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Copia RNA levels are elevated in dunce mutants and modulated by cAMP

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ABSTRACT

Clones carrying sequences expressed at altered abundance levels in dunce mutants were isolated by differentially screening a genomic library with cDNA probes representing the RNA population from dunce$^+$ flies and the RNA population from dunce mutant flies. These mutants have an elevated cAMP content, so some isolates potentially contain cAMP responsive genes. Two classes of clones were isolated. One class contains genes expressed at a higher steady state abundance level in dunce mutants compared to dunce$^+$ flies and the other contains genes expressed at a lower steady state level in the mutants. The recovery of clones from the differential screen demonstrates that in addition to altering normal behavior, fertility, and cAMP metabolism, dunce mutation confers an alteration in the level of expression of certain genes. The class of clones carrying sequences which are overexpressed in the mutants have been characterized. These clones carry a common repetitive sequence which codes for a 5.5 kb poly(A)$^+$ RNA - the RNA species found to be overexpressed in the mutants. Restriction analysis and hybridization experiments show these repetitive sequences to be members of the copia family of transposable elements. Administration of pharmacological agents to normal flies to increase cAMP levels leads to an increased steady state level of copia RNA. Thus, copia RNA metabolism appears to be influenced by cAMP levels.

INTRODUCTION

The dunce (dnc) mutants of Drosophila melanogaster are of special interest for two primary reasons. First, these mutants have a short-lived memory after being conditioned behaviorally (1-5). Second, dnc is the structural gene for the enzyme, cAMP phosphodiesterase (PDE), and when mutated, the flies lose much of their ability to metabolize cAMP (6-10). In flies carrying amorphic alleles, the activity of the cAMP-specific PDE is completely lost and cAMP levels increase about six fold (9, 10). Since cAMP is known to regulate gene activity in procaryotes and

in eucaryotes, genes that are normally responsive to cAMP levels should be expressed at aberrant levels in the mutants.

In this report, we describe the results of using RNA from dnc$^+$ and dnc mutant flies to carry out differential screens in order to isolate genes expressed at altered abundance levels in the mutants and ones potentially regulated by cAMP. The technique of differential screening has been utilized by many investigators to isolate genes expressed at altered abundance levels under certain circumstances. For example, differential screens have provided clones for genes expressed under specific growth conditions of the organism, at different developmental stages, after addition of hormones or other cofactors, or in a sex-specific manner (selected ref. 11-14). Our differential screens employing dnc mutants have provided clones of sequences expressed at higher abundance levels in the mutants as well ones expressed at lower levels. We previously reported the characterization of a family of serine protease genes which are expressed at decreased level in dnc mutants (15). Here, the specific sequences on the clones which have an elevated expression have been identified as members of the copia family of transposable elements, which suggests that copia RNA metabolism is regulated by cAMP. In addition, feeding normal flies with pharmacological agents known to increase cAMP concentration results in an elevation of copia RNA levels, providing direct evidence that copia RNA levels are modulated by cAMP.

MATERIALS AND METHODS

The dnc mutations, dnc^{M14} and dnc^{M11} were induced in a y cv v f chromosome (9). The latter chromosome was used as a control in experiments with y dnc^{M14} cv v f and y dnc^{M11} cv v f, with the chromosomes carrying the mutations abbreviated as dnc^{M14} and dnc^{M11} in the text for simplicity. The mutants do have significant differences in genetic background relative to the control and to each other from balancing the mutants and from the time elapsed since the mutations were induced. Flies carrying a synthetic deficiency of 86-100 kb of the X-chromosome (16), including the dnc locus, were prepared by mating males of the genotype Df(1)N^{64i16};Sm1,CyDp(1;2)w^{+51b7} to C(1)DX,ywf/w$^+$Y females (16). Males of the genotype Df(1)N^{64i16}/w$^+$Y were

selected and used as dnc deficiency flies [abbreviated Df(1)dnc in the text]. The wild type stock, Canton-S, was used as the dnc^+ control for the deficiency flies. The differential screening methods were essentially those of T. St. John and R. Davis (14). Methods for isolating RNA, selecting the poly(A)$^+$ fraction and synthesizing cDNA have been described (15).

Pharmacologial agents were administered by feeding (15) Canton-S flies (3-5 days old) for three days on 3 ml of standard cornmeal food supplemented with: 0.25 ml of water, 50 mg 5'-AMP in 0.25 ml water, 50 mg cAMP in 0.25 ml water, 50 mg isobutylmethylxanthine in 0.25 ml water, 0.25 ml of 95% ethanol, or 2 mg forskolin in 0.25 ml of 95% ethanol.

RESULTS

Differential screens reveal alterations in the level of expression of certain genes in dnc mutants.

Radiolabeled cDNAs were synthesized using y cv v f and dnc^{M11} poly(A)$^+$ RNA and equal counts of the two probes were used to screen duplicate filters from a plating of 32,000 genomic clones. About 30 clones produced a stronger signal to the y cv v f probe than to the dnc^{M11} probe, and about an equal number produced a stronger signal to the dnc^{M11} probe than to the y cv v f probe. These clones were rescreened several times to select those which reproducibly produced signals of differential intensity with the two probes. From these rescreens, we obtained 7 clones which display less intense signals and 4 clones which display more intense signals to the dnc^{M11} probe compared to the y cv v f probe. Since the signal intensity approximates the abundance of the RNAs homologous to the genomic clones, these clones were saved as potentially containing genes expressed differentially in dnc mutants.

The isolated clones might reflect alterations in gene expression between the two fly strains because of the different state of the dnc locus or because of random differences existing at other genetic loci. To control for genetic background, the final screening included a clone blot (Fig. 1), prepared in triplicate, with each probed with equal counts of y cv v f, dnc^{M11}, or dnc^{M14} cDNAs. Those clones which contain genes expressed at altered abundance levels because these flies carry a mutation at

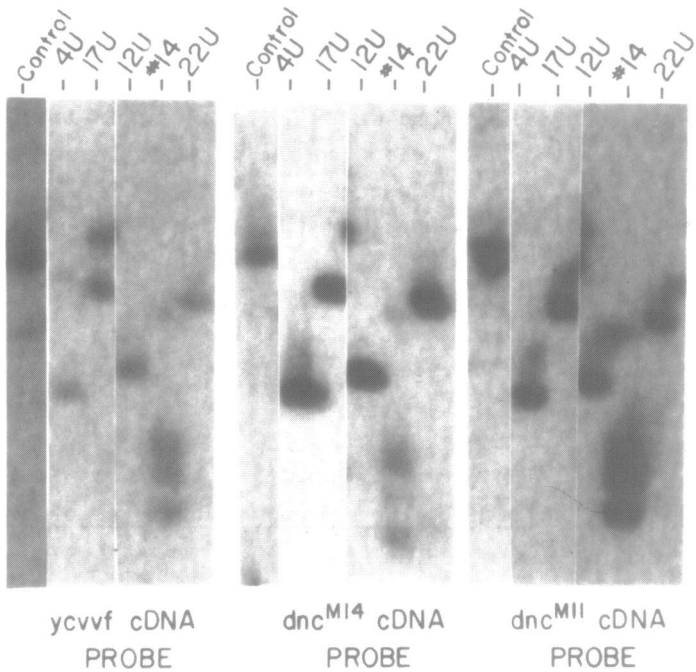

Fig. 1. Clone blots of cARS Up clones hybridized with cDNA probes. Three identical blots of EcoRI-digested phage DNAs hybridized with equal counts of cDNA probes made to poly(A)$^+$ RNA from y cv v f, y dnc^{M14} cv v f, and y dnc^{M11} cv v f male flies. Four clones shown (4U, 12U, 17U, 22U) show an increased signal using dnc mutant cDNAs relative to y cv v f. Clone #14 was picked in the differential screen but failed to show increased signal with both of the dnc mutant cDNA probes.

the dnc locus should display an altered hybridization signal in both dnc mutants relative to y cv v f. Included on the blot is one of several control clones, which we randomly picked in the initial screen from the clones which displayed a signal of equal intensity with the y cv v f and dnc^{M11} probes. Four representative clones clearly show an increase in the signal intensity with dnc mutant cDNA probes (Fig. 1). These are named cARS (cAMP Regulated Sequence) 4U (Up), 12U, 17U and 22U. Densitometry of the autoradiogram reveals that the signals with the dnc mutant cDNA probes are 3-9 times more intense than signals with the y cv v f probe, after normalizing the signal values to that of the control values. We conclude that those clones which behave simi-

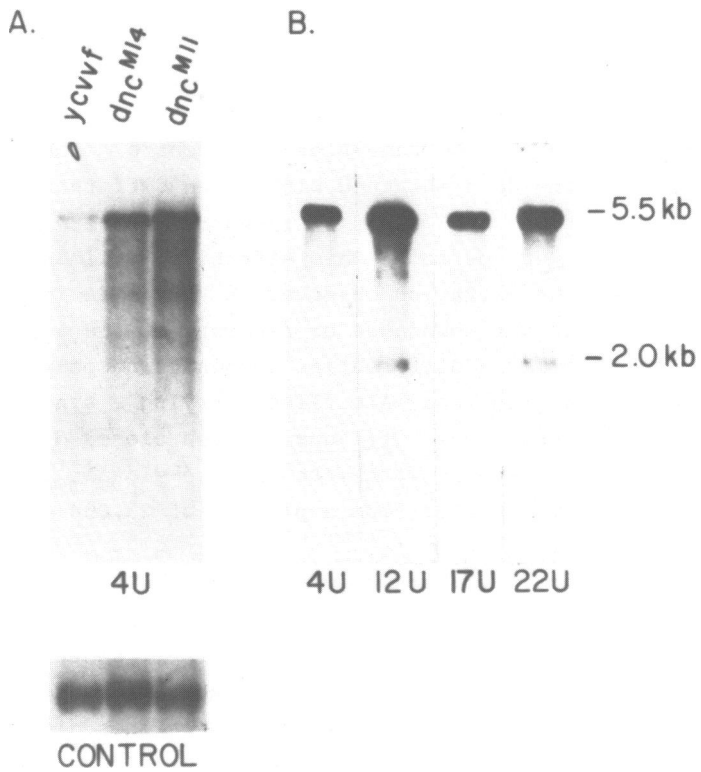

Fig. 2. RNA blots using selected clones as probes. (A) Ten micrograms of poly(A)$^+$ RNA from the three fly strains after blotting and probing with the 2.8 kb EcoRI fragment (Fig. 3) isolated from clone 4U. The bottom panel shows the signal obtained after hybridizing the same blot with control clone 1. (B) Blot strips prepared with Canton-S RNA probed separately with restriction fragments of clones 4U, 12U, 17U, and 22U which hybridized to the total cDNA probe (Fig. 3). Each hybridizes to a ca. 5.5 kb poly(A)$^+$ RNA and some detect a faint band at 2.0 kb.

larly with both dnc mutant probes represent sequences expressed at altered abundance levels because of the absence of dnc$^+$ function. Although several clones were obtained containing sequences underexpressed in dnc mutants (not shown, ref. 15), the remainder of this report is focused on the identity and the regulation of cARS Up clones.

RNA blotting experiments confirm that these clones carry sequences which are differentially expressed in the mutants. The restriction fragment of cARS 4U which hybridizes to total cDNA

(Fig. 3), was used to probe the RNA population from y cv v f, dnc^{M11} and dnc^{M14} flies (Fig. 2A). The signal associated with a ca. 5.5 kb RNA is 4-10 times more intense in the mutant lanes than in the y cv v f lane, after normalizing the signal to that obtained upon reprobing the same blot with control clone 1. Similar results have been obtained with three different batches of y cv v f, dnc^{M11} and dnc^{M14} RNAs isolated on separate occasions over a five year period. This effectively eliminates the possibility that the elevation observed in Figure 2A is due to chance variation in the abundance or recovery of the 5.5 kb RNA.

We also performed a differential screen using poly(A)$^+$ RNA from Canton-S male flies and male flies carrying a synthetic deficiency of the dnc locus, Df(1)dnc. Seven clones displaying less intense signals and 16 with more intense signals to the Df(1)dnc probe were isolated from a plating of 23,000 genomic clones.

cARS Up clones contain members of the copia family of transposable elements.

The clones carrying sequences which are over-expressed in dnc mutants were used in cross-hybridization experiments to detect the existence of any sequence homology. The clones did cross-hybridize (not shown), indicating the existence of a repetitive element common to all clones. This repetitive element resides on the restriction fragment of each clone containing the sequences which are overexpressed (Fig. 1), suggesting that the repetitive element might be the differentially-expressed sequence. When used as probes of RNA blots, each cARS Up clone hybridizes to a ca. 5.5 kb poly(A)$^+$ RNA and occasionally, to a less abundant one of about 2.0 kb (Fig. 2B). Restriction mapping reveals that some clones obtained from separate differential screens overlap and represent the same genomic region (Fig. 3). For example, cARS 22U and 4U were obtained from the y cv v f vs dnc^{M11} screen, and 3U and 1U from the Canton-S vs Df(1)dnc screen, yet 22U overlaps with 3U and 4U overlaps with 1U. The isolation of overlapping clones from screens using different dnc mutants provides additional evidence that the clones carry sequences expressed differentially because of the mutant state of the dnc locus and not from chance differences in genetic background. Furthermore, the

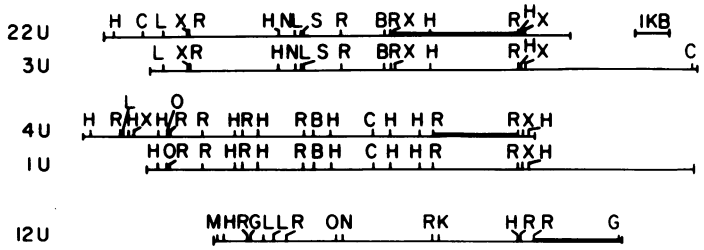

Fig. 3. Restriction maps of several clones, revealing an overlap in restriction site patterns. The broad segment represents the EcoRI fragment which hybridizes to a total cDNA probe (Fig. 1). B = BamHI, O = XhoI, S = SstII, X = XbaI, N = NarI, H = HindIII, R = EcoRI, C = SacI, L = SalI, G = BglII, M = SmaI, K = KpnI. The order of some sites close to one another is not accurate as mapped in bacteriophage clones; i.e., the R-H-X cluster in clones 22U and 3U. Cloned DNA of 12U did not cut well with XbaI, so the predicted XbaI site in the H-R-R cluster was not detected.

maps show that the clones have a similar cluster of restriction sites flanking the EcoRI frament containing the over-expressed and repetitive sequence. This cluster includes one or two EcoRI sites, a HindIII site, and an XbaI site.

Since transposable elements make up the majority of Drosophila middle repetitive DNA (17), we examined known transposable elements to determine whether they were similar to the over-expressed repetitive DNA in the cARS Up clones. Copia elements, which are prototypic for the class of copia-like transposable elements, are known to encode an abundant RNA of about 5 kb and several of lesser abundance, one of which is approximately 2 kb (18-20). In addition, the sequence analyses of copia elements have shown that they contain a restriction site cluster containing a HindIII, an XbaI and three EcoRI sites within a 0.5 kb interval (21, 22). Copia elements, therefore, emerged as excellent candidates for the identity of the repetitive sequences on the cARS clones.

A blot of several Drosophila transposable elements was probed with a representative of the cARS Up clones, 4U. Fig. 4 shows that of five different elements tested, only copia DNA hybridizes. This demonstrates, along with the results of prior experiments, that the cARS Up clones contain copia elements and that it is these elements which are overexpressed in dnc mutants.

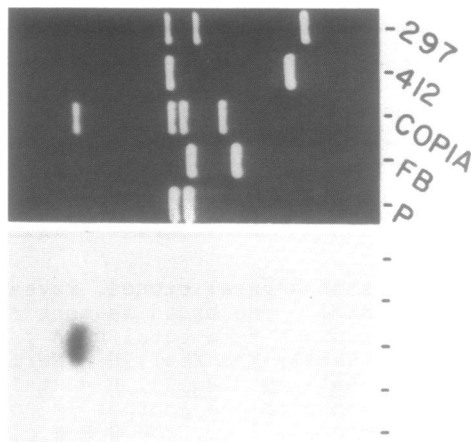

Fig. 4. Blot of transposable element DNAs probed with the 2.8 kb EcoRI fragment of cARS 4U. The following elements were used: 297, a BamHI/XhoI digest of plasmid cDm4006 (38); 412, a BamHI digest of plasmid cDm2042 (38); copia, a BamHI/XbaI digest of plasmid cDm5002 (39); FB, a SalI/BamHI digest of pD75.3 (40); and P, a BamHI digest of pπ25.1 (41). The stained gel is on the left and the corresponding blot on the right.

The most likely explanation for these results is that copia element RNA is expressed at higher abundance levels in dnc mutants due to alterations in transcriptional or post-transcriptional processes. A less-likely alternative is that dnc mutants contain more genomic copies of copia than the control strains producing an elevated RNA level due simply to an increase in copy number. To estimate the copy number of copia elements in the mutants, blots of genomic DNA from the relevant strains were hybridized to a restriction fragment internal to a cloned copia element (Fig. 5A). The signal in the dnc^{M11} and dnc^{M14} lanes was 54 and 60% as intense as the y cv v f lane, after normalization to the signal obtained with a unique sequence probe (Fig. 5B) from the dunce gene (23). In addition, the intensity of the copia band from Df(1)dnc is quite similar to that from Canton-S. These comparable or slightly decreased signal intensities for dnc mutant DNA do not support the possibility that there is an increase in copia copy number sufficient to elevate RNA levels by 5-10 fold. Therefore, we conclude that the increase is due to differences in transcriptional or post-transcriptional processes.

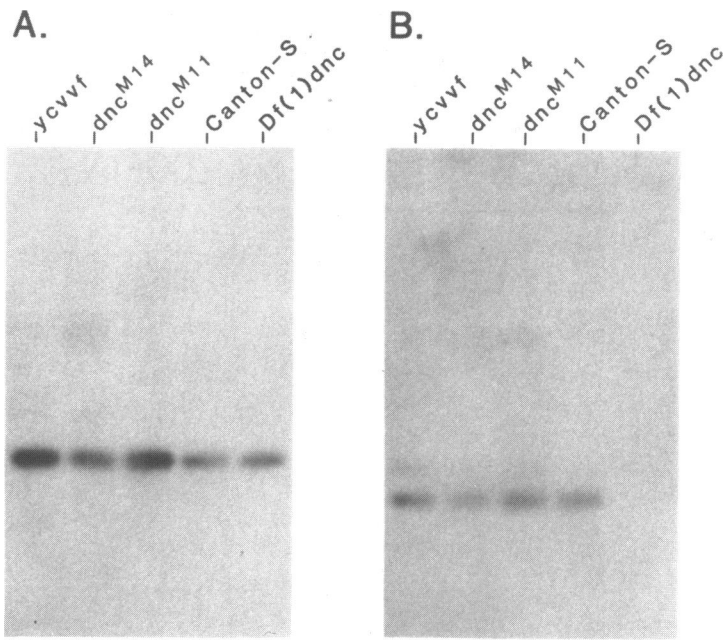

Fig. 5. Genome blots of wild type and dnc mutant strains probed with a restriction fragment from copia or dunce. (A) Genomic DNAs were digested with EcoRI and HpaI, blotted and probed with labeled EcoRI/HpaI (2.0 kb) fragment isolated from cDM5002 (39). The size of the hybridizing bands is identical to that of the probe. (B) The blot shown in (A) was reprobed with a 1.6 kb EcoRI fragment from the dunce gene (23). The size of the detected bands is 1.6 kb.

Administration of pharmacological agents known to increase cAMP levels increases copia RNA abundance in normal flies.

Since dnc mutants have an elevated cAMP content, one attractive hypothesis is that copia RNA levels are increased in the mutants because some aspect of copia transcription or RNA processing or stability is influenced by cAMP concentration. To directly demonstrate that cAMP can influence copia RNA abundance, adult flies were fed cAMP or pharmacological agents known to increase cAMP levels and the abundance of copia RNA in these flies was measured by RNA blotting experiments. The level of copia RNA is not influenced by administration of 5'-AMP, but cAMP, the cAMP PDE inhibitor isobutylmethylxanthine, and the adenylate cyclase activator forskolin, all increase copia RNA abundance approxi-

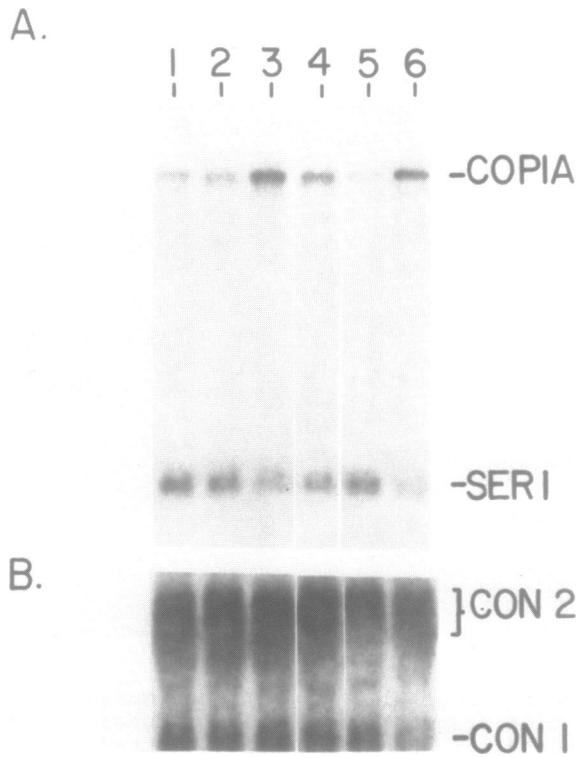

Fig. 6. Blots of RNA from Canton-S flies fed agents to increase cAMP levels. RNA from flies reared on food supplemented with (1) water; (2) 5'-AMP; (3) cAMP; (4) isobutylmethylxanthine; (5) 95% EtOH; and (6) forskolin. The blot was probed with (A) a 2.8 kb XbaI fragment of cDm5002 (39) and a putative serine protease gene (SER1) found to be underexpressed in dnc mutants (15); and subsequently (B) with two control clones (Con 1 and 2) picked in the differential screen.

mately 3-5 fold (Fig. 6). The controls for this experiment included reprobing the blot with two control clones, one of which gives a diffuse signal, and a clone selected in the differential screen as underexpressed in dnc mutants (SER1). This clone encodes a putative serine protease gene (15) whose RNA levels vary inversely with copia levels in the various lanes. This experiment has been repeated with results similar to those shown.

DISCUSSION

Several important conclusions can be drawn from the data presented. First, we have shown that the dnc strains used here

express certain sequences at abundance levels different than normal flies. Therefore, the phenotype of "altered gene expression" can be listed along with the three other classes of phenotype which characterize dnc mutants. These include: (1) alterations in behavioral conditioning (1-5), (2) alterations in cAMP metabolism (6-10), and, (3) female sterility (6, 24). Although it is clear that the amorphic point and deficiency mutants used here exhibit alterations in gene expression, the hypomorphic mutants of dnc may not exhibit this phenotype. Our preliminary experiments have failed to detect any deviation in copia RNA abundance levels in the hypomorphic mutants, dnc^1 and dnc^2, although the deviation in these may be too subtle to be detected. These two mutants retain a considerable portion of cAMP PDE activity and as a consequence, have cAMP levels which are much closer to normal than amorphic mutants (9).

The screening procedures employed detect genes expressed only at high or moderate levels of abundance. A total of 55,000 pfu were screened and approximately 10% of these hybridized to the total cDNA probes. Thirty-four clones were isolated from both screens, so 0.5% (34/55,000 x 10%) of the clones were judged to carry differentially expressed genes of the high or moderate expression classes. We feel that this number is an underestimate of the true number of clones which carry differentially-expressed sequences. The clones ultimately saved were passed through several rescreens and we eliminated many of the initial clones if they displayed a marginal differential signal in any one rescreen.

Second, all of the overexpressed sequences recovered are members of the copia family of transposable elements. We assume that most copies of copia in the genome are expressed at 5-10X their normal level, but we cannot rule out the possibility that the increased expression is due to a few or a single copia element. Assuming that the average transcriptional activity of copia elements in the mutants is equal to that in normal controls, the dnc mutants would require five to ten times more copies of copia in their genomes to explain the increased RNA levels as simply due to copy number differences. The results of genomic blots performed to estimate the copy number of copia in the mutants relative to the controls are not consistent with a

five to tenfold increase in the number of copia elements in the mutant genome.

Third, the results suggest that copia RNA metabolism is under cAMP control although we cannot determine whether the control is direct. When flies were maintained on food supplemented with cAMP, isobutylmethylxanthine, or forskolin, copia RNA levels are observed to increase relative to the appropriate controls. The increases are not of the same magnitude as observed in dnc mutants, but it is expected that the administration of pharmacological agents would be less effective in elevating cAMP levels than a null mutation in the PDE structural gene. We note that not all attempts at pharmacological intervention were successful. For example, our attempts to raise copia RNA levels in Schneider-2 cells with dibutyryl cAMP did not succeed. Copia is expressed at extremely high levels in these cells and has perhaps been released from its normal regulatory contraints. Part of the rationale for directing our attention to feeding experiments and examining expression in the gut is that greater than 90% of the total copia expression in the animal occurs in the gut (unpublished).

Cyclic AMP is known to affect trancriptional rates (25-31) and RNA stability (32, 33) in other eucaryotes. Sequence and mutational analyses of the 5'-flanking regions of several different genes regulated by cAMP have revealed a consensus sequence, TGACGTCA, which usually resides between -30 and -150 of the transcriptional start site and which is contained within a cAMP-responsive enhancer (25-31). A sequence which matches the consensus at six of eight positions, TAACGTTA, resides in the copia LTR at residue 40, approximately 90 residues upstream from the start sites for transcription. Whether this sequence confers cAMP responsiveness upon copia elements is unknown, but it is interesting that the palindromic nature of the cAMP-response sequence is conserved in the putative copia variant. In relation to this, it is intriguing that the retrovirus, HTLV-1, contains three copies of sequences similar to the cAMP response element within its LTR. These sequences contribute basal promoter activity and mediate induction by a viral activator protein (34).

Very little is known about the factors which modulate transposable element expression. These elements show develop-

mental and spatial specificity in expression, and it has been reported that stress, including heat shock, is capable of increasing copia expression (35). It may be that stress and cAMP increase copia RNA expression in the same way. Besides stress and cAMP, hormones and ultraviolet radiation are other factors known to modulate the expression of other transposable elements (12, 36, 37).

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