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ISOLATION AND CHARACTERIZATION OF GENES REGULATED BY CYCLIC AMP IN *Drosophila melanogaster* AND DIFFERENTIALLY EXPRESSED IN *dunce* MUTANTS

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By

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF GENES REGULATED BY CYCLIC-AMP IN *Drosophila melanogaster* AND DIFFERENTIALLY EXPRESSED IN *dunce* MUTANTS.

By

Yungdae Yun

Clones containing sequences expressed at altered abundance levels in *dunce* mutants were isolated by differentially screening a *Drosophila* genomic library with cDNA probes synthesized from the mRNAs of normal *Drosophila* flies and *dunce* mutant flies. Two classes of clones were isolated. One class contains genes expressed at a higher steady state abundance level in *dunce* mutants compared to normal flies and the other contains genes expressed at a lower steady state level in the mutants. Since *dunce* mutants have an elevated cAMP content due to a mutation in the structural gene for cAMP phosphodiesterase, the isolated clones potentially contain cAMP responsive genes.

The clones containing sequences which are overexpressed in the mutants carry a common repetitive sequence which codes for a 5.5 kb poly A+ RNA. Restriction analysis and hybridization experiments show these repetitive sequences to be members of the *copia* family of transposable elements. Administration of pharmacological agents (cAMP, isobutylmethylxanthine or forskolin) to normal flies to increase cAMP levels leads to an increased steady state level of *copia* RNA. Thus, *copia* RNA metabolism is under control of cAMP.

The nucleotide sequence of one clone which is underexpressed in dunce mutants was determined. Within the sequenced genomic region, two complete open reading frames (SER1 and SER2) and part of a third (SER3) exist. All of these were found to be homologous to genes which code for serine proteases. Genome blotting experiments indicate that at least nine genes homologous to these genes are present in a haploid genome. Hybridization experiments with probes representing specifically each of the three sequenced genes suggest that only SER1 (or SER1 related genes) is underexpressed in dunce flies. The SER family of genes are abundantly expressed in the gut, which suggests that a major function of the gene products is probably in digestion. Upon treatment of normal flies with the pharmacological agents cAMP, isobutylmethylanthine or forskolin, the mRNA levels of SER1 (or SER1 related genes) decrease. Thus, mRNA levels of a serine protease gene(s) are regulated negatively by cAMP in Drosophila. This is the first example of regulation of a simple serine protease gene(s) by cAMP.

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ABBREVIATIONS

bp	Base pairs
C	Catalytic subunit of cAMP-dependent protein kinase
cAMP	Adenosine 3', 5'-cyclic monophosphate
cARS	cAMP Regulated Sequence
cGMP	Guanosine 3', 5'-cyclic monophosphate
cDNA	Complementary DNA
CRP	cAMP Receptor Protein
CS	Canton-S
Ddc	Dopa Decarboxylase
DNA	Deoxyribonucleic acid
5'-AMP	Adenosine 5'-phosphate
kb	Kilobases or kilobasepairs
LTR	Long terminal repeat
R	Regulatory subunit of cAMP-dependent protein kinase
RNA	Ribonucleic acid
SDS	Sodium Dodecylsulfate

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cyclic-AMP (cAMP) has been shown to mediate many biological processes in diverse organisms. In procaryotes, cAMP with its receptor protein, CRP, modulates the transcriptional activity of a variety of genes (3, 89). In eucaryotes, the molecule serves as a second messenger through which intracellular events are initiated in response to external stimuli. One important area of cAMP action which is currently under intensive study is its role in modulating eucaryotic gene expression. The genes regulated by cAMP encode proteins with broad range of functions. For example, cAMP has been shown to regulate the mRNA levels of several hormone genes (23, 46, 62, 67, 87), proto-oncogenes (15, 42, 76, 85) and genes encoding key metabolic enzymes (47, 54, 57, 90). The alteration in gene expression, in turn, presumably leads to physiological responses.

Part of my interest in the function of cAMP regulated genes involves their possible role in learning and memory. Previous work on two invertebrates, the sea snail Aplysia (17, 48) and the fruit fly Drosophila (2), has demonstrated that cAMP metabolism is intimately coupled with learning and memory processes. Kandel and his colleagues (39, 40) have proposed a model in which long

term memory is envisioned to result from an alteration in gene expression due to an increased level of cAMP. This model predicts that at least some of the genes regulated by cAMP are functionally involved in long term memory consolidation.

The dunce mutants of Drosophila were originally isolated as learning/memory mutants (32) and subsequently found to have a mutation in the structural gene for the cAMP phosphodiesterase (19, 26). The loss of cAMP degradative capacity results in an increased cAMP content, up to six fold over normal depending on the particular mutation assayed (26). Since cAMP is known to regulate gene expression in procaryotes and in eucaryotes, it is expected that the mRNA levels of certain genes whose expression is modulated by cAMP would be different between normal flies and dunce mutant flies.

Reported here are results of studies designed to isolate genes expressed at altered abundance levels in dunce mutants. The genes were identified by differential screening with labelled cDNA probes prepared from normal and dunce mutant flies and subsequently found to be regulated by cAMP.

This review is divided into four parts. The first part will provide a summary of the general roles of cAMP and its mechanism of action. The second and third parts will be focused on the roles of cAMP in altering gene expression and in learning and memory. The fourth part will provide a summary of dunce work.

Role of cAMP and its mechanism of action.

Since its discovery, cAMP has been shown to regulate numerous cellular processes in both procaryotes and eucaryotes. In E. coli, cAMP bound to its receptor protein (CRP) induces many catabolic enzymes and the represses several biosynthetic enzymes and regulatory proteins (3, 89). The action of cAMP-CRP is apparently mediated at the level of transcription. The best studied systems are operons subject to catabolite repression, like those for utilization of lactose, galactose, or arabinose. For example, the cAMP-CRP complex exerts a positive control by binding to the control region of the lactose operon and increases the efficiency of RNA polymerase transcription (89). The cAMP-CRP complex can also modulate transcription by the control of termination. In the galactose operon, the expression of the promotor-distal genes is reduced with respect to the promotor-proximal gene. This polarity can be relieved by cAMP-CRP (89).

In eucaryotes, cAMP mediates cellular responses to external stimuli in a variety of cells. Peptide hormones, neurotransmitters, and prostaglandins trigger their respective physiological responses by binding to cell surface receptors and changing internal cAMP concentrations in target cells (8). The intracellular cAMP concentration is regulated by a balance between the activities of adenylate cyclase and phosphodiesterase. Guanine-nucleotide-binding proteins (G proteins) appear to play a critical role in the regulation of the adenylate cyclase activity. In the adenylate cyclase system, the

stimulatory and inhibitory receptors are coupled to two G proteins, stimulatory G protein (Gs) and inhibitory G protein (Gi) (37). G proteins are composed of three subunits, α, β, γ subunits. When GTP is bound, the α subunit is dissociated from the $\beta\gamma$ complex and this dissociated α subunit is responsible for the activation or inhibition of adenylate cyclase (37). The cAMP level is also modulated by the degrading capacity of phosphodiesterase. In eucaryotes, multiple forms of cyclic nucleotide phosphodiesterase exist. Some forms specifically degrade either cAMP or cGMP and others degrade both (6).

G proteins that regulate adenylate cyclase activity are members of a large family of guanine nucleotide binding proteins including transducin (82) and Go protein (81). The ras gene family was first found as a cellular homologue of the ras oncogene which have sequence homology to the α subunit of G protein (44). In yeast, two genes, ras1 and ras2, are involved in the cAMP pathway by regulating adenylate cyclase (16). Recent advances in molecular cloning revealed the presence of more G proteins than have been biochemically defined, which suggests a more complex role for G proteins (12, 14).

In eucaryotes, cAMP action is mediated via cAMP-dependent protein kinase, which phosphorylates specific proteins when it is activated by cAMP. The holoenzyme of cAMP-dependent protein kinase is an inactive tetramer composed of two regulatory subunits (R) and two catalytic subunits (C). Binding of two molecules of cAMP to each R subunit releases the C subunits,

which become active and are able to phosphorylate substrate proteins (53). Two major classes (TypeI and TypeII) of cAMP-dependent protein kinases are present in most mammalian cells. These two classes have different R subunits (RI and RII) but seemingly identical C subunits (53). Some C subunit activity is neutralized by the heat stable protein kinase inhibitor, which can inactivate as much as 20% of the total C subunit present (30,91).

Cyclic-AMP regulates several metabolic pathways via cAMP-dependent protein kinase. These include triglyceride synthesis and breakdown, inhibition of fatty acid synthesis, stimulation of gluconeogenesis and glycogen metabolism (22). The best studied system is the control of glycogen metabolism in skeletal muscle. When activated by cAMP, the C subunit of cAMP-dependent protein kinase alters the phosphorylation state of phosphorylase kinase and glycogen synthase (22). Phosphorylation not only changes the catalytic activity of these enzymes but also affects the sensitivity of the enzymes to effectors. For example, the regulation of phosphorylase kinase by two Ca^{++}-binding proteins, calmodulin and troponin-C, depends on the phosphorylation state of the enzyme (22). Calmodulin determines the Ca^{++} sensitivity of the phosphorylated form, while troponin-C determines the sensitivity of the dephosphorylated form. The effects of phosphorylation can be amplified when the initial substrate is a kinase or a phosphatase which in turn modifies a second enzyme. The cAMP-dependent phosphorylation of phophorylase kinase

provides a good example of such a cascade (22).

The role of cAMP in Dictyostelium differentiation is a unique case because in this instance cAMP acts extracellularly like a hormone (36). Dictyostelium grows vegetatively as single cells, but can aggregate to form a multicellular organism when starved. Aggregation is mediated by cAMP pulsing. cAMP is secreted by a cell at an aggregation center and binds to receptors of nearby cells. Nearby cells sense the cAMP concentration and move up the cAMP gradient toward an aggregation center. This aggregate develops into a fruiting body with two terminally differentiated cell types, spores and stalks cells (36). In addition to this role in aggregation, cAMP is involved in the regulation of gene expression throughout the development of Dictyostelium (36). Regulation by cAMP of cell type specific genes in Dictyostelium is mediated by a cell surface cAMP receptor but does not require an increase in intracellular cAMP level by activating adenylate cyclase (41).

Although the influence of cAMP on cell proliferation has been a subject of controversy, recent evidence indicates that cAMP can act as a mitogenic signal both in mammalian cells (13) and in yeast (63). In yeast, cAMP induces mitosis via activation of a cAMP dependent protein kinase. However, an elevated level of cAMP blocks the initiation of meiosis in Xenopus oocytes (61) and in yeast (63).

Cyclic-AMP has also been implicated in the regulation of muscle contractility (31). The phosphorylation of troponin-I in

cardiac muscle fibers contributes to the increased rate of relaxation of cardiac muscle in response to adrenaline (31). Similiarly, phosphorylation of myosin light chain kinase in smooth muscle induces relaxation of the muscle (31). In addition, cAMP controls muscle contractility through the direct modulation of Ca^{++} flux at the membrane level. The phosphorylation of a protein in cardiac sarcoplasmic reticulum, termed phospholamban, by cAMP-dependent protein kinase and by a membrane bound Ca^{++}-calmodulin dependent protein kinase is associated with activation of the sarcoplasmic reticulum ATPase and increased rates of Ca^{++} uptake into these vesicles. This may promote relaxation of the cardiac muscle by adrenaline (22, 31). This example shows that phophorylations stimulated by either cAMP or Ca^{++} are to some degree interdependent.

Regulation of gene expression by cAMP.

One important area of cAMP action which is currently under intense investigation is its effect on the regulation of gene expression. As already stated, cAMP in bacteria acts as an allosteric effector, after binding to CRP, to change the transcriptional activity of many genes including catabolite sensitive operons. This appears to be the only role of cAMP in bacteria (3, 89).

In eucaryotes, various genes have been reported to be regulated by cAMP. Expression of several hormone or neurotransmitter genes are known to be regulated by cAMP. These

include the genes encoding growth hormone (93), prolactin (62), somatostatin (67), gonadotropin (46), vasoactive intestinal peptide (87), proopiomelanocortin (60), and proenkephalin (23). Cyclic-AMP regulates the mRNA levels of these genes in a positive manner and at least in part at the level of transcription. The proto-oncogenes c-myc and c-fos can also be induced at the level of mRNA by increases in cAMP (15, 42, 76, 85). Often these increases in cAMP are intermediary steps in stimulation by growth factors. For example, the induction of c-myc and c-fos by epidermal growth factor in fibroblast cells (76) is cAMP-dependent. The mRNA levels of tubulin and actin, the main protein constituents of microtubules and microfilaments, are also modulated by cAMP (38). Tubulin mRNA levels are modulated biphasically by cAMP, increasing at low concentrations and decreasing at higher concentrations (38). The induction of plasminogen activator by calcitonin and vasopressin is mediated by cAMP at the level of mRNA (69). The mRNA levels of the transferrin receptor gene were recently shown to decrease when levels of cAMP increased (86). In addition, mRNA levels of several genes encoding key enzymes in different metabolic pathways are regulated by cAMP either positively or negatively. The positively regulated genes include those encoding tyrosine hydroxylase (57), phosphoenol pyruvate carboxykinase (54), lactate dehydrogenase (47), tyrosine aminotransferase (72), and alkaline phosphatase (35). Cyclic-AMP regulates the mRNA levels of these genes at the level of transcription. On the other hand,

mRNA levels of pyruvate kinase (90) and dihydrofolate reductase (96) are regulated negatively by cAMP at the level of transcription. Thus, the extent of current knowledge regarding the role of cAMP in the regulation of gene expression is that cAMP modulates the activity of various genes by altering mRNA abundance, either positively or negatively and in most cases, this modulation is at least in part at the level of transcription.

The mechanism by which cAMP alters the expression of genes in eucaryotes is not well understood. cAMP may act by altering the phosphorylation states of nuclear proteins, which in turn affect the transcription rates of specific genes. Alternatively, as in bacteria, cAMP could bind to a receptor protein, like R subunit of protein kinase, which would interact directly with the regulatory region of the DNA wihout being phosphorylated. Both R and C subunits of protein kinase have been suggested as mediators of cAMP action in alteration of gene expression. The R subunit has been proposed as a candidate based on the following rather indirect evidence. First, the amino acid sequence of RII subunit is homologous to that of the bacterial CRP which binds to DNA and modulates transcription (94). Second, RII may have intrinsic topoisomerase activity, and when phosphorylated can alter the degree of DNA supercoiling (24). Third, translocation of the R subunit into the nucleus increases as the intracellular concentration of cAMP increases (52).

Several observations support the alternative hypothesis that

the C subunit of protein kinase mediates cAMP action. First, free C subunit, when microinjected into hepatoma cells, causes an increase in tyrosine aminotransferase synthesis (10). Second, the transfection of the gene which codes for the heat stable inhibitor of cAMP-dependent protein kinase (95) results in inhibition of the cAMP-stimulated expression of a cotransfected reporter gene (43). This result indicates that active C subunit is a necessary intermediate in the stimulation of gene transcription by cAMP, since protein kinase inhibitor specifically binds the dissociated C subunits. However, the result does not exclude R subunit as a necessary phosphoprotein in transcriptional regulation by cAMP.

There have also been efforts to understand the mechanism of cAMP action through the study of trans-activating factors. The study of several cAMP regulated genes in mammalian cells by deletion analysis identified a short stretch of DNA which is responsible for cAMP sensitivity in the 5′ flanking region of the gene. The genes studied include phosphoenol pyruvate carboxykinase (79), somatostatin (67), proenkephalin (23), vasoactive intestinal peptide (87), and gonadotropin genes (46, 80). The sequences of these cAMP responsive elements strongly resemble each other; an 8 base pair consensus sequence 5′-TGACGTCA-3′ can be derived. Montiminy et. al. (68) purified the trans-activating factor which binds to the cAMP responsive element of the somatostatin gene and showed that it is phosphorylated by cAMP-dependent protein kinase.

The work on proenkephalin gene (23) suggests the possible intersection of cAMP and protein kinase C in regulation of gene expression. The expression of this gene is modulated both by cAMP and phorbol ester. The DNA sequences required for regulation by both cAMP and phorbol ester map to the same stretch of DNA, including the cAMP responsive element described above, in the 5′ flanking region of the gene. This finding raises the possibility that phorbol ester, known to act via activation of protein kinase C (7, 71), may act to increase intracellular levels of cAMP, activate cAMP-dependent protein kinase, or phosphorylate an trans-activating factor common to both pathways.

Several groups reported the localization of the phorbol ester responsive elements in the 5′ flanking region of several phorbol ester inducible genes (4, 49, 64) and found that they share a seven base pair motif 5′-TGAGTCA-3′ which is identical to the cAMP responsive element 5′-TGACGTCA-3′ except for the absence of cytosine residue in the middle of the motif. The trans-activating factor which binds to this seven base pair motif of phorbol ester inducible genes has been purified (5, 56) and found to be identical to the mammalian transcription factor AP-1 previously described (55). AP-1 is activated only by phorbol ester but not by cAMP (45). The relationship of AP-1 and the trans-activating factor that is sensitive to cAMP remains to be determined.

Recently, another trans-activating factor, AP-2, was described which mediates both phorbol ester and cAMP responses

(45). AP-2 recognizes a totally different stretch of DNA from the cAMP responsive element described above (45). AP-2 is the first example of a trans-activating factor which mediates gene induction in response to two distinct signalling pathways, cAMP and protein kinase C .

Role of cAMP in learning and memory.

The study of learning and memory in higher forms of animals is difficult due to their complex nervous system. Therefore, the reductionist approach using an organism with a simpler nervous system is justified assuming that elementary molecular events underlying learning and memory are shared throughout phylogeny. One organism that seems particularly useful for a molecular analysis of learning and memory is the marine mollusc Aplysia. This animal has a relatively simple nervous system with large identifiable neurons that are accessible for electrophysiological and biochemical studies. The molecular model of learning and memory established from the study of Aplysia has been described in recent reviews (17, 39, 40, 48). The model predicts that learning and memory occur at the same locus, the synapse. They result from alterations in synaptic connections mediated by changes in cAMP concentration and subsequent changes in neurotransmitter release. Experimental observations indicate that memory has at least two forms: a short-term form that can last seconds, minutes and hours,and a long-term form lasting days, weeks and years.

Short-term memory does not require the synthesis of new proteins and is thought to depend on second messenger-mediated covalent modifications of previously synthesized proteins that modulate the properties of nerve cells and their synaptic connections (17, 48). Molecular mechanisms underlying the short term memory are best illustrated in the sensitization of Aplysia (17, 48). The animal learns to enhance its gill or siphon withdrawal reflex in response to a noxious stimulus applied to its head or tail. At the molecular level, the first step of sensitization involves the release of neurotransmitters, including serotonin, from the facilitating interneurons. Serotonin released from the nerve terminal of the facilitating interneurons binds to receptors on the surface of the nerve terminal of the sensory neuron and activates adenylate cyclase system to increase the level of cAMP. One consequence of the increased level of cAMP is the closure of the S-channel (serotonin sensitive K^+ channel) through phosphorylation by cAMP-dependent protein kinase. As a result, repolarizing K^+ currents are decreased and action potentials become broader (presynaptic facilitation). Consequently, the Ca^{++} influx that normally occurs during the action potential is increased and this enhances the transmitter release and activity of motor neurons. Thus, short-term memory in Aplysia is the result of high levels of cAMP in presynaptic neurons and the subsequent release of neurotransmitter (17, 48).

Long-term memory can be distinguished from short-term

memory at the molecular level, since long-term memory requires new protein synthesis. Inhibitors of RNA synthesis or protein synthesis selectively block the long-term facilitation of the synaptic connection without interfering with the short-term facilitation (66). The long-term facilitation also requires protein synthesis only while the stimulus is being applied (66). Inhibiting protein or RNA synthesis after the period of training fails to block long-term memory. Thus long-term memory is thought to be the result of altered gene expression due to the changes in levels of second messengers including cAMP (61, 62). This model of long-term memory predicts that at least some of the genes regulated by cAMP are involved in long-term memory processes. Recently, it has been shown that other second messengers, inositol trisphosphate and protein kinase C, also may be involved in learning and memory consolidation (9).

The genetic analyses of learning and memory in Drosophila have independently shown that cAMP plays a critical role in learning and memory. Flies can be trained in various learning paradigms, both associative and non-associative. For example, flies can be trained to avoid an odorant associated with electrical shock (73). Several X-linked mutants have been isolated that fail to display this learning. The first mutant isolated was dunce (32) and since then, four other mutants rutabaga, amnesiac, turnip and cabbage (1, 74) have been isolated. In addition, the mutant Ddc with a second chorosome mutation may have the learning defect (84, 58).

For some of the mutants, the biochemical defects are known. The dunce flies have a mutation in the structural gene for cAMP-phosphodiesterase (19). Drosophila has two different phosphodiesterase activities and dunce flies show reduced or eliminated levels of cAMP-phosphodiesterase activity depending on specific mutation assayed (26). The rutabaga flies show reduced adenylate cyclase activity. Adenylate cyclase in Drosophila is heterogeneous and rutabaga lacks the form of adenylate cyclase activity activated at low Ca^{++} concentrations (59). It has been speculated that turnip flies may have a defect in the signal transduction system coupled to serotonin receptors (88). All these biochemical defects mentioned above affect sequential steps in cAMP signalling pathway. These results from Drosophila work together with the findings in Aplysia indicate that cAMP second messenger systems play an essential role in learning and memory.

Some of the mutants described above were subsequently found to perform poorly in other learning paradigms including habituation and sensitization (34), leg position conditioning (11), reward type associated learning (83), and experience dependent courtship behaviour (75). Careful examination of the learning and memory ability of isolated mutants demonstrated that dunce, rutabaga, amnesiac and cabbage flies show appreciable learning initially, but their memory decays very rapidly compared to wild type flies (33, 88). Therefore, the mutants dunce, rutabaga, amnesiac and cabbage can be classified as memory

mutants rather than learning mutants.

The dunce mutants.

Adult flies of Drosophila have at least two different forms of phosphodiesterase activity. Form I phosphodiesterase degrades both cAMP and cGMP as its substrate whereas form II is specific for cAMP and is designated as cAMP-phosphodiesterase (25). Form I phosphodiesterase is activated by Ca^{++}-calmodulin, but form II is not (92). Several observations indicate that dunce flies have a defective form II phosphodiesterase. Six different mutations in dunce have been isolated (26, 32, 65). All reduce or eliminate the activity of form II phophodiesterase. The duncеM14, duncеM11 and duncеML mutants completely lack form II activity, but dunce1, dunce2 and duncеCK exhibit reduced levels of form II activity (26). Moreover, two of the mutations alter the enzyme activity in ways which indicate a structural alteration of the enzyme. The dunce1 allele produces a form II enzyme that is more labile to heat than normal and the dunce2 allele produces an enzyme with higher Km (50). In addition, form II phosphodiesterase activity is proportional to the dosage of polytene chromosome band 3D4, where the dunce mutations have been localized (51, 77). The loss of cAMP degrading activity results in high cAMP levels in the dunce mutants, up to six fold over normal in the amorphic mutants such as duncеM14 or duncеM11.

The dunce gene has been cloned by chromosomal walking and recombination mapping using restriction site polymorphisms as

genetic markers (28). The amino acid sequence of the dunce open reading frame shows extensive homology to those of the mammalian and yeast phosphodiesterases (18). This with the other evidence mentioned above clearly indicate that the dunce gene encodes a phosphodiesterase. The dunce gene locus generates a complex set of mRNAs ranging in size from 4.2 to 9.5 kb (29) and subsequent gene structure characterization revealed that these RNAs are generated by a complex process including alternative splicing (19, 20, 21). The dunce gene extends over 107 kb and contains at least two other genes within one of its intron, including the glue protein Sgs-4 (20, 21).

One other phenotype of dunce is female sterility. The dunceM14 and dunceM11 mutations were originally isolated on the basis of female sterility (65) and later other dunce mutants were also found to have female sterility (77, 78). Female dunce mutants lay few eggs even though the severity of female sterility is highly subject to genetic background (77). The reason for this female sterility is unknown, but cAMP has been reported as a inhibitor of meiosis in Xenopus (61) and yeast (63). The amino acid sequence of a part of the dunce open reading frame was found to have homology to that of egg-laying hormone of Aplysia (19). Whether this has an effect on female sterility is unknown.

Chapter II describes the differential screens employing dunce mutants. The differential screens have provided clones of sequences expressed at higher abundance levels in the mutants as well as ones expressed at lower levels. The specific sequences on

the clones responsible for elevated expresssion have been idenfied as members of the copia family of transposable elements. Feeding normal flies with pharmacological agents known to increase cAMP levels results in elevation of copia RNA levels, indicating that copia RNA levels are directly modulated by cAMP.

Chapter III describes the characterization of a genomic clone under-expressed in dunce mutants. This clone contains genes coding for putative proteins homologous to serine proteases. The mRNA levels of a gene (SER1) contained in this clone are reduced in the dunce mutants. Feeding normal flies with pharmacological agents known to increase cAMP levels leads to reduction of mRNA levels of SER1 gene. This is the first example of a simple serine protease gene regulated by cAMP.

LITERATURE CITED

1. Aceves-Pina, E. O., and W. G. Quinn. 1979. Science. 206:93-96.

2. Aceves-Pina, E. O., R. Booker, J. S. Duerr, M. S. Livingstone, W. G. Quinn, R. F. Smith, P. P. Sziber, B. L. Tempel, and T. P. Tully. 1984. Cold Spring Harbor Symp. Quant. Biol. 48:831-840.

3. Adhya, S., and S. Garges. 1982. Cell 29:287-289.

4. Angel, P., I. Baumann, B. Stein, H. Delius, H. J. Rahmsdorf, and P. Herrlich. 1987. Mol. Cell. Biol. 7:2256-2266.

5. Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Cell 49:729-739.

6. Beavo, J. A., R. S. Hansen, S. A. Harrison, R. L. hurwitz, T. J. Martins, and M. C. Mumby. 1982. Mol. Cell. Endocrinology 28:387-410.

7. Berridge, M. J., and R. F. Irvine. 1984. Nature 312:315-321.

8. Berridge, M. J. 1985. Sci. Amer. 253,4:142-152.

9. Berridge, M. J. 1986. Nature 323:294-295.

10. Bony, C., D. Fink, D. Schlichter, K. Carr, and W. D. Wicks. 1983. J. Biol. Chem. 258:4911-4918.

11. Booker, R. and W. G. Quinn. 1981. Proc. Natl. Acad. Sci. 78:3940-3944.

12. Bourne, H. R. Nature 321:814-816.

13. Boynton, A. L., and J. F. Whitefield. 1983. Adv. Cyclic Nucleotide Res. 15:193-294.

14. Bray, P., A. Carter, C. Simons, V. Guo, C. Puckett, J.Kamholz, A. Spiegel, and M. Nirenberg. 1986. Proc. Natl. Acad. Sci. 83:8893-8897.

15. Bravo, R., M. Neuberg, J. Burckhardt, J. Almendral, R. Wallich, and R. Muller. Cell 48:251-260.

16. Broek, D., N. Samily, O. Fasano, A. Fujiyama, F. Tamanoi, J. Northup, and M. Wigler. Cell 41:763-769.

17. Byrne, J. H. 1985. Trends in Neurosci. 8:478-482.

18. Charbonneau, H., N. Beier, K. A. Walsh, and J. A. Beavo. 1986. Proc. Natl. Acad. Sci. 83:9308-9312.

19. Chen, C-N, S. Denome, and R. L. Davis. 1986. Proc. Natl. Acad. Sci.83:9313-9317.

20. Chen, C-N, T. Malone, S. K. Beckendorf, and R. L. Davis. 1987. Nature 329:721-724.

21. Chen, C-N, and Davis, R. L. 1988. Submitted to EMBO.J.

22. Cohen, P. 1982. Nature 296:613-619.

23. Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman. Nature 323:353-356.

24. Constantinou, A. I., S. P. Squinto, and R. A. Jungmann. 1985. Cell 42:429-437.

25. Davis, R. L., and J. A. Kiger. 1980. Arch. Biochem. Biophys. 203:412-421.

26. Davis, R. L., and J. A. Kiger. 1981. J. Cell. Biol. 90:101-107.

27. Davis, R. L., and Kauvar, L. M. 1984. Adv. Cyclic Nucleotide Res. 16:393-402.

28. Davis, R. L., and N. Davidson. 1984. Mol. Cell. Biol. 4:358-367.

29. Davis, R. L., and N. Davidson. 1986. Mol. Cell. Biol. 6:1464-1470.

30. Demaille, J. G., K. A. Peters, and E. H. Fischer. 1977. Biochemistry. 16:3080-3086.

31. Demaille, J. G., and J-F. Pecheres's Group. 1983. Adv. Cyclic Nucleotide Res. 15:337-371.

32. Dudai, Y., Y-N, Jan, D. Byers, W. G. Quinn, and S. Benzer. 1976. Proc. Natl. Acad. Sci. 73:1684-1688.

33. Dudai, Y. 1983. Proc. Natl. Acad. Sci. 80:5445-5448.

34. Duerr, J. S., and W. G. Quinn. 1982. Proc. Natl. Acad. Sci. 79:3646-3650.

35. Firestone, G. L., and E. C. Heath. 1981. J. Biol. Chem.

256:1396-1403.

36. Gerisch, G. 1987. Annu. Rev. Biochem. 56:853-879

37. Gilman, A. G. 1984. Cell 36:577-579.

38. Ginzburg, I., S. Rybak, Y. Kimhi, and U. Z. Littauer. 1983. Proc. Natl. Acad. Sci. 80:4243-4247.

39. Goelet, P., and E. R. Kandel. 1986. Trends in Neurosci. 9:492-499.

40. Goelet, P., V. F. Castelluci, S. Schacher, and E. R. Kandel. 1986. Nature 322:419-422.

41. Gomer, R. H., D. Armstrong, B. H. Leichtling, and R. A. Firtel. 1986. Proc. Natl. Acad. Sci. 83:8624-8628.

42. Greenberg, M. E., L. A. Green, and E. B. Ziff. 1985. J. Biol. Chem. 260:14101-14110.

43. Grove, J. R., D. J. Price, H. M. Goodman. and J. Avruch. 1987. Science 238:530-533.

44. Hurley, J. B., M. I. Simon, D. B. Teplow. 1984. Science 226: 860-862.

45. Imagawa, M., R. Chin, and M. Karin. 1987. Cell 51:251-260.

46. Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener. 1987. Mol. Cell. Biol. 7:3032-3040.

47. Jungmann, R. A., D. C. Kelly, M. F. Miles, and D. M. Milkowski. 1983. J. Biol. Chem. 258:5312-5318.

48. Kandel, E. R., and J. H. Schwartz. 1982. Science 218:433-443.

49. Karin, M., and R. I. Richards. 1982. Nature 299:797-802.

50. Kauvar, L. M. 1982. J. Neurosci. 10:1347-1358.

51. Kiger, J. A., and E. Golanty. 1979. Genetics 91:521-535.

52. Kondrashin, A. A. 1985. Trends in Biochem. Sci. 10:97-98.

53. Krebs, E. G. and J. A. Beavo. 1979. Annu. Rev. Biochem. 48:923-959.

54. Lamers, W. H., R. W. Hanson, and H. M. Meisner. 1982. Proc. Natl. Acad. Sci. 79:5137-5141.

55. Lee,W., A. Haslinger, M. Karin, and R. Tjian. 1987. Nature 325:368-372.

56. Lee, W., P. Mitchell, and R. Tjian. 1987. Cell 49:741-752.

57. Lewis, E. J., A. W. Tank, N. Weiner, and D. M. Chikaraishi. 1983. J. Biol. Chem. 258:14632-14637.

58. Livingstone, M. S., and B. L. Tempel. 1983. Nature 303:67-70.

59. Livingstone, M. S., Sziber, P. P, and W. G. Quinn. 1984. Cell 37:205-215.

60. Loh, Y. P., B. Myers, B. Wong, D. C. Parish, M. Land, and M. E. Goldman. 1985. J. Biol. Chem. 260:8956-8963.

61. Maller, J. L., and E. G. Krebs. 1980. Curr. Top. Cell. Reg. 16:272-311.

62. Maurer, R. A. 1981. Nature 294:94-97.

63. Matsumoto, K., I. Uno, and T. Ishikawa. 1983. Cell 32:417-423.

64. Matrisan, L. M., P. Leroy, C. Ruhlman, M. C. Gesmal, and R. Breathnach. 1986. Mol. Cell. Biol. 6:1679-1686.

65. Mohler, J. D. 1977. Genetics 85:259-272.

66. Montarolo, P. G., P. Goelet, V. F. Castelluci, J. Morgan, E. R. Kandel, and S. Schacher. 1986. Science 234:1249-1253.

67. Montiminy, M. R., K. A. Savarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Proc. Natl. Acad. Sci. 83:6682-6686.

68. Montiminy, M. R., and L. M. Bilezikjian. 1987. Nature 328:175-178.

69. Nagamine, Y., M. Saudol. and E. Reich. 1983. Cell 32:1181-1190.

70. Nagamine, Y. and E. Reich. 1985. Proc. Natl. Acad. Sci. 82:4606-4610.

71. Nishizuka, Y. 1984. Nature 308:693-698.

72. Noguchi, T., M. Diesterhaft, and D. Granner. 1982. J. Biol. Chem. 257:2386-2390.

73. Quinn, W. G., W. A. Harris, and S. Benzer. 1974. Proc. Natl.

Acad. Sci. 71:708-712.

74. Quinn, W. G., P. P. Sziber, and R. Booker. 1979. Nature 277:212-214.

75. Quinn, W. G., and R. J. Greenspan. 1984. Annu. Rev. Neurosci. 7:67-93.

76. Ran, W., M, Dean, R. A. Levine, C. Henkle, and J. Campisi. 1986. Proc. Natl. Acad. Sci. 83:8216-8220.

77. Salz, H. K., R. L. Davis, and J. A. Kiger. 1982. Genetics 100:587-596.

78. Salz, H. K., and J. A. Kiger. 1984. Genetics 108:377-392.

79. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson. 1986. J. Biol. Chem. 261:9721-9726.

80. Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson. 1987. Proc. Natl. Acad. Sci. 84:2198-2202.

81. Sternweiss, P. C. and J. D.Robishaw. 1984. J. Biol. Chem. 259:13806-13813.

82. Stryer, L. 1983. Cold Spring Harbor Symp. Quant. Biol. 48:841-852.

83. Tempel, B. L., N. Bonini, D. R. Dawson, and W. G. Quinn. 1983. Proc. Natl. Acad. Sci. 80:1482-1486.

84. Tempel, B. L., M. S. Livingstone, and W. G. Quinn. 1984. Proc. Natl. Acad. Sci. 81:3577-3581.

85. Tramontano, D., W. W. Chin, A. C. Moses, and S. H. Ingbar. 1986. J. Biol. Chem. 261:3919-3922.

86. Trepel, J. B., O. R. Colamonici, K. Kelly, G. Schwab, R. A. Watt, E. A. Sausville, E. S. Jaffe, and L. M. Neckers. 1987. Mol. Cell. Biol. 7:2644-2648.

87. Tsukuda, T., J. S. Fink, G. Mandel, and R. H. Goodman. 1987. J. Biol. Chem. 262:8743-8747.

88. Tully, T., and W. G. Quinn. 1985. J. Comp. Physiol. 157:263-277.

89. Ullmann, A., and A. Danchin. 1983. Adv. Cyclic Nucleotide Res. 15:1-53.

90. Vaulont, S., A. Munnich, J-F. Decaux, and A. Kahn. 1986. J.

Biol. Chem. 261:7621-7625.

91. Walsh, D. A., C. D. Ashby, C. Gonzalez, D. Calkins, E. H. Fischer, and E. G. Krebs. 1971. J. Biol. Chem. 246:1977-1985.

92. Walter, M. F., and J. A. Kiger. 1984. J. Neurosci. 4:495-501.

93. Waterman, M., G. H. Murdoch, R. M. Evans, and M. G. Rosenfeld. 1985. Science 229:267-269.

94. Weber, I. T., K. Takio, K. Titani, and T. A. Steitz. 1982. Proc. Natl. Acad. Sci. 79:7679-7683.

95. Whitehouse, S., and D. A. Walsh. 1982. J. Biol. Chem. 258:3682-3687.

96. Wu, J-S. R., L. F. Johnson. 1982. J. Cell. Physiol. 110:183-189.

CHAPTER II

copia RNA LEVELS ARE ELEVATED IN *dunce* MUTANTS AND MODULATED BY CYCLIC-AMP.

This chapter describes the results of a differential screen to isolate genes expressed at altered abundance levels in *dunce* mutants and potentially regulated by cAMP. The differential screening employing *dunce* mutants provided clones containing sequences expressed at either higher or lower abundance levels in the mutants. Clones containing sequences which are overexpressed in the mutants were characterized and shown to carry a common repetitive sequence, the *copia* family of transposable elements. Treatment of normal flies with pharmacological agents known to increase cAMP levels resulted in an increased level of *copia* RNA. The experiments described in Fig. 1, 4, 5 were performed by Ronald L. Davis.

MATERIALS AND METHODS

Fly Strains.

The mutants, dunceM14 and dunceM11 were induced in an X-chromosome (3, 19) carrying the visible markers yellow, crossveinless, vermillion and forked (y cv v f). The only difference in genetic background between these genotypes should be those which incurred during mutagenesis and subsequent manipulation and maintenance of stocks. Therefore, y cv v f chromosome was used as a control in experiments with dunceM14 and dunceM11.

Flies carrying a synthetic deficiency of 86-100 kb of the X-chromosome (9), including the dunce locus, were prepared by mating males of the genotype Df(1)N64i16;SM1, CyDp(1;2)w+51b7 to C(1)DX, ywf/w+Y females (9). Males of the genotype Df(1)N64i16/w+Y were selected and used as dunce deficiency flies (abbreviated as Df(1)dnc in the text). The wild type stock, Canton-S, was used as the dunce$^+$ control for the deficiency flies.

RNA isolation.

Total RNA was isolated from adult flies not older than 5 days as described previously (10). Poly A$^+$ RNA was selected by one pass over an oligo-dT cellulose column. RNA blotting and hybridizations were performed as described (10).

Differential screening.

Complementary DNA probes were synthesized by priming poly A^+ RNA with oligo-dT. The differntial screening methods were essentially those of T. St. John and R. Davis (31). Abundance measurements of RNAs were obtained by scanning autoradiograms with a densitometer and normalizing the experimental signal to that obtained for the control signal.

Treatment of flies with pharmacological agents.

Three to five day old adults of wild type Canton-S flies were grown in vials containing 3 mls of regular corn meal media supplemented with pharmacological agents for 3 days as described (2). Cyclic-AMP (50 mg per vial) and isobutylmethylxanthine (50 mg per vial) were first added in 250 ul of water and then mixed with media. As controls for cAMP and isobutylmethylxanthine, media were supplemented either with 5'-AMP in 250 ul of water or with water alone. Forskolin (2 mg per vial) was dissolved in 250 ul of 95% ethanol and added to the media. As a control for forskolin, media were supplemented with 250 ul of 95% ethanol. Total RNA was isolated by homogenizing flies in 1:1 mixture of Holmes-Bonner buffer and phenol as described (5). The concentration of total RNA was measured with spectrophotometer and equal amounts of RNA samples were loaded in each lane.

RESULTS.

Differential screening reveals alterations in the expression level of certain genes in dunce mutants.

Poly A^+ RNA was isolated from y cv v f male flies and dunceM11 male flies. Radiolabelled cDNAs were synthesized using the two RNA populations as templates and equal counts of the two probes were used to screen duplicate filters from a plating of 32,000 genomic clones. The general scheme of differential hybridization is depicted in Fig. 1. About 30 clones showed a stronger signal to the y cv v f cDNA probe than to the dunceM11 cDNA probe, and a similiar number of clones showed a stronger signal to the dunceM11 cDNA probe than to the y cv v f cDNA probe. These clones were rescreened several times to select those which hybridize reproducibly to the two probes with differential intensity. From these rescreens, 7 clones displaying less intense signals to the dunce mutant probe and 4 clones displaying more intense signal to the dunce mutant probe than to the y cv v f probe were obtained. Since the signal intensity represents the abundance of the RNAs homologous to the genomic clones, these clones potentially contain the genes differentially expressed in dunce mutants.

The isolated clones might reflect alterations of gene expression between the two fly strains because of the different state of the dunce locus or because of random differences in genetic background. To control for genetic background, the clones

Fig. 1. The differential hybridization scheme.

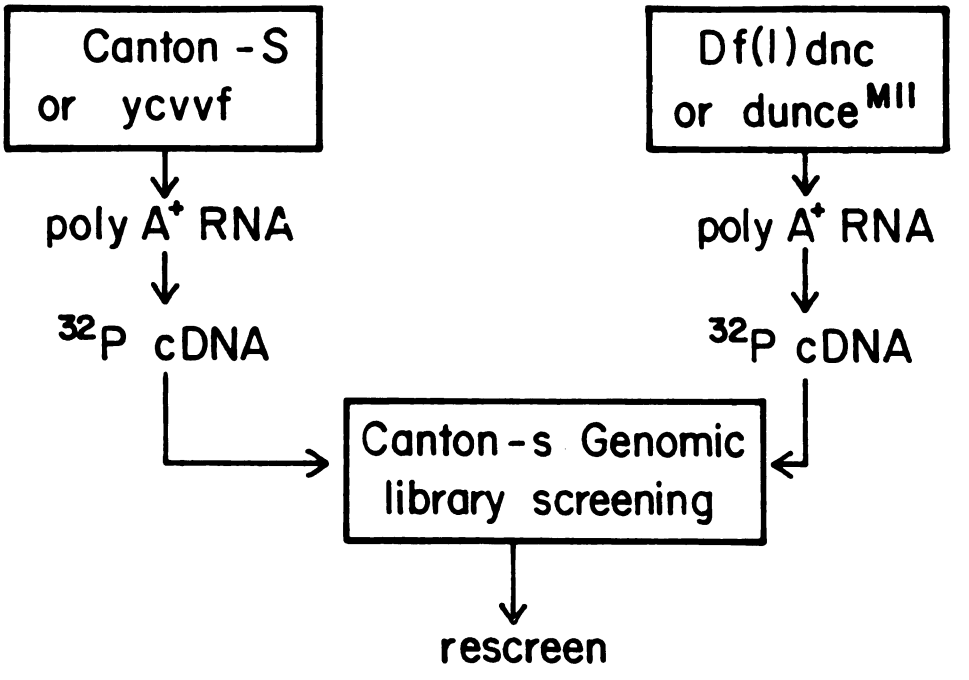

Canton -S
or ycvvf
Df(1)dnc
or dunce M11
poly A+ RNA
poly A+ RNA
32P cDNA
32P cDNA
Canton-s Genomic
library screening
rescreen

Fig. 1

obtained from the differential screening were finally screened by clone blotting experiments (Fig. 2). The clone blots were prepared in triplicate from the phage DNAs of these clones. Each blot was probed with equal counts of cDNA probes made to RNA from y cv v f, dunceM14 or dunceM11. The clones which contained genes expressed at altered abundance levels due to mutation in dunce locus were expected to display an altered hybridization signal in both dunce mutants compared to y cv v f. The control clone included on the blot had shown equal hybridization intensity to both the y cv v f and the dunceM11 cDNA probe during the differential screening procedure. Four representative clones clearly show an increase in the hybridization signal with dunce mutant cDNA probes (Fig. 2). These are named cARS (cAMP Regulated Sequences) 4U (Up), 12U, 17U and 22U. Densitometry of the autoradiogram reveals that the signals with the dunce mutant cDNA probes are 3-9 times more intense than signals with the y cv v f cDNA probes, after normalizing the signal values to that of the control value. This result supports the conclusion that those clones which behave similiarly with both dunce mutant probes represent sequences expressed at altered abundance levels because of the absence of dunce$^+$ function. Although several clones were also obtained containing sequences underexpressed in dunce mutants (refer to Chapter III), the remainder of this chapter is focused on the identity and the regulation of the clones expressed at higher abundance levels in dunce mutants.

RNA blotting experiments shown in Fig. 3A confirm that

Fig. 2. Clone blots of cARS Up clones hybridized with total cDNA probes.

Three identical blots containing EcoRI digested phage DNAs of several cARS Up clones hybridized with equal counts of cDNA probes made to poly A^+ RNA from y cv v f, dunceM14 and dunceM11 male flies. Four clones shown (4U, 12U, 17U, 22U) display an increased signal with dunce mutant cDNA probes relative to y cv v f probe. Clone #14 was isolated in the differential screen but failed to show increased signal with both of the dunce mutant cDNA probes.

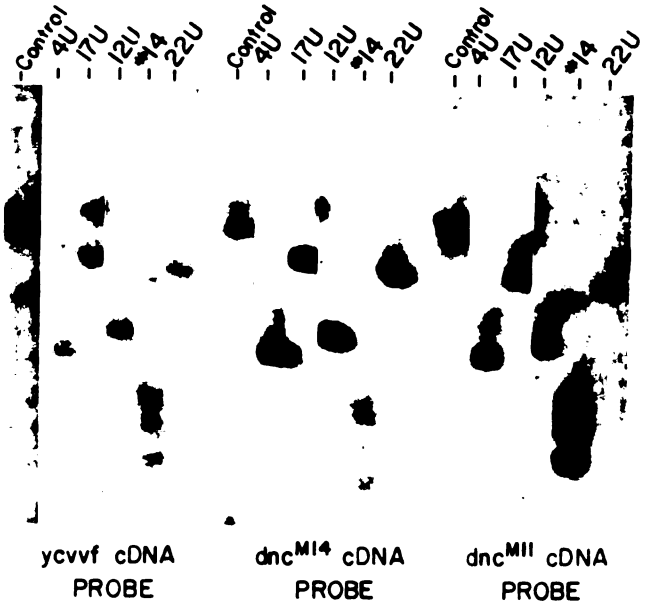

Control
4U
17U
12U
14
22U
Control
4U
17U
12U
14
22U
Control
4U
17U
12U
14
22U
ycvvf cDNA PROBE
dnc^MI4 cDNA PROBE
dnc^MII cDNA PROBE

Fig. 2

these clones carry sequences which are expressed more abundantly in the mutants. The labelled restriction fragment of the cARS 4U clone which hybridizes to the cDNA probe was used to probe the RNA population from parental strain y cv v f and two mutant strains dunceM14 and dunceM11. The signal associated with a 5.5kb RNA in the mutant lanes is 4-10 times more intense than the signal in the y cv v f lane, after normalizing the signal to that obtained upon reprobing the same blot with control phage clone 1.

The differential screens were repeated using RNA from Canton-S male flies and male flies carrying a synthetic deficiency of the dunce 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 transposable elements.

The clones carrying sequences which are over-expressed in dunce mutants were used in cross-hybridization experiments to detect the existence of any sequence homology. The clones did cross-hybridize (data not shown), indicating the existence of a repetitive sequence common to all of the cARS Up clones. This repetitive sequence was localized on the restriction fragment containing the sequences which are overexpressed in each clone (Fig. 2), suggesting that the repetitive sequence itself is differentially expressed. In addition, each cARS Up clone

Fig. 3. Northern blots probed with selected cARS Up clones.

(A). A northern blot prepared with ten micrograms of poly A^+ RNA from dunce mutants and normal strains was probed with the 2.8 kb EcoRI fragment (refer to Fig. 4) isolated from clone 4U. The bottom pannel shows the signal obtained after hybridizing the same blot with control clone 1. (B). Blot strips prepared with Canton-S poly A^+ RNA were probed separately with clones 4U, 12U, 17U and 22U. Each probe hybridizes to a 5.5 kb poly A^+ RNA and some detect a faint band of 2.0 kb.

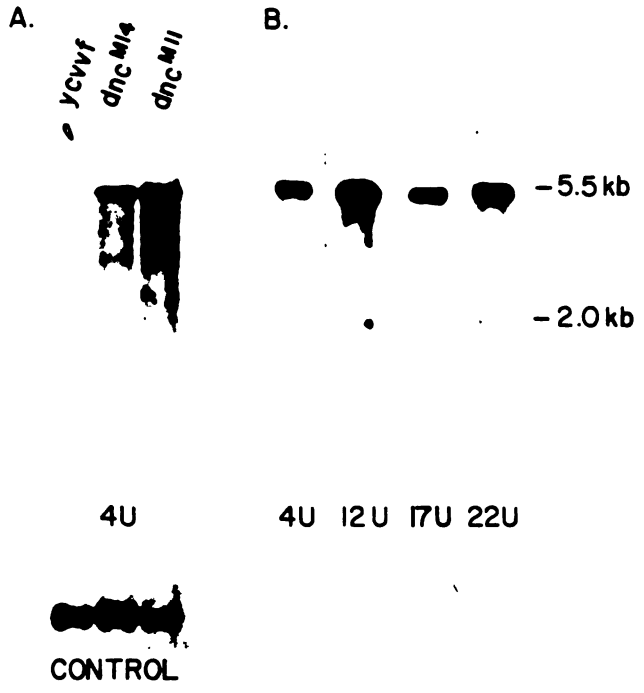


Fig. 3

hybridizes to a 5.5kb poly A^+ RNA and occasionally to a less abundant one of 2.0 kb (Fig. 3B). Detection of the same size of RNA by representative cARS Up clones also suggests the existence of a repetitive sequence common to all of the cARS Up clones. Restriction mapping of the cARS up clone revealed that some clones obtained from separate differential screens overlap and represent the same genomic region (Fig. 4). For example, cARS 4U and 22U were isolated from the y cv v f vs. dunceM11 screen and 1U and 3U were isolated from the Canton-S vs. Df(1)dnc screen, yet 4U overlaps with 1U and 22U overlaps with 3U. The isolation of overlapping clones from separate screens using different dunce mutants provides additional and compelling evidence that the cARS Up clones contain sequences expressed differentially due to a mutation in dunce locus, and not because of differences in genetic background. Furthermore, the maps show that the clones have a similiar cluster of restriction sites flanking the EcoRI fragment containing the repetitive sequence. This cluster includes one of two EcoRI sites, a HindIII site and an XbaI site.

Since transposable elements make up the majority of Drosophila middle repetitive DNA (37), known transposable elements were examined to determine whether they were similiar to the overexpressed DNA in the cARS Up clones. copia elements are known to encode an abundant RNA of about 5.0kb and several of lesser abundance, one of which is approximately 2.0kb (14, 23, 29). In addition, the sequence analyses of copia elements have shown that they contain a restriction site cluster containing a

Fig. 4. Restriction map of several cARS Up clones, revealing an overlap in restriction site patterns.

The broad segment represents the fragment which hybridizes to a total cDNA probe (Fig. 2). O, XhoI; S, SstI; 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; 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.

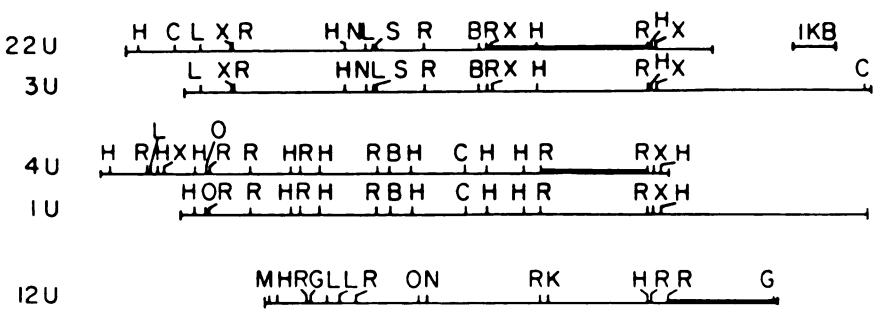

22U
H C L X R
H N L S R
B R X H
R H X
IKB
3U
L X R
H N L S R
B R X H
R H X
C
4U
H R H X H R R
H R H
R B H
C H H R
R X H
1U
H O R R H R H
R B H
C H H R
R X H
12U
M H R G L L R
O N
R K
H R R
G

Fig. 4

Fig. 5. Blots of several different transposable element DNAs hybridized with the 2.8 kb EcoRI fragment of clone 4U. The following plasmids were used: 297, a BamHI/XhoI digest of cDm4006 (26); 412, a BamHI digest of cDm2042 (26); copia, a BamHI/XbaI digest of cDm5002 (12); FB, a SalI/BamHI digest of pD75.3 (22); P, a BamHI digest of $p^{\pi}25.1$ (26).

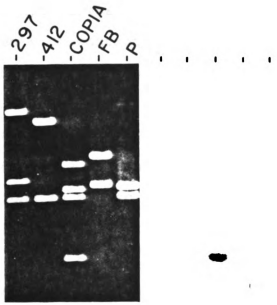


Fig. 5

HindIII, an XbaI and three EcoRI site within a 0.5kb interval (13, 21). Copia elements, therefore emerged as candidates for the identity of the repetitive sequences on the cARS Up clones.

A blot of several Drosophila transposable elements was probed with a representative of the cARS Up clones, 4U. Fig. 5 shows that only copia DNA out of five different transposable elements hybridizes. This along with the results of the prior experiments demonstrate that the cARS Up clones contain copia elements and that these elements are overexpressed in dunce mutants.

The most likely explanation for these results is that the levels of copia element RNA is increased in dunce mutants due to alterations in transcriptional or post-transcriptional processes. A less likely alternative is that dunce mutants contain more genomic copies of copia than the control strains and the elevated RNA level is simply due to an increase in copy number. To eliminate this possibility, genome blots of the control and the dunce mutant strains were probed with a labelled restriction fragment containing a copia element (Fig. 6). Although there are deviations in the hybridization patterns due to restriction site polymorphisms or differences in the location of copia elements within the genome, cumulative hybridization signals are approximately the same. The hybridization signals in the dunceM14 and dunceM11 lanes are increased only by 19% and 7% as compared to y cv v f lane. The signal in Df(1)dnc lane is reduced by 27% as compared to Canton-S lane. This result eliminate the

Fig. 6. Genome blots of DNA from various fly strains.

The genome blots were probed with a 2.8 kb XbaI fragment isolated from cDm5002 (12). The probe contains a part of copia sequence. CS, Canton-S.

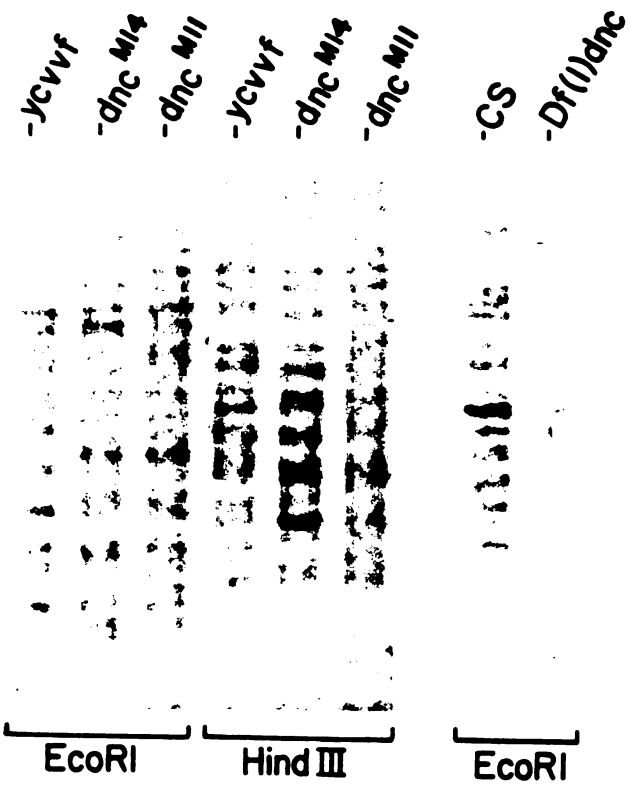

-ycvvf
-dnc M4
-dnc M11
-ycvvf
-dnc M4
-dnc M11
-CS
-Df(1)dnc
EcoRI
Hind III
EcoRI

Fig. 6

possibility that there is an increase in copy number in the dunce mutants sufficient to elevate the levels of copia RNA 5-10 times. Therefore, we conclude that the increase in copia RNA levels in dunce mutants is due to differences in transcription rate or post-transcriptional processes.

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

Since dunce mutants have an elevated cAMP content, one attractive hypothesis is that copia RNA levels are increased in the mutants because some aspects 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 with cAMP, the phosphodiesterase inhibitor isobutylmethylxanthine or the adenylate cyclase activator forskolin and the abundance of copia RNA in these flies was measured by RNA blotting experiments. Typical results are illustrated in Fig. 7. The level of copia RNA is not infuenced by administration of 5'-AMP, but cAMP, isobutylmethylxanthine and forskolin increase the copia RNA level approximately 2-4 fold. The controls for this experiment include reprobing the blot with two control clones, one of which gives a diffuse signal. An additional control was a clone selected in the differential screening as under-expressed in dunce mutants (cARS 11D). This clone (cARS 11D) encodes a putative serine protease (SER1, refer to chapter III) whose RNA levels vary inversely with

copia RNA levels when treated with the phamacological agents (Fig. 7).

Fig. 7. Northern blots of Canton-S flies fed with pharmacological agents to increase cAMP levels.

RNA blots were prepared from flies reared on food supplemented with (1) water; (2) 5′-AMP; (3) cAMP; (4) isobutylmethylxanthine; (5) 95% EtOH; (6) forskolin. (A) The blots were probed with a 2.8 kb XbaI fragments isolated from cDm5002 (12), and a putative serine protease gene (SER1) which is underexpressed in dunce mutants. (B) The same blots were probed with two control clones isolated during the differential screening . Note that copia RNA levels increase when the SER1 RNA levels decrease.

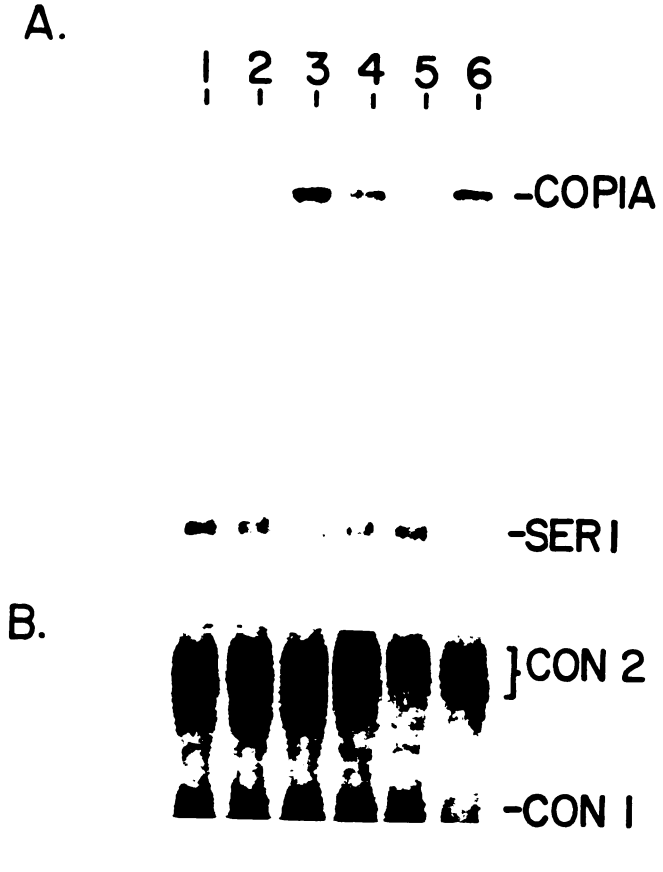


Fig. 7

DISCUSSION

Several important conclusions can be drawn from the data presented here. First, the dunce mutant strains used here express certain sequences at abundance levels different from normal flies. Therefore, the phenotype of 'altered gene expression' can be listed along with the three others which characterize dunce mutants. These include : (1) alterations in behavioral conditioning (1, 11, 18, 33, 35), (2) alterations in cAMP metabolism (3, 4, 6, 7, 8), and (3) female sterility (3, 27). Although it is clear that the amorphic point mutants and a deficiency mutant used here exibit alterations in gene expression, the hypomorphic mutants of dunce may not exibit this phenotype. Preliminary experiments (data not shown) have failed to detect any deviation in copia RNA abundance levels in the hypomorphic mutants, dunce1 and dunce2, although the deviation in these strains may be too subtle to be detected. These two mutant retain a considerable portion of cAMP-phosphodiesterase activity and as a consequence, have cAMP levels which are much lower than amorphic mutants (6, 7)

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 clones were isolated from both screens. Therefore, 0.5% (30/55,000 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 isolated went through several rescreens and many of the clones initially isolated were eliminated if they displayed a marginal differential signal in any one screen.

Second, all of the overexpressed sequences recovered are members of the copia family of transposable elements. It is assumed that most copies of copia in the genome are expressed at 5-10 times their normal level, but the possibility that the increased expression is due to a few or a single copia element can not be ruled out. Assuming that the transcriptional activity of all copia delements is equal, dunce 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. This seems unlikely, since dunceM14 and dunceM11 were induced in the y cv v f chromosome rather recently and their genetic backgrounds must be quite similiar, although they were not controlled strictly. The results of genomic blots performed to estimate the copy number of copia in the mutants relative to controls are not consistent with a 5-10 fold increase in the number of copia elements in the mutant genome.

Third, the copia RNA metabolism is under cAMP control. When flies were maintained on food supplemented with cAMP, isobutylmethylxanthine or forskolin, copia RNA levels increased relative to the appropriate controls. The increases are not of

the same magnitude as observed in the dunce 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 structural gene of the cAMP phosphodiesterase. Thus, the experiments with pharmacological agents support the conclusion that copia RNA metabolism is under cAMP control.

Not all the attempts to increase the RNA levels of copia element by administration of the pharmacological agents were successful. The attempts to raise copia RNA levels in: (1) Schneider line 2 cells with dibutyryl cAMP, (2) cultured larval midgut with several pharmacological agents, and (3) adult flies with dibutyryl cAMP did not succeed. Part of the rationale for directing our attention to feeding experiments and examining expression in the gut is that copia elements are abundantly expressed in the gut (data not shown). There are several explanations for the failure, so we discount these, and emphasize the successful one.

Cyclic AMP is known to affect transcriptional rates (16, 20, 25, 28, 30, 34) and RNA stability (15, 17) in other eucaryotes. Sequence and mutational analyses of the 5′-flanking region of several different genes regulated by cAMP revealed a concensus sequence, 5′-TGACGTCA-3′. This sequence resides between -30 and -150 and is at least partly responsible for conferring responsiveness to cAMP upon a gene (16, 20, 30, 34). A sequence which matches the concensus sequence at six of eight positions, 5′-TAACGTTA-3′, 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.

Very little is known about the factors which modulate transposable element expression. These elements show developmental (23) and spatial specificity in expression, and it has been reported that stress, including heat shock, is capable of increasing copia expression (32). It is unknown whether there is an intersection in the pathways by which stress and cAMP increase copia RNA expression. Besides cAMP, hormones are the only other factor known to modulate the expression of other transposable elements (24) and their evolutionary cousins, retroviruses (36).

LITERATURE CITED

1. Aceves-Pina, E. O., R. Booker, J. S. Duerr, M. S. livingstone, W. G. Quinn, R. F. Smith, P. P. Sziber, B. L. Tempel, and T. P. Tully. 1984. Cold Spring Harbor Symp. Quant. Biol. 48:831-840.

2. Benkel, B. F., and D. A. Hickey. 1986. Genetics 114:137-144.

3. Byers, D., R. L. Davis. and J. A. Kiger. 1981. Nature 289:79-81.

4. Chen, C-N., S. Denome, and R. L. Davis. 1986. Proc. Natl. Acad. Sci. 83:9313-9317.

5. Cohen, R. S., and M. Meselson. 1985. Cell 43:737-746.

6. Davis, R. L. and J. A. Kiger. 1981. Biochem. Biophys. Res. Comm. 81:1180-1186.

7. Davis, R. L. and J. A. Kiger. 1981. J. Cell. biol. 90:101-107.

8. Davis, R. L., and L. M. Kauvar. 1984. in Advances in Cyclic Nucleotide Research and Protein Phosphorylation, eds. Strada, S.J. and W. J. Thompson. (Raven, New York), 16:393-402.

9. Davis, R. L., and N. Davidson. 1984. Mol. Cell. Biol. 4:358-367.

10. Davis, R. L., and N. Davidson. 1986. Mol. Cell. Biol. 6:1464-1470.

11. Dudai, Y. 1983. Proc. Natl. Acad. Sci. 80:5445-5448.

12. Dunsmuir, P., W. J. Brorein, M. A. Simon, and G. M. Rubin. 1980. Cell 21:575-579.

13. Emori, Y., T. Shiba, S. Kanaya, S. Inouye, S. Yuki, and K. Saigo. 1985. Nature 315:773-776.

14. Flavell, A. J., S. W. Ruby, J. J. Toole, B. E. Roberts, and G. M. Rubin. 1980. Proc. Natl. Acad. Sci. 77:7107-7111.

15. Giorgio, M., A. Ceccarelli, and H. F. Lodish. 1983. Nature 301:616-618.

16. Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe.

and J. F. Habener. 1987. Mol. Cell. Biol. 7:3032-3040.

17. Jungmann, R. A., D. C. Kelly, M. F. Miles, and D. M. Milkowski. 1983. J. Biol. Chem. 258:5312-5318.

18. Kyriacou, C. P. and J. C. Hall. 1984. Nature 308:62-64.

19. Mohler, J. D. 1977. Genetics 85:259-272.

20. Montiminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Proc. Natl. Acad. Sci. 83:6682-6686.

21. Mount, S. M. and G. M. Rubin. 1985. Mol. Cell. Biol. 5:1630-1638.

22. Levis, R., M. Collins, and G. M. Rubin. 1982. Cell 30:551-565.

23. Parkhurst, S. M., and V. G. Corces. 1987. EMBO J. 6:419-424.

24. Peronnet, F., J. L. Becker, J. Becker, L. d'Auriol, F. Galibert, and M. Best-Pomme. 1986. Nucl. Acids. Res. 14:9017-9033.

25. Ran, W., M. Dean, R. A. Levine, C. Henkle, and J. Campisi. 1986. Proc. Natl. Acad. Sci. 83:8216-8220.

26. Rubin, G. M. et. al. 1980. Cold Spring Harbor Symp. Quant. Biol. 45:619-628.

27. Salz, H. K., R. L. Davis, and J. A. Kiger. 1982. Genetics. 100:587-596.

28. Sasaki, K., T. P. Cripe, S. R. Koch, T. L. Andreone, D. D. Peterson, E. G. Beale, and D. K. Granner. 1984. J. Biol. Chem. 259:15242-15251.

29. Schwartz, H. E., T. J. Lockett, and M. W. Young. 1982. J. Mol. Biol. 157:49-58.

30. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson. 1986. J. Biol. Chem. 261:9721-9726.

31. St. John, T., and R. W. Davis. 1979. Cell 16:443-452.

32. Strand, D. J., and J. F. MacDonald. 1985. Nucl. Acids Res. 13:4401-4410.

33. Tempel, B. L., N. Bonini, D. R. Dawson, and W. G. Quinn. 1983. Proc. Natl. Acad. Sci. 80:1482-1486.

34. Tsukuda, T., J. S. Fink, G. Mandel, and R. H. Goodman. 1987. J. Biol. Chem. 262:8743-8747.

35. Tully, T. and W. G. Quinn. 1985. J. Comp. Physiol. 157:263-277

36. Von Der Ahe, D., S. Janich, C. Scheidereit, R. Renkawitz, G. Schultz, and M. Beato. 1985. Nature 313:706-709.

37. Young, M. 1979. Proc. Natl. Acad. Sci. 76:6274-6278.

CHAPTER III

THE mRNA LEVELS OF A PUTATIVE SERINE PROTEASE GENE ARE REDUCED IN *dunce* MUTANTS AND ARE MODULATED BY cAMP.

This chapter describes the characterization of the genomic clone, cARS 11D, which contains a gene whose expression level is decreased in *dunce* mutants. The cARS 11D clone has been localized to the polytene chromosome bands 99C-D. The level of mRNAs coded by the gene contained in this clone is decreased 2-6 fold in *dunce* mutants compared to normal flies. Sequence analysis of the genomic region and representative cDNAs revealed that cARS 11D contains genes coding for putative serine proteases (SER1, SER2 and SER3). These serine protease genes are abundantly expressed in the gut, which suggests that a major function of the gene products is in digestion. Administration of the pharmacological agents (cAMP, isobutylmethylxanthine or forskolin) to normal flies resulted in decreased mRNA level of SER1 (or SER1 related genes). This is the first example of a simple serine protease gene regulated by cAMP.

MATERIALS AND METHODS

Fly strains.

The mutants dunceM14 and dunceM11 were induced from the parental strain y cv v f by treating flies with ethylmethanesulfonate and screening for female sterility (40). These two mutants were subsequently found to be allelic to dunce. Canton-S is the wild type strain used throughout the experiments.

Isolation of RNA.

Total RNA was isolated by homogenizing flies in a buffer containing guanidine thiocyanate, followed by cesium chloride density centrifugation as described by Fyrberg et al. (16). Polyadenylated RNA was selected by one pass over an oligo-dT cellulose column. RNA blotting and hybridizations were performed as described by Davis and Davidson (14).

Isolation of cDNA clones.

A Canton-S cDNA library was obtained from T. Bargiello and M. Young and screened by the methods of Benton and Davis (3).

Nucleic acid sequencing and analysis.

The cARS 11D genomic clone and cDNAs were digested with various restriction enzymes and appropriate restriction fragments were subcloned into M13mp18 or M13mp19 vectors. In some cases,

subclones with progressive deletions were generated with exoIII and S1 nuclease as described by Henikoff (22). Sequencing was performed using either ^{32}P or ^{35}S as a label as described (3, 39). The nucleic acid sequences were analyzed with Staden's programs (45) which have been modified to run on an IBM microcomputer (9). The compilation of the codon usage table from several Drosophila genes has been described (9). A protein data base was searched for homology to the conceptualized open reading frames using the FASTP program as described by Lipman and Pearson (35).

Isolation of genomic DNA.

Genomic DNA was isolated from adult flies by cesium chloride density centrifugation as described (14).

S1 mapping and primer extension experiments.

The 5' ends of mRNA were identified by S1 nuclease mapping and primer extension experiments. S1 experiments were performed as described by Telford et al. (47) with minor modifications. 32P-labelled single-stranded DNA complementary to the coding strand was synthesized from the M13 subclones by primer extension using the Klenow fragment of DNA polymerase I. The resulting product was linearized by digestion with appropriate restriction enzymes and electrophoresed on a 0.8% alkaline agarose gel. After autoradiography, the radioactive band corresponding to the desired probe was eluted from the gel. Approximately $5x10^5$ cpm of

labelled probe was added to 3 ug of poly A^+ RNA, heated to 75°C for 10 min, and then hybridized at 54°C for 3 hrs in 20 ul of annealing buffer (80% formamide, 40 mM piperazine-N,N-bis(2 ethane sulphonic acid) pH 6.4, 1 mM EDTA and 0.4 M NaCl). After hybridization, samples were digested with 1,000 units of S1 nuclease in 300 ul of S1 nuclease buffer (0.3 M NaCl, 0.05 M Na-Acetate; pH 4.6, 4 mM ZnSO4, 15 ug/ml denatured salmon sperm DNA).

Primer extensions were performed using an end labelled oligonucleotide as a primer. The labelled primer was annealed with 2 ug of poly A^+ RNA in the same annealing buffer used for S1 experiments for 3 hrs at 42°C following a 10 min, 75°C denaturation step. After precipitation, Avian Myeloblastosis Virus reverse transcriptase (Life Science) was used to synthesize the complementary strand. Reactions were analyzed by electrophoresis on a 5% acrylamide sequencing gel, followed by autoradiography.

Synthesis of oligonucleotide and hybridization.

Oligonucleotides were synthesized by the solid phase phosphotriester chemistry methods on an automated synthesizer (Applied Biosystems) at the Macromolecular facility of Michigan State University. Oligonucleotides were purified by electrophoresis on an 18% polyacrylamide gel containing 7 M urea or by HPLC chromatography on a Vydac C4 reverse phase column according to the methods described in the Applied Biosystems

technical bulletin. Fifty pmol of each oligonucleotide was labelled with T4 polynucleotide kinase and γ-[32P]-ATP. The labelled oligonucleotides were hybridized to RNA blots as described with minor modifications (54). Blots were hybridized for several hours at 42°C in 5x SSC, 20 mM sodium phosphate (pH 7.0), 10x Denhardt's solution, 7% SDS, and 100 ug per ml denatured salmon sperm DNA, and then hybridized overnight at 42°C in the above solution following the addition of dextran sulphate to a final concentration of 10% and 5 ng per ml of end-labelled oligonucleotide probe. Formamide was added to adjust the theoretical temperature of hybridization to Tm-7°C. Tm was calculated according to Lathe (32). After hybridization, filters were washed for 1 hr at the hybridization temperature in 3x SSC, 10 mM sodium phosphate(pH 7.0), 10x Denhardt's solution and 5% SDS and for 1 hr at the hybridization temperature in 1x SSC, and 1% SDS.

Isolation of tissue specific RNA.

The identification and dissection of larval organs were performed as described by Bodenstein (5). Brain, salivary glands, gut, fat body and malpighian tubules were separated and total RNA was isolated as described (10) with minor modifications. Organs were immediately homogenized in 1:1 mixture of Holmes-Bonner buffer (7 M urea, 2% SDS, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl; pH 7.4) and buffer saturated phenol (25:24:1 mixture of phenol:chloroform:isoamyl alcohol) with small glass

homogenizers. After several phenol extractions, the nucleic acids were precipitated with ethanol. The pellet was washed with 70% ethanol, then redissolved in a small volume of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2% SDS followed by addition of three volumes of 4 M sodium acetate (pH 5.0). The mixture was kept on ice for 2-4 hrs, centrifuged, and the pellet was washed with 70% ethanol and redissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA.

Treatment of flies with pharmacological agents.

Three to five day old adults of wild type Canton-S strain were maintained in 8 dram shell vials containing 3 mls of regular corn meal media supplemented with cAMP, isobutylmethylxanthine or forskolin for 3 days as described (1) with some modifications. Cyclic-AMP (50 mg per vial) and isobutylmethylxanthine (50 mg per vial) were first added in 250 ul of water and mixed with media. As controls for cAMP and isobutylmethylxanthine, media were treated either with 5'-AMP in 250 ul of water or with water alone. Forskolin (2 mg per vial) was dissolved in 250 ul of 95% ethanol and added to the media. As a control for forskolin, media were treated with 250 ul of 95% ethanol. Total RNA was extracted from the flies by the same method used for isolation of tissue specific RNA as described above. The concentration of RNA was measured with spectrophotometer and equivalent amounts of RNA samples were loaded in each lane of the gel.

RESULTS

The levels of mRNAs homologous to cARS 11D are decreased in dunce mutants.

In chapter II, the isolation of phage clones from the Canton-S *Drosophila* genomic library by differential hybridization techniques was described. One of the clones, cARS 11D, showed consistently less hybridization to a cDNA probe synthesized from poly A^+ RNA isolated from *dunce*M11 than to a cDNA probe synthesized from poly A^+ RNA from parental flies. This result was confirmed by probing poly A^+ RNA blots from two *dunce* mutant flies with a probe made from the cARS 11D clone (Fig. 8). If the differential expression of cARS 11D clone is due to a mutation in the *dunce* locus, the probe should detect reduced hybridization signals to RNAs of both *dunce* mutants relative to parental strain. The leftmost EcoRI fragment (G50E, Fig. 9A) of the cARS 11D insert was used as a probe, since only this restriction fragment hybridized to the total cDNA probe (data not shown). Two mRNA species of 1.0kb and 3.5kb were detected with this probe and both showed decreased hybridization in two amorphic *dunce* mutants, *dunce*M14 and *dunce*M11, compared to the parental strain *y cv v f*. In order to verify that equal amounts of RNAs were loaded in each lane, the same blots were hybridized with nick translated DNA of a control phage. Control phages are those phages isolated during the differential screening procedure that displayed equal hybridization to cDNA probes from both the parental and *dunce*

Fig. 8. Differential expression of cARS 11D clone in dunce mutants.

Poly A^+ RNA blots prepared from y cv v f, dunceM14, and dunceM11 flies were probed with the 5.0 kb EcoRI fragment (G50E, Fig. 9A) of the cARS 11D phage clone. The sizes of the two mRNA species are indicated. The several bands larger than 3.5kb mRNA are from previous hybridization experiments.

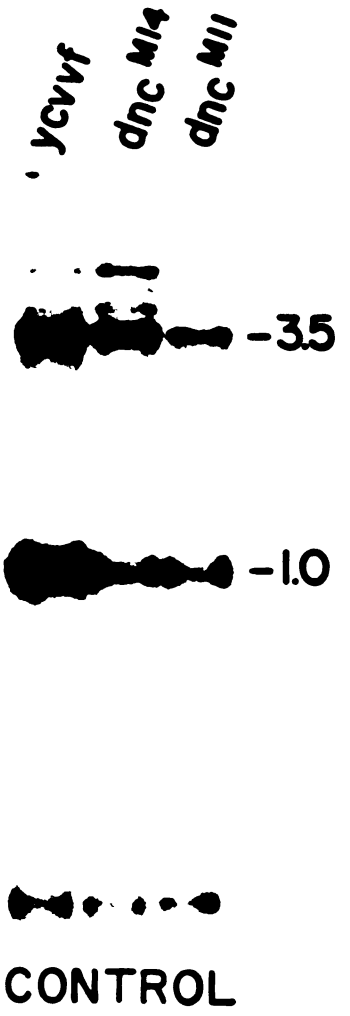


Fig. 8

mutant flies. The intensities of hybridization of the 1.0kb and the 3.5kb bands to the cARS 11D probe were determined by densitometry, and were normalized to the intensity of the control band. Expression of the 1.0kb transcript is reduced by 65% and 83% in the dunceM14 and dunceM11 respectively as compared to parental type flies. Expression of the 3.5kb transcript is reduced by 30% and 70% in the dunceM14 and dunceM11 respectively as compared to parental type flies. These results support the conclusion that cARS 11D contains genes expressed at reduced abundance levels because of the absence of the dunce$^+$ function.

Characterization of the genomic region and cDNAs.

About 4 kb of the 5.0 kb EcoRI restriction fragment from the insert of cARS 11D clone (G50E, Fig. 9A) was sequenced and analyzed for the presence of open reading frames. Two complete open reading frames (SER1, SER2) and part of a third (SER3) were identified. A diagram of the region is depicted in Fig. 9. The nucleic acid sequences of SER1 and SER2 are identical except for a difference in 3 bases (shown in Fig. 9B) and the presence of additional 23 bp in the 3' untranslated region of SER1 (Fig. 9B). SER3 is approximately 60% homologous to SER1 or SER2 at the nucleic acid level throughout the sequenced region. The nucleic acid sequence of the genomic region is shown in Fig. 10 together with the putative amino acid sequences of the conceptualized open reading frames. All three genes have the conserved polyadenylation signal 'AATAAA' 15-51 nucleotides downstream of

Fig. 9. Structural organization of the cARS 11D genomic region and corresponding cDNA clones. (A). Schematic organization of the genomic DNA insert of cARS 11D is depicted. At the top is a restriction map of the cARS 11D insert. EcoRI sites at both ends were generated during the construction of the genomic library by attachment of EcoRI linkers. A detailed restriction map of the leftmost 5.0 kb EcoRI fragment (G50E) is also shown as well as the sequencing strategy of the region. Transcription units SER1 and SER2, and a part of SER3 are depicted as solid arrows above the restriction map of G50E. The direction of the arrows indicates the direction of transcription. The 0.55 kb SalI fragment used for generation of the single stranded probe for S1 experiments is shown as a hatched box. The 0.8 kb EcoRI/SalI and 0.3 kb BamHI/SalI fragments used as probes in subsequent experiments are indicated as open boxes. R, EcoRI; X, XhoI; H3, HindIII; B, BamHI; Sal, SalI; Sac, SacI. (B). Structure of the cDNA clones. The position of the 3 bases which differ between SER1 and SER2 are numbered by counting the putative cap site as number 1. Also, 23 bases of additional sequence in the 3′ untranslated region of SER1 is indicated by a triangle. The structure of the cDNAs are drawn under the corresponding genomic sequence. The poly A^+ tails are marked by 'AAAA'. The clones C10 and C8 were sequenced completely on both strands and C12, C6 and C16 were sequenced on one strand. The broken lines indicate the unsequenced portion of C6 and C16. Note that the sequence of C8 differs at position 693 from SER1.

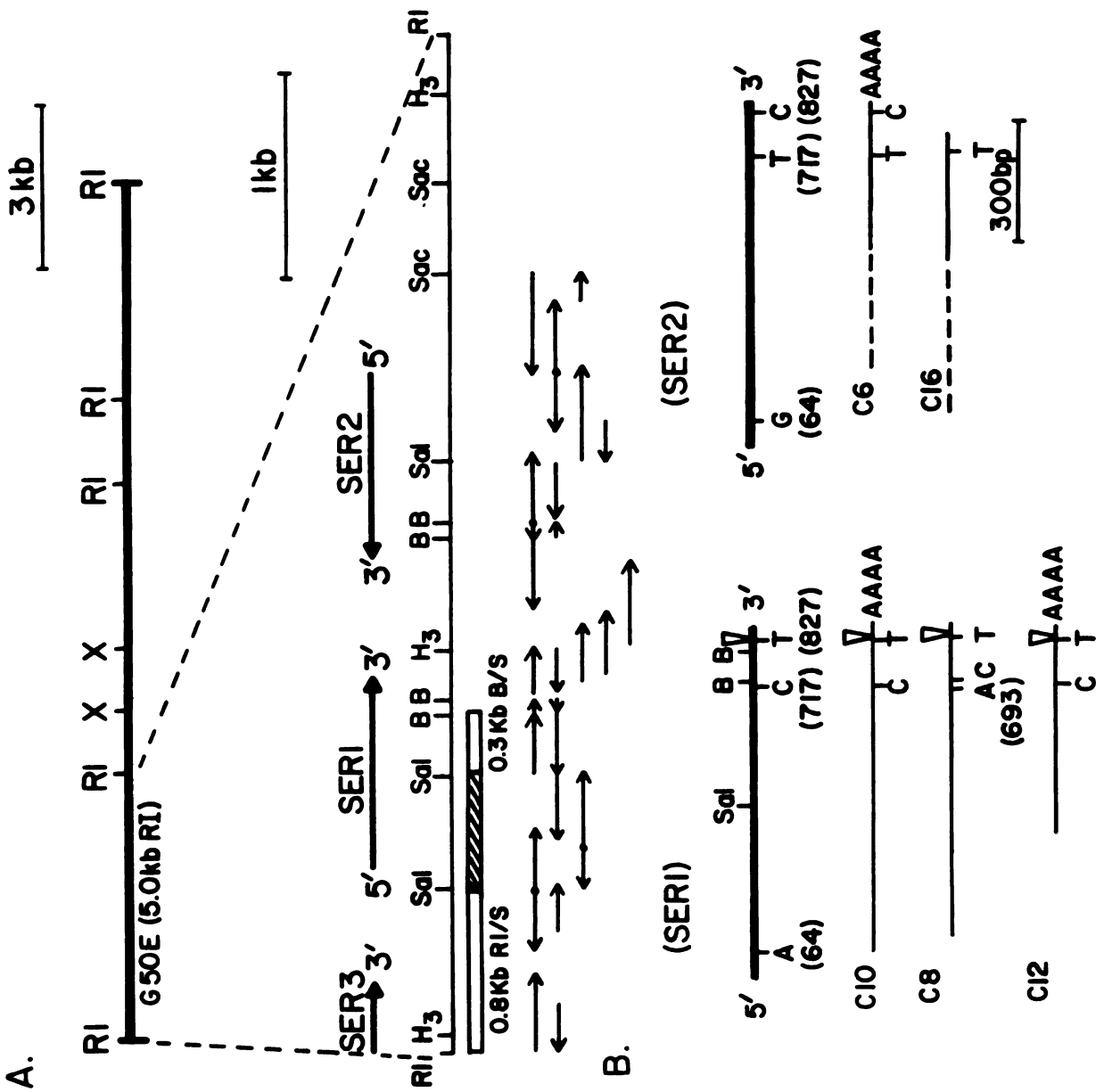

A.
3kb
1kb
RI
X
G50E (5.0kb RI)
SER3
SER1
SER2
5'
3'
Sal
BB
H3
Sac
0.8Kb RI/S
0.3Kb B/S
B.
(SER1)
(SER2)
B
Bg
C
T
A
G
(64)
(717)
(827)
AAAA
C10
C8
C12
C6
C16
AC
(693)
300bp

Fig. 9

Fig. 10. The nucleotide sequence of the genomic region containing SER1, SER2 and SER3.

Nucleotide sequence and putative amino acid sequence of SER1,SER2, and SER3 are shown. Open arrows indicate the direction of translation. Potential 'TATA' boxes and poly A^+ tail addition signals for each transcription unit are boxed. The putative transcriptional start sites of SER1 and SER2 are indicated by arrows above the nucleotide sequence and the ends of protected fragments in the S1 experiments are underlined. The sequence complementary to that of the synthetic oligonucleotide used for primer extension is underlined and labelled as 'primer'. Asterisks indicate those single residues which differ between SER1 and SER2. The predicted cleavage sites of the signal peptides and the activation peptides are marked with filled arrowheads.

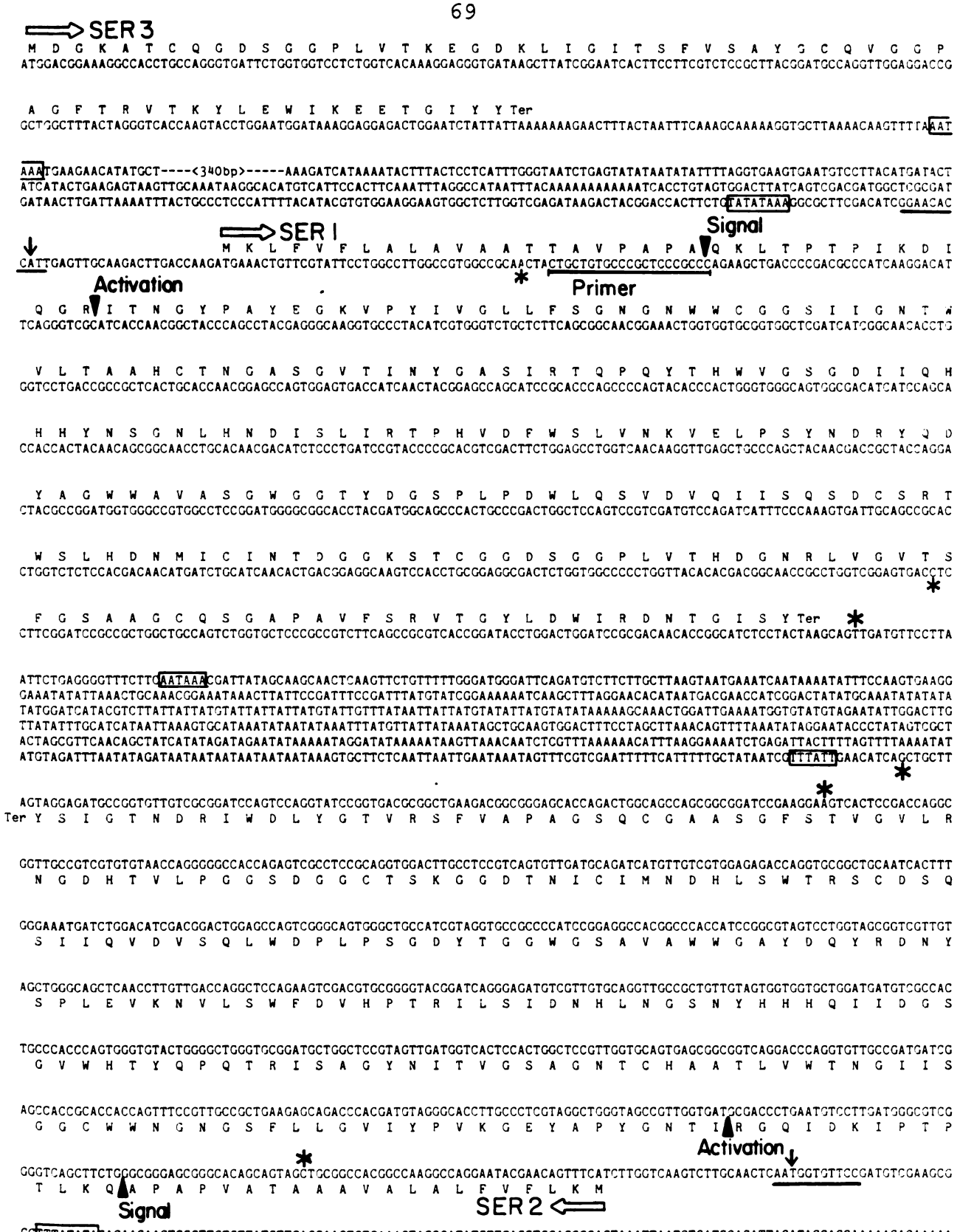

SER 3
SER I
Signal
Activation
Primer
Activation
Signal
SER 2

Fig. 10

the translation termination codons. SER1 and SER2 code for conceptualized proteins with 265 amino acids. They are identical except for one amino acid at the 14th residue from the amino terminal methionine, which is threonine in SER1 and alanine in SER2.

A cDNA library prepared from poly A^+ RNA of Canton-S flies was probed using the 5.0 kb EcoRI fragment (G50E). Thirty out of $1.5X10^5$ phages screened hybridized to this probe. The inserts from several of these phages were sequenced and compared with the sequence obtained from the genomic clone. SER1 and SER2 contain no introns within their coding sequences, and are transcribed from opposite strands. Three cDNA clones, C10, C8, C12, were classified as SER1-homologous, since the sequences are identical to SER1 throughout the sequenced region, with a single exception. The nucleotide sequence of C8 differs from that of SER1 by a cytosine to adenine substitution at position 693 (Fig. 9B). This substitution alters the sequence of corresponding amino acid from aspartic acid to glutamic acid. Presently, it is not clear whether this is due to allelic differences of a single SER1 gene or whether a number of highly homologous genes exist, each exibiting very slight sequence variation. Two cDNA clones, C6 and C16, were classified as SER2-homologous. Since cDNA clones complementary to both genes have been found, it is expected that both SER1 and SER2 genes are transcribed into functional mRNAs and are translated.

Determination of the transcriptional start site.

The transcriptional start sites of SER1 and SER2 were mapped by S1 nuclease mapping and primer extension experiments (Fig. 11). For S1 experiments, a single-stranded probe was synthesized from a 0.55kb SalI restriction fragment (Fig. 9A) and subcloned into M13 vector. The sizes of the protected fragments range from 419-428 bases. Although S1 nuclease digestion can produce protected DNAs of heterogeneous sizes, the possiblility also exists that mRNAs with heterogenous 5' ends are derived from highly homologous genes.

Primer extension was performed using a synthetic oligonucleotide as a primer. The sequences complementary to oligonucleotide and the predicted transcriptional start sites are indicated in Fig. 10. The sizes of the extended products are again heterogenous, ranging from 84-88bp. It is presumed that the longest extension product corresponds to the transcriptional start site for SER1 and SER2. The sequence around this 'A' residue fits well with the proposed weak concensus transcriptional start site sequence, 'CA' followed preferentially by a short stretch of pyrimidines (7). The heterogeneity of the extended products again suggests the possible existence of the genes highly homolologous to SER1 or SER2. Both SER1 and SER2 have a putative 'TATA' box sequence 22bp upstream from the predicted transcriptional start site.

Fig. 11. Mapping of the transcriptional start site.

(A). S1 experiments. Fragments remaining after S1 digestion were fractionated on a 5% polyacrylamide sequencing gel. The right four lanes show a sequencing ladder of known sequence used as size markers. The size of the protected fragment is indicated. Lane 1. Probe; Lane 2. Control using yeast tRNA instead of poly A^+ RNA; Lane 3. Product of S1 experiment.

(B). Primer extension. The last four lanes are the sequencing ladder of known sequence used as size markers. The lengths of the extended products are indicated. The signals of the oligonucleotide probe are not included in the photo. Lane 1. Probe; Lane 2. Control using yeast tRNA instead of poly A^+ RNA ; Lane 3. Primer extension products.

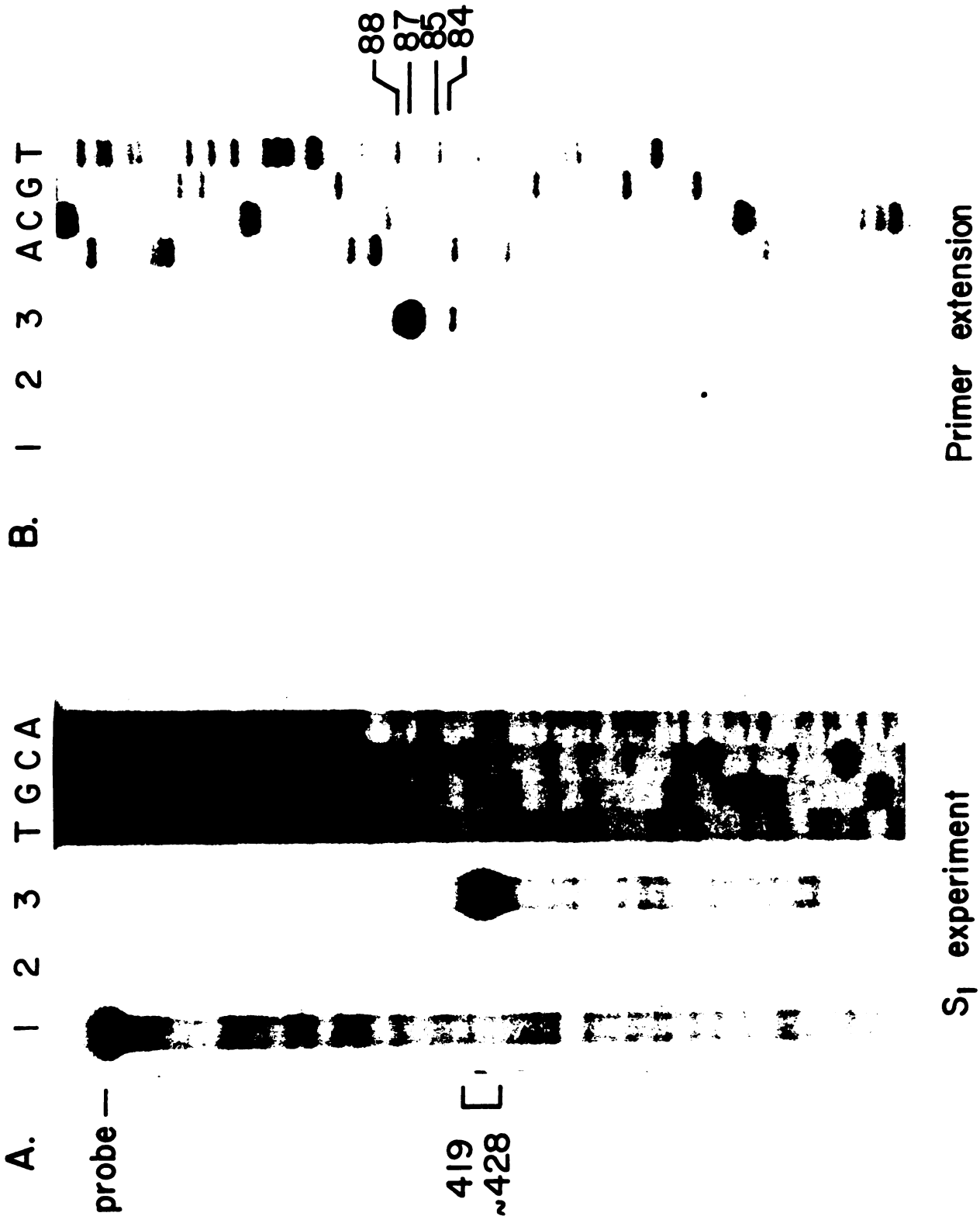

A.
1 2 3 T G C A
S1 experiment
probe
419
~428
B.
1 2 3 A C G T
Primer extension
88
87
85
84

Fig. 11

The predicted amino acid sequences of the genes contained on cARS 11D are homologous to serine proteases.

Comparison of predicted protein sequences of SER1, SER2 and SER3 with the protein data base revealed a homology to members of the family of serine proteases. An alignment of the putative amino acid sequences of SER1/SER2 and SER3 with those of known serine proteases is shown in Fig. 12. SER1 has significant homology with other known serine proteases through the entire protein sequence. In particular, the amino acid sequence surrounding the active site residues (His-57, Asp-102, Ser195)(29) as well as the six cysteine residues (Cys-42, 58, 168, 182, 191, 220) characteristic of serine proteases and important for determination of structure (4, 43) are well conserved. SER1, SER2, and SER3 are new members of the serine protease gene family. The conservation of important amino acids suggests that gene products of SER1 and SER2 are likely to be active serine proteases. However, not all members of the family have enzyme activity. For instance, the α-subunit of 7S nerve growth factor (23) and haptoglobin (30) lost their enzyme activity. In haptoglobin, the active site residues, His-57 and Ser-195, are replaced with other amino acids (30).

The active enzyme forms of simple serine proteases have highly conserved amino terminal sequences starting with isoleucine in most cases (Ile-16 in Fig. 12 and the 36th residue of amino acid sequences of SER1 and SER2 in Fig. 10). Based on this, the predicted amino acid sequences of SER1 and SER2 have an

Fig. 12. Alignment of putative amino acid sequences of SER1/SER2 and SER3 with those of other serine proteases.

Amino acids identical to those at same position in SER1 are boxed. Numbering is according to the Bovine chymotrypsin system. Gaps were allowed to align the sequences with maximum homology and are indicated by dashes. HORCHY, Hornet chymotrypsin (25); CRACOL, Crab collagenase (18); DMTRY, Drosophila trypsin like enzyme (12); DMSNAK, Drosophila snake gene product (15); BOVCHY, Bovine chymotrypsin; BOVTRY, Bovine trypsin.

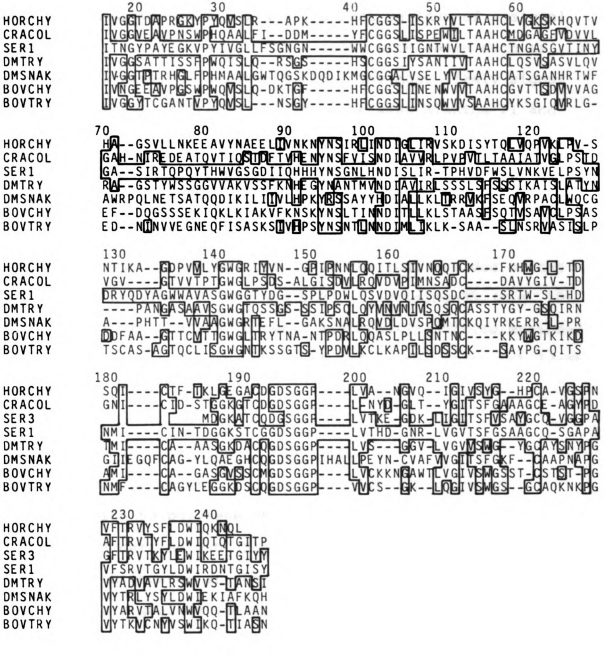


Fig. 12

additional 35 amino acids at the amino terminus that most likely represent the signal (pre) and activation (pro) peptides. Many signal sequences have hydrophilic residues near the beginning and end of a hydrophobic core, and the last residue of a signal sequence is an amino acid with a short side chain such as alanine, glycine, or serine (8, 36). The most likely signal sequence cleavage site is therefore between Alanine-21 and Glutamine-22 (Fig. 10). After secretion through the rough endoplasmic reticulum, many pancreatic serine proteases are activated by a trypsin-like cleavage and the last residue of the activation peptide is ususlly a lysine or arginine (36). This is consistent with the prediction that the last residue of the activation peptide of SER1 and SER2 is arginine-35 (Fig. 10). Cleavage of SER1 or SER2 between Arginine-35 and Isoleucine-36 would result in active proteins of 230 amino acids with identical sequence.

Cytological localization of cARS 11D.

To map the genomic location of cARS 11D, *in situ* hybridization to polytene chromosome spreads of wild type Canton-S flies was carried out (Fig. 13). The cARS 11D was localized to the third chromosome at the boundary of regions 99C and 99D. We believe the signal is located on band 99D1. The *serendipity* locus, which codes for a blastoderm stage specific RNA, and the *rp49* gene, encoding the large subunit of ribosomal protein 49, have been mapped nearby at 99D2-9 (28, 50). In addition, a *kayak*

Fig. 13. Cytological localization by in situ hybridization to polytene chromosomes.

The cARS 11D phage DNA was labelled by nick translation using biotin-dUTP (ENZO Biochem) and hybridized to polytene chromosome spreads from wild type Canton-S flies (31). The signal was detected by an avidin-horseradish peoxidase complex followed by histochemical detection with diaminobenzidine (Vector Laboratories). The hybridization signal is indicated with an arrow.

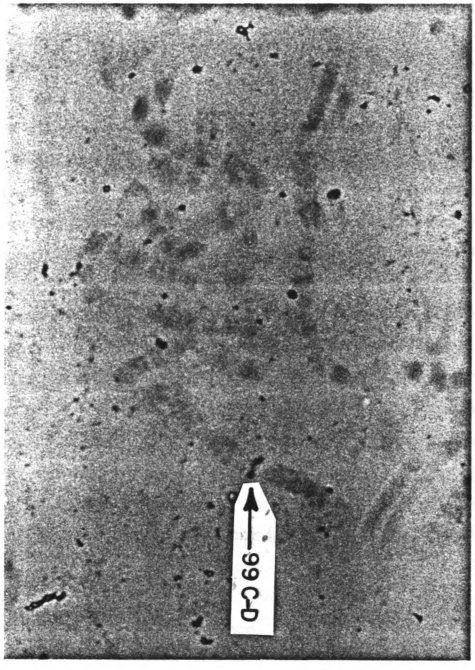


Fig. 13

mutation which shows an altered morphology of the embryonic cuticle has been mapped near this region (27).

Gene copy number of SER family genes.

Genome blotting experiments were performed to determine the number of genes homologous to the SER family. Genomic DNA of Canton-S wild type fly was digested with EcoRI, BamHI and HindIII restriction enzymes, electrophoresed in agarose gels and blotted. The blots were hybridized with probes representing SER family genes (Fig. 14). The 0.3 kb BamHI/SalI fragment was used as a probe representing both SER1 and SER2, and the 0.8 kb EcoRI/SalI fragment was used as a probe representing SER3. These two probes were specific to their corresponding genes under the hybridization conditions employed. The labelled 0.3 kb BamHI/SalI restriction fragment hybridized to 6, 7, and 9 distinct bands in EcoRI, BamHI, and HindIII lanes respectively. This result suggests that at least seven genes homologous to SER1/SER2 may be present in the haploid genome of Drosophila, since G50E contain two members. The 0.8 kb EcoRI/SalI probe representing SER3 detected two bands in each lane, suggesting the presence of at least one additional gene homologous to SER3.

Two EcoRI fragments of 12.0 kb and a 9.0 kb hybridized to both the SER1/SER2 and SER3 probes. In addition, one BamHI band and one HindIII band also hybridized with both probes. These results, and the fact that only one polytene chromosome band is detected by in situ hybridization, raise the possibility that all

Fig. 14. Genome blot analysis of SER family genes.

Genomic DNA (3 ug) isolated from Canton-S flies was digested individually with the restriction enzymes EcoRI, BamHI, or HindIII. The blots were probed with the labelled 0.3 kb BamHI/SalI restriction fragment (Fig. 9A) representing both the SER1 and SER2 genes (A) or with the labelled 0.8 kb EcoRI/SalI restriction fragment (Fig. 9A) representing SER3 (B). Sizes of the EcoRI bands detected with the probes are indicated.

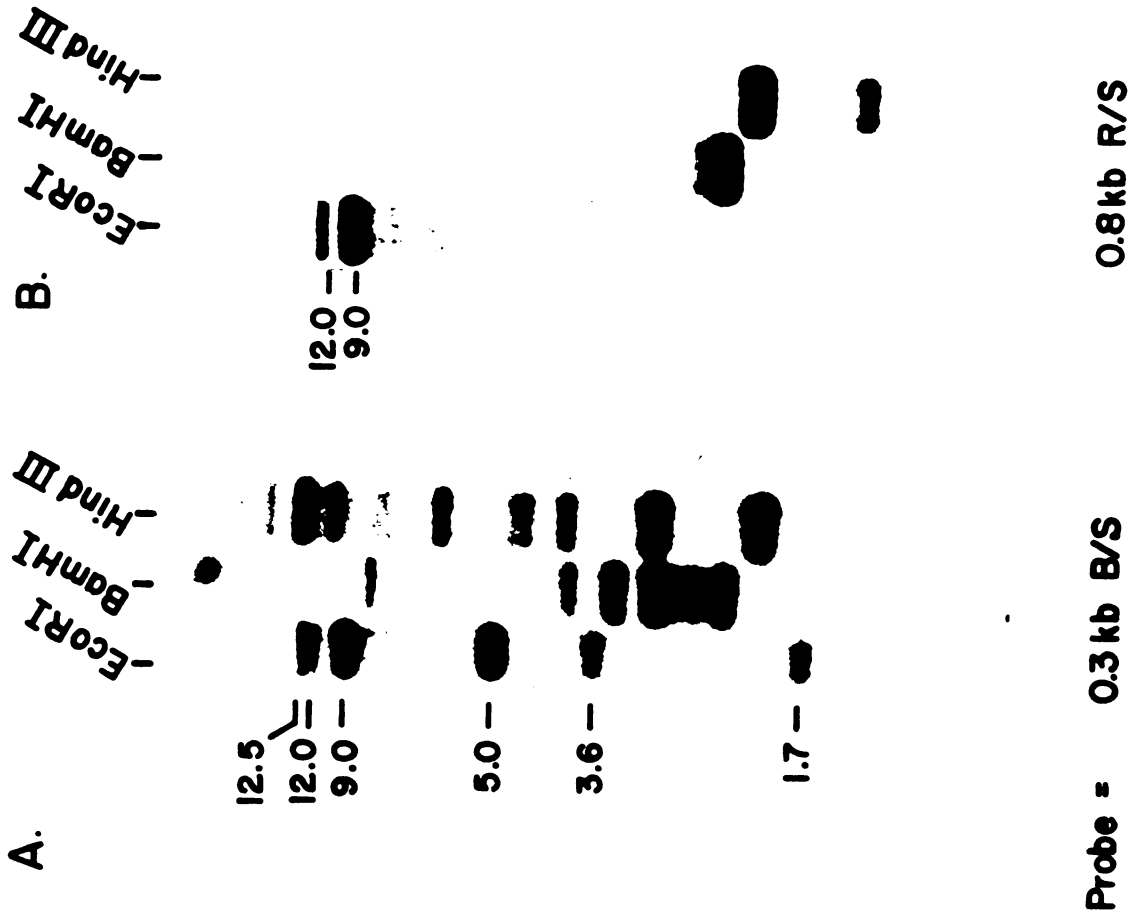

A.
-EcoRI
-BamHI
-HindIII
12.5
12.0
9.0
5.0
3.6
1.7
Probe = 0.3kb B/S
B.
-EcoRI
-BamHI
-HindIII
12.0
9.0
0.8kb R/S

Fig. 14

of the members of the SER family are clustered at the 99C-D region. However, the use of whole phage DNA as a probe for *in situ* hybridization may not be sensitive enough to detect limited homology at other sites.

Only SER1 (or SER1-related genes) is differentially regulated in dunce mutants.

To distinguish which of the sequenced genes are responsible for differential expression of SER family genes, poly A+ RNA blots prepared from *y cv v f* and two *dunce* mutant strains were hybridized separately with probes representing SER1, SER2 or SER3. The SER1-specific probe was an oligonucleotide complementary to the additional 23 bases in the 3′ untranslated region of SER1 that are not present in SER2. The SER2-specific probe was an oligonucleotide complementary to the sequence in SER2 that corresponds to the regions flanking the additional 23 bases observed in SER1 (Fig. 15A). These oligonucleotide probes hybridize specifically to their corresponding genes under the hybridization conditions employed (Fig. 15B). As a probe representing SER3, the 0.8kb EcoRI/SalI fragment was used. The intensities of the bands were normalized to that of the control. The probe representing SER1 detected 4-10 fold weaker hybridization signal to the 1.0kb RNA species, and 2-5 fold weaker signal to the 3.5kb RNA species in *dunce* mutants than in normal (Fig. 15C Panel 1). The probes representing SER2 or SER3 detected about equal hybridization signal to RNAs from both *dunce*

Fig. 15. RNA blotting experiments with gene specific probes.

(A). The sequences of the 3′ untranslated regions of SER1 and SER2 are aligned. The putative termination codon and poly A^+ addition signal sequence are marked with asterisks. Those sequences complementary to the oligonucleotides used as gene specific probes are enclosed in boxes.

(B). Clone blotting experiments with SER1-OLIGO and SER2-OLIGO as probes. Southern blots of the HindIII digested G50E of the cARS 11D insert were prepared and hybridized to end-labelled oligonucleotides to confirm that the probes are specific for each gene. The 1.8 kb fragment contains the SER1 gene and 2.7 kb fragment contains the SER2 gene (Fig. 9A). Each oligonucleotide probe recognizes specifically either SER1 or SER2.

(C). The poly A^+ RNA was isolated from y cv v f, dunceM14 and dunceM11 male flies and blots were prepared. These blots were probed with end-labelled SER1-OLIGO and SER2-OLIGO as well as the labelled 0.8 kb EcoRI/SalI restriction fragment representing SER3. Probe; 1. SER1-OLIGO 2.SER2-OLIGO 3. 0.8 kb EcoRI/SalI.

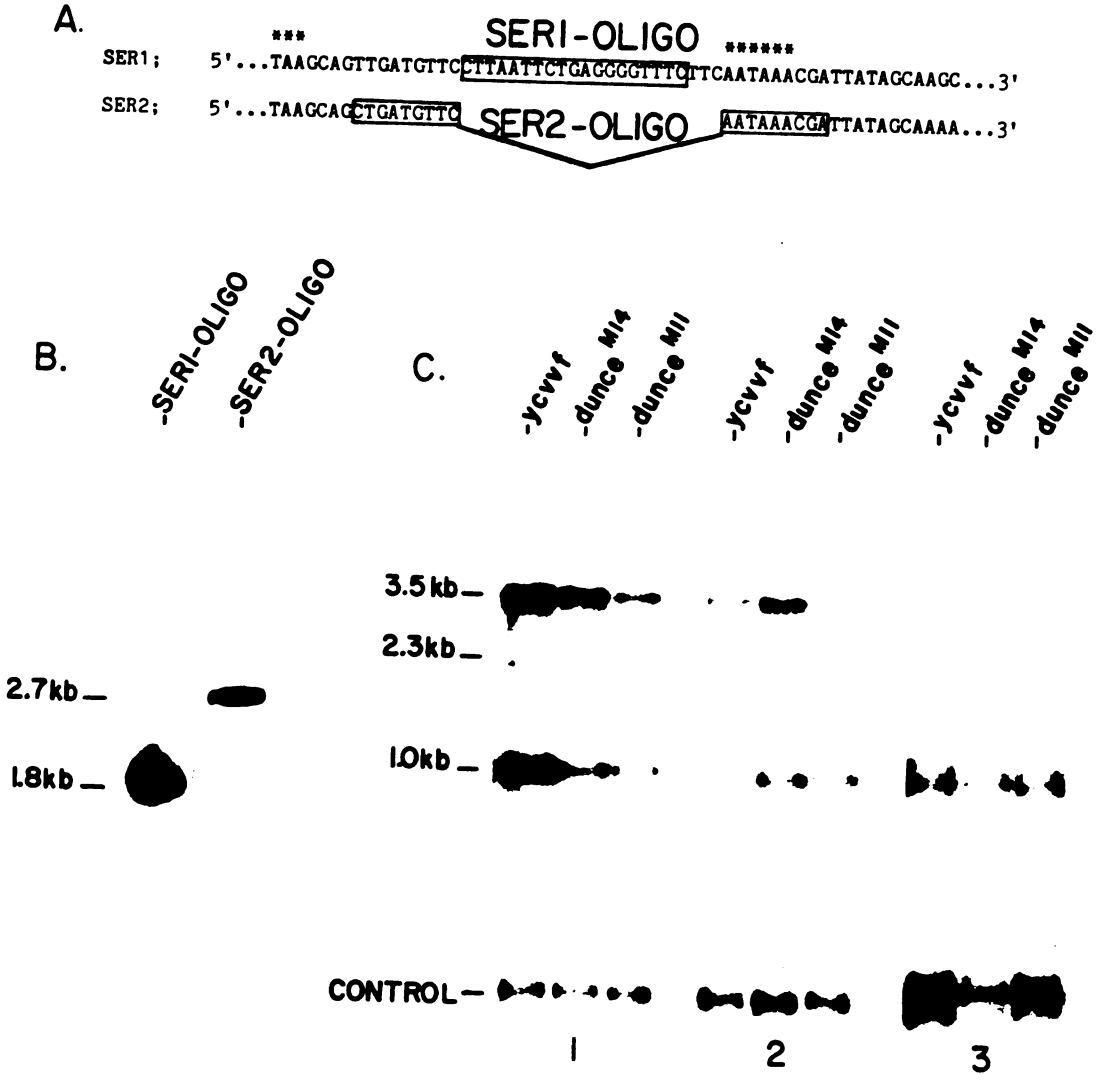

A.
***
SER1-OLIGO
******
SER1;
5'...TAAGCAGTTGATGTTCCTTAATTCTGAGGGGTTTCTTCAATAAACGATTATAGCAAGC...3'
SER2;
5'...TAAGCAGCTGATGTTC
SER2-OLIGO
AATAAACGATTATAGCAAAA...3'
B.
-SER1-OLIGO
-SER2-OLIGO
C.
-ycvvf
-dunce M14
-dunce M11
-ycvvf
-dunce M14
-dunce M11
-ycvvf
-dunce M14
-dunce M11
3.5kb
2.3kb
2.7kb
1.8kb
1.0kb
CONTROL
1
2
3

Fig. 15

mutants and the normal strain (Fig. 15C Panel 2 and 3). Thus, only SER1 (or SER1 related genes) is down-regulated in dunce flies. Furthermore, the hybridization signal of both mRNA species detected by the SER1-specific probe is greater than 10-fold stronger than the signal detected by the SER2-specific probe in y cv v f lanes, suggesting that SER1 is expressed more abundantly than SER2 in normal flies.

Tissue and developmental expression of the SER family genes.

In order to gain clues as to the function of the protein products of the SER family genes, the expression as a function of tissue type and developmental specificity was monitored in wild type Canton-S flies (Fig. 16A). The SER family genes are abundantly expressed in the larval gut, which suggests that a major function of the gene products is to aid in digestion. Tissue RNA blots were also hybridized with probes specific to each of SER1-, SER2- and SER3-related genes (SER1-OLIGO, SER2-OLIGO, and the 0.8 kb EcoRI/SalI fragment) and each hybridized to RNAs from larval gut (data not shown). In addition, northern blots prepared from head and body RNAs of Canton-S adult flies were also probed with 0.3 kb BamHI/SalI and 0.8 Kb EcoRI/SalI fragments (Fig. 17). The 0.3 kb BamHI/SalI probe detected strong hybridization signals to 1.0 kb and 3.5 kb RNAs, and faint signal to a 2.3 kb RNA in body RNA lane. The 0.8 kb EcoRI/SalI probe detected the signal to 1.0 kb RNA in body RNA lane. Neither probes hybridized to RNA from the head of the flies. This result

Fig. 16. Tissue and developmental expression of SER family genes.

(A). Tissue specific RNA blot probed with G50E. Organs were dissected from ten larvae and total RNA was isolated as described in Materials and Methods. S. Salivary glands; B. Brain; G. Gut; M. Malpighian tubules; F. Fat body; T. Total larvae.

(B). Developmental expression. Northern blots containing 10 ug of poly A^+ RNA from early embryos (Ee, 0-4 hrs), late embryos (El, 16-20 hrs), first instar larvae (L1), third instar larvae (L3), pupae (P), and adult flies (A) were probed with the labelled G50E fragment of the cARS 11D insert.

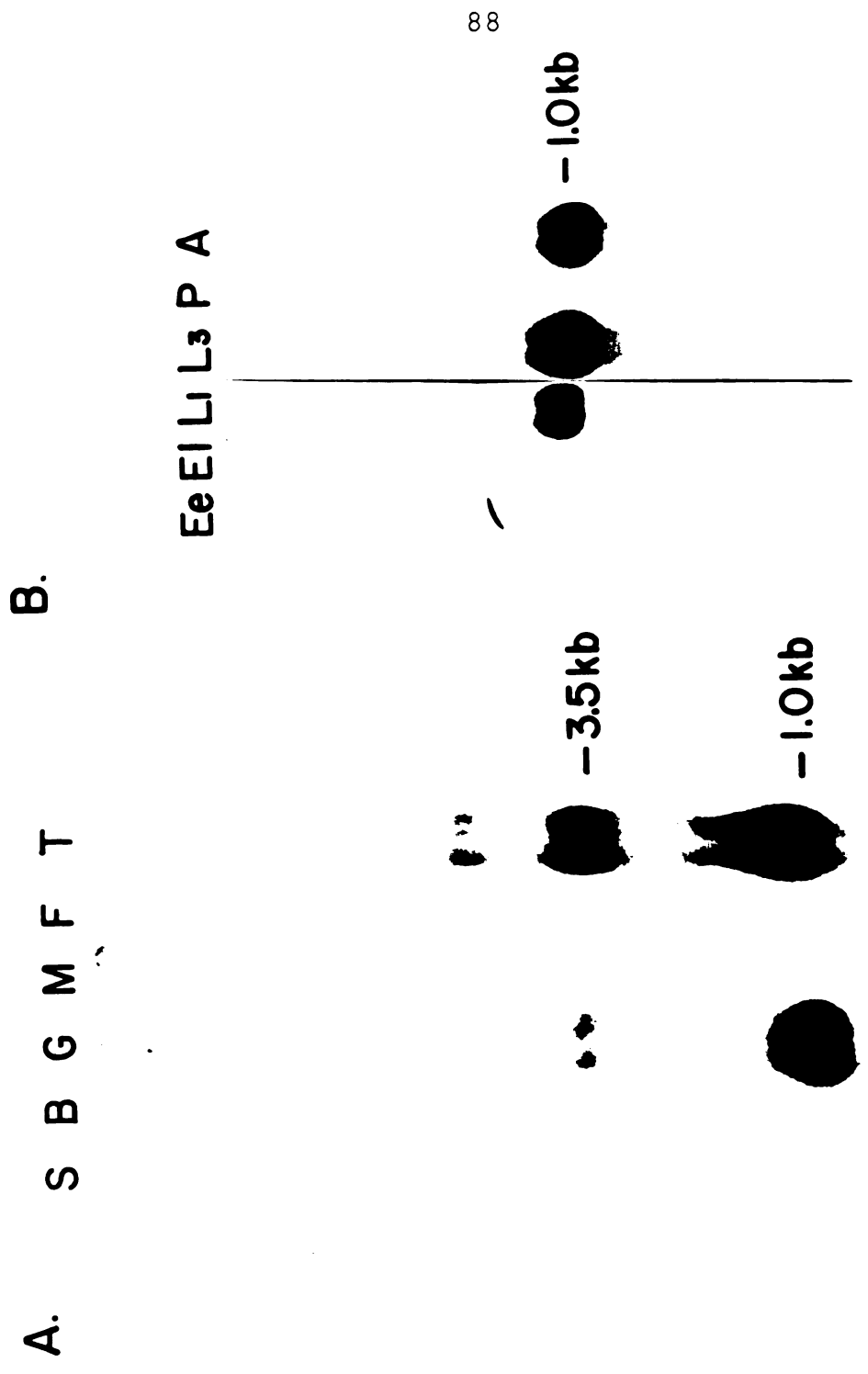


Fig. 16

Fig. 17. Expression of SER family genes in the head and body of adult flies.

Three micrograms of total RNA either from head or body of Canton-S adult flies were loaded on each lane. Probes are indicated at the bottom. H, Head; B, Body.

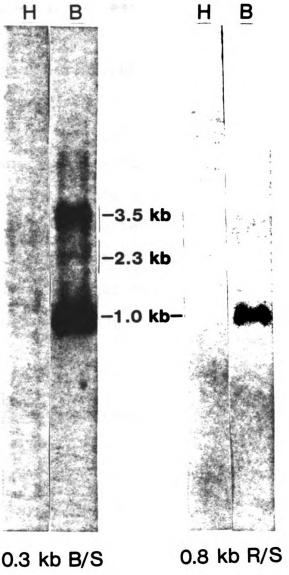


Fig. 17

suggests that SER family genes are not expressed in the central nervous system and so are probably not related to the memory defect phenotype of dunce mutants.

The developmental expression of the SER family genes was also examined (Fig. 16B). The transcripts begin to appear at the late embryo stage and continue to increase in abundance throughout the larval stages. The transcripts of these genes disappear during the pupal stage then reappear in the adult. Hybridization experiments with the probes specific to SER1-, SER2- and SER3-related genes (SER1-OLIGO, SER2-OLIGO, and 0.3kb BamHI/SalI fragment) each detected the same temporal expression patterns as above (data not shown). The matching tissue and developmental expression pattern of these three genes together with their sequence homology imply that they may function similiarly.

Effect of cAMP on the expression of the SER1 (or SER1-related genes in normal flies.

Since the SER family genes were isolated on the basis of differential expression in dunce mutants, and these flies have an elevated cAMP content, the decreased expression of SER1 (or SER1 related genes) might be due to the high levels of cAMP. To determine if cAMP modulates RNA abundance, wild type Canton-S flies were fed cAMP, phosphodiesterase inhibitor isobutylmethylxanthine, or adenylate cyclase activator forskolin. The total RNA blots prepared from these flies were probed with

Fig. 18. Effect of pharmacological agents on expression of SER1 genes.

The blots of total RNA prepared from Canton-S flies fed pharmacological agents were hybridized with labelled 0.3 kb BamHI/SalI restriction fragment (Fig. 9A). The same blots were reprobed with two control phage DNAs to confirm that equal amounts of RNAs were loaded. The pharmacological agents used are indicated on top of each lane. IBMX; isobutylmethylxanthine. (A). Short exposure (B). long exposure

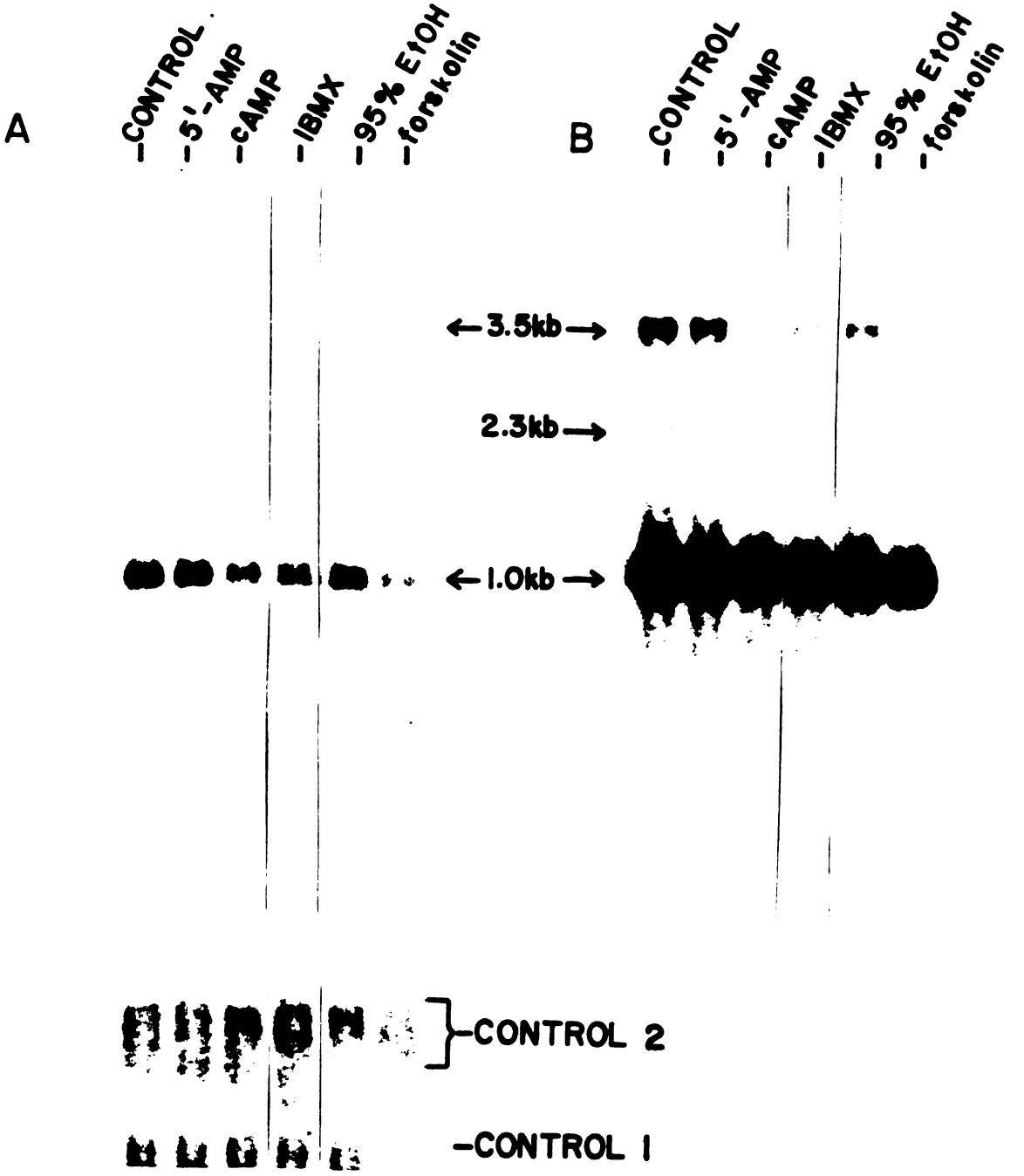

A
-CONTROL
-5'-AMP
-cAMP
-IBMX
-95% EtOH
-forskolin
B
-CONTROL
-5'-AMP
-cAMP
-IBMX
-95% EtOH
-forskolin
←3.5kb→
2.3kb→
←1.0kb→
-CONTROL 2
-CONTROL 1

Fig. 18

the 0.3kb BamHI/SalI fragment. The results are shown in Fig. 18. The abundance of the 1.0 kb transcript decreased approximately 2-3 fold and that of 3.5kb transcript decreased 3-5 fold in flies treated with the pharmacological agents. Again, to normalize more precisely for variations in the amount of RNA loaded per lane, the same blots were reprobed with the two control clones which hybridize equally with total cDNA probes from normal and dunce mutant strains. These results support the conclusion that the decreased expression of SER1 is mediated by incresed cAMP. The probe also detected a weak hybridization to a 2.3 kb RNA. The signal of this band was about the same in all lanes.

DISCUSSION

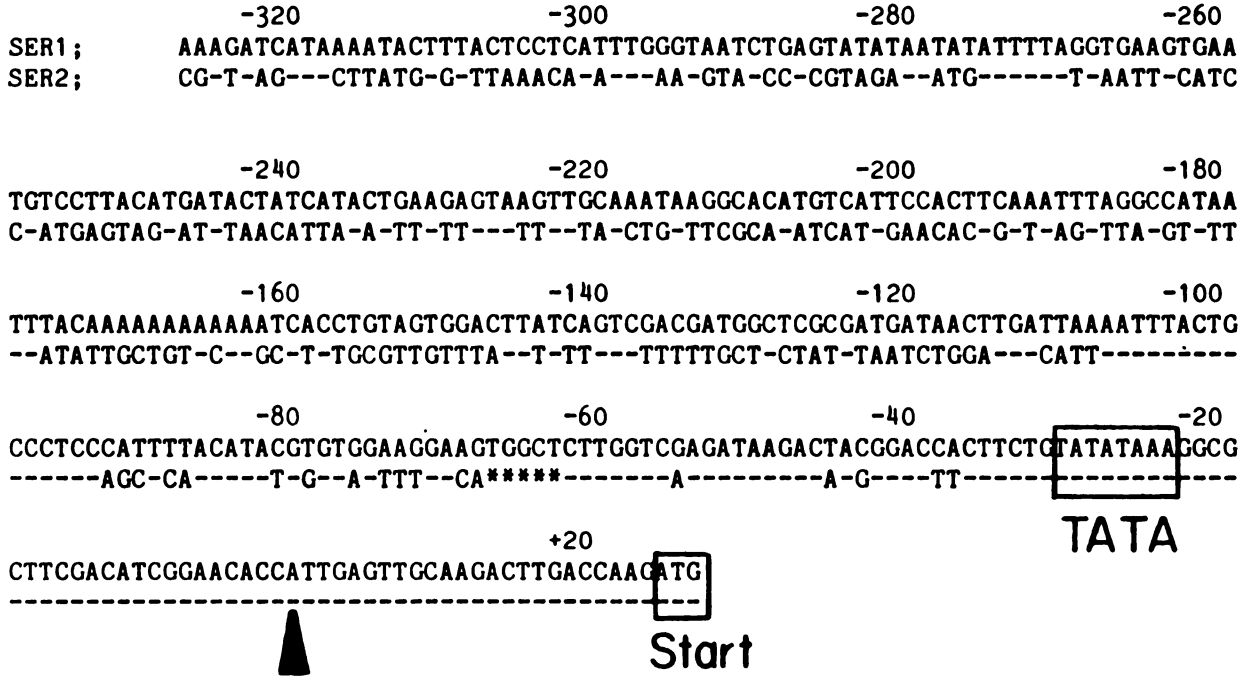
Our analyses show that the mRNA levels of a putative serine protease gene(s) are reduced in dunce mutants compared to wild type flies, and that this reduction is due to the elevated cAMP level. The following observations support this conclusion. First, the mRNA levels of SER1 (or SER1-related genes) are reduced in both of the dunce mutants tested. Since dunceM14 and dunceM11 were induced from y cv v f relatively recently by EMS treatment (40), their genetic background is expected to be quite similiar except for the difference in dunce locus. If the reduced expression of SER1 (or SER1-related genes) is due to a difference in genetic background outside of the dunce locus, it would be unlikely that such a change would occur in both of the mutants. Therefore, the differential expression of the SER1-related genes is presumably the result of the mutation in the dunce locus (cAMP- phosphodiesterase). Second, when normal flies were treated with pharmacological agents known to increase levels of cAMP, the mRNA levels of SER1 (or SER1-related genes) are decreased relative to appropriate controls. This result demonstrates that reduced mRNA levels of SER1 (or SER1-related genes) in the mutants are the result of the direct action of increased levels of cAMP and are not secondary effects caused by the mutations in the dunce locus.

cAMP has been shown to regulate mRNA levels of various genes including hormone genes (11, 24, 38, 41, 51), proto-

oncogenes (6, 20, 42, 48) and other genes of key metabolic pathways (17, 21, 26, 34, 44, 53) by either modulating the transcription rate or mRNA stability. To our knowledge, this report is the first example of a member of the serine protease gene family regulated by cAMP. In mammalian pancreatic cell lines, cAMP treatment has been reported to have no significant effect on the expression of the genes for the serine proteases, trypsin and chymotrypsin (46).

It appears that at least some SER family genes are clustered in the genome. Other serine protease genes, such as the Drosophila trypsin-like gene family (12) and the mammalian kallikrein subfamily (33) are also clustered in the genome. The high sequence homology between SER1 and SER2 suggests that these two genes diverged very recently. Despite the high sequence homology, the experimental results with gene specific probes indicate that the two genes are regulated differentially. Only the expression of SER1 is decreased by cAMP and in normal flies, it is expressed more abundantly than SER2. The 5′ flanking sequences of SER1 and SER2 are quite homologous up to the -60 position (Fig. 19) which suggest that the sequence of SER1 responsive to cAMP is likely to reside further upstream of the -60 position. In mammalian cells, the study of several genes positively regulated by cAMP has revealed an eight base pair consensus motif, 5′-TGACGTCA-3′, which is responsible for the cAMP mediated increase in expression (11, 24, 41, 44, 49). This sequence does not exist in the 5′ flanking region of SER1, which

Fig. 19. Alignment of 5′ flanking sequences of SER1 and SER2. The putative transcription start site is marked by an arrowhead and assigned position number 1. The sequence of SER2 that is identical to SER1 is indicated by dashes. Asterisks indicate the gap allowed for maximum homology. Putative 'TATA' sequences and translation start codons are enclosed in boxes.


-320
-300
-280
-260
SER1; AAAGATCATAAAATACTTTACTCCTCATTTGGGTAATCTGAGTATATAATATATTTTAGGTGAAGTGAA
SER2; CG-T-AG---CTTATG-G-TTAAACA-A---AA-GTA-CC-CGTAGA--ATG------T-AATT-CATC
-240
-220
-200
-180
TGTCCTTACATGATACTATCATACTGAAGAGTAAGTTGCAAATAAGGCACATGTCATTCCACTTCAAATTTAGGCCATAA
C-ATGAGTAG-AT-TAACATTA-A-TT-TT---TT--TA-CTG-TTCGCA-ATCAT-GAACAC-G-T-AG-TTA-GT-TT
-160
-140
-120
-100
TTTACAAAAAAAAAAAAATCACCTGTAGTGGACTTATCAGTCGACGATGGCTCGCGATGATAACTTGATTAAAATTTACTG
--ATATTGCTGT-C--GC-T-TGCGTTGTTTA--T-TT---TTTTTGCT-CTAT-TAATCTGGA---CATT---------
-80
-60
-40
-20
CCCTCCCATTTTACATACGTGTGGAAGGAAGTGGCTCTTGGTCGAGATAAGACTACGGACCACTTCTGTATATAAAGGCG
------AGC-CA-----T-G--A-TTT--CA*****-------A---------A-G----TT----------------
TATA
+20
CTTCGACATCGGAACACCATTGAGTTGCAAGACTTGACCAAGATG

Start

Fig. 19

is to be expected, since SER1 is negatively regulated and the motif is found in genes positively regulated by cAMP.

It is not clear if reduced expression of a serine protease gene(s) in the dunce mutants has any relationship to the dunce phenotypes, memory defects or female sterility. Tissue localization of the mRNA to the gut suggest this relationship is unlikely. However, Greenberg et. al. (19) recently reported the possible involvement of an undefined protease in the memory retention of Aplysia.

The extensive homology of the amino acid sequence of SER family to that of other known serine proteases and the conservation of the key amino acids important for catalysis and structure determination leaves little doubt that SER1 and SER2 encode serine proteases. When the sequences are optimally aligned, the sequence of SER1 shows highest homology to crab collagenase (40%). It also has similiarity to hornet chymotrypsin (37%), Drosophila trypsin-like enzyme (34%), Drosophila snake gene product (29%), bovine chymotrypsin (35%), and bovine trypsin (36%). These levels of homology are about the same as those between the members of the serine protease family listed above. The residues forming the charge relay system of the active site (29) in serine proteases (His-57, Asp-102, Ser-195) are found in corresponding position in SER1 and SER2 and the sequences around these residues are also highly conserved. The amino-terminal Ile-16 and Asp-194 which are known to form a salt bridge stabilizing the catalytic site in the other serine proteases (4) are also

conserved in SER1 and SER2. Since the six cysteine residues present in SER1 occur at the same positions as in other serine proteases, it is likely that similiar disulfide bridges are formed in SER1. These correspond to Cys42-Cys58, Cys168-Cys182 and Cys191-Cys220 (4, 43). Three disulfide bridges are characteristic of invertebrates serine proteases including Drosophila trypsin, hornet chymotrypsin, and crab collagenase. Vertebrate serine proteases, with the exception of group specific protease (52), always have more than three disulfide bridges (25).

The amino acids in serine proteases that line the binding pocket and determine the substrate specificity are at position 189, 216 and 226 (29). These positions are occupied by amino acid with small side chains in SER1/SER2 (serine, glycine and alanine respectively), which should result in a wide binding pocket like that of chymotrypsin. In contrast, SER3 is expected to have an occluded binding pocket like elastase (37), since Val-216 has bulky side chain.

LITERATURE CITED

1. Benkel, B. F., and D. A. Hickey. 1986. Genetics 114:137-144.

2. Benton, W. D., and R. W. Davis. 1977. Science 196: 180-182.

3. Biggin, M. D., T. J. Gibson, and G. F. Hong. 1983. Proc. Natl. Acad. Sci. 80:3963-3965.

4. Birktoft, J. J., and D. M. Blow. 1972. J. Mol. Biol. 68:187-240.

5. Bodenstein, D. 1950. The postembryonic development of Drosophila. In Biology of Drosophila, M. Demerec. ed. (New York: Hafner Publishing Co.):pp 275-367.

6. Bravo, R., M. Neuberg, J. Burckhardt, J. Almendral, R. Wallich, and R. Muller. 1987. Cell 48:251-260.

7. Bucher, P., and E. N. Trifonov. 1986. Nucleic Acid Res. 14:10009-10026.

8. Carne,T., and G. Scheele. 1982. J. Biol. Chem. 257:4133-4140.

9. Chen, C-N., S. Denome, and R. L. Davis. 1986. Proc. Natl. Acad. Sci. 83:9313-9317.

10. Cohen, R. S., and M. Meselson. 1985. Cell 43:737-746.

11. Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman. 1986. Nature 323: 353-356.

12. Davis, C. A., D. C. Riddell, M. J. Higgins, J. A. Holden, and B. N. White. 1985. Nucleic Acids Res. 13:6605-6619.

13. Davis, R. L. and J. A. Kiger. 1981. J. Cell. Biol. 90:101-107.

14. Davis, R. L., and N. Davidson. 1984. Mol. Cell. Biol. 4:358-367.

15. Delotto, R., and P. Spierer. 1986. Nature 323:688-692.

16. Fyrberg, E. A., K. L. Kindel, N. Davidson, and A. Sodja. 1980. Cell 19:365-378

17. Ginzburg, L., S. Rybak, Y. Kimhi, and U. Z. Littauer. 1983. Proc. Natl. Acad. Sci. 80:4243-4247.

18. Grant, G. A., K. Henderson, A. Eisen, and R. Bradshaw. 1980. Biochemistry 19:4653-4659.

19. Greenberg, D. M., V. F. Castellucci, H. Bayley, and J. H. Schwartz. 1987. Nature 329:62-65.

20. Greenberg, M. L., L. A. Green, E. B. Ziff. 1985. J. Biol. Chem. 260:14101-14110.

21. Hashimoto, S., W. Schmid, and G. Schutz. 1984. Proc. Natl. Acad. Sci. 81:6637-6641.

22. Henikoff, S. 1984. Gene 28:351-359.

23. Isackson, P. J., and R. A. Bradshaw. 1984. J. Biol. Chem. 259:5380-5383.

24. Jameson, J. L., P. J. Deutch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener. 1987. Mol. Cell. Biol. 7:3032-3040.

25. Jany, K-D., G. Beckler, G. Pfeiderer, and J. Ishay. 1983. Biochem. Biophys. Res. Comm. 110:1-7.

26. Jungmann, R. A., D. C. Kelly, M. F. Miles, and D. M. Milkowski. 1983. J. Biol. Chem. 258:5312-5318.

27. Jurgens, G., E. Wieschaus, C. Nusslein-Volhard, and H. Kluding. 1984. Roux's Arch. Dev. Biol. 193:283-295.

28. Kongsuwan, K., Q. Yu, A. Vincent, M. C. Frisardi, M. Rosbash, J. A. Lengyel, and J. Merriam. 1985. Nature 317:555-558.

29. Kraut, J. 1977. Ann. Rev. Biochem. 46:331-358.

30. Kurosky, A., D. R. Barnett, T-H. Lee, B. Touchstone, R. E. Hay, M. S. Arnott, B. H. Bowman, and W. M. Fitch. 1980. Proc. Natl. Acad. Sci. 77:3383-3392.

31. Langer-Safer, P. R., M. Levine, and D. Ward. 1982. Proc. Natl. Acad. Sci. 79:4381-4385.

32. Lathe, R. 1985. J. Mol. Biol. 183:1-12.

33. Leeuwen, B. H., B. A. Evans, G. W. Tregear, and R. I. Richards. 1986. J. Biol. Chem. 261:5529-5535.

34. Lewis, E. J., A. W. Tank, N. Weiner, and D. M. Chikaraishi. 1983. J. Biol. Chem. 258:14632-14637.

35. Lipman, D., and W. R. Pearson. 1985. Science 227:1435-1441.

36. MacDonald, R. J., S. J. Stray, and G. H. Swift. 1982. J. Biol. Chem. 257:9724-9732.

37. MacDonald, R. J., G. H. Swift, C. Quinto, W. Swain, R. L. Pictet, W. Nikovits, and W. J. Rutter. 1982. Biochemistry 21:1453-1463.

38. Maurer, R. A. 1981. Nature 294:94-97.

39. Messing, J. 1983. Methods. enzymol. 101:20-78.

40. Mohler, J. D. 1977. Genetics 85:259-272.

41. Montiminy, M. R., K. A. Savarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Proc. Natl. Acad. Sci. 83:6682-6686.

42. Ran, W., M. Dean, R. A. Levine, C. Henkle, and J. Campisi. 1986. Proc. Natl. Acad. Sci. 83:8216-822

43. Sawyer, L., D. M. Shotton, J. W. Campbell, P. L. Wendell, H. Muirhead, H. C. Watson. 1978. J. Mol. Biol. 118:137-208.

44. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson. 1986. J. Biol. Chem. 261:9721-9726.

45. Staden, R. 1984. Nucl. Acid. Res. 12:521-538.

46. Stratowa, C., and W. J. Rutter. 1986. Proc. Natl. Acad. Sci. 83:4292-4296.

47. Telford, J., J. Burckhardt, B. Butler, and V. Pirotta. 1985. EMBO J. 4:2609-2615.

48. Tramontano, D., W. W. Chin, A. C. Moses, and S. H. Ingbar. 1986. J. Biol. Chem. 261:3919-3922.

49. Tsukada, T., J. S. Fink, G. Mandel, and R. H. Goodman. 1987 J. Biol. Chem. 262:8743-8747.

50. Vincent, A., M. V. Colot, and M. Rosbash. 1985. J. Mol. Biol. 186:149-166.

51. Waterman, M., G. H. Murdock, R. M. Evans, and M. G. Rosenfeld. 1985. Science 229:267-269.

52. Woodbury, R. G., N. Katunuma, K. Kobayashi, K. Titani, and H. Neurath. 1978. Biochemistry 17:811-819.

53. Wu, J-S. R., and L. F. Johnson. 1982. J. Cell. Physiol. 110:183-189.

54. Zeff, R. A., and J. Geliebter. 1987. Focus 9:1-2.