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Insulating DNA Directs Ubiquitous Transcription of the *Drosophila melanogaster* α1-*tubulin* Gene

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We identify DNA regions that are necessary for the ubiquitous expression of the *Drosophila melanogaster* α1-*tubulin* (α1*t*) gene. In vitro transcription showed that two upstream regions, tubulin element 1 (TE1 [29 bp]) and tubulin element 2 (TE2 [68 bp]), and a downstream region activate transcription. Germ line transformation demonstrated that these three regions are sufficient to direct the α1*t* core promoter to begin transcribing at the stage of cellular blastoderm formation and to continue thereafter at high levels in all tissues and developmental stages. Remarkably, mutation of any one of these regions results in high sensitivity to chromosomal position effects, producing different but reproducible tissue-specific patterns of expression in each transformed line. None of these regions behaves as an enhancer in a conventional germ line transformation test. These observations show that these three regions, two of which bind the GAGA transcription factor, act ubiquitously to insulate from position effects and to activate transcription. The results also provide vectors for ubiquitous expression of gene products and for examining silencer activities.

Cell viability depends on a large number of ubiquitous proteins, such as tubulins, histones, and enzymes, involved in basic cellular metabolism. This raises the question of how genes encoding these ubiquitous proteins are regulated in multicellular organisms whose cells undergo dramatic changes in gene regulation during differentiation. Expression of ubiquitous genes may be entirely independent of the regulatory programs that direct differential gene expression. For example, expression could result solely from the affinity between the core promoter and general transcription factors present in all cell types. Alternatively, ubiquitous transcription of a gene may be directed by these regulatory programs. For example, expression could result from the activity of a large collection of cell-type-specific enhancers.

In light of these widely different possibilities, it is surprising that relatively little attention has been focused on the regulation of ubiquitously expressed genes. The investigations that have been conducted suggest that, in general, such genes are expressed at low levels, lack a TATA promoter element, and contain GC-rich regions upstream of the transcription initiation site (11). However, there are almost as many exceptions to these generalizations as there are agreements with them. For example, the mouse Pgk-1 promoter is expressed at high levels, the human triosephosphate isomerase gene contains a TATA element, and the *Drosophila melanogaster* α1-*tubulin* (α1*t*) gene has a TATA element and lacks GC-rich regions (3, 36, 51). Furthermore, understanding of ubiquitous regulation has been limited because in vivo analyses have been done with cell culture assays which do not test the full range of potential tissue specificities of the DNA elements involved (for example, see references 3, 6, and 36).

This report examines DNA regions that regulate the *Drosophila* α1*t* gene (20). This gene encodes α-tubulin protein, a subunit of microtubules which are necessary for cell division, cell motility, intracellular transport, and maintenance of cell structure. The α1*t* gene is one of the four α-tubulin genes of *D. melanogaster* and is the only one that appears to be expressed at high levels in all tissues and stages of development (21, 35, 38).

We identify DNA regions that activate α1*t* transcription in vitro and are sufficient for ubiquitous expression in vivo. The positions, numbers, and activities of these regions indicate that they are promoter elements necessary and sufficient for ubiquitous expression and that the affinity between the core promoter and general transcription factors is insufficient for ubiquitous expression. Further, the results show that these regulatory regions not only activate transcription but also prevent chromosomal position effects. Finally, these results provide vectors for expressing proteins ubiquitously throughout *Drosophila* development and for testing and identifying silencer activities.

MATERIALS AND METHODS

Templates for in vitro transcription. All templates were made from plasmids pα1*t*(−2000/+696) and pα1*t*(−61/+696), subcloned from pTα1(3′-5′) (51) into pUC8 or pUC18 (55). In these and all other constructs, the numbers within parentheses refer to the α1*t* DNA endpoints relative to the transcription initiation site (51). Unless otherwise noted, endpoints were determined by sequencing with Sequenase (U.S. Biochemical). Standard methods were used for DNA constructions and cloning (33, 44) with restriction enzymes, T4 DNA ligase, and the Klenow fragment of *Escherichia coli* DNA polymerase purchased from New England Biolabs and Boehringer-Mannheim.

Deletions between −2000 and −157 were made from pα1*t*(−2000/+696) using *Bal* 31 nuclease (International Biotechnologies, Inc.), producing structures with 5′ endpoints as determined by agarose gel electrophoresis of restriction enzyme-digested DNAs. Deletions between −157 and +1 were made from pα1*t*(−157/+696) by using the Double-Stranded Nested Deletion kit (Pharmacia) or PCRs (43) with synthetic

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primers and Vent DNA polymerase (New England Biolabs). In all PCR constructions, the entire PCR-synthesized fragment was sequenced after cloning. PCR was also used to make the ΔTE1 substitution by changing TE1 from 5′-CTTCAGTT ATCGGTTATGCGGCGTTTAAG-3′ to 5′-GCTAGCCATA TCCCATCCTCCGCACATTG-3′. The 3′ deletions with and without TE1 were made by standard restriction digestion, ligation, and cloning methods.

A ψ-wt (pseudo-wild-type) template with a 5′ endpoint of −61 was constructed by inserting an 8-bp *Nhe*I linker (no. 1069; New England Biolabs, Inc.) into the *Bst*BI site at bp +39 by blunt-end ligation after the 5′ ends had been filled in by the Klenow fragment of DNA polymerase I. The resulting plasmid had a net insertion of 10 bp (5′-CGGGCTAGCC-3′), as confirmed by sequencing.

Construction of fusion genes for germ line transformation. The basic *α1t/lacZ* P-element transformation vector was made in several steps. *α1t* DNA (−2000 to +696) was ligated in frame to the protein-coding portion of a modified *E. coli lacZ* gene from pPW716. This produced a junction of 5′-TGGGCG GAT-3′, in which the first three nucleotides were the complement of the 21st codon of *α1t* and the final three were the complement of the 7th codon of *lacZ*. The *lacZ* gene in pPW716 was from pMLB1034ΔAvaI (45) but had been modified by inserting a *Hpa*I fragment (+2175 to +2800) containing the *α1t* polyadenylation site (51) into the *Bal*I site at kb +3.5 relative to the *lacZ* translation initiation site. This gene fusion was then placed into the transformation P-element vector CP20.1 (46), yielding the basic *α1t/lacZ* P-element vector.

Deletions with 5′ endpoints at −600, −320, −250, and −157 were moved into the *α1t/lacZ* P-element vector by substituting the −2000-to-+333 *Xba*I restriction fragment with the appropriate *Xba*I fragment from in vitro transcription templates. The −61 deletion construct was made by changing the *Sal*I site at bp −61 of pTα1(3′-5′) (51) to *Xba*I by linker mutagenesis and then substituting the product −61-to-+333 *Xba*I fragment for the *Xba*I deletion of the *α1t/lacZ* construct. ΔTE1 was subcloned into *α1t/lacZ* by replacing the *Xba*I fragment from −600 to +333 in the −600 *α1t/lacZ* fusion vector with the −157-to-+333 *Xba*I fragment from the ΔTE1 template. ΔTE2 was subcloned into the *α1t/lacZ* fusion vector in a similar manner after the *Hin*dIII site at bp −89 was changed to an *Nhe*I site, which has sticky ends that are compatible with *Xba*I ends.

The construct Δint/lacZ replaces the DNA fragment from +40 to +696 of the −157 deletion construct with a PCR product containing an exact intron deletion. The construct hseΔint/lacZ was made by inserting heat shock elements (−256 to −38 of *hsp70*) from the HIC-UP cassette (26) into the Δint/lacZ construct at bp −61. Each of these constructions was placed into the transformation vector CP20.1. The constructions were sequenced to confirm the exact junctions and substitution sequences.

The *α1t/hsp70/lacZ* fusion genes were made by inserting *α1t* fragments into a P-element vector (−44 *hsp/lac*ZCP20.1 P-element vector [7]) at unique *Sal*I and *Xba*I sites in a polylinker at bp −44 upstream of the initiation site of the *Drosophila hsp70* gene which had been fused in frame to the protein-coding portion of the *E. coli lacZ* gene. The upstream region (UR) (−157-to−34) insert was made by PCR with primers that created *Xba*I sites at both ends; the TE2/TE1 (−157-to-−61) insert was made by digesting the −157 template with *Xba*I and *Sal*I restriction endonucleases; the downstream region (DR) (+20-to-+696) insert was made by replacing the *Sal*I (bp −61) and *Bst*BI (bp +39) fragment of pTα1(5′-3′) with a synthetic duplex (bp +20 to +40) with a *Sal*I at the +20 end, changing the *Sac*I site at bp +696 to *Sal*I by linker mutagenesis; the DR/UR insert was made by inserting the DR fragment into the UR/*hsp/lacZ* construct.

Tetramers of TE1 were inserted upstream of −44 *hsp/lacZ*. Multimers of TE1 were formed by synthesizing, phosphorylating, annealing, and ligating 5′-AGCTTGCATCTTCAGTT ATCGGTTATGCGGCGTTTAAG-3′ and 5′-TCGACTTAA ACGCCGCATAACCGATAACTGAAGATGCA-3′. This sequence spans TE1 (−89 to −61 [underlined]) and a small portion of the pUC polylinker sequence immediately upstream of TE1 in the −89 deletion. The annealed oligonucleotides have *Hin*dIII and *Sal*I 5′ overhanging ends, so that ligation with T4 DNA ligase (Boehringer-Mannheim) generated head-to-head and tail-to-tail multimers. Multimerized oligonucleotides were inserted upstream of the *hsp70* promoter in the *Sal*I site of −44 *hsp/lacZ*. Restriction maps of the resulting construction, designated $(TE1)_4$/*hsp/lacZ*, identified a structure with four copies of TE1.

Preparation of nuclear extracts. Nuclear extracts were prepared from *Drosophila* embryos with minor modifications of standard methods (2, 47) (typical yield, 2.5 mg/100 g of embryos) and from Kc0 cells (18, 40) (typical yield, 30 mg/liter of cells harvested at approximately 5×10^6 cells per ml). Kc0 cells, a *Drosophila* cell line of embryonic origin, were purchased from the MIT Cell Culture Center or from Harvard University.

In vitro transcription and primer extension. In vitro transcription assays were done by standard methods (2, 47) with the following modifications (see Results). The reaction mixtures contained 0.75 nM supercoiled test template (approximately 20 ng), 0.75 nM ψ-wt template, and a total DNA concentration of 25 μg/ml, using supercoiled pUC plasmid as nontemplate DNA, unless otherwise specified.

The primer extension assay (37) used a primer, 5′-GTGTA GAGCTACGATTTCGAGG-3′ (+88 to +67), with a noncoding *α1t* sequence. Transcripts were hybridized to the radiolabeled primer (200 fmol; approximately 10^5 cpm), and the primer was extended with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) under conditions recommended by the manufacturer. Samples were denatured and electrophoresed on 6 or 8% acrylamide–8 M urea gels.

Endogenous RNA from 2- to 20-h embryos was generously provided by S. Hardin.

DNA sequencing. TE1 and TE2 were sequenced on both strands with the Sequenase DNA sequencing kit (U.S. Biochemical).

Germ line transformation. Reporter gene constructs were integrated into the genome by P-element-mediated transformation (49). *Drosophila* ry^{506} embryos were coinjected with P-element reporter constructs and pπ25.7wc (22) at concentrations of 0.35 and 0.15 mg/ml, respectively. All transformed lines were homozygosed with *CyO/Sco*; ry^{506}/ry^{506} or *TM2, Ubx*, ry^{506}/*MKRS, Sb*, ry^{506} fly strains when possible. However, many lines were either homozygous lethal or homozygous sterile, so all transformants were analyzed as single-copy heterozygotes. The copy numbers of reporter gene inserts were determined by Southern blot analysis (48).

To increase the number of ΔTE1/TE2 lines and to test for position effects, constructs were transposed from the X chromosome (42) and the second chromosome (1) with a *w*/Y; *CyO/Sp*; *Dp*, P(*ry*+ Δ2-3)(99B)/*Ubx*, *TM6* fly strain as the source of transposase.

Histochemical and spectrophotometric assays of β-galactosidase activity. Heterozygous flies were assayed by histochemical staining for expression of β-galactosidase as described

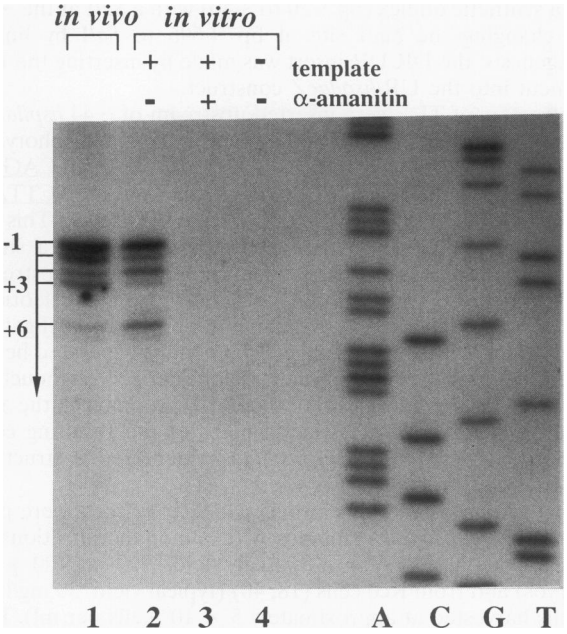

FIG. 1. In vitro transcription of $\alpha 1t$ by embryonic nuclear extracts; primer extension analysis of mRNA (12 μg) from 2- to 20-h embryos (lane 1) and in vitro-synthesized RNA (lane 2). In vitro transcription reaction mixtures contained embryonic extract (lanes 2 to 4), 1.0 nM supercoiled plasmid template p$\alpha 1t$(−2000/+696) (lanes 2 and 3), and α-amanitin (1 μg/ml [lane 3]). Initiation sites were mapped by comparison with sequencing reactions (lanes A, C, G, and T) using p$\alpha 1t$(−98/+696) as template and the $\alpha 1t$ primer that was used in transcription assays. The arrows to the left indicate the transcription initiation sites.

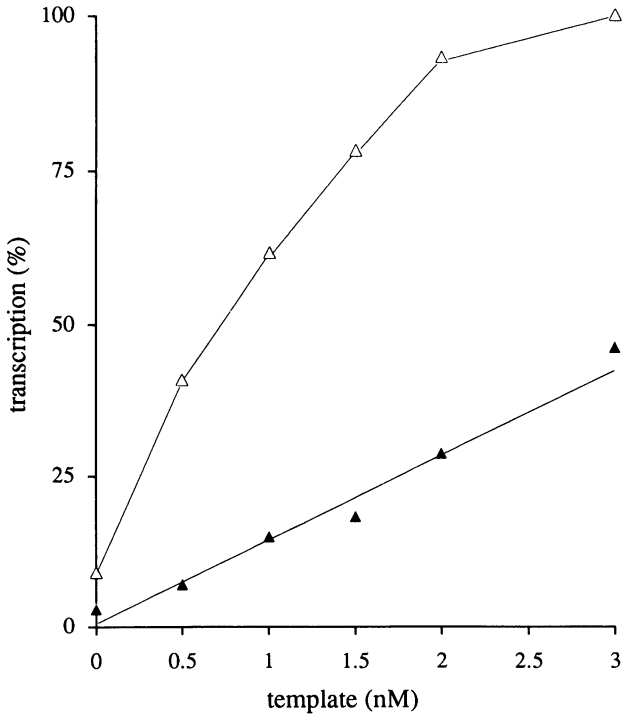

FIG. 2. Titration of template in the presence or absence of competitor. Primer extension analysis of transcription reaction mixtures with different concentrations of supercoiled plasmid $\alpha 1t$ template (−157/+696) either in the absence (closed triangles) or presence (open triangles) of supercoiled, nontemplate competitor DNA added to yield a total DNA concentration of 30 μg/ml. Transcription is relative to the reaction with competitor and 3 nM template (100%).

previously (7, 29). Dissected flies were stained for approximately 15 h.

Spectrophotometric assays of β-galactosidase activity in whole-fly lysates were done as described previously (7).

RESULTS

In vitro transcription of $\alpha 1t$. To rapidly identify DNA regions that are likely to regulate the ubiquitous expression of $\alpha 1t$, we examined transcription of $\alpha 1t$ templates in an in vitro transcription system prepared from embryos. Embryos represent many stages and tissues of development, suggesting that embryonic extracts may use many of the regulatory regions necessary for ubiquitous transcription. To investigate the accuracy of initiation in this assay system, we compared the 5′ ends of transcripts made in vitro with those made in embryos (Fig. 1, lanes 1 and 2). In each case, initiation occurs at five predominant sites (−1, +1, +2, +3, and +6), although in common with many other genes, the frequency of initiation at alternative sites is somewhat different in vitro and in vivo (for example, see reference 41). Additional reactions demonstrated that transcription in vitro depends on both RNA polymerase II and the $\alpha 1t$ template (Fig. 1, lanes 3 and 4).

Template and total DNA concentrations were optimized to allow both positive and negative regulatory elements to be detected. An initial titration experiment demonstrated that transcription increases linearly with template concentrations of up to at least 3 nM (Fig. 2, closed triangles). To reduce the effects of nonspecific repressors in the nuclear extract, for example, histone H1 (8, 27), we examined the effect of adding nontemplate DNA as competitor. At a fixed template concentration, transcription was maximal when total DNA was 25 to 30 μg/ml and decreased as the concentration was decreased or raised (data not shown). At this maximum, transcription increases nearly linearly with a template concentration of up to approximately 2 nM (Fig. 2, open triangles). The slope then appears to decrease, suggesting that at least one protein necessary for transcription becomes rate limiting. On the basis of these results, subsequent transcription reactions were performed at subsaturating template concentrations (0.75 nM test template and 0.75 nM ψ-wt included as an internal control) and with sufficient nontemplate DNA to give a total DNA concentration of 25 μg/ml.

***α1t* is positively regulated by an upstream region.** To identify $\alpha 1t$ regions important for transcription, a 5′ deletion series was assayed. Minor changes in transcription were detected upon deletion of DNA from −2000 to −157 (Fig. 3). In contrast, deletion into the region between −157 and −35 (UR) gave more substantial changes that could be attributed to small DNA segments. Deletions revealed the 5′ ends of two strong positive elements: one between −139 and −114 and the other between −89 and −77 (Fig. 3). Deletion from bp −77 to −35 had no effect, suggesting that this DNA has no regulatory elements. Finally, deletion from bp −35 to −26 reduced transcription to background levels. This last region contains the TATA element, a core promoter component essential for transcription of many genes (17, 34).

These experiments reveal three regulatory elements within the UR, one weak negative and two positive (Fig. 3 and 4A), thereby providing the first evidence that $\alpha 1t$ is regulated by sequences outside the core promoter. This observation rules

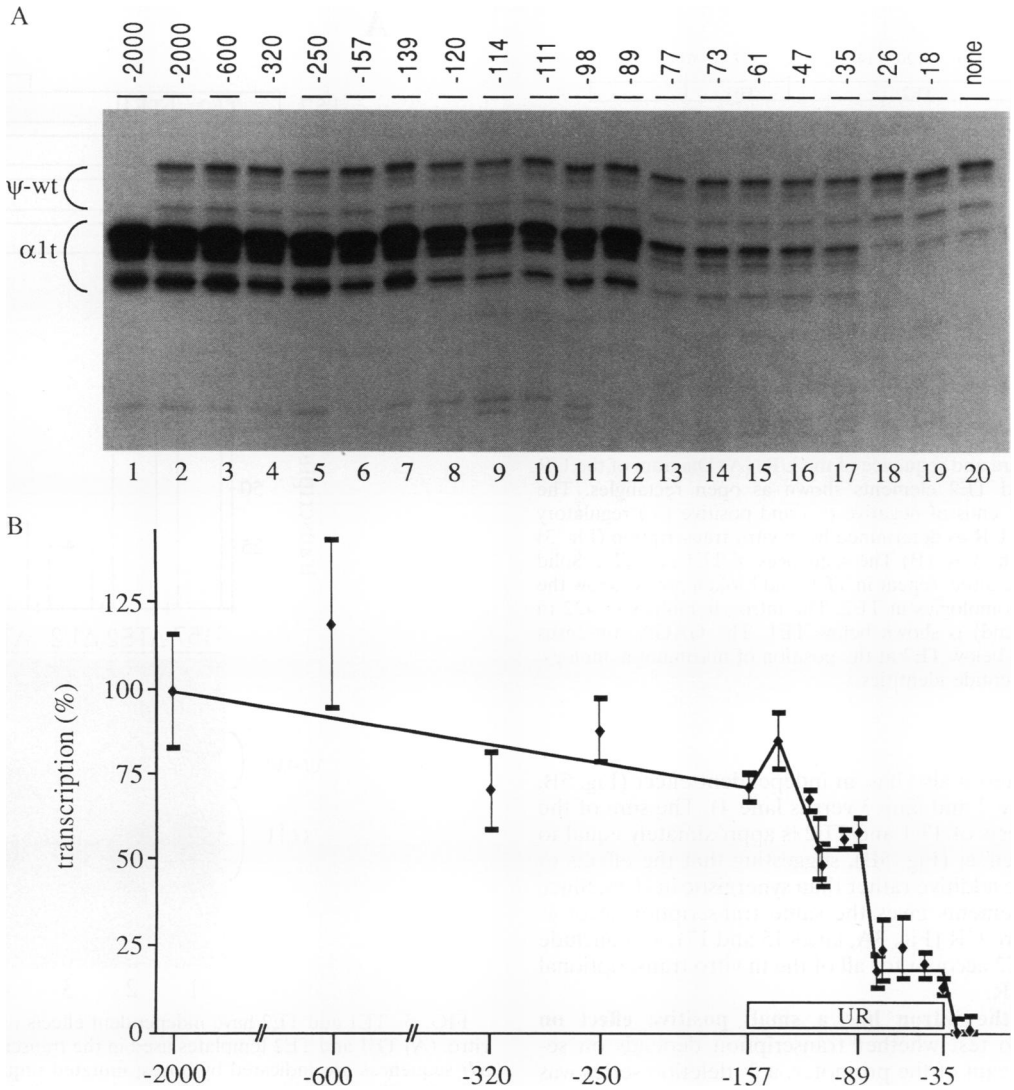

FIG. 3. In vitro transcription of an α*1t* 5′ deletion series. (A) Primer extension analysis. Transcription reactions were done with embryonic extract and supercoiled templates with α*1t* DNA that had different 5′ ends (shown above each lane) and the same 3′ end (+696). All reaction mixtures except those shown in lanes 1 (no ψ-wt) and 20 (no template) contained the indicated template, ψ-wt internal control template, and nontemplate competitor DNA. The residual signal in lane 20 is presumably due to endogenous α*1t* mRNA in the nuclear extract. The α*1t* and ψ-wt extension products are indicated on the left. (B) Quantitation of transcription. The average signal and standard deviation of at least three assays of each template are plotted relative to transcription from the −2000 template (100%). UR location is indicated by the rectangle.

out the hypothesis that α*1t* expression is controlled solely by general transcription factors binding to the core promoter.

TE1 and TE2 are independent regulatory elements that account for all UR activity in vitro. Examination of DNA sequence suggested the locations of UR regulatory elements (Fig. 4). For example, the 5′ end of the strongest UR regulator is just upstream of a short tandem repeat. Deletion of one copy of this repeat decreases transcription substantially (Fig. 3A, lanes 12 and 13). In addition, a sequence homologous to the repeat occurs in the intron, and homology extends upstream of the repeat (Fig. 4B). This suggests that the strongest regulator includes the tandem repeat and its immediately surrounding DNA, together called tubulin element 1 (TE1 [29 bp]; Fig. 4B).

The second positive regulator detected by in vitro transcription is upstream of TE1 in a region we call tubulin element 2 (TE2 [68 bp]; Fig. 4A). Although TE2 has no apparent homology to TE1, it does have two matches to the consensus binding site for the GAGA transcriptional regulatory protein (Fig. 4B) (2). Moreover, the 5′ end of the best match to the GAGA consensus sequence coincides with the 5′ end of a positive element detected in the transcription experiments (Fig. 4). GAGA sites are known to regulate expression of many *Drosophila* genes expressed in many tissues (2, 6, 25, 28). Therefore, the TE2 GAGA sites are good candidates for regulators of ubiquitous expression. Additional GAGA sites occur immediately downstream of the TE1 homologous sequence in the α*1t* intron, raising the possibility that regions upstream and downstream of the promoter have similar functions.

To test whether TE1 and TE2 stimulate transcription independently, we compared transcription levels of several substitution templates (Fig. 5A). TE1 alone stimulates transcription threefold in the absence of TE2 (Fig. 5B, lanes 2 and 3). TE2 gives a small stimulation in both the presence and the absence

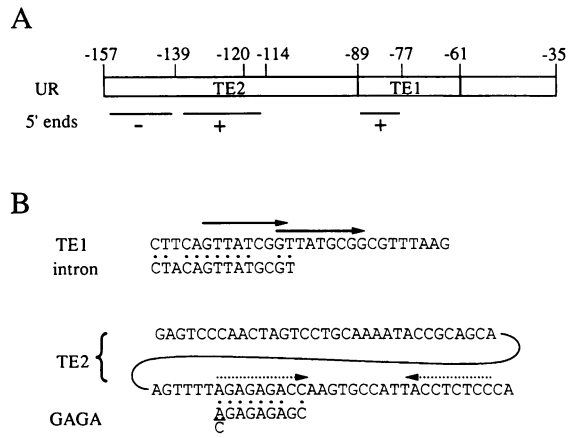

FIG. 4. Structure and sequence of the UR. (A) Diagram of the UR with the TE1 and TE2 elements shown as open rectangles. The locations of the 5′ ends of negative (−) and positive (+) regulatory regions within the UR as determined by in vitro transcription (Fig. 3) are shown below the UR. (B) The sequences of TE1 and TE2. Solid arrows indicate the direct repeat in TE1, and broken arrows show the inverted GAGA homologies in TE2. The intron homology (+422 to +435, bottom strand) is shown below TE1. The GAGA consensus sequence is shown below TE2 at the position of maximum homology. Dots indicate nucleotide identities.

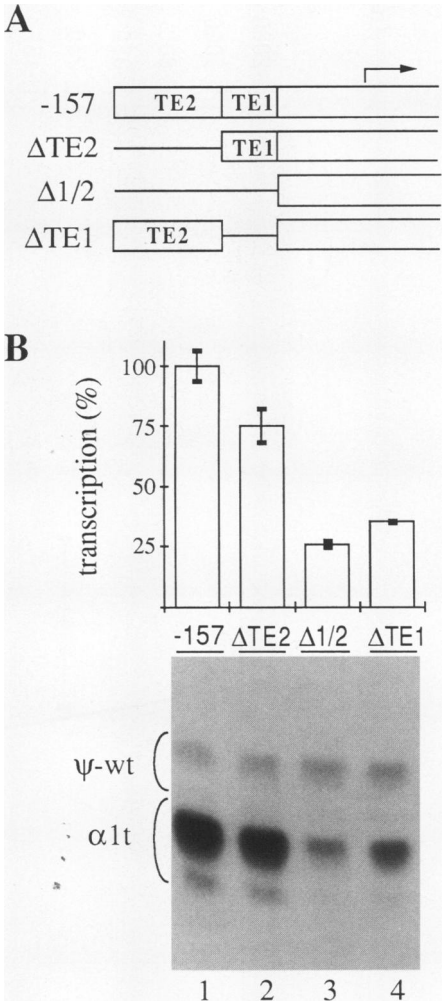

FIG. 5. TE1 and TE2 have independent effects on transcription in vitro. (A) TE1 and TE2 templates used in the transcription assay. The *α1t* sequences are indicated by boxes; mutated sequences are represented by lines. The arrow indicates the transcription initiation site. ΔTE2 is equivalent to p*α1t*(−89/+696), and Δ1/2 is equivalent to p*α1t*(−61/+696). (B) Primer extension analysis of transcription reactions performed with extracts from Kc0 cultured cells. The results from reactions performed with embryonic extract are the same. The results from three experiments are summarized in the histogram, with standard deviations indicated. Transcription is relative to the reaction with p*α1t*(−157/+696) (100%). A representative autoradiogram is shown below.

of TE1 and therefore also has an independent effect (Fig. 5B, lane 1 versus lane 2 and lane 3 versus lane 4). The sum of the independent effects of TE1 and TE2 is approximately equal to their combined effect (Fig. 5B), suggesting that the effects of TE1 and TE2 are additive rather than synergistic in vitro. Since deleting both elements gives the same transcription level as deleting the entire UR (Fig. 3A, lanes 15 and 17), we conclude that TE1 and TE2 account for all of the in vitro transcriptional activity of the UR.

DNA within the intron has a small positive effect on transcription. To test whether transcription depends on sequences downstream of the promoter, a 3′ deletion series was examined. Deleting sequences between +696 and +333 had no statistically significant effect on transcription, whereas deleting to +121 reduced transcription slightly and had no effect on the threefold activation by TE1 (data not shown). Therefore, in vitro, the intron DNA between +121 and +333 is necessary for the highest level of transcription but is unnecessary for the regulatory effect of the upstream TE1.

DNA from −157 to +696 is sufficient for ubiquitous expression in vivo. The results described above have identified positive regulatory elements that are potential regulators of *α1t* expression in vivo. To establish an in vivo assay for the role of these *α1t* regulatory regions, we fused the *α1t* gene (−2000 to +696) in frame to a β-galactosidase reporter gene and introduced the product (termed −2000 *α1t/lacZ*; see Fig. 10) into the *Drosophila* germ line by P-element-mediated transformation. In addition to establishment of an in vivo assay system, these transformation experiments also aimed to use a more refined histological staining assay to substantiate the hypothesis that the *α1t* gene is expressed ubiquitously. Previous Northern (RNA) blot assays had shown only that *α1t* transcripts are present at many stages of the life cycle and in several dissected tissues (21, 35, 38).

All five of the lines transgenic with a single copy of −2000 *α1t/lacZ* expressed a high level of β-galactosidase activity at all developmental stages and, at the level of resolution of a dissecting microscope, in all living tissues. As shown in Fig. 6, β-galactosidase was detected in every organ and in almost every body structure of the adult. Staining occurred in external structures, such as the wing veins, halteres, maxillary palps, and legs, and in internal organs, such as the crop and all components of the digestive system and genitalia. The eyes and the wing surfaces do not stain unless further dissected (Fig. 6g and h), indicating that the dye does not readily penetrate these structures. The only unstained structures detected were the adult exoskeleton, pericardial membrane, and some connective tissues, all likely to be metabolically inactive tissues (Fig. 6). Similar ubiquitous staining was observed in embryos (Fig. 7b to e), larvae, and pupae (data not shown).

Since *α1t* is expressed in ovarian nurse cells that contribute

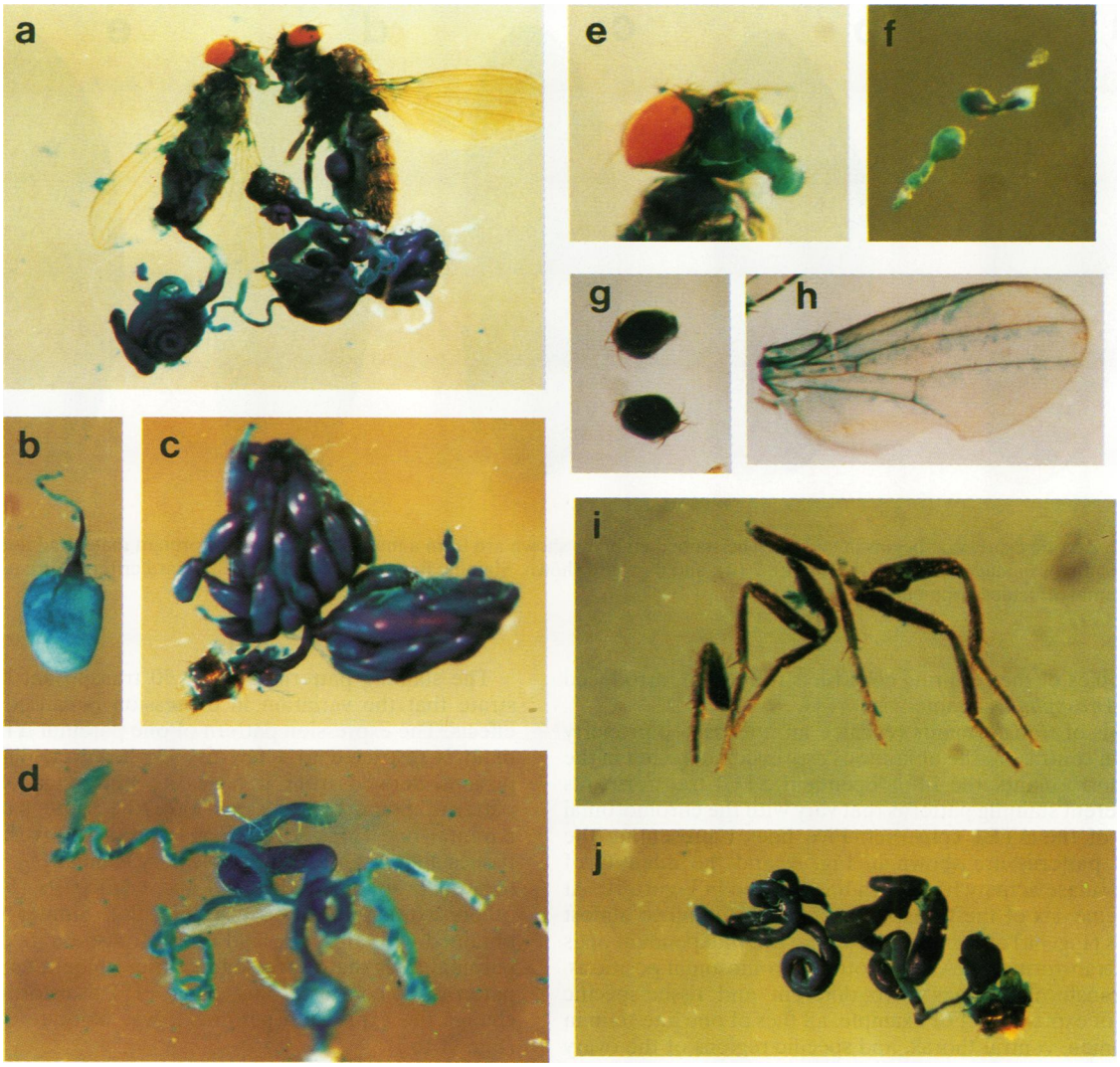

FIG. 6. Ubiquitous expression pattern of the −2000 α1t/lacZ fusion. (a) Dissected flies stained for β-galactosidase activity (blue) with a male to the left and female to the right. (b to j) Individual parts of stained flies including crop (b), ovaries (c), intestine and Malpighian tubules (d), head (e), halteres (f), eyes (g), wing (h), legs (i), and testes (j).

much RNA and protein to the oocyte, transcripts and protein from the fusion gene are likely to be inherited maternally. Thus, β-galactosidase staining at early stages in development could derive either from maternal products or from early zygotic transcription. To distinguish between these possibilities and to determine the developmental stage at which expression begins, a genetic cross was performed. When transgenic males are mated with nontransgenic females, fusion gene products will not be inherited maternally. In embryos produced by this cross, β-galactosidase first appears at stage 5 of embryonic development, the time when cellularization begins in the blastoderm (Fig. 7b). Staining occurs throughout the embryo at this stage and becomes more intense subsequently (Fig. 7c to e). This onset of staining reflects new synthesis of β-galactosidase rather than changes in dye permeability or other assay artifacts, because staining was observed at all early stages when matings were done with females transgenic for the same chimeric gene. Thus, zygotic expression of the gene begins at stage 5 and thereafter appears to occur in all living tissues for the remainder of the life cycle.

To identify the upstream DNA that regulates α1t in vivo, several of the 5′ deletion templates assayed by in vitro transcription were introduced into the germ line. As with in vitro transcription, deletion from bp −2000 to −157 (−2000, −600, −320, −250, or −157) has no significant effect on expression, either quantitatively (see Fig. 10) or in the tissue distribution of staining. The single exception was one of the five −157 lines which stains less strongly in the adult thorax (data not shown). Therefore, DNA between bp −157 and +696 is sufficient for ubiquitous expression in the organism. This DNA includes the UR (−157 to −35) identified in vitro, the promoter region (−34 to +19), and the DR (+20 to +696), which includes the downstream activator identified in vitro.

TE1 and TE2 regulate in all tissues and prevent chromosomal position effects. The roles of TE1 and TE2 in regulating ubiquitous transcription were investigated by germ line transformation. Mutations that eliminated the positive effects of TE1 and TE2 in vitro (Fig. 5A; ΔTE1, ΔTE2, and ΔTE1/TE2) were introduced into the −157 α1t/lacZ fusion construct.

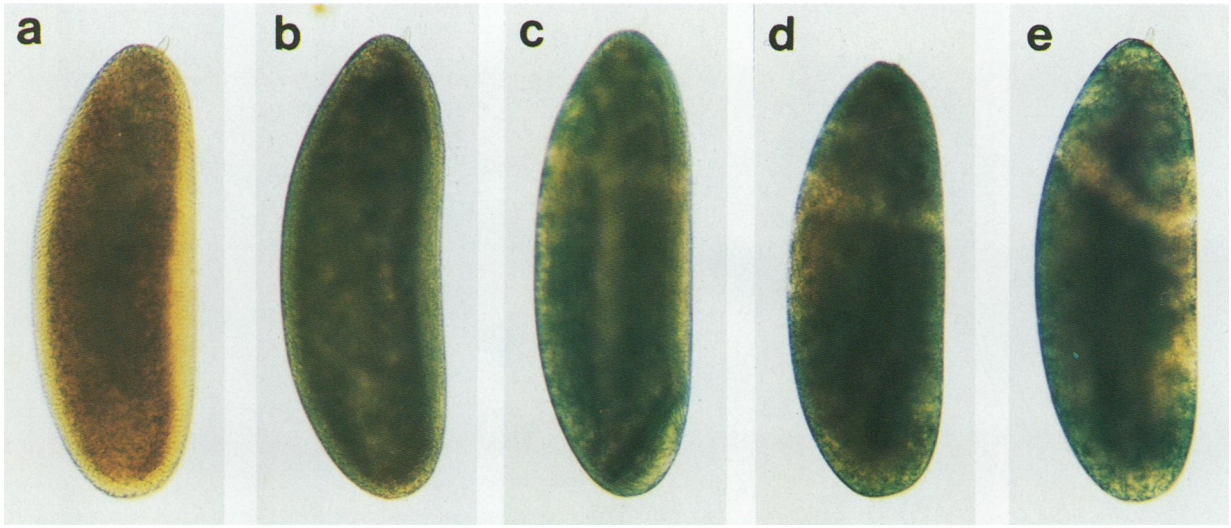

FIG. 7. Zygotic expression begins at stage 5. The stained embryos shown are from a mating between transformant males and nontransformant females. Embryos at stages 4b (a), 5 (b), 6b (c), 7 (d), and 8 (e) are shown. Staining first occurs at stage 5. Standard criteria were used to identify stages of embryonic development (4).

Single copies of the resulting constructs were then introduced into the *Drosophila* genome.

Analysis of transformants revealed an unexpected property of TE1. In contrast to the ubiquitous expression detected in the −157 transformants, the 14 independent ΔTE1 transformants have different staining patterns that vary with the chromosomal position of the ΔTE1 construct. Five lines representing the range of patterns are shown in Fig. 8, and the quantity of β-galactosidase activity from each line is listed in Figure 10. At one extreme, six of the transformants express in all or almost all tissues (Fig. 8a) and are similar to the five independent lines of −157 transformants. At eight other chromosomal positions, ΔTE1 transformant lines have different and tissue-specific patterns of expression. For example, all flies of one line stain in the abdomen, ventral thorax, and specific regions of the ovary (Fig. 8b). In all flies of another line, the abdomen and thorax are essentially unstained, whereas all regions of the ovaries stain strongly (Fig. 8c). At the other extreme, all flies of two ΔTE1 transformant lines stain only in testes (Fig. 8e). In the last two lines, expression in almost all tissues has been affected by the TE1 mutation. These results demonstrate that this 29-bp transcriptional regulator protects the *αlt* promoter from chromosomal position effects and that it does this in all tissues.

To test the in vivo transcriptional effects of TE2 and of the combination of TE1 and TE2, lines with ΔTE2 and ΔTE1/TE2 constructs were also examined. Surprisingly, 9 independent lines of ΔTE2 and 20 independent lines of ΔTE1/TE2 exhibited the same position-dependent range of staining patterns as that which was observed with ΔTE1 transgenics.

The experiments described above suggest that both TE1 and TE2 are necessary to protect the promoter from the positive and/or negative effects of transcriptional regulators that are located near the site of insertion. However, it is also possible that the variation in expression between transgenic lines is due to mutations introduced into the transforming DNA during the transformation process. To investigate this possibility, ΔTE1 and ΔTE2 constructs in established lines were transposed to other chromosomal positions by transient expression of P-element transposase (42). Flies with repositioned constructs are called transposants (1).

The staining patterns of over 30 transposant lines demonstrate that the variation in expression is caused by position effects. The expression pattern of one parental ΔTE2 transformant is restricted to a few tissues; however, different transposants derived from it have the full range of expression patterns. Some transposants have the same pattern as the parental line (Fig. 9e). Others express in several additional tissues and organs, such as accessory glands (Fig. 9d), ejaculatory duct, abdomen (Fig. 9c) and head parts (Fig. 9b). Still others express in all tissues (Fig. 9a). Similar results were obtained with all ΔTE1 and ΔTE2 transposants derived from parental lines that expressed in either ubiquitous or restricted patterns. By contrast, transposing −157 transformants did not change the expression pattern; it was ubiquitous in all cases (Fig. 9f to j).

These results suggest that TE1 and TE2 act in all tissues to protect the *αlt* promoter from regulation by elements in the surrounding chromosomal DNA. There are two simple hypotheses for the way TE1 and TE2 prevent outside regulation. First, conventional positive and negative regulatory elements directing ubiquitous expression may be located elsewhere in the −157 to +696 region and TE1 and TE2 may protect only against the regulatory influences of more distal regulators. Second, TE1 and TE2 may be positive, ubiquitously acting regulators of *αlt* with a strength that overwhelms the influence of more distal regulators. The positive regulation by both TE1 and TE2 observed in our in vitro experiments leads us to favor the second hypothesis.

The intron also prevents position effects. To search for additional regulatory elements between −157 and +696, we investigated the role of the intron in regulating ubiquitous expression. In vitro transcription experiments showed that the intron was necessary for the highest level of transcription, and preliminary experiments demonstrated that DNA between +20 and +696 (DR), which includes the intron, is required for ubiquitous expression in vivo. To further localize this DNA, a precise deletion of the intron (+144 to +635) was made (Δint; Fig. 10). Like mutations in TE1 and TE2, deletion of the intron leads to sensitivity to position effects. Thus, the intron has an effect similar to TE1 and TE2 and is therefore another

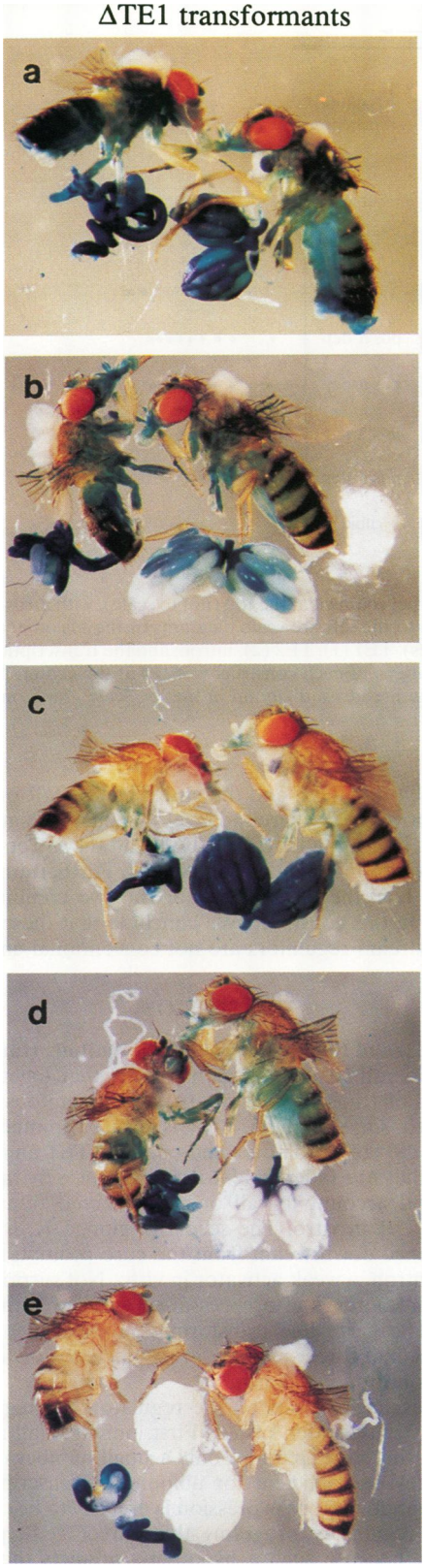

FIG. 8. ΔTE1 transformant expression patterns at different chromosomal positions. Flies transformed with ΔTE1 were dissected and assayed for β-galactosidase activity. Males are on the left, females are on the right, and testes or ovaries are shown below each fly. Each photograph shows a different transformed line.

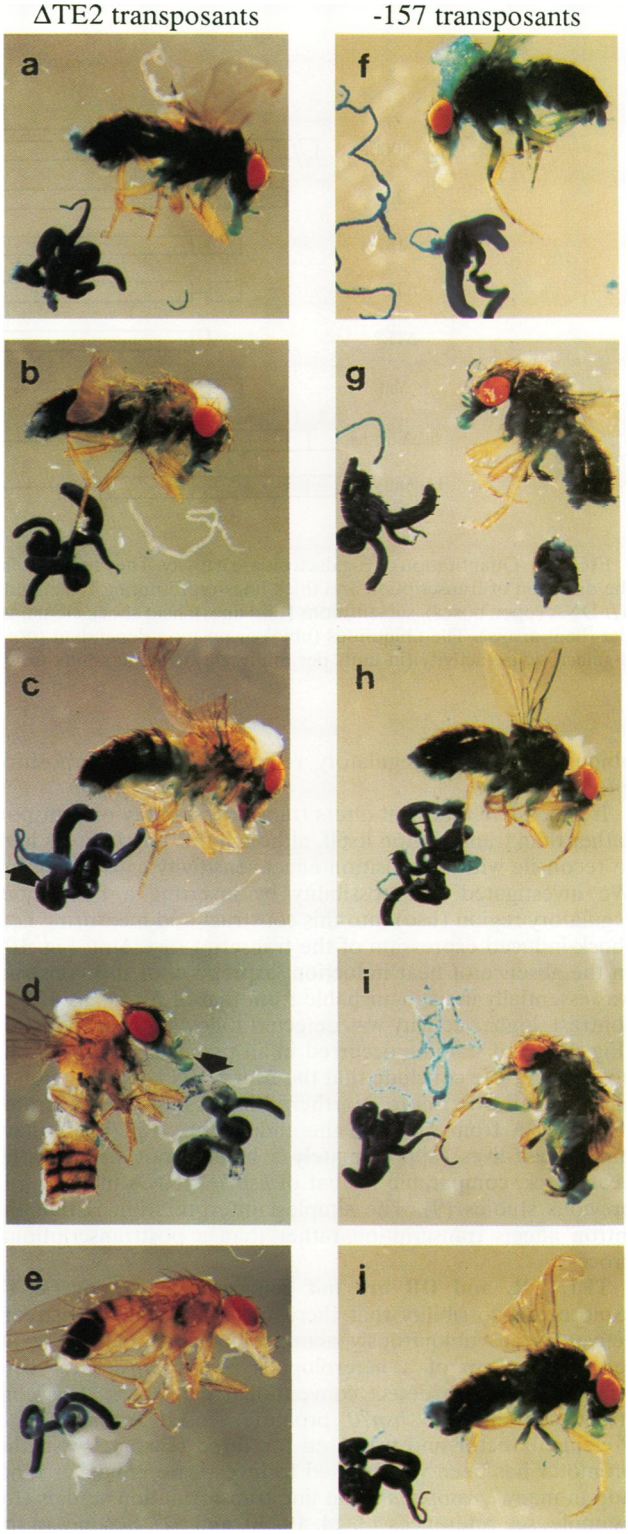

FIG. 9. Expression patterns of ΔTE2 and −157 transposants. Transposants (male only) of ΔTE2 (a to e) and −157 (f to j) were dissected and stained for β-galactosidase activity. The arrows point to the ejaculatory duct (c) and accessory gland (d). Each photograph shows a different transformed line.

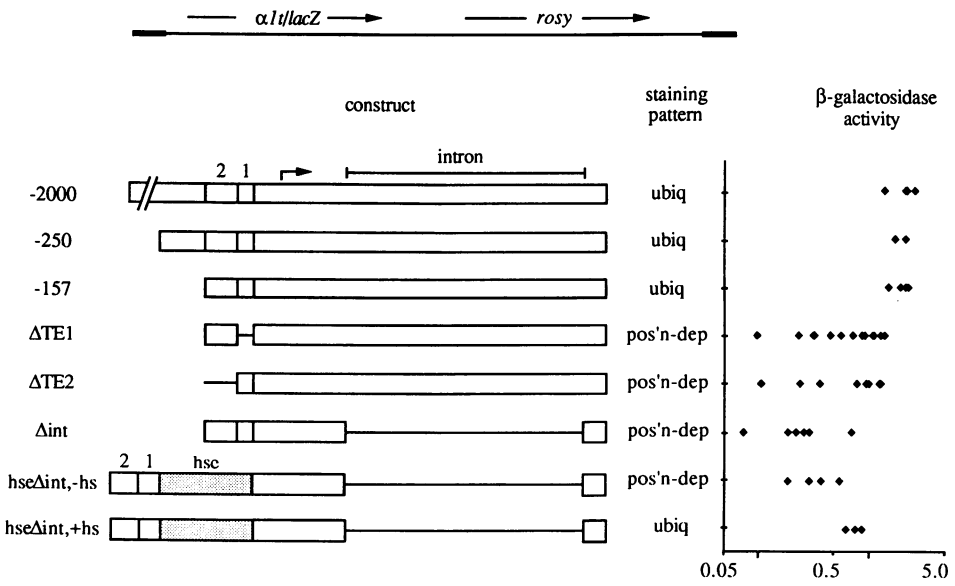

FIG. 10. Quantitation of β-galactosidase activity. The entire *αlt/lacZ* reporter construct is drawn at the top (not to scale), with arrows indicating the direction of transcription and thick lines representing P-element DNA. Below and to the left are scale diagrams of the fusion genes showing *αlt* DNA (open boxes), substitutions (thin lines), heat shock elements (hse, shaded boxes), TE1 (1), TE2 (2), intron, and the transcription initiation site (bent arrow). The ubiquitous (ubiq) or position-dependent (pos'n-dep) staining pattern of each construct is listed at the center. The average β-galactosidase activity (in units per milligram) of male adults from each line is represented by a diamond in the log scale graph on the right.

ubiquitously acting regulatory region that prevents position effects.

It is possible that Δint alters transcript stability or transport rather than transcription itself, although this possibility is hard to reconcile with the position effect sensitivity caused by Δint. We investigated this possibility by inserting a heat shock regulatory region (hse) into this construct and measuring heat shock-induced expression of the transcript (hse/Δint; Fig. 10). In the absence of heat induction, expression of this construct was essentially indistinguishable from that of Δint (Fig. 10). In contrast, higher activity was detected following heat induction (Fig. 10), and staining occurred in all tissues and stages (data not shown). We conclude that the Δint mRNA can function in all tissues. Furthermore, Northern blot analysis demonstrated that mRNA from Δint and the endogenous *hsp70* gene have similar half-lives (approximately 5 h; data not shown) after heat shock, comparable to that of *hsp70* mRNA measured in previous studies (9). The simplest interpretation is that the intron affects transcription rather than a posttranscriptional process.

TE1, UR, and DR are not conventional enhancers. To explore the possibility that these upstream and downstream activators are ubiquitously acting enhancers, they were inserted upstream of a heterologous promoter/reporter construct designed to detect conventional enhancers. This construct contains the *hsp70* promoter fused to *lacZ* in a P-element vector and was used for three reasons. First, this promoter has been widely used to investigate enhancer function in many *Drosophila* germ line transformation studies (for example, see references 13, 14, 19, 30, and 52). Second, in the absence of an enhancer, it is inactive in all tissues. Third, it shows low sensitivity to chromosomal position effects (7), presumably because of increased distance from chromosomal DNA resulting from the presence of the upstream *rosy* gene and the downstream *lacZ* gene (54).

Several combinations of *αlt* regions were tested: a tetramer of TE1, TE1 and TE2, and the entire UR or DR or both. No staining above the background level was seen with any of these constructs, indicating that TE1, TE2, and the intron activation regions are not independent enhancers. Furthermore, neither the entire UR, the entire DR, nor the two combined act as enhancers in this construct. We conclude that these upstream and downstream regulators are not conventional enhancers.

DISCUSSION

TE1, TE2, and the intron regulate ubiquitous transcription. We have identified DNA regions of *αlt* that together direct high levels of transcription in vitro and ubiquitous transcription in vivo. One of them, UR, is located immediately upstream of the core promoter and contains TE1 and TE2, two elements that are independent activators of *αlt* transcription in vitro and account for all of the observed transcriptional activity of UR in vitro. The second region, DR, is located a short distance downstream from the core promoter and contains the intron, a weak activator in vitro. Not surprisingly, the *αlt* core promoter is also necessary for in vitro transcription (Fig. 3). The germ line transformation experiments showed that the same three regions are sufficient for ubiquitous transcription in the organism.

These results set limits on the regulatory strategy used for ubiquitous transcription of *αlt*. First, only a small amount of DNA and, therefore, perhaps only a small number of protein-binding sites are sufficient for ubiquitous transcription. This suggests that ubiquitous expression is unlikely to be directed by the same regulatory programs that direct cell-type-specific expression, because such a hypothesis predicts the need for many enhancers with different stage and tissue specificities and, therefore, a large amount of DNA. Second, the locations of the regulatory regions rule out the hypothesis that the *αlt* core promoter alone regulates ubiquitous transcription through its affinity for general transcription factors. On the

contrary, their locations suggest that ubiquitous transcription requires factors acting immediately upstream and downstream of the core promoter. Finally, the α1t regulatory regions behave as promoter elements rather than conventional enhancers according to several criteria (34): they activate transcription in vitro, they are located in the immediate vicinity of the core promoter, and they do not stimulate transcription of a heterologous promoter. Therefore, we conclude that the regulatory strategy for ubiquitous transcription of α1t is limited to a small number of promoter elements flanking the core promoter. This contrasts with the regulatory strategies used for differential gene transcription, which typically require proteins bound to promoter elements close to the core promoter and proteins bound to enhancers scattered over many kilobases.

TE1, TE2, and the intron prevent position effects. We find that the three α1t promoter elements are also necessary to prevent chromosomal position effects. Deletion of any one of the elements produces the line-to-line variation in expression that is diagnostic of position effects. Moreover, if a TE1 or TE2 deletion construct that expresses in a tissue-specific or ubiquitous pattern is transposed to other chromosomal sites, it reproduces the full range of line-to-line variation in expression. This demonstrates that position effects rather than mutations are the source of the expression variability. In contrast, when all three elements are present, position effects are virtually eliminated in all tissues at all chromosome positions tested. We conclude that these elements protect the α1t promoter from distal enhancers or silencers and, further, that they act in all tissues.

Activators, insulators, or both? Several hypotheses can account for the in vivo effects of the α1t regions. In one, activation of transcription and protection from position effects are mediated by the same proteins. In this case, TE1, TE2, and the intron would prevent position effects by binding one or more activator proteins that successfully compete with distal regulators for interactions with general transcription factors that are bound at the α1t core promoter. This hypothesis is similar to one recently described to explain the activities of the locus control region of the human β-globin gene (10). This locus control region protects against position effects; however, in contrast to the α1t promoter elements, it is very large and is a strong enhancer.

An alternative hypothesis is that activation of transcription and protection from position effects are mediated by different proteins. DNA regions that protect against position effects and lack enhancer activity have been observed previously and are called boundary regions (5, 12, 16, 24, 50, 54). The mechanism of boundary region function remains speculative, but the favored model is that two boundary regions generate a chromatin domain in which the intervening DNA is an independent unit of transcriptional regulation (12, 15, 16, 23, 24). With both the *su(Hw)* and scs boundary regions, it appears that the barrier is most effective when boundaries occur both upstream and downstream of the promoter (16, 24). This property is similar to the need for both upstream and downstream promoter elements in α1t, suggesting that a similar mechanism may operate.

Although both hypotheses are plausible, we favor the first. One reason is that transcriptional activation in vitro and insulation in vivo are inseparable at a resolution of as high as 29 bp, in the case of TE1. This small size does not eliminate but certainly reduces the probability that two proteins with separate functions operate from TE1. Resolution also appears high for TE2. We have recently reported that the GAGA transcription factor binds to TE2, producing DNase footprints that occupy at least 70% of TE2 DNA, leaving only three short (6- to 12-bp) regions sensitive to nuclease digestion (39). Thus, with each upstream element it appears most likely that activation and insulation are due to a single protein.

The first hypothesis and the apparent function of GAGA factor provide a simple model for the ubiquitous regulation of α1t. A variety of in vitro and in vivo studies lead to the conclusion that GAGA activates transcription by binding specific DNA sites and removing nearby nucleosomes in an ATP-dependent process called antirepression (25, 31, 32, 53). The uncovered DNA then becomes available for binding by proteins that more directly activate transcription. We speculate that the activation and insulation effects that we have observed with TE2 are due to antirepression by GAGA factor, rather than formation of independent chromatin domains by some unknown protein. A simple model for upstream α1t regulation is that GAGA protein uncovers TE1 DNA, allowing a transcriptional activator to bind. A similar model has been proposed for *Drosophila* heat shock genes which also bind GAGA factor (32, 53). Since GAGA binding sites also occur in the α1t intron (39), we presume that a similar process occurs downstream. Thus, strong, ubiquitous activation by the combined action of these upstream and downstream elements may insulate α1t transcription by competing with the weaker activation and repression from more distal regulators.

Finally, our results identify an expression vector that could be used to direct ubiquitous expression of a protein throughout *Drosophila* development. By analogy with the use of the *hsp70* promoter to test enhancer activity in vivo, this vector could also be used to test silencer activity in any tissue.

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