The Promoter of the Heterochromatic Drosophila Telomeric Retrotransposon, *HeT-A*, Is Active When Moved Into Euchromatic Locations

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ABSTRACT

The Drosophila telomeric retrotransposon, *HeT-A*, is found only in heterochromatin; therefore, its promoter must function in this chromatin environment. Studies of position effect variegation suggest that promoters of heterochromatic genes are very different from euchromatic promoters, but this idea has not been tested with isolated promoter sequences. The *HeT-A* promoter is the first heterochromatin promoter to be isolated and it is of interest to investigate its activity when removed from telomeric heterochromatin. This promoter was initially characterized by testing reporter constructs in transient transfection of cultured cells, an environment that may approximate its endogenous heterochromatin. We now report *P*-element-mediated transpositions of these constructs, testing the function of different parts of the putative promoter in euchromatin. Expression of endogenous *HeT-A* RNA shows marked developmental regulation and accumulates preferentially in replicating diploid tissues. *HeT-A* promoter constructs are active in all euchromatic locations tested and some display aspects of endogenous *HeT-A* stage- and cell-type expression programs. The activity of each promoter construct in euchromatic locations is also generally consistent with its activity in the transient transfection tests; a possibly significant exception is one sequence segment that appreciably enhanced activity in transient transfection but repressed promoter activity in euchromatin.

A continuing question for genetics and cell biology is the significance of the deeply staining blobs and flecks found in interphase nuclei. An important step toward finding the answer came from work of Heitz (1928). By following this staining through the cell cycle, Heitz showed that the densely stained structures were specific chromosomes or chromosome regions that did not decondense as did the rest of the chromatin at the end of mitosis. This indicated that the chromatin structure of these regions differed from that of the rest of the genome. Heitz coined the name "heterochromatin" for the darkly staining material and "euchromatin" for the rest of the genome. Using Drosophila polytene chromosomes, he was able to show that heterochromatin was divisible into at least two classes, α and β, on the basis of their staining ability. α- and β-Heterochromatin were present in the pericentric regions of the chromosomes (Heitz 1934). Later, when Muller recognized that telomeres have special functions, he pointed out that the terminal regions of Drosophila polytene chromosomes share the morphological features of these pericentric regions (Muller 1938). Sequences at the extreme end resemble β-heterochromatin in their diffuse structure and tendency to pair ectopically. Thus, they fit the Heitz morphological description of heterochromatin.

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Understanding how the large regions of altered chromatin structure that make up heterochromatin affect genetic activity is obviously important. However, the question is experimentally difficult to approach. Studies in several experimental systems led to a composite view of heterochromatin in which many different traits were associated with the diagnostic morphology. However, few, if any, particular regions of heterochromatin were shown to have all the traits. Heterochromatin was considered genetically inert: Few conventional genes map to heterochromatin and genes are shut off when a chromosome (*e.g.*, the mammalian X) becomes heterochromatic (Lyon 1972). Heterochromatin is also rich in repeated sequences with no obvious function, which led to the idea that heterochromatin was "junk" DNA. The many transposable elements found in heterochromatin were thought to be dead elements that had been trapped by an environment that had inactivated them. Heterochromatin has effects on neighboring euchromatin: When a chromosomal rearrangement brings a gene into proximity to heterochromatin, that gene is frequently inactivated in a mosaic pattern (Spofford 1976). This phenomenon is called position effect variegation (PEV). Still other attributes of heterochromatin are a tendency toward ectopic pairing and late replication.

Few experimental systems for heterochromatin can be used to study the complete array of characteristics; therefore, much of the general picture of heterochromatin has been based on extrapolation from a small number of systems. As a result, little is known about the

range of variability of these characteristics in different regions of heterochromatin or about different species of organisms. As experimental techniques have improved, exceptions have been found to many of the generalizations about heterochromatin. Genes mapping to heterochromatin have been identified and found to be active (Hilliker 1976). Many of these genes are essential (Devlin *et al.* 1990; Sinclair *et al.* 2000). Many, but not all, of these genes show PEV when a rearrangement moves them away from, rather than close to, heterochromatin (Wakimoto and Hearn 1990; Eberl *et al.* 1993; Weiler and Wakimoto 1995, 1998; Clegg *et al.* 1998). Some genes on the mammalian X are not inactivated when the X becomes heterochromatic (Gilbert *et al.* 2000). The repeated telomere sequences in heterochromatin are not junk but have functions in addition to buffering the end of the chromosome (Blackburn 2001). The two retrotransposons that form the Drosophila telomeres, *HeT-A* and *TART*, are found only in heterochromatin, but they are important for telomere-specific functions (see Pardue and DeBaryshe 2002 for review). These new findings extend the range of experimental models for study of heterochromatin. They also emphasize the importance of considering different kinds of heterochromatin to obtain a complete understanding of this chromatin.

The contrasting PEV responses shown by euchromatic and many heterochromatic genes have suggested that the promoters of these two classes of genes may be significantly different. However, no promoter native to heterochromatin has been characterized at the level of its nucleotide sequence. In this report, we analyze a promoter from telomeric heterochromatin. The promoter is from the Drosophila telomeric transposon, *HeT-A*, one of the two retrotransposons that make up the telomeres in Drosophila. (Two non-LTR retrotransposons, *HeT-A* and *TART*, form long head-to-tail arrays by successive transpositions to the ends of chromosomes.)

The telomeric *HeT-A/TART* arrays are heterochromatic. Their localization in the polytene regions that Muller (1938) identified as heterochromatic is most easily seen by *in situ* hybridization to nuclei in which telomeres are ectopically paired with each other and with pericentric heterochromatin. In such pairing, a hybridized *HeT-A/TART* sequence can be stretched for long distances between chromosome ends (*e.g.*, see Young *et al.* 1983). The telomere-associated sequences (TAS) immediately interior to *HeT-A/TART* arrays are hotspots for transgene insertions (Karpen and Spradling 1992; Levis *et al.* 1993; Cryderman *et al.* 1999) and cause PEV, although, surprisingly, they do not respond to most of the genes shown to modify PEV produced by other kinds of heterochromatin (Cryderman *et al.* 1999). In contrast, no insertions in *HeT-A/TART* arrays are reported. However, an interesting study of a *white*$^{+}$ transgene in the *2L* TAS showed that terminal *HeT-A* arrays had an activating influence on the transgene. The authors point out that this activation could be due to readthrough from a *HeT-A* promoter, but is more likely an effect of a strong enhancer elsewhere in the element (Golubovsky *et al.* 2001). Their experiments show that the extent of the activation is affected by the amount of telomere array distal to the transgene as well as by the telomere on the homologous chromosome. These results raise the intriguing possibility that *HeT-A/TART* arrays produce a new variety of PEV.

The *HeT-A* promoter was characterized initially by analyzing its ability to drive transcription of a reporter gene transiently transfected into cultured Drosophila cells (Danilevskaya *et al.* 1997). The transient transfection system was chosen with the hope that the transfecting DNA, because it forms aggregates within the cells, would mimic the native heterochromatic environment of *HeT-A* for the transgene. This system was selected in preference to *P*-element-mediated insertion into chromosomal heterochromatin because there is no evidence that *P* elements insert into *HeT-A/TART* arrays. Searches for insertion in heterochromatin have found *P* elements in the TAS proximal to *HeT-A/TART* arrays but not within those arrays (Karpen and Spradling 1992; Levis *et al.* 1993; Cryderman *et al.* 1998, 1999; Golubovsky *et al.* 2001).

Both *HeT-A* and *TART* are non-long-terminal-repeat (non-LTR) retrotransposons, yet the *HeT-A* promoter defined by transient transfection experiments is very different from the promoters that had been found for other non-LTR retrotransposons, probably because *HeT-A* has evolved into an essential component of Drosophila chromosomes. The promoter for typical non-LTR elements is located in the 5′ untranslated region (5′ UTR) and has an upstream transcription start at the element's 5′ end rather than the downstream transcription start seen for most polymerase II promoters (Mizrokhi *et al.* 1988; Swergold 1990; Minchiotti and DiNocera 1991; McClean *et al.* 1993). The entire promoter sequence of these retrotransposons is contained within each transcript. *HeT-A* is clearly different from these elements. Its 5′ UTR has very weak promoter activity, but this activity is enhanced by at least a factor of 10 when the 3′-most sequence of the adjacent upstream element is added to the 5′ UTR sequence. Thus, the upstream neighbor provides sequence for the promoter of the downstream element and much of the promoter is not contained in the transcript (Danilevskaya *et al.* 1997).

Not only is the *HeT-A* promoter located within the sequence of the upstream neighboring element, but also the transcription start is located within the sequence of this neighbor (but only ~60 nucleotides into the neighbor's sequence) so that each transcript has a short segment of its neighbor's 3′ end attached to its own 5′ end (see Figure 1A). Thus, the *HeT-A* promoter resembles an evolutionary intermediate between the typical promoter of non-LTR retrotransposons and that of the

LTR elements. The 3′ sequence of the neighbor providing the promoter is identical to the 3′ end of the element being transcribed. Thus, the promoter is structurally and functionally equivalent to the 5′ LTR, which contains the promoter for LTR retrotransposons and for retroviruses. The significant difference is that the *HeT-A* promoter requires the collaboration of two *HeT-A* copies, while LTR elements are self-contained.

In the studies reported here, we have characterized the tissue- and stage-specific expression of *HeT-A* transcripts. This description was used to guide analyses of the ability of segments of the *HeT-A* promoter sequence to drive expression of a reporter gene when transposed into euchromatic regions of the chromosome. The minimal promoter defined in other chromatin environments (Danilevskaya *et al.* 1997; Kahn *et al.* 2000) is active in these euchromatic locations and is able to reproduce at least part of the normal transcription pattern. However, one segment of the sequence, which significantly enhanced activity in transient transfection, has the opposite effect in the euchromatic environment, suggesting that increased flanking sequence could increase the sensitivity of the promoter to its surroundings.

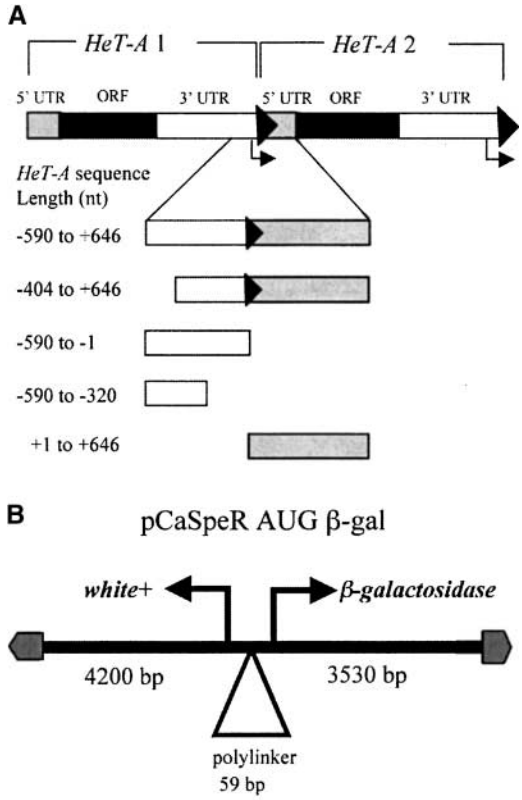

Figure 1.—Constructs used to generate transgenic Drosophila lines. (A) Diagram showing *HeT-A* sequences tested in the transgene constructs, drawn below a tandem array of *HeT-A* elements. Arrows below these elements indicate transcription start sites ~60 nt from the 3′ end of each element. Arrowheads indicate oligo(A) sequence at the 3′ end of each element. For each *HeT-A* fragment used in a construct, nucleotide limits are shown on the left. The first nucleotide of the 5′ UTR is +1, with the numbers increasing 5′–3′. The last nucleotide of the upstream 3′ UTR [not including the poly(A) tail] is −1 and numbers become more negative moving 3′–5′. The constructs used in these experiments were originally described in Danilevskaya *et al.* (1997). (B) Schematic diagram of the *P*-element vector, *CaSpeR AUG*, β-*gal* (Thummel *et al.* 1988) used to generate transgenic Drosophila lines. The *white* gene, driven by its own promoter, is present to allow identification of flies carrying the construct. The *HeT-A* sequence to be tested, inserted into the polylinker, drives the β-*galactosidase* (*lacZ*) gene. Arrows denote the direction of transcription from the *white* and *lacZ* reporter genes. Shaded arrowheads represent the *P*-element ends.

MATERIALS AND METHODS

Drosophila stocks: Our standard stock, Oregon-R, was used for Northern blot and tissue *in situ* hybridizations of endogenous *HeT-A* RNA. Transgenes were in *Df(1) w*^{67c23}, *y* flies. Tissue hybridizations for comparison to transgene expression were repeated in the *Df(1) w*^{67c23}, *y* stock and those results are shown here.

Transgenic flies: Lines were generated by standard protocols (Bartoszewski and Gibson 1994). The lines used in this report were each homozygous for a single insert of pCaSpeR AUG β-galactosidase (β-gal). The constructs used were those described by Danilevskaya *et al.* (1997) and are shown in Figure 1A. The construct insertion sites are summarized in Table 1.

***In situ* hybridization of endogenous transcripts:** RNA probes were labeled with digoxigenin by *in vitro* transcription of cloned sequences with T7 polymerase. The *HeT-A* probe used was from the coding region to avoid hybridization to small RNAs with 3′ UTR sequence. Probe was from element 23Zn-1 (GenBank accession no. U06920), nucleotides (nt) 1746–4421. The histone probe was transcribed from a cloned Drosophila H2B gene (Lifton *et al.* 1978). Larval tissues were dissected and hybridized by the technique of Kozopas *et al.* (1998). Hybridized RNA was detected by the activity of alkaline phosphatase conjugated to antidigoxigenin.

Sequences for RNA probes: The *HeT-A* 3′ UTR probe was from element 23Zn-1 (GenBank accession no. U06920), nt 4851–6481. The *TART* open reading frame (ORF) 2 probe was from *TART-A* (GenBank accession no. U02279), nt 434–2683. For each probe used in this study, sense and antisense strands were transcribed from DNA fragments of identical length.

Northern hybridization: RNA samples (20 μg/lane) were treated with glyoxal, separated on an 0.8% agarose gel, and transferred to Hybond-N nylon membrane according to the method of Sambrook *et al.* (1989). ^{32}P-labeled riboprobes were transcribed *in vitro* from DNA fragments inserted into Bluescript II SK with T7 or T3 RNA polymerases, according to the Promega (Madison, WI) protocol. Hybridization was performed at 65° in 4× SET [1× SET is 0.15 m NaCl, 0.03 m Tris-HCl (pH 7.0), and 2 mm EDTA], 5× Denhardt's solution, 0.5% SDS, and 50 μg of salmon sperm DNA/milliliter (Sambrook *et al.* 1989). The filters were washed twice with 2× SSC (1× SSC is 0.15 m NaCl plus 0.015 m sodium citrate) plus 0.5% SDS and twice with 1× SSC plus 0.5% SDS at 65° and then treated with 100 units/ml of RNase T1 (Boehringer Mannheim, Indianapolis) in buffer [10 mm Tris-HCl (pH 7.5), 300 mm NaCl, 5 mm EDTA] for 1 hr at 37°, rinsed with 1× SSC-0.5% SDS, and exposed for autoradiography.

Staining of larval tissue for β-galactosidase activity: Third instar larvae were dissected in cold phosphate buffered saline (PBS), fixed at room temperature in PBS containing 0.5%

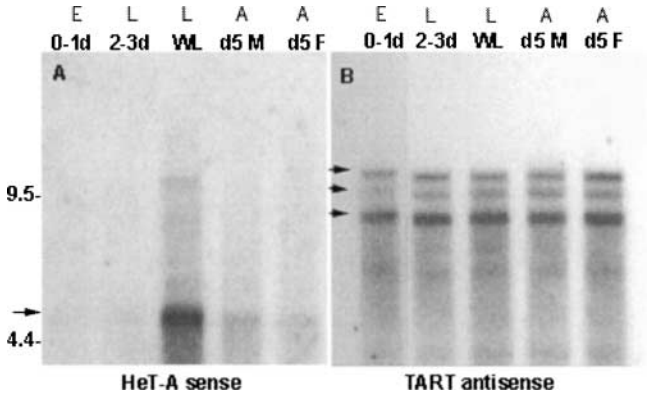

FIGURE 2.—Expression of *HeT-A* changes markedly during development. Autoradiographs of Northern blots of RNA from E (embryo), L (larva), or A (adult) Oregon-R. Each lane contains 20 μg of total RNA from 0- to 1-day embryos, first and second instar larvae, wandering third instar larvae, day 5 adult males (M), or day 5 adult females (F). Single-strand probes detecting sense-strand RNA were used for lanes marked "sense," while "antisense" lanes were probed with the opposite strand. (A) Blot probed with *HeT-A* 3′ UTR sequence to detect sense transcripts (3-day exposure). The probe detects a major band of ~6 kb, corresponding to full-length *HeT-A* elements (arrow). Larger transcripts (>9.5 kb) may correspond to read-through transcripts of tandem *HeT-A* elements. Antisense *HeT-A* transcripts have not been detected. (B) Duplicate blot probed with *TART* ORF 2 to detect antisense transcripts. We use this RNA as a loading control because we find that it is as unchanging through development as any housekeeping gene and because its large size makes it a rigorous measure of RNA quality (DANILEVSKAYA *et al.* 1999).

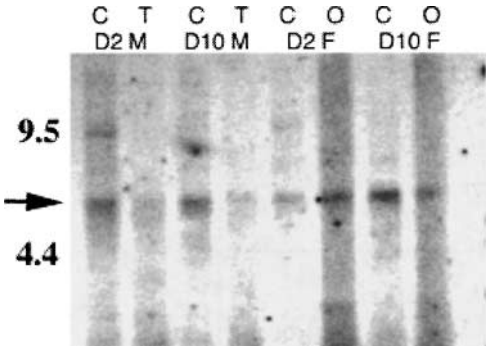

FIGURE 3.—*HeT-A* transcripts are present in both somatic and reproductive tissues from adult males and females. An autoradiograph of a Northern blot of total RNA from either the testes (T) or the ovaries (O) and their remaining carcasses (C) from day 2 and day 10 adult male (D2 or D10 M) or female (D2 or D10 F) Oregon-R flies, respectively. Each lane was loaded with 20 μg of RNA. The blot was probed with *HeT-A* 3′ UTR sequence to detect sense transcripts (2-day exposure). The major transcript (arrow) is ~6 kb in length.

glutaraldehyde, and stained for β-galactosidase activity overnight at 4° in a solution using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; LU *et al.* 1998). The stained tissue was rinsed in PBS and mounted in 80% glycerol with 0.15 M NaCl.

RESULTS

Expression of *HeT-A* transcripts is developmentally regulated: Before beginning the study of the isolated *HeT-A* promoter sequences, we analyzed the expression of *bona fide HeT-A* transcripts. Northern hybridizations of RNA extracted from intact animals and isolated tissues were used to measure relative levels of transcript accumulation as a function of developmental stage. *In situ* hybridization to RNA in intact animals was used to determine spatial patterns of accumulation within specific tissues. These techniques gave us an approximate picture of the cell type and tissue specificity to expect from a correctly functioning promoter. (Of course, they did not rigorously measure promoter activity because RNA accumulation is determined not only by synthesis but also by turnover.) RNA extracted from animals of different developmental stages shows dramatic differences in the levels of *HeT-A* transcripts (Figure 2A). Transcripts are barely detectable in RNA from embryos and first and second instar larvae; however, the quantity of RNA increases abruptly during the third instar. Adults still contain significant amounts of *HeT-A* RNA but much less than that present in the late third instar larvae. Although it is difficult to experimentally determine how many *HeT-A* elements are transcribed at any time, the available evidence suggests that multiple elements contribute to the RNA pool (PARDUE *et al.* 1996).

We considered the possibility that the decreased levels of *HeT-A* RNA in adults might indicate loss of transcripts from somatic tissues while RNA continued to be produced in the gonads. To test this possibility, we isolated RNA separately from dissected ovaries or testes and the remaining carcasses. We tested one group of flies 2 days after they had eclosed and a second group 10 days after eclosion to look for effects of aging (Figure 3). Northern hybridization clearly shows that adult flies, both females and males, express *HeT-A* RNA both in somatic tissues and in the gonads. Comparison of RNA extracted 2 days after eclosion with that extracted after 10 days shows some small differences but no consistent trend. We have seen variable differences in other experiments and suspect that these differences reflect nutritional status and growth conditions of the culture rather than aging.

Antisense *HeT-A* transcripts were not found at any time; however, we occasionally detected smaller RNAs in Northern hybridizations (for examples, see Figure 4). As expected from their size, these RNAs do not contain the complete *HeT-A* sequence. They tend to be tissue specific and may differ from stock to stock. They could be degradation products of full-length *HeT-A* transcripts, but we think it more likely that they are products of truncated or fragmented elements. Truncated elements are not uncommon in telomere arrays and fragments of elements have been found in other types of heterochromatin (DANILEVSKAYA *et al.* 1993; CASACU-

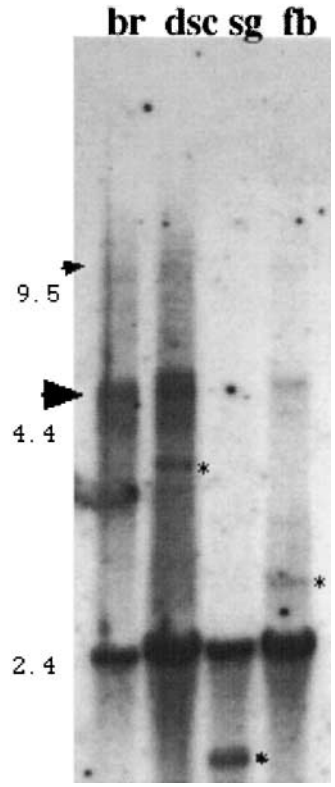

FIGURE 4.—*HeT-A* transcript levels are elevated in the diploid tissues of wandering third instar larvae. Autoradiograph of a Northern blot of total RNA isolated from tissues dissected from *Df(1) w*^{67c23} wandering larvae. br: brain; dsc: wing, haltere, and eye discs; sg: salivary gland; and fb: fat body. Discs and brains are diploid tissues; other tissues are predominantly polyploid. Each lane has the RNA of the indicated parts of 20 larvae. Smaller arrow denotes probable readthrough transcripts from tandem elements. Larger arrow indicates full-length *HeT-A* transcripts. The blot was probed with the *HeT-A* 3′ UTR to detect sense transcripts (overnight exposure). Asterisks indicate smaller tissue-specific transcripts thought to be products of truncated or fragmented elements (see text).

BERTA and PARDUE 2002). Most of the smaller transcripts hybridized only with probes from the 3′ UTR; however, one of the small RNAs was detected with probes from both coding region and 3′ UTR although it was smaller than either of these regions. These small RNAs will not be considered further in this report.

***HeT-A* transcripts are found preferentially in diploid tissue:** The abrupt increase in levels of *HeT-A* RNA in the third instar larvae suggests that the RNA accumulates in replicating diploid tissue. Nearly all of the increase in size of the larvae is accomplished by changes in cell size rather than by changes in cell number. As these larval cells enlarge, they continue to replicate their DNA but do not undergo divisions to separate the new genomes. In contrast, during the third larval instar, the small groups of diploid cells that form the primordia of adult tissues undergo rapid cycles of growth and division in preparation for metamorphosis (SMITH and ORR-WEAVER 1991; EDGAR and ORR-WEAVER 2001). To explore the hypothesis that the accumulation of *HeT-A* RNA is associated with these replicating diploid tissues, we isolated RNA from tissues of wandering third instar larvae and measured the relative amounts of *HeT-A* RNA (Figure 4). We also performed *in situ* hybridization to tissues to analyze the distribution of *HeT-A* RNA with higher spatial resolution.

Our Northern hybridization experiments compared brain, imaginal discs (eye-antennal, legs, wing, and haltere), salivary glands, and fat body (Figure 4). The brain and disc complex is composed largely of diploid, rapidly replicating cells that will make up adult tissues. The other tissues are made up mostly of larval cells that will not survive metamorphosis; however, they also contain small numbers of cells that will go on to make adult tissues (*e.g.*, the nests of histoblasts in the gut and the imaginal ring of the salivary gland; BRYANT and LEVINSON 1985). Northern hybridizations show that the brain and disc complex contains essentially all of the *HeT-A* transcripts detected in the wandering larvae. The experiment shown in Figure 4 compared body parts from an equal number of larvae. Another experiment, not shown, compared body parts on the basis of equal amounts of total RNA and gave the same result. That experiment included gut RNA. We detected very little full-length *HeT-A* RNA in RNA from gut and from fat body and have never seen full-length transcripts in RNA from salivary glands.

***In situ* hybridization to transcripts in larval tissues shows *HeT-A* expression in replicating regions:** *In situ* hybridization to tissues from third instar larvae shows patterns of hybridization indicating regional concentrations of transcripts within discs and brain (Figure 5, a–c). Two of the most distinctive patterns are seen in the eye disc and the brain. In the eye disc a very prominent band of dark staining is seen behind the morphogenetic furrow, immediately in front of the region where the eight-cell clusters have formed (Figure 5b, arrowheads). This is the region of the second mitotic wave, a tight band of cells undergoing simultaneous replication and division (TOMLINSON 1985). A second example of distinctive *HeT-A* RNA enrichment is seen in the proliferation centers of the brain where patterns of circular lines are seen (Figure 5a, arrowheads). Both of these patterns seem to be due to regions of high concentrations of cells in S phase because similar patterns are produced when these tissues are hybridized with probes for histone mRNA (Figure 5, d–f), although histone mRNA is much more abundant than *HeT-A* RNA. Histone transcripts provide a marker for cells in S phase because this RNA, controlled by both transcription and turnover, is abundant only during this stage of the cell cycle (ANDERSON and LENGYEL 1980; BAUMBACH *et al.* 1987).

Histone probes also detect some hybridization to salivary glands, gut, and fat body in these experiments. The patterns are dynamic; larvae in the same experiment

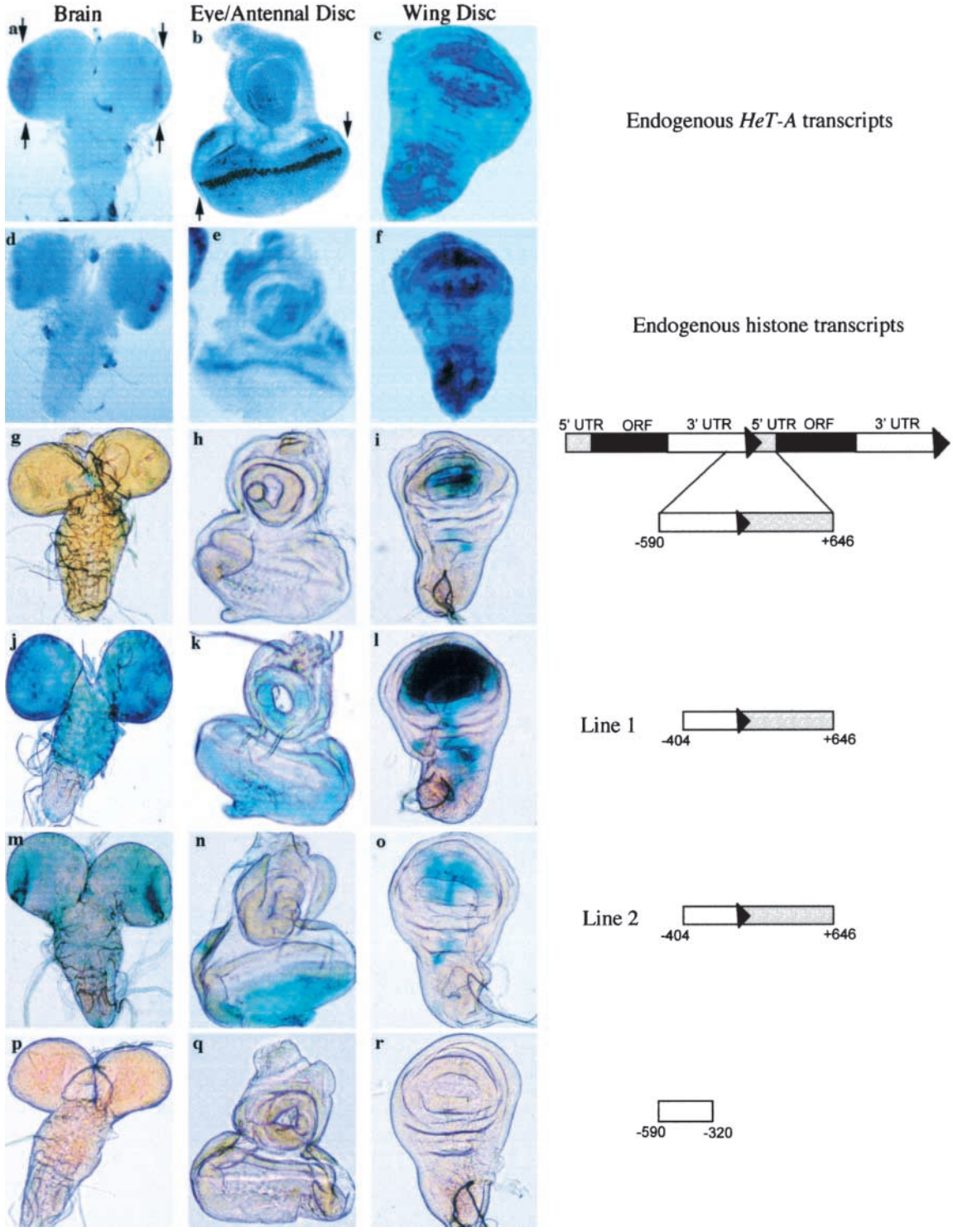

FIGURE 5.—Comparison of expression of endogenous *HeT-A* RNA with histone mRNA expression and with expression from *HeT-A* promoter constructs. Tissues are from wandering third instar larvae of the *Df(1) w*^{67c23}, *y* stock. The proliferation region in a and the second mitotic wave in b are flanked by arrows. Diagrams of the promoter sequences in the transgene carried by each line are shown to the right of the appropriate row. Left column shows brains. Middle column shows eye-antennal discs. Right column shows wing discs. (a–f) *In situ* hybridization probed to detect endogenous *HeT-A* transcripts (a–c) and histone transcripts (d–f), reflecting the distribution of S-phase cells. All tissues are from the same experiment for each probe, except the brain tissue probed for *HeT-A* RNA, which was taken from an experiment in which staining was less intense to better display the pattern in the proliferation region. The remaining rows show tissues dissected from transformed *P*-element lines (g–r) stained for β-galactosidase activity produced by the reporter transgene. Activity of the construct shown in p–r is not detectably different from the nontransformed control (not shown).

may show slightly different patterns of hybridization, probably indicating that they are at slightly different developmental stages. In spite of the variation, patterns are consistent for a particular larva. For example, when salivary glands show hybridization to the imaginal ring, the hybridization is seen on both members of the pair of glands from that animal. When heavy staining of histoblast clusters is seen in the gut, that staining is usually seen over many clusters in a contiguous region of the gut. In some cases, scattered polytene nuclei of the salivary gland show heavy hybridization. A variable background staining in these polyploid tissues does not obscure detection of the very abundant histone mRNA, but confounds analysis of the low-level *HeT-A* RNA, preventing conclusions from being drawn about *HeT-A* RNA in this set of tissues from *in situ* hybridization. However, Northern hybridization results show that these tissues have little, if any, full-length *HeT-A* RNA (Figure 4).

Constructs used to test the *HeT-A* promoter sequences in euchromatic locations: The constructs used to define the *HeT-A* promoter in transiently transfected cultured cells (Danilevskaya *et al.* 1997) were made in a *P*-element vector, pCaSpeR AUG β-gal (Thummel *et al.* 1988; see Figure 1). Thus, we were able to use the same constructs to transform embryos for the studies reported here. The set of sequences used by Danilevskaya *et al.* (1997) had gradually increasing lengths of *HeT-A* 3′ UTR extending from nt −1, the last nt before the poly(A), to nt −590. (For the upstream element providing part of the promoter, we designate nt by negative numbers beginning just before the poly(A) and running 3′ → 5′.) Each of these sequences was attached to nt +1 to +646 of the 5′ UTR of the element being transcribed. Thus, the largest sequence of this set was nt −590 to +646 and the shortest was nt +1 to +646. Danilevskaya *et al.* (1997) found that nt −133 to −1 contained sequence necessary for promoter activity, while the addition of more 3′ UTR sequence enhanced the activity of the promoter. The activity seemed to increase in three steps as increasing lengths of 3′ UTR sequence were added, suggesting that several enhancing elements were in this sequence. The absolute requirement for nt −133 to −1 is probably due to the transcription start sites that we have found at −62 and −31.

Analysis of promoter sequence activity in euchromatic sites: For the study reported here, we made transgenic lines carrying the strongest promoter, nt −590 to +646, and the promoter with the second highest level of activity, nt −404 to +646, from the study of Danilevskaya *et al.* (1997). We also made a line carrying the construct lacking the transcription start site (nt −590 to −320) expected to be a negative control. Additional lines were made to test the sequence from the 5′ UTR by itself (nt +1 to +646) and by its absence when deleted from the largest promoter (to give nt −590 to −1). All insertions (Figure 1A) were single copy and carried in lines homozygous for the insertion. *In situ* hybridization to polytene chromosomes confirmed that each insertion was in a typical euchromatic, banded region. The insertion sites are summarized in Table 1. Southern hybridization of DNA from transgenic flies was also used to confirm that the *HeT-A* inserts in the constructs were not altered during the creation of the transgenic lines.

TABLE 1

Summary of transgenic insertion sites

HeT-A construct nucleotides	Chromosomal insertion-site polytene bands
−590 to +646	85A-C
	64-66
	49F-50A
	51A
−404 to +646	43D
	89-91
−590 to −1	16-18A
+1 to +646	67F
−590 to −320	53F-54A

The promoter activity of each of these constructs was consistent with results of both the transient transfection experiments and the analyses of endogenous RNA expression. In considering the results, we take into account the fact that the promoter in the construct is single copy while the endogenous promoters are almost certainly multicopy. In addition, our experiments with cultured cells have given us the impression that the reporter RNA is unstable in Drosophila cells, perhaps because of its bacterial origin. Both the single copy nature of the construct and the instability of the reporter RNA may explain why we have been unable to detect *lacZ* transcripts on Northern blots of RNA from whole organisms. Because brain-disc complexes from wandering larvae have abundant expression from the *HeT-A* promoter, we isolated RNA from these tissues for Northern analysis and found a small amount of *lacZ* RNA (data not shown).

We found β-galactosidase activity to be a more useful assay of reporter gene expression. We analyzed this activity in two developmental stages, embryos and third instar larvae. Little, if any, endogenous *HeT-A* RNA can be detected in embryos (Figure 2A). None of the constructs yielded detectable β-galactosidase expression in embryos, a result that would be expected of sequences giving proper stage-specific expression.

Third instar larvae have a very high level of endogenous *HeT-A* RNA expression (Figure 2A). At this stage, constructs active in transient transfection were also active as chromosomal transgenes (Figure 5, g–o). Thus, the lack of detectable activity of these transgenes in embryos is consistent with proper developmental regulation of the promoter. As expected, the transgene lacking the transcription start site (−590 to −320) was inactive in both embryos and larvae (Figure 5, p–r).

In transient transfections, constructs differed in the strength of their activity. Analyses of β-galactosidase expression in larval tissues showed parallel differences in the transgenic lines, with the notable exception of the longest construct (discussed below). In addition, analyses of larval tissues allowed us to assess the tissue-specific patterns of promoter activity.

The longest promoter sequence construct (−590 to +646) had weak but detectable promoter activity in one of the larval tissues, the wing discs, where the endogenous promoter is active (Figure 5, g–i). This construct showed no inappropriate expression. Four lines carried independent insertions of this construct in different chromosomal sites. Each line showed expression of the β-galactosidase reporter only in wing discs of third instar larvae. Because the four lines had similar expression patterns, we suggest that this pattern was determined by the sequence in the construct rather than by its chromosomal location.

Surprisingly, the shorter sequence, −404 to +646, although a weaker promoter than −590 to +646 in transient transfection, was much more effective in transgenic flies. In these euchromatic sites, the −404 to +646 construct showed strong promoter activity that reproduced several aspects of the tissue distribution typical of *bona fide HeT-A* RNA (Figure 5, j–l and m–o). In addition to expression of β-galactosidase in wing discs seen with the −590 to +646 construct, the −404 to +646 construct produced β-galactosidase expression in other discs and in the brain.

The β-galactosidase expression driven by the −404 to +646 sequence reflects some of the distinctive cell-type patterns seen for endogenous *HeT-A* RNA. There was marked β-galactosidase activity in the circular patterns on the proliferation centers of the larval brain. In addition, β-galactosidase activity was detected over most of the eye disc behind the second mitotic wave. We suggest that this broad region of β-galactosidase activity is the result of promoter activity in cells of the second mitotic wave, leading to synthesis of the long-lived β-galactosidase protein, which remains after the wave of mitosis has passed through. The two lines carrying independent insertions of the −404 to +646 construct show the same general pattern of promoter activity, but differences in the relative expression in different regions suggest that the chromosomal sites of these two transgenes modulate their expression. This modulation is more complex than a general effect on the level of expression. In Figure 5, j–l, line 1, brains and wings show strong activity while the eye-disc expression is lower. In this line, expression in the brain is in the proliferation centers and also in many clusters of cells on the anterior regions of the brain hemispheres and the anterior two-thirds of the ventral ganglion. In line 2, the eye-disc activity is strong while the other tissues have less activity than in line 1 (Figure 5, m–o).

The 5′ UTR sequence is of interest because this region contains the promoter of other non-LTR retrotransposons; one scenario for evolution of the *HeT-A* promoter suggests that it contains the promoter for the ancestral *HeT-A* element (Danilevskaya *et al.* 1997). To evaluate the contribution of the 5′ UTR, Danilevskaya *et al.* (1997) divided the longest promoter sequence (−590 to +646) into two parts, the 3′ UTR (−590 to −1) and the 5′ UTR (+1 to +646). Transient transfection experiments showed that the contributions of the two regions to reporter strength were approximately additive. The 5′ UTR alone had 10% of the activity of the full-length construct, while its removal left the 3′ UTR with 90% of that activity.

We used these same constructs to explore the contribution of the 5′ UTR to stage- and tissue-specific expression. The expression pattern of these transgenes suggests that specific expression requires cooperation between the 5′ and 3′ sequence. Both constructs showed inappropriate expression that was not seen with other constructs. For the 5′ UTR sequence (+1 to +646) staining was observed (but not consistently) in cells in the region of the foregut imaginal ring (data not shown). No other β-galactosidase expression was seen. The only β-galactosidase expression seen in larvae carrying the 3′ UTR (−590 to −1) construct was in a segment of the posterior larval midgut (data not shown). We did not detect any appropriate expression from either construct; however, this may be due to the limited sensitivity of the β-galactosidase assay. The 5′ UTR construct had very weak activity in transient transfection. The 3′ UTR construct contains the −590 to −405 sequence that severely reduced activity of the −590 to +646 construct.

***HeT-A* segments do not affect the adjacent *white* gene:** In planning this experiment we had some concern that the promoter sequences might have enough heterochromatic character to induce silencing of the *white*+ gene, the reporter gene used in the constructs to identify transgenic animals. In the studies reported here we have seen no evidence that this occurs; none of the transgenic flies carrying these constructs have the variegating eyes that might be expected if the reporter were influenced by neighboring heterochromatin. The number of transgenic lines recovered was consistent with expected frequencies. Because transgenic flies were identified by eye color, low recovery would suggest that the *white*+ reporter was completely silenced in some constructs.

DISCUSSION

The *HeT-A* promoter is interesting for several reasons: (i) It regulates the synthesis of a major component of telomeres; (ii) it has features suggestive of an evolutionary intermediate between promoters of non-LTR retrotransposons and those of LTR retrotransposons; and (iii) it is located in telomeric heterochromatin and is the first heterochromatic promoter to be characterized

at a molecular level. [We do not discuss ribosomal RNA genes because they are transcribed by a dedicated polymerase, pol I (Moss and Stefanovsky 2002). In addition, rRNA genes are located in heterochromatin in some species but not in others (Pardue *et al.* 1970).]

The heterochromatic location of the *HeT-A* promoter was the motivation for the present study. This promoter can shed new light onto heterochromatin, one of the most enigmatic features of eukaryotic cells. Heterochromatin differs from euchromatin in a number of aspects but there are several categories of heterochromatin and little evidence that any of them has the entire set of these characteristics. This variety adds to the enigma and requires investigation. Transcription of most euchromatic genes is inhibited when they are moved near heterochromatin (Spofford 1976; Weiler and Wakimoto 1995). In contrast, transcription of most of the very few genes known to be in heterochromatin is inhibited by chromosomal rearrangements that move them away from the main body of heterochromatin [most studies have been done on *rolled* (Eberl *et al.* 1993) and *light* (Wakimoto and Hearn 1990; Howe *et al.* 1995; Weiler and Wakimoto 1998) from *Drosophila melanogaster*]. These genetic analyses are difficult to interpret in molecular terms because large segments of heterochromatin are involved in the rearrangements. Nevertheless, the results raise the possibility that promoters native to heterochromatin may be very different from those native to euchromatin.

***HeT-A* and *TART* are intermingled in heterochromatin yet have different patterns of expression:** These two retrotransposons form telomeres, chromosome regions that have the cytological hallmarks of heterochromatin (Muller 1938). Heterochromatization is sometimes thought of as a mechanism for coordinately regulating expression of an entire chromosome or chromosome region. *HeT-A* and *TART* are closely associated in telomeres, yet our study shows that these elements have very different patterns of expression during development (Figure 2). The promoters of the two elements appear to respond to their environment in different ways.

***HeT-A* appears to be expressed predominantly in rapidly dividing diploid cells:** Our tissue hybridizations suggest that, like histone mRNA, *HeT-A* RNA is expressed in replicating cells and turns over rapidly. An RNA involved in maintaining telomeres might be expected in dividing cells, but *HeT-A* RNA, although not very abundant, is nevertheless present at higher levels than would appear to be needed to maintain chromosome length. It has been calculated that Drosophila chromosomes lose an average of two nucleotides per round of replication (Biessmann *et al.* 1990; Levis *et al.* 1993). Each *HeT-A* transposition can add 6 kb of new sequence, so transpositions should be needed only rarely. It is interesting that the RNA component of *Mus musculus* telomerase also undergoes developmental regulation although the long telomeres of this species do not appear to need constant replenishment (Blasco *et al.* 1995; Martin-Rivera *et al.* 1998).

Activity of *HeT-A* promoter sequences transposed into euchromatin: Although the −404 to +646 segment of the *HeT-A* promoter is completely removed from its native environment, it is active and its activity is consistent with behavior of this sequence in two other contexts. This construct showed strong promoter activity in the transient transfection experiments (Danilevskaya *et al.* 1997). Kahn *et al.* (2000) have studied a terminally deleted chromosome with a promoterless *yellow* gene exposed on the end. They have shown that "healing," by *HeT-A* transposition onto this end, will provide a promoter capable of directing *yellow* gene expression. Only 400 bp of 3′ *HeT-A* sequence (*i.e.*, −400 to −1) was needed to produce wild-type expression of the yellow gene.

The −404 to +646 promoter not only was active in euchromatic environments but also reflected some of the temporal and cell-type regulation seen with the endogenous promoter, suggesting that the region contains not only a core promoter but also some of the regulatory enhancers. As shown in Figure 5 (j–o), there are some differences in the patterns of expression produced by this construct in different chromosomal sites, indicating that this regulation could be modulated by the transgene's surroundings. The −404 to + 646 sequence may also be reflecting appropriate regulation in the transient transfection experiments of Danilevskaya *et al.* (1997) and healed chromosome experiments of Kahn *et al.* (2000). The cultured cells studied by Danilevskaya *et al.* express endogenous *HeT-A* RNA, as expected for cycling diploid cells. Expression of the *yellow* gene from the *HeT-A* promoter studied by Kahn *et al.* was detected in body parts formed from imaginal discs where *HeT-A* RNA is expressed.

The −590 to +646 construct showed a marked response to euchromatic environments. In transient transfection, this was the strongest promoter, yet in the transgenic larvae, where the shorter −404 to +646 sequence had strong promoter activity and at least some of the appropriate regulation, this longer sequence produced β-galactosidase expression only in larval wing discs (Figure 5, g–i). The failure to detect the reporter in other discs may be a reflection of low promoter strength rather than tissue-specific control. This level of activity seems to be due to the sequence in the construct since four independent insertions were tested and each gave only wing-disc expression. The −590 to +646 construct differs from the −404 to +646 construct only by the −590 to −405 sequence. This additional 186 bp of *HeT-A* 3′ sequence appears to have caused the −590 to +646 construct to become sensitive to the euchromatic environment (see below).

What distinguishes heterochromatic promoters? Studies of some other heterochromatic genes, transposed by chromosomal rearrangement, have found that their

expression was progressively repressed as they were associated with less heterochromatin. Those results suggested that we might find that the *HeT-A* constructs were completely inactive in the euchromatic sites studied here. That was not the case. Instead we found that one sequence from the *HeT-A* promoter, −404 to +646, not only is active but also shows at least part of the appropriate developmental regulation. Surprisingly, increasing the length of flanking sequence by adding nt −590 to −405 repressed that activity. The repression by −590 to −405 contrasts with the increased activity it produced in the transient transfection assay, suggesting that this sequence is functioning differently in the euchromatic location.

The contrast between this study of *HeT-A*, where added flanking sequence is making the promoter less effective, and studies of *light* and *rolled*, where increased flanking sequence makes transcription more effective, could indicate that the studies examine qualitatively different types of heterochromatic transcription units. For instance, Eissenberg and Hilliker (2000) have proposed two models, adaptation and coexistence, for gene expression from heterochromatic locations and the resulting variegation of expression when these genes are moved to euchromatic locations. In the adaptation model, genes that are currently in heterochromatic locations were once in euchromatic locations. When the accumulation of repetitive sequence (or multiple copies) resulted in a folded state, which was replaced by heterochromatic compaction, they adapted to this altered environment. These promoters would then cease to function when they are again placed in a euchromatic environment. In the coexistence model, the promoter is of a sufficiently simple structure to allow for activation that tolerates the accumulation of heterochromatin. When the promoter sequences are placed again in euchromatin, if heterochromatin is still associated with them but the heterochromatin is somehow disrupted, the disrupted heterochromatin creates an abnormal environment that interferes with transcription. Although either adaptation or coexistence could explain the studies of *rolled* and *light*, we prefer the coexistence model for *HeT-A*. This model would explain our observation that, while the −404 to +646 construct is an effective promoter, adding an additional 186 bp of 3′ UTR sequence significantly reduces its activity. The implication is that the added sequence is enough to create the disrupted heterochromatin. We can imagine several ways in which the postulated disruption could occur. Nevertheless, the intriguing possibility is that the −590 to −405 sequence is the region of the promoter/enhancer complex where the *HeT-A* promoter begins to respond to its broader chromatin environment. This region and more distal sequence may be necessary to more completely recapitulate the normal expression pattern.

There is an important difference between this study and the studies of genes moved by chromosomal rearrangement. Our study examines changes in the micro-environment of the promoter (~1 kb around the transcription start site), while the chromosomal rearrangement studies examine changes in the macro-environment in megabases. This difference suggests that the basic promoters may not be qualitatively different when removed from their macro-environment. It seems reasonable to expect that pol II interacts with DNA at the start of transcription in much the same way in euchromatin and in heterochromatin. Thus, the core promoters, and possibly other sequence of the promoter, may not differ much in the two environments. The difference would be the neighborhood in which they find themselves.

Studies with transgenes have shown that euchromatic genes can be expressed from at least some regions of heterochromatin and that regions of heterochromatin are not entirely equivalent in the ways in which they affect the nearby transgene (Cryderman *et al.* 1998, 1999). These effects could be transmitted by the chromatin structure, subnuclear localization, or replication timing determined by the local heterochromatic environment (see Wallrath 2000 for recent review). Heterochromatin is clearly not a single entity. The *HeT-A* promoter gives us one example of a promoter that functions in one heterochromatic environment.

In conclusion, the behavior of the minimal *HeT-A* promoter in foreign locations suggests that its native telomeric heterochromatin is an environment that permits, and possibly cooperates with, this promoter's inherent activity rather than an environment that is necessary for basic activity and regulation.

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