for the control embryos. Cuticle preparations from the unhatched embryos carrying p*hs-ttk* were analyzed. All of the heat-shocked embryos examined showed a range of cuticular abnormalities that were not seen with the non-heat-shocked controls or with the heat-shocked *Df(1)w67c2,y* line. The most predominant class of defects observed (about 50%) had naked cuticles completely devoid of denticle belts (Fig. 6D). Other cuticular abnormalities included defects in the number, spacing or appearance of the denticle belts (Fig. 6B,C); some embryos did not secrete any cuticle at all. In addition, many of the embryos also showed severe abnormalities of the mouthparts. This highly nonspecific phenotype indicates that the ectopic expression of *ttk* throughout the blastoderm-stage embryo must interfere with the normal expression or function of a number of genes besides *ftz* that are important for embryogenesis. The wide range of phenotypes observed could be due to the precise developmental age of the individual embryo when p*hs-ttk* was

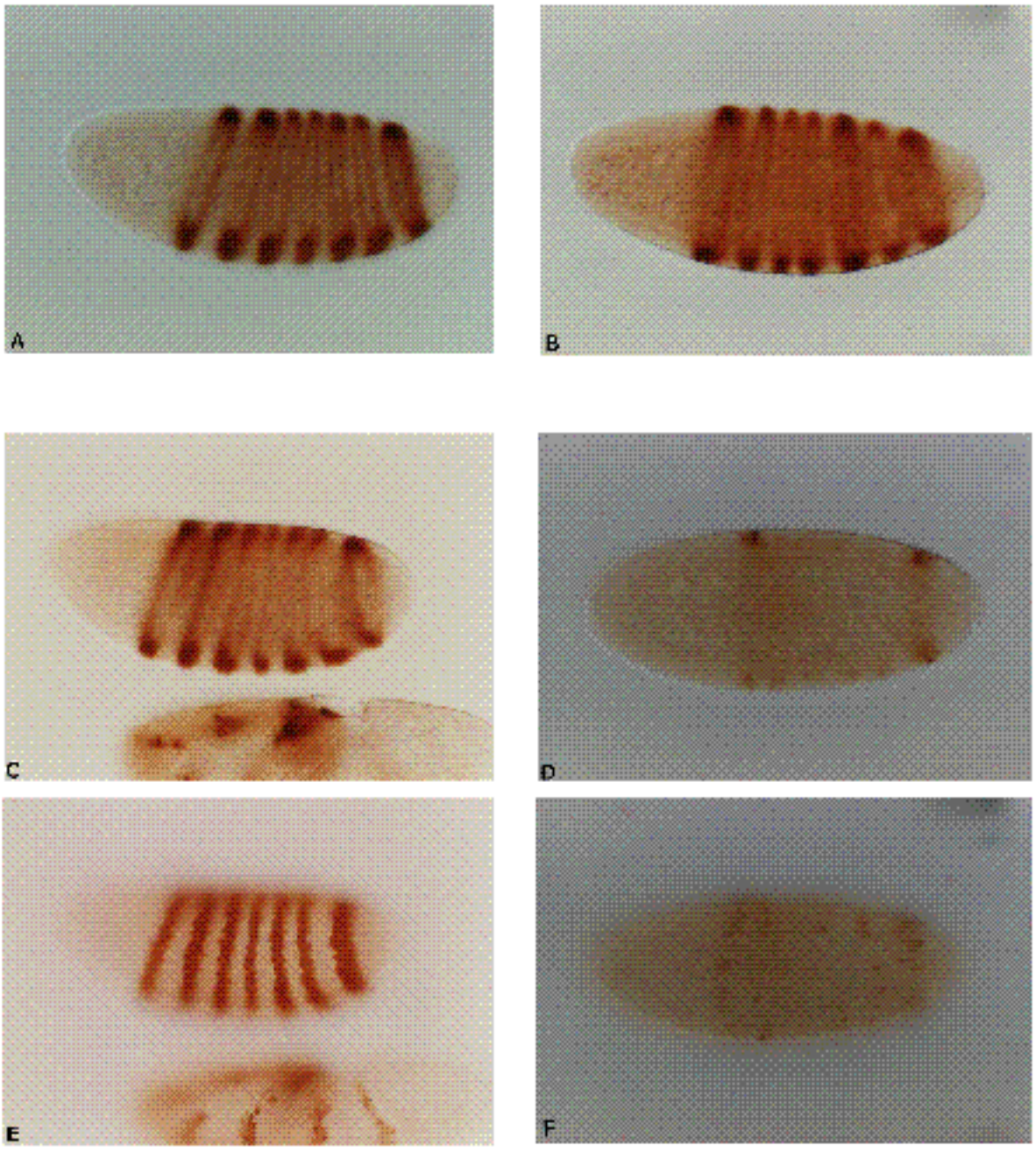

Fig. 5. Effect of ectopic *ttk* expression on *ftz* protein distribution, analyzed by immunohistochemical staining with antibodies to *ftz* protein. (A) Unshocked syncytial blastoderm-stage embryo from the untransformed *Df(1)w67c2,y* strain; (C,E) same stage unshocked embryos from the line 4C carrying p*hs-ttk*; (B) heat-shocked *Df(1)w,67c2,y* embryos; (D,F) heat-shocked line 4C embryos. (E,F) Surface views. The same results were obtained with transformant line 4B.

expressed, leading to a different or overlapping set of affected genes or functions.

Effects of ectopic *ttk* expression on pair-rule and gap genes

In order to test the possibility that *ttk* might be involved in the regulation of genes other than *ftz*, we analyzed the expression of four other members of the pair-rule class of segmentation genes. As shown in Fig. 7, embryos expressing p*hs-ttk* cause significant repression of *runt* and *eve* and also showed effects on *hairy* and *odd skipped*. Like *ftz*, the *runt* and *eve* RNA expression patterns are severely repressed in the heat-shocked embryos, although some residual expression is observed in stripes 1,2 and 7 (see Fig. 7A-D). More than 90% of the heat-shocked embryos show these effects whereas the non-heat-shocked line carrying p*hs-ttk*, and the non-heat-shocked or heat-shocked *Df(1)w67c2,y* embryos showed the normal expression patterns for *runt* and *eve*, as described by Gergen and Butler (1988), and MacDonald et al. (1986), respectively.

In the case of *hairy* and *odd skipped* the repression is more localized. For *hairy*, 92% of the heat-shocked

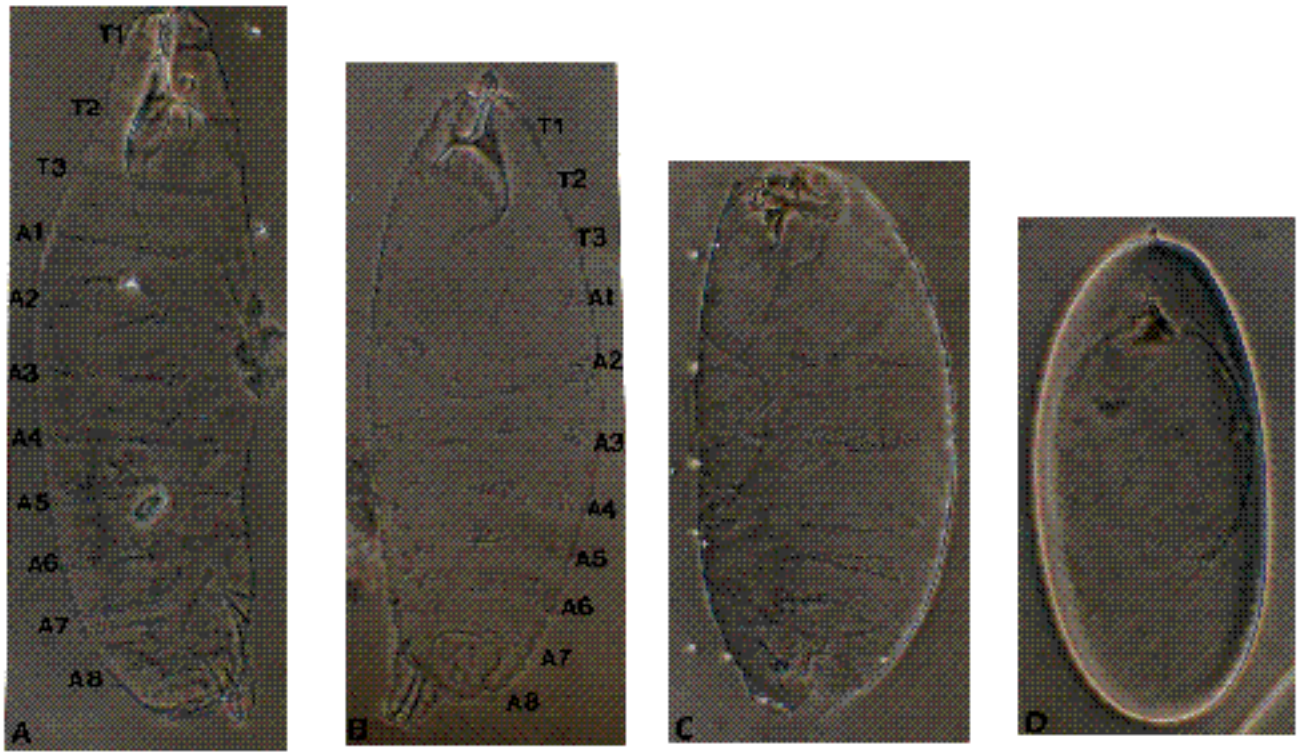

Fig. 6. Examples of the cuticular abnormalities induced by ectopic expression of *ttk*. (A) Cuticle preparation of an unshocked embryo from the transformant line 4C. (B-D) Cuticular defects induced by ectopic expression of *ttk* p69. Heat-shocked *Df(1)w67c2,y* control embryos did not reveal any systematic pattern abnormalities.

embryos carrying p*hs-ttk* showed a severe repression of stripes 3 and 4 but virtually no effect on the other stripes (Fig. 7F). This effect was not observed with any of the heat-shocked *Df(1)w67c2,y* embryos, or the unshocked line 4C carrying p*hs-ttk* (Fig. 7E). For *odd skipped*, repression is seen in stripes 1, 5, 6 and 7, but not stripes 2, 3 and 4 (Fig. 7H); there also appears to be increased *odd skipped* expression in the regions between stripes 2, 3 and 4. We have investigated the possibility that the ectopic expression of *ttk* also leads to repression of the gap genes. As shown in Fig. 8, we failed to observe any effects of ectopic *ttk* on the expression of the gap genes *Kruppel* and *hunchback*.

DISCUSSION

In this paper, we have tested the hypothesis that *ttk* p69 acts as a direct, maternal repressor whose decay and dilution in relation to the exponentially dividing cleavage nuclei leads to the onset of *ftz* transcription in blastoderm-stage embryos (Brown et al., 1991). We show that the endogenous distribution of *ttk* p69 protein as revealed by immunohistochemical staining of preblastoderm-stage embryos is consistent with its proposed role as a maternal repressor of *ftz*. The absence of *ttk* p69 at the cellular blastoderm stage is also consistent with the peak of *ftz* expression at that stage. Most importantly, the ectopic production of *ttk* p69 in blastoderm-stage embryos carrying a *heat shock-ttk* construct causes complete or near-complete repression of the endogenous *ftz* gene, and a significant repression of *even skipped*, *odd skipped*, *hairy* and *runt*. No effects were observed on the expression of the gap genes *Kruppel* and *hunchback*. In an independent study, Read and Manley (1992b) have also found that expression of *ttk* p69, but not *ttk* p88, leads to repression of *even-skipped* and *ftz*, but not *hunchback*, *Kruppel* and *giant*. The combined results offer strong support for the hypothesis that maternal *ttk* p69 functions as a specific repressor of *ftz* and suggest further that this repression may extend to at least several other pair-rule segmentation genes.

That there should be a requirement for specific repression of *ftz* in preblastoderm-stage embryos is unusual, since it has been generally accepted that there is no zygotic transcription prior to nuclear division cycle 9 or 10 (Lamb and Laird, 1976; McKnight and Miller, 1976; Anderson and Lengyel, 1979; Edgar and Schubiger, 1986). A priori, a lack of transcription could be caused either by a lack of activators (general or specific) or by the presence of repressors. To our knowledge, current evidence on transcriptional controls at the preblastoderm stage do not preclude specific repression as one mechanism (among others) for the overall lack of preblastoderm transcription. Nonetheless, it is generally assumed that the extreme rapidity with which cleavage nuclei divide during the first nine nuclear division cycles could be sufficient to prevent significant transcription in preblastoderm embryos. Indeed, early cleavage nuclei divide approximately every nine minutes, with an interphase or S phase of about 4 minutes (Rabinowitz, 1941; McKnight and Miller,1977; Foe and Alberts, 1983). Despite this narrow time window available for transcription, it is

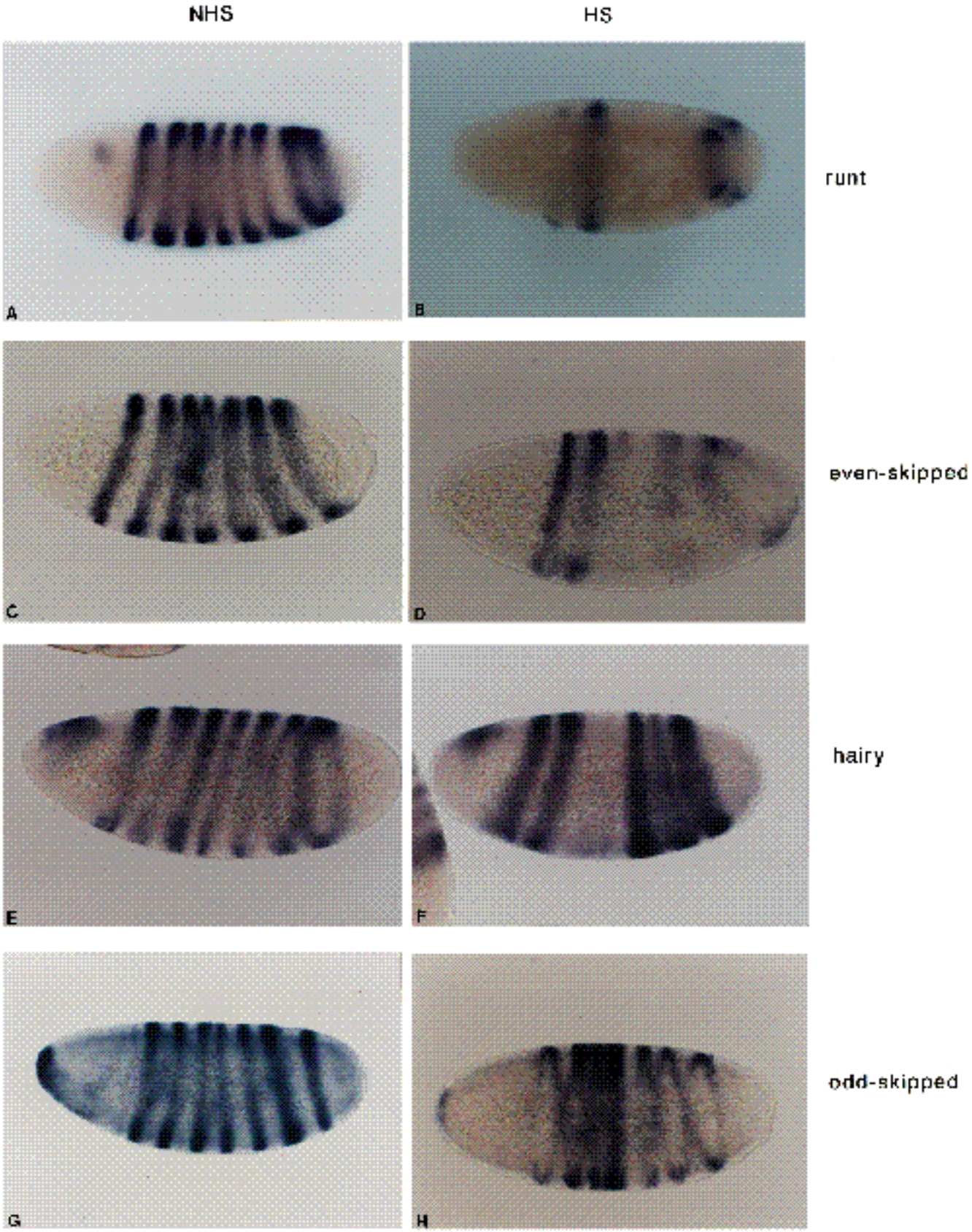

Fig. 7. Effect of ectopic *ttk* expression on the RNA expression patterns of *runt*, *eve*, *hairy* and *odd-skipped*. (A,C,E,G) Unshocked syncytial blastoderm-stage embryos from the transformant line 4C hybridized in situ with *runt*, *eve*, *hairy* and *odd-skipped* DNA probes respectively. (B,D,F,H) Heat-shocked embryos from the transformant line 4C probed similarly.

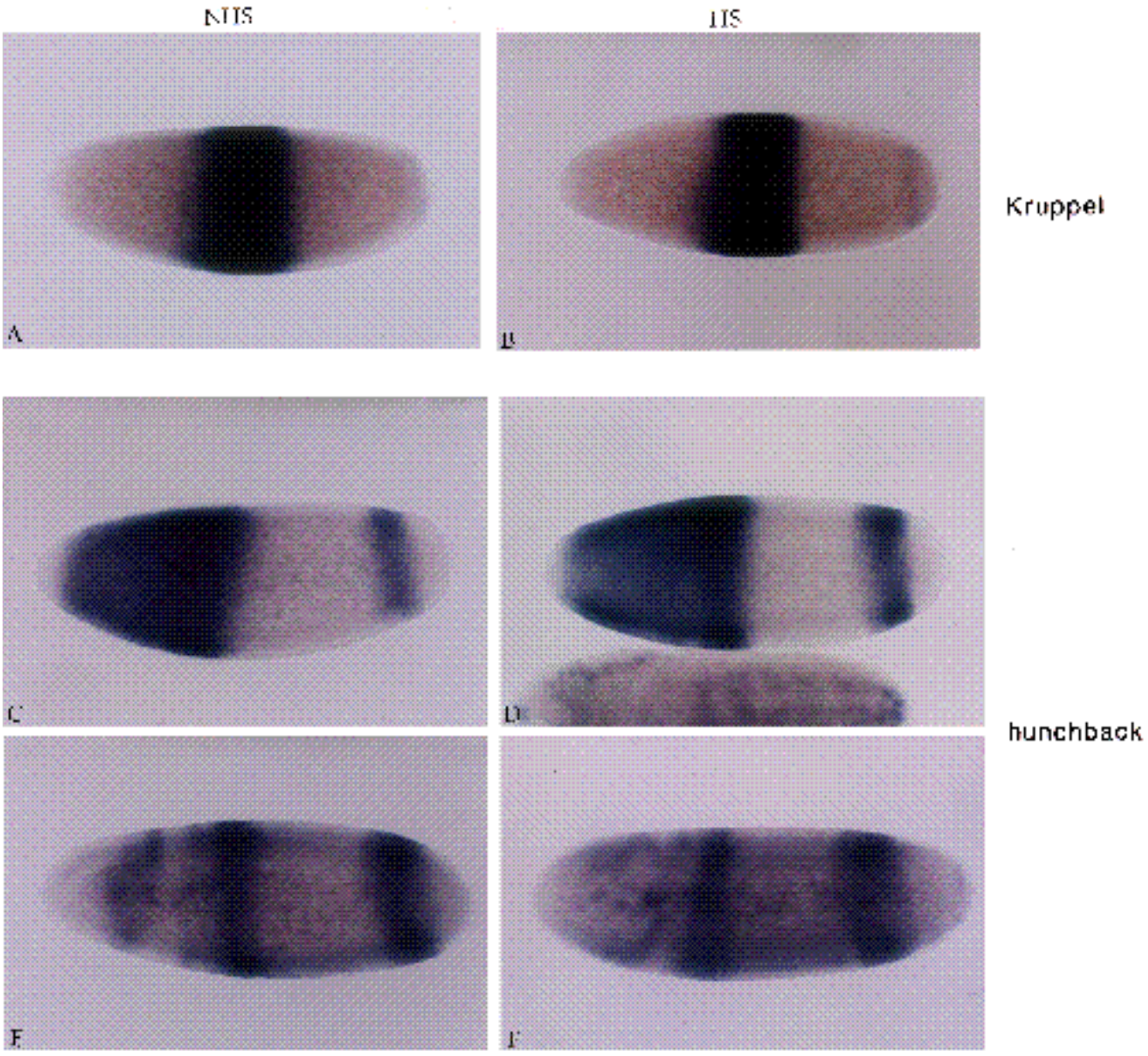

Fig. 8. Effect of ectopic *ttk* expression on the RNA expression patterns of the gap genes *Kruppel* and *hunchback*. (A,C,E) Unshocked and (B,D,F) heat-shocked syncytial blastoderm or cellular blastoderm-stage embryos from the transformant line 4C, hybridized with a *Kruppel* (A,B) or *hunchback* (C-F) DNA probe. (C,D) Embryos show both the maternal *hunchback* expression in the anterior domain and the initiation of the zygotic expression in anterior and posterior domains. (E,F) Embryos show the zygotic pattern of *hunchback* expression. Minor differences between the unshocked and heat-shocked embryos are a reflection of the dynamic nature of the *hunchback* RNA expression pattern (Tautz and Pfeifle, 1989).

possible that small genes (less than 5 kb) could be fully transcribed in under 4 minutes, assuming a rate of 1.1 to 1.4 kb per minute for RNA polymerase II [Thummel et al. (1990); Shermoen and O'Farrell, (1991)]. While transcripts from large developmental genes such as *Ubx* would be aborted by the interference of rapid mitotic cycles (Shermoen and O'Farrell, 1991), other repressive mechanisms must prevail for small genes, which constitute a significant fraction of the *Drosophila* genome.

In addition to *ftz*, the four pair-rule genes affected by ectopic *ttk* expression are all under 5 kilobases in size (Coulter et al., 1990; Kania et al., 1990; MacDonald et al., 1986, Laughon and Scott, 1984; Rushlow et al., 1989). Hence, they could in principle undergo complete transcription in preblastoderm embryos and therefore require specific repression by *ttk* until the blastoderm stage has been reached. For *hairy* and *odd skipped*, which are affected by ectopic *ttk* expression in only a subset of their stripe patterns, it would be necessary to invoke negative regulators other than *ttk* that would ensure the complete repression of those genes in the preblastoderm stage. It should be emphasized, however, that a direct interaction between *ttk* p69 and

DNA has only been shown for *ftz* and *even skipped* (Brown et al. 1991; Read and Manley, 1992a). For *even skipped*, the location of negative *cis*-elements that interact directly with *ttk* has not been determined. Further studies will be required to ascertain whether the repression of *hairy*, *runt*, *even skipped* and *odd skipped* that we have observed is actually dependent on direct interactions with *ttk* p69 or is the result of cross-regulatory interactions between pair-rule genes. The results could also be caused by artefactual interactions between *ttk* p69 and stripe-specific activators that are unrelated to the normal function of *ttk*. It is interesting to note that *hairy* stripes 3 and 4, which are repressed by ectopic *ttk* expression, are located in the domain of action of the *Kruppel* gene, raising the possibility of competition between these two zinc finger proteins. The lack of *ttk* repression of gap genes indicates that the subjects of *ttk* repression are somewhat restricted, and leave open the question whether other early embryonic genes are under repression by a different mechanism in the preblastoderm stage.

Our previous observation of the expression in preblastoderm embryos of a zebra element-*lacZ* construct with mutated *ttk* binding sites suggests that general or specific activators must be present in preblasotoderm embryos that interact with the 740 bp zebra element and drive expression of β-galactosidase. Two maternally provided activators that bind to the zebra element have been identified so far: *caudal* (Dearolf et al., 1989b) and *FTZ-F1* (Ueda et al 1990). The orphan hormone receptor *FTZ-F1* is uniformly distributed throughout the preblastoderm embryo (G. Lavorgna and C. Wu, unpublished results) and behaves as an activator of all the *ftz* stripes, with greater effects on stripes 1,2,3 and 6 (Ueda et al., 1990). The *caudal* protein is expressed in the posterior of the embryo (MacDonald and Struhl, 1986) and has an effect on *ftz* activation in this region of the embryo (Dearolf et al., 1989b). It is possible that *ttk* p69 may act to antagonize the positive influences of *FTZ-F1* and *caudal* on RNA polymerase II by blocking the binding of these factors to DNA, to other components of the transcriptional machinery, or indirectly by forming nonproductive interactions with general transcription factors. Whatever the mechanism of preblastoderm repression by *ttk*, such repression should be necessary to prevent premature initiation of the *ftz*-dependent, positive autoregulatory loop until the necessary positional cues are in place (Hiromi and Gehring, 1987; Pick et al., 1990).

The reemergence of *ttk* p69 in a highly complex pattern of expression subsequent to the cellular blastoderm stage indicates that the zygotically expressed p69 protein has additional regulatory functions aside from *ftz* repression. The pleiotropic nature of *ttk* is also indicated by the wide range of cuticular abnormalities observed for embryos that have undergone ectopic expression of *ttk* p69. Such embryos die before hatching and do not exhibit a specific pair-rule phenotype. In addition, a *ttk* null mutation has recently been isolated and reveals an embryonic-lethal phenotype (Xiong and Montell, personal communication). In order to test genetically the regulation of *ftz* by *ttk*, it will be necessary to eliminate the maternal contribution by the creation of germline clones homozygous for the *ttk* null mutation.

The model that we have proposed for preblastoderm repression of *ftz* by maternal *ttk* protein is not the only system of repression for this pair-rule segmentation gene. Previous microinjection experiments using the protein synthesis inhibitor cycloheximide have revealed two systems by which *ftz* transcription is repressed in early embryos. A polar repression system has been proposed to repress *ftz* expression in the anterior 35% and posterior 15% of the embryo, and a periodic system of repression is responsible for repression in the interstripe regions (Edgar et al., 1986). Our studies have elucidated a previously unforseen requirement for repression at a time when a need for repression was not generally anticipated. We suggest that the number of genes in the *Drosophila* genome that may be subject to preblastoderm repression by *ttk* or by other maternal repressors should not be underestimated.

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