Two separable promoters control different aspects of expression of a *Dictyostelium* gene

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ABSTRACT

A single copy *Dictyostelium* gene was dissected and elements responsible for its complex pattern of regulation were defined by transcript analysis of gene fusions. Two overlapping promoters responsible for the transcription of an 'L' and an 'S' mRNA could be defined. Further dissection of the P8A7 L promoter resulted in the identification of a sequence necessary for stress induction and an element required for vegetative expression. The P8A7 S promoter could be reduced to 449 bp which were sufficient for expression in developing cells. The sequence element required for this transcriptional activity was shown to reside in a 51 bp fragment. Our results show that differential expression of the P8A7 gene is mediated by two independently functioning promoters which, however, share some regulatory elements. A third nuclear RNA species 'P' was due to the stress-sensitivity of the 3' processing signal.

INTRODUCTION

Differential gene expression in *Dictyostelium* has been studied extensively by analyzing the patterns of specific RNAs throughout development (1). More recently, promoters of genes displaying a simple regulation in development have been characterized (2,3), and *cis* acting elements responsible for regulated expression have been identified (4, May *et al.*, in preparation, Vauti *et al.*, in preparation). Multiple transcripts detected by a single probe could mostly be attributed to multigene families (5,6). In only one well documented case, a single copy gene gives rise to several mRNAs (7).

In contrast to *Dictyostelium*, higher eucaryotes extensively exploit posttranscriptional mechanisms, like alternative splicing (8), to generate multiple transcripts from a single copy gene, which in turn give rise to distinct gene products. The use of different promoters has been found in the regulation of tissue specific gene expression (9). These mechanisms were also described for *Dictyostelium*, where Podgorski *et al.* (10, Faure *et al.*, Mol. Cell. Biol. in press) show that three promoters direct the expression of the phosphodiesterase gene.

In a previous paper we considered the use of alternative promoters as a mechanism for the generation of two distinct,

divergently regulated mRNAs, P8A7 L and P8A7 S, from a single copy gene (11). A genomic clone, containing the whole 5' region of the gene now enabled us to demonstrate that two tandemly arranged and partly overlapping promoters direct the differential expression of the *Dictyostelium* P8A7 gene. The promoters are composed of modules, thus allowing the definition of elements with specific effects on transcriptional regulation.

MATERIAL AND METHODS

1. Cloning and sequencing of the genomic fragment

Analysis of restriction fragments of genomic DNA by Southern blotting suggested that a 2.5 kb *Sau3A* fragment should contain the whole P8A7 gene from *Dictyostelium*.

Digested genomic DNA was separated on an agarose gel and electrophoresed onto DE 81 paper (Whatman). The adherent DNA was isolated according to Dretzen *et al.* (12). Dot blots of DNA isolated from gel slices in the 2–3 kb region were hybridized with a labelled cDNA fragment, and an appropriate fraction was ligated into the *Bam*HI site of a pGEM 4 vector (Promega Biotech). This partial genomic library was screened, using the same probe as above. Positive bacterial clones were confirmed by restriction analysis and one was chosen for further studies.

For the purpose of sequencing, unidirectional deletions were generated from both ends of the insert using the Erase-A-Base kit from Promega according to the instructions. The resulting staggered subfragments were sequenced with T7 polymerase from Pharmacia, following the instructions of the manufacturer. By this, the full sequence of both strands was obtained.

2. Northern blot analysis of transformants

RNA was extracted and Northern blots were performed as described previously. (13). For hybridizations in vitro transcripts prepared as described by Maniak and Nellen, (11) were used as probes.

3. *Dictyostelium* transformations

Stably transformed *Dictyostelium discoideum* A $\times$ 2–214 clones or populations were obtained following the protocol of Nellen and Saur, (14).

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Fig 1. Transcription map of the P8A7 locus. In the upper half of the figure, Northern blot stripes are shown. They were hybridized with fragments (A,B,...E), located between the triangles shown beneath. Filled triangles denote *Sau3A* sites, the ends of the genomic clone. Open triangles represent the locations of *DraI* sites. The fragments in between were subcloned in pGem3Z vectors and in vitro transcripts used for hybridization. Arrows give the approximate positions of the mRNA start sites. The lower half of the figure summarizes the results in a transcription map. P, L, S are the P8A7 specific transcripts, U denotes the upstream gene.

4. Other molecular techniques

Molecular cloning was done according to Maniatis *et al.* (15). RNase Protections and other molecular techniques were adopted from Nellen *et al.* (16).

RESULTS

1. Transcript analysis of the genomic region

The genomic fragment is represented graphically in the middle of Fig. 1. *In vitro* transcripts, generated from a number of restriction fragments, were used to determine the transcribed regions of the gene. The most 5' fragment (A) hybridized to a 1.5 kb mRNA, which was constitutively expressed during development (data not shown). It belongs to an upstream gene, that is transcribed in the same direction as the P8A7 gene of *Dictyostelium*. The subsequent fragment (B) does not hybridize to any RNA on a Northern blot, indicating a non-transcribed intergenic portion.

The following probes were used to detect transcripts of the P8A7 gene in total RNA from cold-shocked cells, known to express all three specific RNAs. The first probe used (C) detects only L and P RNA (Introduction and 11) indicating that both share a common 5' end. The next fragment (D), which also contains the intron, detects all three (S,L,P) transcripts. The most 3' probe (E), however, hybridizes only to the precursor (P RNA), demonstrating that this transcript does not contain the same 3' end as the S and L mRNA. It has previously been shown that this molecule is exclusively localized in the nucleus, and thus not involved in the production of the encoded protein (11).

2. Sequence analysis and high resolution map

Both strands of the entire fragment were sequenced, using unidirectional deletions (see Materials and Methods). Interpretation of the sequence (Fig. 2A.) lead to the following conclusions:

(i) The genomic fragment should contain all sequences required in *cis* for promoter activity, since part of the coding region of

an upstream gene is present. (ii) The coding region of the upstream gene ends with the highly preferred TAA stop codon. In addition, the codon usage is typical for *Dictyostelium* (17). The amino acid composition indicates a soluble protein with a high content of aromatic amino acids.

(iii) The ATG found in the previously published P8A7 cDNA clone (11) is the start of translation. Thus both, S and L mRNAs, encode the same gene product, since no upstream exon was detected. (iv) As predicted from comparative analysis of cDNA and Southern blots of genomic DNA (11), we find a typical *Dictyostelium* intron with a length of 103 nts and a GC content of 3%. (v) Comparison with the sequence of the 3' end of the cDNA allowed to identify the end of the transcribed region of the gene.

RNase protection assays indicate the position of the two different start sites of the L and S mRNA (Fig 2b). Since we find two differently regulated mRNAs transcribed from a single copy gene, and the 5' ends appear to be too far separated for two different start sites, we suspected that the gene is controlled by two promoters.

3. The L promoter controls stress induction and expression in vegetative cells

Using the promoter analysis vector described by May *et al.* (4), gene fusions of the presumptive promoters to the bacterial CAT reporter gene were constructed. Promoter-deletion-CAT constructs were transformed into *Dictyostelium* cells and the effect of the deletions was prescreened by performing CAT assays, where the activity of the promoter is monitored on the protein level (data not shown). For closer analysis Northern blots were performed. A fusion of the sequences including the start site for the L mRNA described above (1 to 1476) results in appearance of only one CAT specific transcript in transformed cells. This CAT mRNA displays the characteristics previously defined for the L mRNA of P8A7 (11), i.e. inducibility by stress and development.

5' deletions performed on this construct allow for analysis of elements that are constituents of the L-promoter (LP). A set of

ten staggered deletions was subsequently analyzed on the level of CAT RNA. Multiple clones of these transformants revealed that the deletions down to position -798 have no effect. Deletion -600 is no longer inducible (Fig. 3.) by developmental and stress-related stimuli described before (11). Within the deleted region, referred to as i(nducible)-element, we are unable to detect a consensus heat-shock box (18). The remaining -600 promoter region is however sufficient to direct transcription of the CAT gene in vegetative, untreated cells, in suspension culture and in cells attached to a surface. Furthermore, a deletion to pos. -486 resulted in the complete loss of transcriptional activity, the 114 bp element between -600 and -486 constitutes the b(asal)-element responsible for vegetative expression.

4. The S promoter controls expression in development

The appearance of only one CAT RNA with the above constructs was expected, since the second *in vivo* start site was deleted in this construct. It was also possible, however, that the 300 bp fragment between the L and S start sites contained a second promoter. The fusion of a fragment containing the 5' region from pos. 382 (corresponding to -1348) down to the ATG and the first 17 codons to the CAT gene, results in two CAT specific transcripts in stably transformed *Dictyostelium* cells. Analysis of progressive deletions of 5' end sequences of this (double) promoter demonstrates that the expression of the larger transcript is lost with deletion -539, while the S specific transcript is still detectable. This strongly argues for the existence of two promoters (LP and SP) (Fig 4.).

The deletion to pos. -842 still gives rise to two transcripts in vegetative cells. A deletion to approximately -486 should result in loss of the LP function. Indeed, we detect only S-CAT mRNA with deletion -539 of the double promoter. Surprisingly this SP displays activity only in developing cells. This result is contrary to what one would predict from the expression pattern of the endogenous gene, from which an S mRNA is transcribed also during vegetative growth and early development. Transcription during development is still detected with deletion -449, but is abolished with deletion -398. These results do not only show that a d(evelopmental) expression element is located between -449 and -398, overlapping the L mRNA start site, but also that sequences necessary for vegetative expression are located between -600 and -539. The results are summarized in Fig. 5.

5. The downstream 3'processing site is sensitive to stress

Stressing cells by heat and cold is known to result in the appearance of nuclear transcripts whose retention can mostly be attributed to the fact that they are not spliced. This was previously also assumed for the P8A7 P transcript (11), which is not 3'processed at the usual site to a mature mRNA under these conditions (section 1). We tested whether this 3'processing site could also function independent of the P8A7 gene. Therefore, transcripts of the constructs shown in Fig.6 were assayed in transformed cells. Northern blot analysis revealed that in untreated cells the wild-type polyadenylation site is used, whereas in stressed cells about 50% readthrough products are observed which terminate in the subsequent actin terminator (Fig 6, construct T1).

Inverting the fragment demonstrates that the processing site can also function in the opposite orientation (Fig 6, construct T3), although with an obvious difference in the specificity of the stress sensitivity (compare hs lanes in T1 and T3). This is

expected, since the opposite strand of this fragment also carries the AAUAAA and GT/T rich sequences which have been shown to be required for 3'processing (19).

The read-through transcripts also showed a sensitivity to recovery from heat-shock, similar to that of the P transcript of the endogenous gene. Read-through transcripts are not restricted to the nuclear compartment (data not shown), probably because they do not require splicing.

DISCUSSION

Stress sensitivity of 3'processing is an intrinsic feature of the 'terminator'

The gene structure shown in Fig. 1 is similar to that of two loci described for *Drosophila* (20, 21). In both cases three mRNAs are generated from a single copy gene, one of those being a transcript that is not processed at the usual 3' site in heat-shock. For two of the *Drosophila* transcripts (21) also nuclear localization was reported, due to the failure to remove an intron at elevated temperatures. In both reports the inability of 3'processing is attributed to heat-shock. Since we find that 3'processing is also sensitive to cold stress, a simple temperature-mediated reduction of factor-affinity is unlikely.

Recent reports indicate that splicing precedes polyadenylation (22, 23, 24). In agreement with that, we do not observe spliced molecules in the cytoplasm that are incorrectly 3'processed. We therefore considered the possibility that inability to splice out the intron is the reason for the lack of 3'processing observed under stress conditions (see section 1). Our results from section 5, however, demonstrate that this is not the case, since the terminator also displays leakiness in a heterologous environment, which does not require splicing. Other results from these constructs indicate that incorrect 3'processing is also not the reason for retention in the nucleus (data not shown).

On the other hand, Vincent *et al.* (25) discuss, that active transcription of a downstream gene interferes with the efficiency of 3'processing of the gene lying upstream. Although we can not rule out this possibility in the *in vivo* situation, we have demonstrated by transplanting the 3'processing site into a different genetic environment, that leaky termination is independent of a nearby promoter, but rather a property of the processing site itself.

We conclude that stress sensitive 3'processing is an intrinsic feature of the 'terminator' fragment and not due to a failure of splicing or to effects of neighbouring genes.

Two overlapping promoters drive the P8A7 gene

By deletion analysis we have studied a double promoter transcribing two mRNAs from a single-copy gene. Both RNAs encode the same gene product, a very hydrophobic membrane protein. Tandemly arranged promoters have previously been implicated in the production of stage- or tissue-specific gene products (reviewed in 9).

In dissecting the functions of the *Dictyostelium* PDE triple promoters, it was found that all three promoters retained full functionality by themselves and that they conferred stage specific mRNA expression (10, Faure *et al.* Mol. Cell. Biol. in press). In contrast, the elements constituting the P8A7 promoters are partly overlapping. At first sight, a clear-cut function can be attributed to the two elements of the L promoter: the i-element mediating induction and the b-element being responsible for basal expression in vegetative cells. By the deletion analysis of the

Elements shared by two promoters have been reported for several divergently transcribed genes (26,27). In the case reported here, the two tandemly arranged promoters are interdigitated by common elements.

In most culture conditions, like cells growing in contact with a solid surface, or developing cells in suspension, we detect activity of both promoters in parallel. In heat-shock we find

a

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1 GATCAATTGGGTAATTGGGACCCAAAGAAATCATTCCCATTTTTCAGAGGGGATACATGGGAAGGTGAAATTTATTTGGGCACAAAGTGGAAAGAA
1 D Q L G N W D P K K S F P L K F L Q G D T W E G E I I L A Q S G R I
101 TAGAATATAAATATTGTATTAGTTATTATGATACTGGTGAATTGGTGTATTGGGAAGCTGGTCCAAATAGAGTTATTTTAATTTCTGGTTCTTCAATTTA
35 E Y K Y C I S Y Y D T G E L V Y W E A G P N R V I L I S G S S I Y
201 TAGAAGAGATATCTTTAAAAATCCTTAAAAATAATTTAATAATAATAATAATAAAAAATAATAAAAAATAATAATATGATATGCAAAAAAATA
68 R R D I F K N P *
301 AAAAAATAAAAAATAAAAAAATCATAGTCACAAATAGTCAATTTTTATAATAATAGAATATACATATATCTTAAGGATATCATATCTTATTGTAA
401 AAAAAATAAAAAAACAATAAAAAATAAAGATGTTTAGAAAAAATAAAAAAATAAATAATGGGAAATACAAACCATTCCGACTATTAA
501 ATTATCATGCCAGCCAATTTAGTCAAATTTTGAAAAATTTAAAAAATAATTTTTTATTTTTTTTTTATTTTTTTTAAATTTTCAAAGAAAAAGTT
601 GAGGAAAACCATCGGTAATTCAAAAAATAAAAAAATAAATTTATGCAATGGTACTAGTAATAGGTTATTATATATATTCTCAATTCAATTAACCAAG
701 TATTTGGGTAAACACCTGTCACCTTTTTTATTTTGTGTATCATGATAACAACATTAAATTTACTGATTGCCCAATCAGCCTTTTACCAAAATAAAAAAT
>DP-842
801 TCAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATA
>LP-789
901 GCACCACAACATGATTATTTTAATTTTTTTTTTTTTTTTTTATTTTATTTTGTTTTCAAACGTGTTTGAAGTTTGTAGACTTTTGGAGAAAAAACATT
1001 GTTTTTATTGAAAAAGAAAAAATAAAAAAATAAATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTT
>LP-600 >DP-539
1011 CCATCCAAATATAAGGTAATAACTTTCTCTTTCTTTCTTTTATTTTAAAAATAAAAAATAATTTTATTATAATGTTTTTCTTTTAAAAAATAAAAAA
>LP-486 >DP-449
1201 CAAATCATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTT
>>>LP start >DP-398
1301 TTTTATATTTTCAATTATTATCTAAAAATAAAATCCTATTTCCTATCCGACATTATAAAATTTTCAACATACAATAAATATAAAAAAGTTTAAATATA
<LP CAT fusion point
1401 CAAATTATAATCATATAAATTGTAAAATTAACAACCCACACCACACAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAA
1501 TTTATAGTCTGAAATTGCCAAAATAATCAACACTTCATTATTTAATTTTTTTTTTATTATAATTTAATTAATAATATTATATAAAATTTGTTTTTTTT
>>>SP start
1601 TTTTTTATTTTCTGATATTAATAAAAAAATAAAGAAATTAGAAAAATCATTATATTAATAATTTAAACGTTTAAAAAATAAAAAAATAAAAAAATA
<DP CAT fusion point
1701 AAGAAATAAAAAAATAAAGAACTTTAAAAATGAGCCAAATCAACTGCATATATTATTTTAAATTTTATTTAGTCTGGTTGTTTTATAACAGC
1 M S Q S T A Y I I L N I L V I L A G C F I T A
1801 ATGTGGTATTATTTATTCGTTAATGGTTTATTCATAGTATTATTGGATTGTACTTGGTATTATTATCTTTTGGCTGGTGGTTGATTGTACTCTCTC
24 C G I Y L F V N G L F H S I I G F V L G I Y Y L L A G V C I V L L
1901 GAAATCGTCTTCCACAAAAATTAGTAAATCTTTTGGTGTATATACATATTGGTTTGGTAAAGGTGCTCTTATTTCTTTGtaagtataaaaaaattaa
57 E I V F P Q K L V N L F G F Y T Y W F G K G A L I S L
2001 aaaaaaatattaattattaataaaaaaaaataattatttattaataaaaattttttttttttttttttttttataatatagAATTGGTTTATTAATCC
85 I G L L I L
2101 TTGGAATAGTGGATTCTTTTTCAGCTGGTATTATTGTAATGCGGTGGTATTGTTGTATGATTTTCCATTTCCTTTTAGGTTGTCCAGTCCATT
91 G N S G F F L A A G I I V I A V G I V C M I F H F L L G C P R P L
2201 AATAAATCGTAGTGTGAAAGAAAACCAGAACCAAGGTCAACATGCTTAATCAATTTTTTAAATTAATTTTAAAAAATAAAAAAATAAAAAAATA
124 I N R S V E R K P E P Q G Q H A *
>poly A addition
2301 AGAAACAGTCATGTTTGTATATATATATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATA
2401 TTAATTGATTTTAAATAATTTTTTGATTTTTCAGATTTTAAAAATATTTAATAAAATTTGAAATGGATAAAAAATTAATAATAAAAAAAGTTTAATT
2501 CAAATGGTAAAGTTATTCTTTGTTTTCTCATTTTCAATTGCAGATATGGTAGCTTTTGAAATTTGATC
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Fig. 2. High resolution map of the P8A7 gene. Part a gives the full sequence of the genomic clone. The translated region from pos. 1 to 218 is the open reading frame of the upstream gene, followed by several poly (A) sites. It is unknown which of them is used. The second region of translation is the coding region of the P8A7 gene extending from 1733 to 2252 interrupted by the single intron typed in small letters. Arrow symbols indicate the positions of the start sites mapped in part b. pos 1321 and 1647 are L and S start sites, respectively. Comparison of cDNA and genomic DNA sequence reveals the location of the poly (A) addition site at pos 2373. The AATAAA consensus is underlined, it is followed by a GT/T rich element required for the 3' end processing reaction. The point of the poly (A) tail addition is marked (pos. 2373). Part b shows an autoradiogram of the mapping of the S and L mRNA 5' ends. The left panel uses a probe (P) the *Dra*I fragment from 1272 to 1665 to protect total RNA from heat-shocked cells. The protected fragment (F) indicated by an arrowhead, was determined to be 345 nts in comparison to marker (M) and a sequencing reaction, mapping the L mRNA start site to pos. 1321. The right panel shows the same type of experiment, but the *Ssp*I fragment 1576 to 1765 was used to map the S mRNA 5' end using total RNA from cells developed on filters for 14 hrs. The symbols are used as above. the S mRNA was calculated to start at pos. 1647 since the protected fragment was 118 nts in size. The resolution of the methods used is ± 2 nts. For convenience and to avoid further confusion we propose to number the A (pos. 1733) from the ATG of the P8A7 coding region as nucleotide number 1. Thus the start points of the L mRNA and the S mRNA are at -412 and -86 respectively.

transcriptional shut-down of the P8A7 SP and concurrent activation of the L promoter (11). Like in other organisms, most *Dictyostelium* promoters are also inactivated (e.g. *Dictyostelium* actin 6 and discoidin Ic, Maniak and Nellen, unpublished results), possibly to avoid the accumulation of unprocessed transcripts (28).

Under the conditions used for our heat-shock experiments, we also find an unprocessed transcript, the precursor (P RNA), in the nucleus. We suggest that the reduced efficiency of 3' processing and the shut-down of the S promoter are

Fig. 3. Deletion analysis of the L Promoter. An *Eco*RI to *Hind*III fragment bringing some restriction sites 5' of the genomic clone and sequences to pos 1476 was fused to the CAT reporter gene of vector PAV-CAT (*Xba*I site). The structure was confirmed by sequencing, and after cutting with *Pst*I and *Hinc*II upstream of the promoter, unidirectional deletions were introduced, using the Promega Erase a base system. Cells transformed with construct -798 (left half of the figure) were treated as indicated above the figure, times are in minutes, att is attachment of cell from suspension culture to a solid plastic surface, hs denotes heat-shock treatment in a 30°C waterbath. Development was 8 hrs in phosphate buffered suspension, without the addition of cAMP. The right half of the figure shows the L CAT mRNA (LC) expression of a deletion down to pos. -600 under the same conditions as described above. The lower panel is a shorter exposure of the upper half.

Fig. 4. Deletion analysis of the double promoter. A fragment of the genomic clone from *Eco*RV pos 382 to *Alu*I pos 1781 was cloned into pIC, cut out with *Sau*3A, and ligated into the *Bam*HI site of PAV-CAT to maintain the reading frame between the P8A7 and the CAT coding region. RNA analysis of populations and later clones demonstrated the presence of two CAT specific mRNAs L CAT (LC) and C CAT (SC) in vegetative cells growing on a plastic dish (panel V). Transcript appearance is identical to the complete (full) construct in deletions down to pos. -842. Further deletions retain only S CAT expression in cells developed for 14 hrs on filters (panel D).

compensated by the induction of the L promoter in cells exposed to stress. Indeed, the mature L mRNA could even have an advantage in translation, since it has been reported by Kozak (29) that increasing length of the 5' nontranslated leader favours its translation during stress, while normal cellular messengers are

excluded from translation (30).

We have defined the -798 to -600 region as being responsible for stress induction of the P8A7 L promoter. Though heat-shock genes and heat-shock elements are known in *Dictyostelium* (31,32), we did not find a heat-shock consensus sequence in this region. Together with the observation that stimuli other than heat-shock also lead to induction via this sequence, this may indicate

Fig 5. Schematic representation of the promoter deletions described. The first line denotes the structure of the endogenous P8A7 gene. The deletion constructs of the double promoter (LP and SP) are given in the upper panel. The deletions that were used for characterization of the LP alone are shown in the lower panel. The transcripts generated from these constructs are indicated in the right side columns. The characteristics are added in brackets. The numbers in the first row give the deletion breakpoint with respect to the ATG (see legend of Fig. 2). The size of the boxes representing the promoter deletions are approx. drawn to scale. The right part shows the transcript generated, L & S are endogenous mRNAs, L CAT and S CAT their marker gene homologues. The text in brackets gives characteristics of the transcript: (dev) detected in development, (dead) no transcript, (induc) and (basal) refer to expression in vegetative cells.

that heat-shock is only one aspect of stimulation mediated by an element responsive to multiple signals. Since the induction of the L promoter is mostly accompanied by the downregulation of the S promoter, we suggest that the i-element is the target for a feedback mechanism.

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Fig. 6. The downstream 3' processing site is sensitive to stress and functions in both orientations. To test the ability of the P8A7 3' processing site a 420 nts 3' fragment of the genomic clone was inserted in two orientations into the *HindIII* site of the plasmid pVE (obtained from P. Morandini) in which the discoidin I c promoter drives the expression of the tetracyclin repressor gene from *E. coli*. To render this vector transcriptionally active under stress conditions, the discoidin promoter was replaced by the complete L promoter described before. The right sketch gives the resulting constructs. T1 carries the 'terminator' in wildtype orientation, whereas T3 has the 3' processing site in opposite orientation (denoted by a filled arrow). Northern blots of RNA extracted from cells, which were differently pretreated (times in minutes, hs=heat-shock, cs=cold-shock, Cd=100 μ M Cd⁺⁺), were hybridized with tet-repressor specific *in vitro* transcripts. mRNAs terminating at the two P8A7 3' processing sites are labelled P. In construct T1 a second, minor processing site (larger transcript) is detected. Letter A denotes transcripts passing beyond this point terminating in the actin terminator encountered thereafter.

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