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MESSENGER RNA DEGRATION IN PROKARYOTES

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RESUMO

A maioria dos processos biológicos não poderá ser plenamente compreendida sem um conhecimento detalhado do metabolismo do RNA. A contínua degradação e síntese de RNA mensageiro (mRNA) permite a produção de novas proteínas, e por isso os níveis de mRNA podem regular a síntese proteica e o crescimento celular. Em procariotas, a análise do mecanismo da degradação do mRNA tem sido particularmente difícil porque a maioria dos mRNA são degradados rapidamente com uma meia vida de 1-2 min. Os mRNAs bacterianos diferem na sua susceptibilidade à degradação por endoribonucleases e exoribonucleases e isto poderá ser devido à sua sequência e estrutura diferentes.

Pretende-se que esta revisão faça uma abordagem global do problema pondo em foco os aspectos do metabolismo do mRNA que parecem ser mais importantes na regulação da expressão génica.

INTRODUCTION

The level of expression of a gene in prokaryotes is determined primarily by three elements: the rate of transcription, the stability of the RNA transcript, and the efficiency of translation. Clearly, factors that modify mRNA or alter its decay may play a major role in controlling gene expression. This review is primarily intended as an overview of the current understanding of mRNA decay in prokaryotes.

Analyzing mRNA degration in prokaryotes has been particularly difficult because most mRNAs undergo rapid exponential decay, with an average mRNA having a chemical half-life of 1.3 min at 37°C (Ingraham *et al.,* 1984)). This mRNA instability best explains the rapid adaptation of microorganisms to a changing environment. An important consequence of the rapid kinetics in microorganisms is that a large fraction of molecules in a message population are decaying. The coupling of transcription, translation and mRNA degradation in prokaryotes also makes mRNA metabolism less amenable to investigation because the perturbation of many cellular processes can indirectly influence mRNA degration (Blundell *et al.,* 1972).

Individual mRNA species differ widely with respect to metabolic stability. Previous studies have established four general facts. First, the rate of turnover has no relation to the length of the gene (Blundell *et al.,* 1972). Second, the segments of the message that decay most rapidly may be anywhere on the mRNA (Achord and Kennell, 1974; Von Gabain *et al.,* 1983). Third, the rates of decay of some species can be altered in response to physiological signals such as changes in growth rate (Nilsson, *et al.,* 1984). Fourth, the stability of gene transcripts seems to be dependent on determinants localized to specific mRNA segments but little is understood about these determinants (Belasco *et al.,* 1986).

In spite of all these studies little is known about the process of mRNA degradation. It is hoped that this overview will enable more recent data to be viewed in context, and in addition, will highlight those aspects of mRNA metabolism which seem most likely to be important in the regulation of gene expression.

PARTICULAR CONFIGURATIONS AT THE 3′ TERMINI AND ROLE OF SECONDARY STRUCTURE IN mRNA DECAY

Secondary structural features at the 3′ termini could serve as a barrier to exonucleases and influence the rate at which these exonucleases degrade a given species of mRNA. It is known that *in vitro* secondary structures are resistant to attack by the 3′−5′ exonucleases present in the cell (Gupta *et al.,* 1977). The role for secondary structure *in vivo* has also been demonstrated to affect mRNA stability (Mott *et al.,* 1985; Newbury *et al.,* 1987a,b; Chen *et al.,* 1988).

The stem-loop structures associated with *rho*-independent terminators appear to play some role in RNA protection. For example, in the *trp* operon there are two transcription terminators, *trp-t* and *trp-t′*. The major site for transcription termination is *trp-t′* but most of the transcripts have 3′ termini at the proximal site *trp-t,* because it is located at a GC-rich hairpin structure. This 3′ terminus is thought to be a point at which the secondary structure

arrests 3′ exonucleolytic cleavage (Mott, *et al.*, 1985). Deletion of the *rho* independent terminator, distal to the *E. coli* ribosomal protein S20 coding sequences, results in an accelerated decay of S20 mRNA and is another example of mRNA protection from 3′ degradation (Mackie, 1987).

Upstream mRNA segments in *E. coli* can also be stabilized by insertion of a foreign transcription terminator derived from the crystal protein (*cry*) gene of *Bacillus thuringiensis* (Wong and Chang, 1986) or from a bacteriophage T7 DNA segment encoding an RNase III cleavage site (Panayotatos and Truong, 1985). These sequences have the potential to form stem-loop structures, and deletion analysis suggests that such a structure is the significant feature. The *cry* terminator is considered a positive retroregulator that enhances the expression of the upstream genes in both *E. coli* and *Bacillus subtilis*, independent of the insertional orientation. Transcription of the retroregulator sequence leads to the incorporation of the corresponding stem-loop structure at the 3′ end of the mRNA. The presence of this structure apparently protects the mRNAs from exonucleolytic degradation from the 3′ en and, therefore, increases the mRNA half-life and enhances protein synthesis of the target genes. However, it does not appear that all potentially stable stem-loop structures serve to stabilize mRNA (Wong and Chang, 1986), nor that the stable mRNA is necessarily more translationally active.

In two mRNA species of bacteriophage ∅X174 there is a sequence with a potential six-base-pair hairpin structure, upstream of a common termination site TJ (Hayashi and Hayashi, 1985). This structure confers stability to the upstream mRNAs, and the stabilized mRNAs are functionally active. Most mutations that reduce a 3′ sequence's potential for stem-loop formation also reduce its stabilizing effect. The effect of this element on mRNA half-life is independent of other ∅X174 encoded activities. When cloned at the 3′ ends of different genes in a pBR322 derivative plasmid, the cloned sequences were functional. This study shows that mRNA-stabilizing sequences can be an important tool to maximize gene expression from cloned genes.

Repetitive extragenic palindromic (REP) sequences are large inverted repeats which are highly conserved in sequence and have the potential to form a stable stem-loop structure (Higgins *et al.*, 1982). These sequences are not terminators of transcription and they have been identified in a large number of genes both in *E. coli* (present in about 25 % of all transcripts) and *Salmonella typhimurium* (Gilson *et al.*, 1984; Stern *et al.*, 1984). The REP sequences are invariably transcribed and are located either in intergenic regions of multicistronic operons or in the 3′-untranslated regions upstream of the terminator. They can stabilize upstream RNA by protection from 3′−5′ exonucleolytic attack and they are frequently responsible for the differential stability of different segments of mRNA within an operon (Newbury *et al.*, 1987a). At least in certain cases, the mRNA is stabilized in a translationally active form, and thus by altering RNA

stability it is possible to alter gene expression (Newbury *et al.,* 1987a). Deletion of REP sequences from the *E. coli malEFG* operon not only destabilizes opstream *malE* mRNA, but also results in a ninefold reduction in the synthesis of MalE protein (Newbury *et al.,* 1987b).

Other hairpin structures also appear to stabilize selected regions of polycistronic mRNAs. In the *Bacillus subtilis pur* operon several degradation products of the attenuated mRNA are protected from rapid degradation by secondary structures. Some products accumulate to an estimated steady state level up to 40-fold greater than the intact attenuated transcript (Ebbole and Zalkin, 1988). In the *rxcA* operon of *Rhodopseudomonas capsulata* the 5′ region of the polycistronic transcript, corresponding to the first two genes, is more stable than the 3′ region. There is a potential stem-loop that appears to impart stability to the 5′ terminal transcripts (Belasco e*t al.,* 1985; Klug *et al.,* 1987). The same result was obtained when a DNA fragment containing the *rxcA* locus was fused to a plasmid promoter and transcribed in *E. coli.* This differential stability appears to play a role in the differential expression of genes in this operon, since there is a 10:1 ratio of polypeptides synthesized from the 5′ and 3′ genes of the operon. However, Chen *et al.,* (1988) showed that decay of the 3′ segment begins with endonucleolytic cleavage in which the intercistronic stem-loop structure does not participate. They concluded that this mRNA hairpin is necessary but insufficient for the stability of the upstream mRNA, and that it functions in message degradation solely as an mRNA decay terminator that protects upstream mRNA segments from degradation by 3′−5′ exoribonucleases. Their findings may explain why *E. coli* mRNA segments upstream of REP elements are found to decay at different rates (Newbury, 1987a). These disparate mRNA half-lives probably reflect differential susceptibility to endonucleolytic cleavage.

In the *E. colli rpsU-dnaG-rpoD* operon, the transcript for DNA primase is in the middle of the operon and decays more rapidly than the other two regions. It has been suggested that the polycistronic transcript is first cleaved at the primase-sigma boundary, and that the exposed 3′ terminus of the primase region is quickly degraded until the stem-loop in the S21 protein region is reached (Burton, e*t al.,* 1983).

These examples illustrate that secondary structure can be very important in determining the relative stabilities of different mRNA moleculus. It is known to affect the stability of mRNA in two ways. First, such structures are often sites for endonucleolytic cleavage. Second, secondary structure can provide a barrier to processive exonucleases.

In bacteriophage retroregulation, the destruction of the *sib* stem-loop structure by RNase III leaves the transcript with an exposed 3′ terminus that leads to its rapid decay. In the absence of active enzyme or in *sib* mutants the transcript is more stable, apparently because the intact stem-loop structure

acts as a barrier against 3′ exonucleolytic cleavage (Schindler and Echols, 1981; Guarneros *et al.*, 1982; Schmeissner *et al.*, 1984). However, RNase III cleavage at a bacteriophage T7 site, which was cloned downstream of a human interferon gene, has the opposite effect of stabilizing the upstream mRNA (Panayotatos and Truong, 1985). Panayotatos and Truong (1985) proposed that the mode of cleavage within the RNase III site determines mRNA stability. A single cleavage leaves part of the phage T7 RNase III site in a folded structure at the generated 3′ end and stabilizes the upstream mRNA that is active, thereby increasing protein levels. Two cleavages at the *int* site removes the folded structure and accelerates degradation. Thus, RNase III cleavages may either stabilize mRNA and stimulate gene expression or destabilize mRNA and prevent protein synthesis. This is also an example where the differential expression of genes within an operon is controlled by differential mRNA stability.

ROLE OF 5′ TERMINAL REGIONS IN mRNA DEGRADATION

Sequences at the 5′ end of mRNA may also be involved in the decay process. Minor variations in the leader region not only result in a differential rate of translation, but also dramatically affect the steady-state amount of full-length mRNA (Stanssens *et al.*, 1986). Single base changes in the 5′ region preceding the β-galactosidase message, which may disrupt a putative secondary structure, caused a faster functional and mass decay of that message (Cannistraro and Kennell, 1979). Sequence changes preceding a Shine-Delgarno region (Shine and Delgarno, 1974) also influence *trpE* mRNA translation and decay (Cho and Yanofsky, 1988).

McCarthy *et al.*, (1986) showed that RNA sequences upstream of the ribosome binding site influence the loading of ribosomes, and depriving a transcript of its ribosomes can destabilize the transcript (Nilsson *et al.*, 1987). However, these leader regions apparently do not seem to share any other sequence besides the Shine-Dalgarno sequence, and they can have very different secondary structures. The ribosome loading site would be an excellent candidate for an inactivating target. Also, it could be preferred because this region must have specificity since it is recognized for initiation from other regions of the mRNA.

If decay commences only after the 3′ end of the transcript is completed then there will be a delay between synthesis and degradation. In 1975, Yamamoto and Imamoto studied *trp* mRNA, moleculus and on the basis of their results they inferred that the rate-limiting step for initiating bulk mRNA degradation was determined by a sequence located at or near the 5′ end of the messenger RNA. Subsequently it was reported that this 5′ proximal segment was the target of endonucleolytic cleavages (Kano and Imamoto, 1979). Size analysis has demonstrated that *lacZ* mRNA is degraded by a net directional

process. Cannistraro and Kennell (1985a) decided to investigate this system and the results of their experiments showed that the 5′ end starts to decay very soon after it is made.

Belasco *et al.,* (1986) have shown that the relatively long half-life of the *E. coli ompA* transcript is determined by a 5′ leader segment that includes the ribosomal binding site and the first few codons. Melefors and von Gabain (1988) showed that the 5′ non-coding region of *ompA* mRNA is a target for growth--dependent endonucleolytic attacks and the half-life of this mRNA follows the rate of endonucleolytic cleavage. *tac-ompA* fusions with 54 additional 5′terminal nucleotides are more susceptible to degradation than the wild type (Melefors and von Gabain, 1988). When the 5′ non-coding region of the β-lactamase (*bla*) transcript is replaced by the same region of the *ompA* message the resultant hybrid mRNA is four times more stable than the normal *bla* mRNA (Belasco *et al.,* 1986). The stabilization effect of this monocistronic message appears to involve ribosome interaction, as suggested by the fact that a hybrid transcript with a termination codon at the end of the 5′ *ompA* segment was not stabilized. Consequently, the stabilization appears to be dependent upon readthrough across the hybrid mRNA junction by ribosomes that initiate translation within the 5′ *ompA* segment

In the *rpsO-pnp* operon the sequence involved in the stabilization of the *pnp* mRNA is also located at the 5′ end of this message (Portier *et al.,* 1987). RNase III cleavage apparently triggers the decay of the transcripts downstream, since in ribonuclease III deficient mutants the *rpsO-pnp* transcript has a considerably longer half-life.

In *B. subtilis* the stabilization of the *ermC* gene occurs independently of induced translation. The regulatory sequences required for stability are promoter-proximal and can confer stability on large mRNAs having diverse 3′ ends (Bechhofer and Dubnau, 1987). Since stalling of ribosomes while translating the leader peptide was essential for induced stability, they concluded that the effect of ribosomes on mRNA stability was exerted by the protection or alteration of specific sites rather than by a general coating of the message.

The relative stability of some bacteriophage T4 transcripts can also be attributed to a 5′ leader sequence (Gorski *et al.*, 1985) In gene *32* the active sequence appears to consist of 40 nucleotides located close to the initiation codon. Although sequences involved in both gene *32* translational regulation and mRNA stabilization appear to be localized in the vicinity of the ribosomal binding site, the sequences responsible for these phenomena are probably not identical, since gene *32* mRNA is stable in the absence of detectable translational repression. Transfer of this 5′ leader sequence to an otherwise unmodified *lac* operon mRNA confers 10-fold stabilization on the hybrid RNA. Duvoisin *et al.* (1986) took advantage of these properties and constructed hybrid plasmid vectors that contain the promoter region and start codon of T4 gene *32,* a

contiguous multiple cloning site, and translation and transcription termination signals. These vectors can be expressed at high levels after phage infection and they also permit stabilization of both mRNAs and proteins encoded by the cloned genes. However, the 5′ stabilization required interaction of the leader sequence with factor(s) produced only in the infected cells (Gorski *et. al.,* 1985).

Thus 5′ terminal regions can be very important in mRNA degradation but their role is not completely defined. The half-lives of mRNAs may vary depending on the susceptibility of sites near the 5′ ends to endonucleolytic attack, and cleavage at these sites may be influenced by the efficiency of ribosome binding and other factors that can bind to these 5′ regions.

DIRECTIONALY OF mRNA DECAY

The chemical decay of many mRNA cistrons occurs in a net 5′−3′ direction, even though no 5′−3′ riboexonuclease has been identified in *E. coli* (Apirion and Gegenheimer, 1984). For example, the mRNA enconding the promoter proximal (5′) genes of the *trp* operon is degraded before that encoding the promoter-distant (3′) genes (Morikawa and Imamoto, 1968; Morse *et al.,* 1968; Schlessinger *et al.*, 1977) and the 5′ end of *lac* mRNA is degraded before the 3′ end is synthesized (Cannistraro and Kennell, 1985a). These results provide good evidence that decay is not simply due to the processive activity of 3′−5′ exonucleases degrading mRNA from the 3′end.

The 5′ end is vulnerable to attack longer than any other part of the molecule. Endonucleolytic cleavage at the 5′ end of a molecule, followed by 3′−5′ direction of decay. This could be a reasonable explanation for the results observed (Apirion and Gegenheimer, 1984). An assumption of the overall 5′−3′ decay mechanism is that 3′−5′ exonucleolytic decay beginning at the 3′ terminus is slow relative to endonucleolytic cleavages, perhaps because structural features at the 3′ termini serve as a barrier to exonucleases. In the *gal* operon, mRNA from the middle cistron, *galT,* has a shorter half-life than of either the two flanking genes (Achord and Kennell, 1974). These data also support the ideia that degradation occurs by the concerted action of endonucleases and exonucleases.

The 5′ end of most messages includes a nontranslated leader which is not covered by ribosomes. If the presence of ribosomes on mRNA confers protection from endonucleolytic attack, then the 5′ end will be particularly vulnerable. A 5′ clevage in the Shine-Dalgarno sequence would block ribosome initiation and the unprotected mRNA would be progressively exposed to nucleases in a 5′ to 3′ direction. By analogy, in *trp* mRNA, nonsense mutations leading to premature ribosome release destabilized mRNA sequences distal to the mutation (Morse and Yanofsky, 1969).

Decay of mRNA exclusively in a 3′−5′ direction would produce many

incomplete proteins. An inactivation at the 5′ end would block new functional protein synthesis immediately, while attack at the 3′ end would result in cessation of synthesis. However, in bacteriophage infection the most important factor can be to shut off certain proteins rapidly, even if that is inefficient biologically. In fact, the *int* message is inactivated by RNase III cleavage at the 3′ end and then the message is degraded 3′ to 5′ (Gottesman *et al.*, 1982; Schmeissner *et al.*, 1984).

Certain monocistronic messages may be completely synthesized before decay is initiated. In such cases degradation might proceed linearly from the 3′ end with no endonucleolytic involvement. The exonuclease would be moving against the movement of ribosomes flowing 5′ to 3′. Von Gabain *et al.* (1983) proposed that this is the case for the *ompA* mRNA. However, with the *ompA* mRNA most of the message decays at the same rate and only a leader at the 5′ end clearly decays more slowly. Another interpretation could be that an initial 5′ cleavage generates a very stable 5′ fragment. In the *trp* operon there is a leader that is cleaved and has a slower decay (Kano and Imamoto, 1979). However the general mode of decay is 5′ to 3′ since the proximal mRNA is mainly degraded before the distal mRNA is even synthesized (Morikawa and Imamoto, 1969; Morse, *et al.*, 1969). Melefors and von Gabain (1988) suggested that 5′ endonucleolytic cleavages provide the initial step in *ompA* mRNA degradation and this supports the fact that the decay does not proceed linearly from the 3′ end.

The pattern of fragmentation of *B. subtilis cat-86* mRNA is consistent with the presence of at least two internal cleavage sites (Ambulos *et al.*, 1987). These sites are highly specific both at the 5′ and at the 3′ ends and fragmentation appears to precede the net loss of mRNA mass. In this case there was no evidence of any defined directionality during the decay process. In the *B. subtilis pur* operon, Ebbole and Zalkin (1988) proposed that the degradation of the attenuated transcript is initiated by an endonucleolytic cleavage in a single--stranded region in the middle of the transcript. The half-life measurements indicated that the degradation of the 3′-half proceeds faster than that of the 5′ end and the proposed scheme of decay involves a series of endonucleolytic cleavages followed by processive 3′ exonucleolytic trimming up to a base-paired secondary structure.

The degradation of *E. coli trxA* mRNA also involves specific endonucleolytic cleavages (Arraiano and Kushner, 1990). The cleavages occur at many internal sites. The majority of the degradation products have intact 5′ ends during decay, suggesting that the 3′ ends are preferentially cleaved. The 3′ ends also seem to be degraded sequentially. The turnover probably results from the combined action of endo — and exonucleases. Most of the molecules follow this pattern of decay but there are exceptions, in which the 5′ end is cleaved early in the decay process. Taken together it would appear that the directionality of the decay process depends on the transcript analyzed.

Stem-loop structures are believed to contribute to mRNA stability (Schmeissner *et al.,* 1984; Newbury *et al.,* 1987a) but other factors might also contribute to stabilize the transcripts (Gorski, e*t al.,* 1985; Belasco *et al.,* 1986). The structural determinants which have been claimed as stabilizing influences are located at either end of the messenger. This implies that degradation can start either at the 5′ or 3′ end depending on the RNA species. The mRNA decay can be a multicomponent process where many RNases could intervence. In any multihit process the rate will only become faster if the slowest event, or rate--limiting step, becomes faster. The directionality of the decay process will be determined by the localization of the rate limiting target sites. It is this apparent combination of endo — and exonucleolytic degradation which leads to the wide variety of degradation patterns which are proving so difficult to solve.

TRANSLATION AND mRNA STABILITY

The presence or absence of ribosomes and the rate of translation may also affect the half life or mRNA molecules. The reduction in translation rates in streptomycin-dependent cells (Gupta and Sschlessinger, 1978), the depletion of ribosomes by heat shock (Har-El *et al.,* 1979), and the blocking of translation with chloramphenicol (Graham, *et. al.,* 1982) or kasugamycin (Schneider e*t al.,* 1978) have all suggested that translation may play an active role in mRNA degradation. However anbiotics and heat shock may well perturb the cell and make the results difficult to interpret. For example chloramphenicol causes ribosomes to stall but they remain bound to the mRNA, and they could stabilize mRNA by protecting the RNA from nuclease attack. Consistent with this notion, mRNA is degraded when ribosomes are released by puromycin action (Cremer, *et al.,* 1974). It was proposed that messages which load ribosomes less efficiently decay faster (Kennell and Riezman, 1977). If this is a means of control then differing codon usage might also affect some mRNA′s stability (Varenne *et al.,* 1984; Pedersen, 1984). Ribosome pausing has been suggested to occur at positions of rare codons. Thus, a mRNA that is being translated does not have a uniform distribution of ribosomes across its length; instead densely stacked clusters of ribosomes (caused by the pausing of the leading ribosome) alternate with mRNA regions that are only sparsely populated by ribosomes.

The fact that mRNA being made at the time of translation inhibition continues to be synthesized complicates the analysis of the results. This «naked» RNA, like the RNA after a nonsense codon, probably decays faster because it is not protected by ribosomes. Also, residual polarity makes it difficult to measure a functional decay on reversal of a translational block (Pastushok and Kennell, 1974).

Studies of translational inhibition are also complicated by the generation of polarity distal to the peptide bond site that is affected by the inhibitor. The message distal to the translation block may not be completed (Imamoto and Kano, 1971) and decays abnormally (Hirago and Yanofsky, 1972), thus obscuring normal decay processes. Furthermore, the sizes of nucleic acid precursor pools change during a translation block (Nierlich, 1968) and there is difficulty in achieving adequate inhibition of transcription initiation by rifampicin (Kennell and Simmons, 1972). This leads to an apparent slow mass decay because of concomitant synthesis of mRNA.

It was mentioned above that 5′ leader sequences can be very important in mRNA degradation. Leader variations possibly influence the rate of translation initiation by altering the RNA secondary structure (Stanssens *et al.*, 1985). Stanssens *et al.*, (1986) showed that translational efficiency can control the mRNA level. Nevertheless, when they compared the stability of *lacZ* transcripts that differed in their ability to initiate translation, and hence in the number of ribosomes covering their coding region, they found no differences in their decay rates. In addition von Gabain *et al.* (1983) have shown that the most stable portion of the *E. coli ompA* transcript lies in the 5′ leader region, which is not covered by ribosomes.

The ribosome structure surrounding the mRNA could prevent some enzymes from penetrating but smaller enzymes might possibly still have access to cleave the mRNA at specific sites. Actually, enzymatic accessibility experiments show that a few sites along the ribosome-bound mRNA are still accessible to ribonucleases (Kang and Cantor, 1985). The position and acessibility of these sites reveal unusual features of ribosome-bound mRNA structure and ribosome--mRNA interactions.

The stability of ribosomal protein (r-protein) mRNAs in *E. coli* decreases under the conditions in which their translation is feed-back inhibited by repressor r-protein (Singer and Nomura, 1985). Thus translational repression can change the half-life of messenger RNA. In the L11 r-protein operon, altering the site on the «L11 mRNA» where the L1 (the operon-specific translational repressor protein) interacts, results in a five-fold increase in half-life (Cole and Nomura, 1986). The half-life of the *spc* ribosomal protein operon mRNA, which is not translationally regulated by L1, stays constant. Furthermore, the half-life of L11 operon mRNA carrying an additional mutation in the ribosome binding site that abolished translation remains short. This indicates that the observed change in half-life is due to translational repression by L1 and not due to some nucleolytic activity mediated by L1. Under conditions of increased transcriptional repression there was an increase in the decay rate of the L11 mRNA that is probably a consequence of inhibition of translation by L1. With the elucidation of the translational regulation of r-protein synthesis, it is now apparent that measurement of remaining mRNA after blocking transcription with rifam-

picin or other inhibitors may be affected by complications and inaccuracies in feedback regulated operons. These inhibitors may have other effects on regulatory mechanisms that are important in determining mRNA half-life.

The 5′ segment of the *ompA* transcript can act as a cis-dominant determinant of mRNA stability that increases the half-life of otherwise short-lived *bla* mRNA segments fused downstream (Belasco *et al.*, 1986). Such stabilization is dependent upon readthrough across the hybrid mRNA junction by ribosomes that initiate translation within the 5′ *ompA* segment. The premature termination of translation at a location that allows ribosomes to traverse only a short segment of *bla* mRNA can lead to destabilization of both the translated and ribosome coverage of mRNA can affect transcript stability. Conversely, premature termination of translation beyond a site located early in the protein coding region of *bla* mRNA has no effect on the half-life of the entire transcript (Nilsson *et al.*, 1987).

The mRNA encoded by the *ermC* gene of *B. subtilis* seems to be affected by a similar mechanism (Bechhofer and Dubnau, 1987). The induced *ermC* mRNA stability occurs even without translation of the methylase coding sequence. However, the induced stability requires ribosome stalling in the leader peptide and the effect of ribosomes on mRNA stability is exerted by the protection or alteration of specific sites rather than by a general coating of the message. It seems that the 5′ end is the target and ribosome stalling protects that end. The molecular mechanism by which degradation of an mRNA that lack ribosomes is affected by ribosome coverage of an upstream segment of that transcript is presently unclear, given the uncertainty of the endonucleases that might be responsible for it and the absence of known 5′ exoribonucleases in *E. coli* (Deutscher, 1985).

In 1978 Schneider *et al.* wrote: «The results suggest that neither ribosomes nor translation play an active role in the degradative process. Rather, targets can be protected by the proximity of a ribosome, and without nearby ribosomes the probability of cleavage becomes very high. During normal growth there is a certain probability that any message is in such a vulnerable state, and the fraction of vulnerable molecules determines the inactivation rate of that species». These comments are still baluable more than 10 years later; many experiments have given suggestive results but so far there is no compelling evidence demonstrating that ribosomes play anything more than a passive role in mRNA degradation.

FUNCTIONAL INACTIVATION AND CHEMICAL DECAY

Distinguishing between functional inactivation and chemical decay has been useful in studies of mRNA turnover. Functional inactivation is an alteration of a mRNA species that renders it unsuitable for further translation. Chemical

decay, on the other hand, refers to the degradation of an mRNA species to oligo — or mononucleotides. It was first shown in 1970 that the two processes are temporally distinct (Schwartz *et al.,* 1970). The analysis of *trp* mRNA molecules suggested that functional inactivation of each messenger is regulated independently of bulk chemical degradation of the message (Yamamoto and Imamoto, 1975). Pedersen *et al.* (1978) calculated the functional half-life of individual mRNA species from *E. coli* by measuring the decay of the capacity to synthesize proteins after complete inhibition of transcription. They found that the decay rates measured showed a large spectrum of half-lives ranging from 40 seconds to approximately 20 min. Moreover, there was no correlation between the abundance of a protein and the half-life of its mRNA.

Chemical decay is thought to result largely from the action of 3′ to 5′ exonucleases, starting from the 3′ end of the message or from internal, endonucleolytic clevages or from both (Lim and Kennell, 1979). Translational inactivation probably involves specific endonucleolytic cleavage at the 5′ end of an mRNA molecule, resulting in the destruction (or blockage) of the ribosome-binding site or proximal coding sequences (Cannistraro *et al.,* 1986).

When streptomycin was used to alter the rate of ribosome movement it was shown that mRNA decay depended on the rate of translation while functional inactivation was unaffected (Gupta and Schlessinger, 1976). The functional inactivation of *trp* mRNA was unaffected by replacement of the *trp* promoter with the lambda PL promoter, while the chemical stability of the mRNA was increased (Yamamoto and Imamoto, 1975). The pattern of fragmentation of *B. subtilis cat-86* mRNA involves endonucleolytic cleavages that cause functional that cause functional inactivation, and fragmentation appears to precede the net loss of mRNA (Ambulos *et al.,* 1987). Furthermore, a temperature sensitive mutant has been isolated in which the chemical mRNA half-life was increased at the non-permissive temperature but the functional half-life was unaltered (Ono and Kuwano, 1979). This mutation was called altered message stability (*ams*) and was mapped at 23′ in the E. coli chromosome (Ono and Kuwano, 1980). These findings imply that functional inactivation precedes mRNA degradation.

If functional inactivation is the initial event in mRNA degradation then inactivated messages could have increased chemical half-lives. This could explain why increased stability does not necessarily lead to increased expression. The isolation of RNase mutants with slower functional inactivation of messages would be extremely useful to understand mRNA decay.

It is important to mention that mechanisms of functional inactivation other than endonucleolytic cleavage are possible. For example, the binding of a protein or RNA to the ribosome-binding site of an mRNA could block further ribosome addition. Nomura *et al.* (1984) showed that certain ribosomal proteins bind to initiation sites, inhibiting further translation of the mRNAs for those proteins.

The expression of the *regA* gene in bacteriophage T4 is controlled by translational autoregulation and affects translation of several T4 early genes (Miller *et al.*, 1985). The functional and chemical half-lives of mRNA species controlled by *regA* are increased in *regA*-deficient mutants (Karam *et al.*, 1977). Processing of mRNA by RNase III regulates expression of gene 1.2 of bacteriophage T7. Cleavage exposes the 3′ end of the mRNA which can hybridize to an upstream ribosome binding site, inhibiting translation (Saito and Richardson, 1981). In the T4 bacteriophage gene *32* mRNA, the gene product shows autoregulation, providing another example of non-nucleolytic functional inactivation (Gorski *et al.*, 1985). Another type of functional inactivation can be mediated by antisense RNA. A small RNA (MIC-mRNA interfering complementary RNA) has been shown to inhibit translation of *E. coli ompF* mRNA by hybridizing to its riboso-me-binding site (Mizuno *et al.*, 1984). This form of regulation by translational inhibitors may apply to other groups of mRNAs.

ENZYMES AND OTHER FACTORS INVOLVED IN mRNA DEGRADATION

The enzymes involved in mRNA degradation are poorly characterized. Most of the nucleases involved in the maturation of tRNA and rRNA precursors do not seem to play a major role in mRNA decay (Apirion, 1984; Deutscher, 1988). It is possible that the loss of one RNase is compensated for by the activities of others, and it would be assumed erroneously to be a non-participant. With so many ribonucleases released in cell extracts as well as changes in compartmentation, enzymes that degrade RNA *in vitro* may have little importance *in vivo*.

Deutscher (1988) proposed that there are a variety of means for achieving RNA stabilization against RNases and for ensuring specificity. The means described were: 1) the inherent three-dimensional structure of the RNA, 2) the addition of moieties to RNA termini, 3) the association of RNA with protein, 4) compartmentalization of RNases, 5) activation or inhibition of RNases, 6) highly-specific RNases, 7) repair of RNase damage. A combination of some of these strategies is probably involved in mRNA decay in order to ensure a certain pattern of decay for each mRNA or to minimize the effect of any unwanted cleavages.

We have to reject the possibility that each message is recognized by a unique enzyme because it would necessitate thousands of different enzymes for several thousand of different messages in the cell. Another possibility would be sequences or structures that are common to all messages and that are attacked by a few RNases. These messages will have different decay rates, reflecting the degrees of protection or vulnerability.

a) EXONUCLEASES

While endonucleases can certainly cleave mRNA, bulk degradation of mRNA must depend on exonucleolytic activities. All the exonucleases so far identified in *E. coli* degrade RNA progressively from the 3′ en and no 5′−3′ exonuclease activity has yet been identified (Deutscher, 1988). The functional significance of many exoribonucleases is not certain since mutants lacking these enzymes often have normal phenotypes. Probably there are other undiscovered exoribonucleases that can take over the function of the missing enzymes.

Several exonucleases have been partially characterized in *E. coli,* but only two are believed to be the principal degradative enzymes (Deutscher, 1985). These two exonucleases are polynucleotide phosphorylase (PNPase) (Reiner, 1969a,b) and ribonuclease II (RNase II) (Spahr, 1964; Singer and Tolbert, 1985) and both degrade single-tranded mRNA processively in the 3′ to 5′ direction. PNPase degrades mRNA via a reversible phosphorolytic reaction and generates mononucleotide 5′-diphosphates. RNase II irreversibly hydrolyzes mRNA to 5′-monophosphates.

Donovan and Kushner isolated a mutant *rnb* allele (*rnb-500*) that encodes a thermolabile RNAse II protein (Donovan and Kushner, 1986). Double mutant strains *(pnp-7 rnb-500)* ceased growth within 30 minutes after shift to 44°C. Cessation of growth was accompanied by the *in vivo* accumulation of mRNA fragments of 100 to 1500 nucleotides (Kushner, *et al.,* 1985; Donovan and Kushner, 1986). Little or no change was observed in cellular levels of rRNA. At the non-permissive temperature, the half-life of total pulselabeled RNA was two-fold greater in a double mutant strain than in single mutants. Single mutant strains deficient in either PNPase or containing thermolabile RNase II activity grew well at 44°C and did not accumulate partially degraded RNA species. These results suggested that the two enzymes are largely complementary. However, in a different study, the absence of PNPase alone was enough to stabilize the *Neurospora crassa* catabolic dehydrogenase *(qa-2)* mRNA in *E. coli* strains (Hautala *et al.,* 1979).

There seems no doubt that RNase II and PNPase play an important role in bulk mRNA degradation (Kaplan and Apirion, 1974; Donovan and Kushner, 1986). However, the possibility remains that additional exonucleases also exist. If they are regulated and/or have specificity for some transcripts then they could be important factors in determining gene expression.

b) ENDONUCLEASES

The first evidence for endonucleolytic cleavage as an initial event in mRNA degradation came from the *gal, lac,* and *trp* operons (Achord and Kennell, 1974; Blundell and Kennell, 1974; Schlessinger *et al.,* 1977). Specifically, in

the *lac* operon it was shown that the full-length molecule is lost much faster than the mRNA mass, which could occur only from internal cleavages (Blundell and Kennell, 1974; Lim and Kennell, 1979). In cells starved for various nutrients the ribosomal ribonucleic acids also appeared to be degraded endonucleolytically (Kaplan and Apirion, 1975). If mRNA degradation were exclusively from the 3′ terminus, degradation would be blocked until the last ribosome was released. Yet, Cannistraro and Kennel (1985a) showed that the 5′ end of *lac* mRNA starts to decay as soon as it is synthesized. Thus, endonucleolytic cleavages may be important in increasing the rate of mass breakdown.

The experiments referred to abovev shoed an accumulation of intermediates in degradation. However, these intermediates might have resulted from endonucleolytic cleavages or, alternatively, could have been due to a blockage in the activity of a processive exonuclease. It was necessary to map the precise endpoints of the degradation intermediates and see if there were any sequences and/or structures that could be recognized by specific endonucleases.

Some of these ends were found in the intercistronic region between *lacZ* and *lacY* (Cannistraro *et al.*, 1986) and in *lacZ* and *lacI* (Cannistraro *et al.*, 1985b,c; Subbarao and Kennell, 1988). The results indicated that the cleavages usually occur between Pyr-A residues, in single-stranded regions. The attenuated transcript of the *B. subtilis pur* operon is endonucleolytically cleaved possible single-stranded regions and the degradation intermediates were identified (Ebole and Zalkin, 1988). Melefors and von Gabain (1988) showed that the 5′ noncoding region of *E. coli ompA* mRNA was a target for site-specific, growth-dependent endonucleolytic attacks.

Recently, Arraiano and Kushner (1990) analized the decay of *E. coli trxA* mRNA and provided the first map of cleavage sites during the degradation of an entire transcript. They identified complementary upstream and downstream cleavage products of internal endonucleolytic cleavage sites and determined the nucleotide sequence of the cleavage sites. There was no apparent consensus sequence. Secondary-structure analysis suggested that the cleavages occurred both in single and double stranded regions. It remains to be demonstrated whether the specificity resides in the primary sequences, in the spatial structure or both.

If mRNA decay were due to a random endonucleolytic process, some diversity could be provided by the target size. However, there is no relationship between mRNA size and decay rate. For exemple the *lacA* message decays twice as fast as the four-fold longe *lacZ* message (Blundell *et al.*, 1972). Belasco *et al.* (1986) constructed internal in-frame deletions in the *E. coli bla* gene and gene and compared the stability of the derived transcripts. The transcripts differed in size by 50 %, yet had similar decay rates.

If endonucleolytic cleavage within coding regions often occurs during mRNA degradation, a significant number of ribosomes translating a message should be blocked on mRNA fragments with no termination codon. This would

result in the release of peptidyl tRNA species partially completed proteins attached, that could be released by peptidyl tRNA hydrolase and subsequently degraded (Menninger, 1976). It is estimated that up to 10 to 20 % of the translational products end prematurely (Manley, 1978; Yen *et al.*, 1980).

In phage retroregulation (Guarneros *et al.*, 1982), the RNase III cleavage demonstrates that endonucleolytic cleavage may expose free 3′ ends, which are substrates for exonucleases.

Polycistronic mRNA molecules may also be endonucleolytically cut to monocistronic units prior to further degradation. In the *lac* operon, specific 5′ and 3′ terminated *lacY* mRNA molecules could be recovered from cells. Direct sequencing of the cleavage sites showed that the mRNA sequence 5′-UUAU-3′ is especially vulnerable and it was suggested that cleavages may occur in single--stranded regions (Cannistraro *et al.*, 1986).

RNase III is an endonuclease that can cleavve double-stranded RNA and can therefore recognize stem-loop structures in mRNA (Robertson *et al.*, 1968; Robertson and Dunn, 1975). It was shown to attack T7 early mRNA (Dunn and Studier, 1973), mRNA (Lozeron *et al.*, 1977), and rRNA precursors (Dunn and Studier, 1973; Nikolaev *et al.*, 1973). *E. coli* strains deficient in RNase III accumulated both mRNA and rRNA precursors (Robertson, 1982). However no single role has emerged for its possible function in mRNA decay. RNase III appears to control several genes as indicated by alterations in patterns of protein synthesis in cells lacking this enzyme (Gitelman and Apirion, 1980; Talkad *et al.*, 1978). However, there are very few examples of post-transcriptional cleavage of *E. coli* mRNA by RNase III.

In the *rplJL-rpoBC* operon RNase III cleaves an intergenic site separating cistrons coding for ribosomal proteins from those coding for RNA polymerase subunits, but the reason for this cleavage is unclear (Barry *et al.*, 1980; Dennis, 1984). RNase III cleaves the intercistronic region of the *E. coli rpsO-pnp* operon (Takata, *et al.*, 1985; Regnier and Portier, 1986). The half-life of the *pnp* mRNA is considerably increased in a ribonuclease III-deficient strain (Portier *et al.*, 1987; Takata *et al.*, 1987). Moreover, PNPase activity increased 10-fold in the mutant strain, which shows that the unprocessed *pnp* mRNA is functional. Portier *et al.* (1987) suggested that the sequence involved in the stabilization of *pnp* mRNA is located in the 5′ end of the message and that the RNase III processing triggers the decay of the transcripts downstream. Discrete cleavage products are produced when *lac* mRNA or *trp* mRNA are incubated with RNase III *in vitro* (Shen *et al.*, 1982). It is not certain which if any of these *in vitro* cleavages is important for mRNA inactivation *in vivo*, and it is difficult to visualize the formation of some of the secondary structures during translation.

As mentioned above, RNase III plays a role in the processing of mRNA from bacteriophages. It cleaves phage, exposing sites for 3′ exonucleolytic attack (Schmeissner *et al.*, 1984). Cleavage of T7 early RNA exposes RNA sequences

which hybridize to an upstream region (Saito and Richardson, 1981). These two examples are quite opposite in effect. Panayotatos and Truong (1985) proposed that the mode of cleavage determines mRNA stability since RNase III single cleavage produces stable, functionally active T7 mRNA whereas double cleavage allows the rapid decay of mRNA. Consequently, RNase III cleavages may either stabilize mRNA and stimulate gene expression or destabilize mRNA and prevent protein synthesis.

A variety of mRNA moleculus have been found to be degraded independently of RNase III. It remains to be established RNase III has an important role in bulk mRNA decay, in addition to the particular effects referred to above.

RNase I is an endonuclease involved in rRNA degradation (Gesteland, 1986) but the possibility remains that this enzyme can also play a role in mRNA degradation. The expression of the *Neurospora crassa ga-2* gene in *E. coli* was increased two-fold in *rna-19* (RNAse I-) strains, 20-50 fold in *pnp-7* (PNPase-) strains, and 100-fold in *rna-19, pnp-7* (RNAse I$^-$, PNPase$^-$) strains (Hautala *et al.*, 1979). There was no increase in the specific activity of either chromosomal or plasmid-borne prokaryotic genes tested. The results of this study reflected that structural differences between prokaryotic and eukaryotic mRNAs probably make them differentially susceptible to degradation.

It seems probable that several different endonucleases remain to be identified. For example, there are at least five sites in the mRNA of bacteriophage f1 which can be cleaved by an unidentified *E. coli* enzyme (Blumer and Steege, 1984)). The M1 RNA subunit of RNAse P is processed (Reed and Altman, 1983), and there is a RNA processing site between *dnaG* and *rpoD* (Burton *et al.*, 1983). In *B. subtilis* the pattern of fragmentation of *cat-86* mRNA is consistent with the presence of at least two internal cleavage sites, and fragmentation appears to precede the net loss of mRNA mass (Ambulos, et al., 1987). A site-specific endonucleolytic activity was identified that cleaves *ompA* and *bla* mRNA. In the case of *ompA* the endonucleolytic cleavages seem to regulate the growth-rate-dependent stability of this mRNA (Melefors and von Gabain, 1988). The clevages occur *in vitro* and *in vivo* and most of the clevage sites map in the 5′ non-coding region (Nilsson *et al.*, 1988). Analysis of mutants revealed that the responsible activity (enzyme) might reflect a classe of endoribonucleases which have not yet been discovered to be involved in mRNA degradation.

c) OTHER FACTORS

Other factors can also influence the rate at which a given species of mRNA is degraded.

The altered message stability (*ams*) gene was mapped at 23′ in the *E. coli* chromosome, and *ams* single mutants have increased mRNA stability (Ono and

Kuwano, 1980). Although *ams* does not seem to code for any known RNases, it plays a role in the degradation of RNA (Ono and Kuwano, 1979). In a triple mutant deficient in Ams, PNPase and RNase II, discrete mRNA breakdown products were dramatically stabilized during the decay of specific messages (Arraiano *et al.,* 1988). The function of the Ams protein is still unknown.

The relative stability of some bacteriophage T4 transcripts can be attributed to a 5′ leader sequence, but the stabilization requires interaction of the leader sequence with trans-acting factor(s) produced only in the infected cells (Gorski *et al.,* 1985).

Translational repression causes changes in the half-life or ribosomal protein mRNA (Singer and Nomura, 1985; Cole and Nomura, 1986). For example the half-life of L11 mRNA increased five-fold when the translational feedback regulation by L1 was absolished. The change in half-life seems to be a consequence of L1 blocking translation of L11 mRNA and not due to some nucleolytic activity mediated by L1. Similar effects can exist in other operons where encoded proteins may regulate its own synthesis and the decay of their mRNA.

In *Klebsiella pneumoniae* the production of active nitrogenase requires the expression of at least 15 linked genes (the *nif* genes) arranged in 7 operons (Brill, 1980). The *nif* transcripts (except *nifLA*) are very stable under derepressing conditions. The specific posttranscriptional control of *nif* mRNA stability is an important feature on the regulation of *nif* gene expression, and this specificity requires a *nif*-encoded protein, the *nifL* product, which is involved in *nif* mRNA destabilization in response to O_2 and to a lesser extent NH_4 (Collins *et al.,* 1986). However, it should be noted that the means by which the *nifL* product recognizes and responds to O_2 and NH_4 is not known.

Carter and Newton (1971) isolated polarity suppressors in *E. coli* that suppressed polarity in the *trp* and *lac* operons without showing codon specificity for suppression. It was proposed that these suppressors act by interference with messenger breakdown because they protect *lac* mRNA and total mRNA from an accelerated rate of degradation in the presence of puromycin.

Autocalytic splicing is well established in eukaryotes (Bass and Cech, 1984) and it has been shown that an RNA molecule from bacteriophage T4 can undergo a specific self-cleavage reaction (Watson *et al.,* 1984). We can only speculate at present, but the autocatalytic properties of certain RNA molecules may also be important in mRNA decay.

Anti-sense mRNAs could also be involved in protecting some regions of mRNA by making them a double-stranded. A small RNA (mRNA interfering complementary or MIC RNA) has been shown to inhibit translation by hybridizing to the E. coli ompF mRNA (Mizuno *et al.,* 1984). This form of regulation may apply to other mRNAs but so far we lack evidence to support it.

CONCLUDING REMARKS

The degradation of mRNA can depend on determinants localized on specific mRNA fragments. Determinants which have been claimed as stabilizing influences can be located at either end of a transcript. However, other factors such as the presence of ribosomes or bound proteins can possibly be involved in the regulation of the decay of specific mRNAs. The selectivity of mRNA decay is best explained by the action of factors that recognize unique sites on the mRNA chains. A rate-limiting endonucleolytic cut triggered by such an interaction would be followed by rapid destruction of the mRNA. It is the combination of endo — and exonucleolytic degradation which leads to the wide variety of degradation patterns which are proving so difficult to unravel.

Secondary structure can modulate gene expression and the combination of the mRNA sequence with its potential for secondary structures will give the particular degradation profile of that message. Secondary structures can be cleaved by double-strand-specific RNases like RNase III but in some cases secondary structures protect the RNA. This apparently is the case of the tRNAs and rRNAs which otherwise might be unstable. Particular configurations at the 3′ termini appear to impart stability to upstream mRNA segments probably because they provide a barrier to processive exonucleases. However, it is not known whether the stem-loop structures do in fact occur *in vivo* and the precise nature of the protective terminator structures remains to be established. Sequences located proximally within a transcript can also influence its half-life. These need not be mutually exclusive possibilities and conceivably sequences at the 3′ end and at the 5′ end could operate within a single transcript.

Every mRNA has a sequence that is complementary to some sequence at the 3′ end of the 16S rRNA, but the exact sequence, as well as the number of nucleotides to which it is complementary varies from mRNA to mRNA; that is, it is a specific sequence but not a unique one. The availibility of this sequence as a target could depend on several factors such as secondary structure or protection by ribosomes, and this could modulate its vulnerability. So far there are no consensus sequences or structures that would provide a common target near the starts of *E. coli* messages, to make them functionally inactive. Related to this finding, efforts to isolate RNase mutants with slower functional inactivation of messages have been unsuccessful.

To analyze mRNA turnover it is extremely useful to construct single and multiple isogenic mutants deficient in factors involved in this process. Factors that control mRNA decay can be important elements in the post-transcriptional regulation of gene expression. Comparing the wild type with each mutant constructed will help assign specific functions for the genes studied.

The puf operon of *Rhodobacter capsulatus* illustrates the importance of mRNA stability to differential gene expression. An intercistronic stem-loop

accounts for segmental differences in transcript stability within the polycistronic
accounts for segmental differences in transcript stability within the polycistronic
transcripts. Consequently, there is differential gene expression and the polypeptides from the 5′ and 3′ ends of the operon are synthesized in a 10:1 ratio (Belasco *et al.,* 1985; Klug *et al.,* 1987). The absence of RNase III cleavages in an RNase III- strain leads to the strong stabilization of fully active *pnp* mRNA that has a 10- fold overexpression of PNPase (Portier, *et al.,* 1987). Attaching the *cry* terminator sequence of *Bacillus thuringiensis* to the 3′ end of cloned genes increases the half-lives of the gene transcripts and overproduces the gene products (Wong and Chang, 1986). As there are many prokaryotic operons in which the genes are expressed at disparate levels, it seems likely that there will be many other examples in which differential mRNA stability determines relative expression. The analysis of the stabilizing sequences can lead to the construction of new vectors that can stabilize mRNAs and maximize gene expression from cloned genes.

Future work will involve the identification and study of the mechanism of action of more RNases, relating these RNases to mRNA decay through the isolation of mutants, and assessing whether the various reactions are regulated. Novel approaches involving the use of cell-free systems, and the identification of cleavage sites that trigger mRNA decay will be required to achieve further understanding of this important aspect of gene expression.

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ARTIGOS DE INVESTIGAÇÃO

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ACTUAL COMPLEXITY OF TRANSCRIBING DOMAINS IN DROSOPHILA POLYTENE CHROMOSOMES

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SUMMARY

The complexity of actual transcribing domains in polytene chromosomes was studied by analysis of chromosomal DNA:RNA hybrids in autoimmunograms following a drastic repression of RNA synthesis caused by a heat shock treatment. After 3 min at 25°C RNA polymerase II molecules and nascent RNA transcripts forming hybrids in situ with the chromosomal DNA may be detected in all nuclei of the salivary gland and in 85-90% of the chromosome subdivisions. After 15 min of recovery the amount of antihybrid reactivity significantly increased but the distribution of the DNA-RNA epitopes over the chromosomes was similar to that detected after 3 min of recovery and to that of non heat shocked larvae. The 0-order like kinetics of DNA-RNA hybridization may be explained by the fact that the chromosomal RNA molecules detected by the anti-DNA:RNA antibodies are bound to the DNA template through a protein linker. The termal stability of these hybrids was that expected for DNA:RNA duplexes. The experiments show, therefore, that all chromosomal domains in which DNA:RNA hybrids may be detected during a steady state growth of the larvae are opened for RNA pol II binding and that RNA transcription reassumes shortly after release of the impediment caused by a heat shock.

INTRODUCTION

A number of reports have measured the complexity of total poly+ and nuclear RNA from different tissues using kinetic measurements of the rate of annealing between cDNA and poly A+ RNA and high resolution saturation

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hybridization techniques [1, 2, 3, 4, see for review 5]. In Drosophila melanogaster, Turner and Laird [6] showed that between 20 and 30 % of the single copy DNA is transcribed into RNA at various stages of development. In addition, Bishop et al [7], demonstrated that during the developmental stages of Drosophila melanogaster about 7000 different codifying poly A+ RNA genes are active. Because this number fits the average number of bands and based also on genetic data it was predicted that the number of genes should be close to that of the bands of the polytene chromosomes. Recent information, however, indicates that the one band-one gene hypothesis can not be literally true and that there are more vital genes than bands (see [8] for review). It is likely that more than one gene is present in each structural units since a band of the polytene chromosomes of Drosophila may contain from 20 to 200 kb [9] and that, therefore, they may be considered as transcriptional domains some of which codify several mRNAs [10, 11] probably involved in determining a complex metabolic pathway or phenotype [12].

The polytene chromosomes have offered unique possibilities for the measurement of the complexity of transcribing loci using autoradiography of 3_H uridine incorporation. It was shown that puffs are not the only subdivisions which transcribe RNA but that labeled precursors incorporate also into many other chromosomal subdivisions including diffuse bands and interbands [13, 14, 15, 16, 17]. Moreover, Mott and Hill [18] have also reported that all decondensed regions of the polytene chromosomes of Drosophila melanogaster are members of a continuum of transcriptionally active structures because RNP particles are found in all interbands, puffs and diffuse bands. In addition, RNA pol II molecules can also be detected in all interbands of the polytene chromosomes of Drosophila melanogaster [19]. In recent years we developed a methodology based on an indirect immunocytochemical staining of DNA:RNA hybrids to study the distribution of RNA molecules in polytene chromosomes [20, 21]. Since DNA:RNA epitopes were observed over most of the chromosomal subdivisions it was suggested that the complexity of transcribing domains may be as high as the number of band-interband units. It was also observed that for the definition of RNA synthesizing domains the resolution given by autoimmunograms of DNA:RNA hybrids is significantly higher than that given by autoradiograms [22].

We think, however, that the actual complexity of transcribing domains could not be revealed, by any of the studies indicated above because it was not possible to detected whether or not the RNPs and the chromosomal RNA molecules forming hybrids in situ reppresented actual transcripts. It is known, moreover, that even the presence of RNA polymerase II over a particular chromosomal subdivision is not per se a sign of actual transcription [23]. The results presented in this paper show that immediately after recovery from a severe heat shock and a drastic repression of RNA synthesis most of the chromosome subdi-

visions are open for RNA polymerase II binding and that upon binding transcription reassumes in these domains and that the pattern of transcribing domains after 15 min at 25°C is identical to that observed during normal growth. Thus, as suggested by Mott and Hill [18] and by Alcover et al., [21], at least 85-90 % of the units of band-interband of the polytene chromosomes form a continuum of actual transcribing structures.

MATERIAL AND METHODS

Animals

Laboratory cultures of Drosophila hydei were used. The larvae were raised at 25°C and 75 % humidity.

Thermal denaturation curve of chromosomes, chromatin and DNA

The melting profiles of DNA and chromatin of Drosophila hydei were recorded in a spectrophotometer ACTA III (Beckman) equipped with a Tm programmer. The chromicity was recorded at a constant temperature increase of 1°C per min. The DNA and chromatin were obtained from isolated nuclei [24]. Denaturation was carried out in 0.1 × SSC containing 2 % formaldehyde. The melting profile of chromosomes was determined by measurement of the increase in hyperchromicity relative to the total extinction value (Etot 265 nm) at 45°C [25, 26]. The chromosomes were fixed either for 2 min in 0.1 × PBS (PH 7.5) containing 2 % formaldehyde or in 45 % acetic acid on quartz slides. The chromosomes were denatured in 0.1 × SSC-2 % formaldehyde.

Immunofluorescence reaction of DNA:RNA hybrids

The method described by Alcover et al. [21] was used. The concentration of formamide was lowered to 30 % instead of 50 % to maintain the temperature of annealing at 45°C [27]. The antihybrid reactivity was visualized under glycerol/1M Tris (9:1, PH 8) and photographed with a Zeiss 40 × oil immersion lens. The chromosomes were afterwards stained with acetoorcein and all the negatives from each particular experiment were developed simultaneously with HC 110D.

Proteinase K digestion of chromosomes

Acetic acid squashed chromosomes were digested for 15 min with proteinase K at a concentration of 10 and 40 μg/ml in 10 mM Tris-HC1-2x SSC (pH 7.5) at 37°C. Afterwards the chromosomes were rinsed in the same buffer,

dehydrated in ethanol for 3 hr, dried by air and prepared for the DNA:RNA immunofluorescence reaction. The enzyme preparation was self digested for 1h at 37°C and did not have any DNase or RNase activity.

Thermal stability of the DNA:RNA hybrids

The thermal stability of the hybrids induced was determined by melting them at different temperatures ranging from 45°C to 90°C in 0.1 × SSC-2 % formaldehyde for 30 sec. Afterwards the chromosomes were stained with the antibody. The relative overal intensity of fluorescence between chromosomes incubated at the temperatures indicated above was done by determination of the time needed to eliminate the fluorescence upon illumination at 450 nm using a 40x objective lens. Cytophotometric determinations in a segment of the 4th chromosome indicated that the alteration effect (change intensity of fluorescence upon illumination) in each band is proportional to initial intensity of fluorescense.

Immunofluorescence of RNA polymerase II

Salivary glands dissected in 0.1%PBS-O.15 M NaC1.0.5% Triton X-100, were squashed and fixed in the same buffer containing 1% formaldehyde for 30 sec in 45 % acetic acid. Afterwards they were postfixed in ethanolic formaldehyde [28]. The chromosomes were then incubated for 45 min with mouse monoclonal anti RNA polymerase II at a dilution of 1:15 and with a rabbit anti-mouse FITC labeled antibody (dilution 1:50).

Heat shock treatment

Middle third instar larvae were placed in petri dishes containing solidified 1% agar preheated at 37°C (± 0.1°C) in order to control the temperature and the period of incubation (5, 20, 35, and 50 min). At the end of the incubation period the salivary glands were isolated in 0.1x PBS buffer or ethanol-acetic acid (3-1), fixed in the same solution for 2 min, squashed in 45 % acetic acid and prepared for the immunofluorescence reaction with anti-DNA:RNA hybrid antibodies. For the analysis of transcriptional activity folowing recovery from a 50 min heat shock treatment, the larvae were transferred to petri dishes containing 1 % agar precooled at 25°C in order to allow immediate recovery from the 37°C treatment. At different intervals from recovery the chromosomes were analyzed for the presence of DNA:RNA hybrids and RNA polymerase II.

RESULTS

Chromosomal DNA: RNA hybrids can only be visualized after an in situ hybridization.

In order to test whether the anti-hybrid reactivity was a result of DNA:RNA hybridization we determined whether the amount of reactivity was a function of the degree of DNA melting. If the hybrids formed resulted from DNA-RNA annealing the intensity of reactivity should be proportional to the amount of single stranded chromosomal DNA. Fig. 1 shows the melting profiles of native

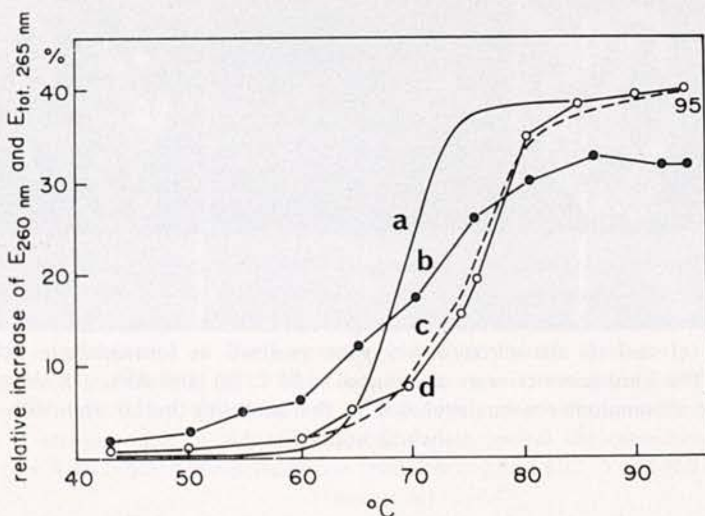

Fig. 1 — Thermal denaturation curves of native DNA (a), acetic acid fixed chromosomes (b), chromatin (c) and formaldehyde prefixed chromosomes (d). Denaturation was carried out in 0.1 x SSC-2 % formaldehyde. Abscissa: relative increase of E tot 265nm (for chromosomes) and of E 260nm (for native DNA and chromatin)

DNA (a), acetic acid fixed chromosomes (b), chromatin (c) and formaldehyde prefixed chromosomes (d). The melting curves of chromatin and chromosomes were similar with a Tm of 77°C and 78°C respectively. The Tm of native DNA was 69°C. Since the chromosomal DNA, in chromosomes prefixed in formaldehyde, had an hyperchromicity similar to that of native DNA we think that it was completely melted at 95°C. When the chromosomes were not prefixed in formaldehyde the hyperchromicity was lower as an indication that during the acetic acid squashing phase a fraction of the DNA was denatured.

Fig. 2 shows that indeed the level of anti-hybrid reactivity is proportional to the amount of denatured DNA since the level of fluorescence increases as a function of the amount of DNA in single stranded form. As expected, on

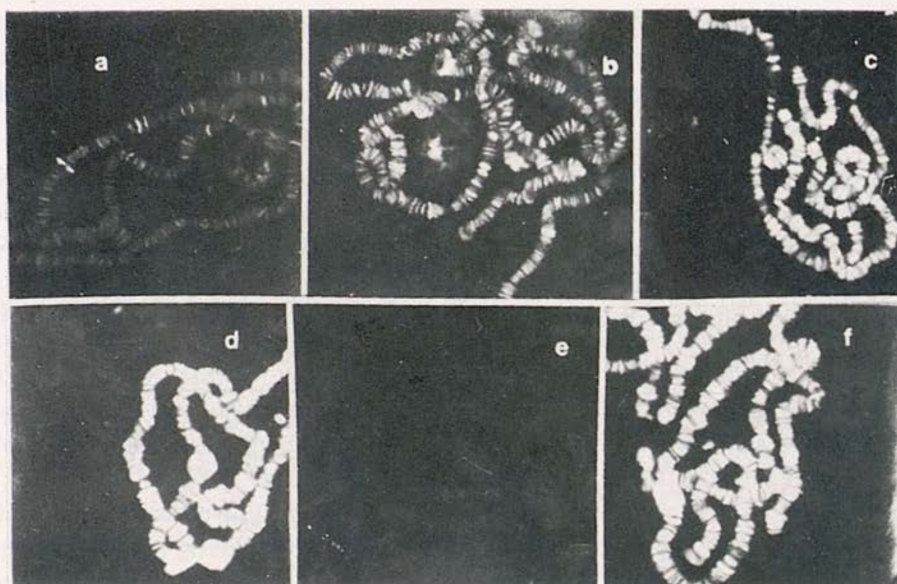

Fig. 2 — Autoimmunograms of chromosomal DNA-RNA hybrids as a function of the temperature of denaturation of the chromosomal DNA. In (a) the acetic acid fixed chromosomes were denatured at 55°C, in (b) at 65°C, in (c) at 75°C and in (d) at 85°C. In (e) and (f) the chromosomes were prefixed in formaldehyde before acid fixation. The chromosomes were denatured at 65°C (e) and 85°C (f). After denaturation the chromosomes were incubated in the annealing buffer and with the anti-hybrid antibodies.

chromosomes prefixed in formaldehyde the intensity of fluorescence increased upon denaturation but only when the chromosomes were heated at 85-90°C the pattern of fluorescent bands was similar to that observerd over acetic acid fixed denatured chromosomes. Since about 25 % of the DNA of acetic acid fixed chromosomes melts at 65°C a low level of fluorescence was observed on many chromosomal subdivisions when the chromosomes were heated at this temperature. No fluorescence was observed, however, on chromosomes prefixed in formaldehyde after denaturation at 65°C. Moreover, if the reannealing process was blocked by incubation of the chromosomes with formaldehyde, during melting, DNA:RNA hybrids were not observed. Thus, anti-hybrid reactivity only occurs when the DNA is in single stranded form and allowed to reanneal with the chromosomal RNA.

Repression of RNA synthesis during a heat shock treatment

In agreement with previous observations [29, 30, 31] we have detected that the number of chromosomal transcriptionally active sites decreases with the period of incubation at 37°C. Fig. 3 shows that after 5 min at 37°C the hs

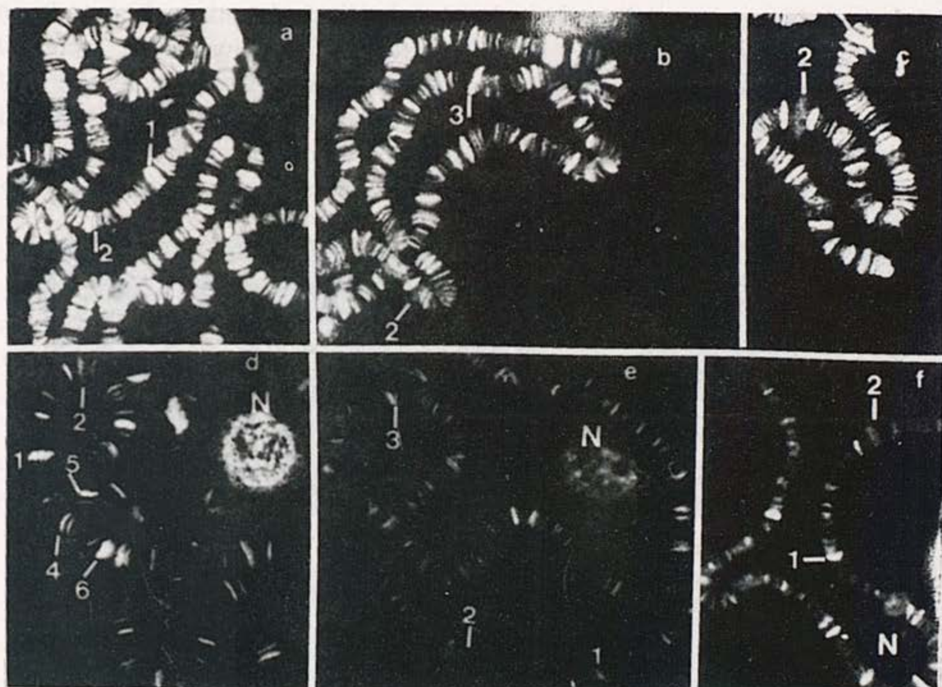

Fig. 3 — Autoimmunograms of chromosomal DNA-RNA hybrids and anti-polymerase II after a heat shock treatment at 37°C. The chromosomes were treated for 5 min (b); for 20 min (c); for 36 min (d); and for 50 min at 37°C (e, 4th and 5th chromosomes); anti RNA polymerase II staining after 50 min at 37°C (f). In (a) control chromosomes. 1= 4-78B; 4= 4-75D; 5= 4-91C; 6= 4-90AB (ecdysone inducible); 2= 4-81C; 3= 2-48B (heat shock inducible)

puffs begin to be formed but that still most of the subdivisions of the chromosomes do have transcripts (3b). Only after 35 min a large number of the chromosomal subdivisions were depleted of RNA molecules (3d). Extensive repression of RNA synthesis was observed after 50 min (3e) when even the fluorescence over the hs puffs was very low (except in the hs inducible subdivision 2-48B). A low level of fluorescence was detected over the 5S region and other few loci. After 50 min at 37°C the chromosomal distribution of the RNA polymerase II was the same as that of the chromosomal hybrids (3f). The anti-hybrid reactivity over the nucleolus was found dispersed over its entire area (3d) in contrast with what it occurs in control chromosomes in which the anti-hybrid reactivity distributes in fibers

RNA synthesis during recovery

After 3 min of recovery at 25°C RNA polymerase II molecules and DNA--RNA hybrids were detected in all subdivisions of the chromosomes (Fig. 4b and c respectively). Although the anti-hybrid reactivity was low in all chromo-

somal sites, the pattern of fluorescent bands was identical to that of control non heat treated chromcsomes (Fig. 4a). The intensity of fluorescence increased with the length of incubation at 25°C. Ater 15 min at 25°C the intensity of anti-hybrid reactivity was similar to that of non heat treated chromosomes (Fig. 4d). Moreover, the nucleolar DNA:RNA hybrids distributed in fibers as it occurs in control glands. Since the fluorescence did not increased after incuba-

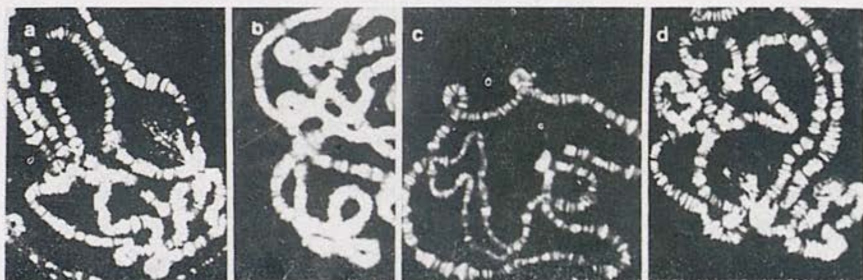

Fig. 4 — Autoimmunograms of chromosomal DNA-RNA hybrids and anti-polymerase II after recovery at 25°C. In (b) anti RNA polymerase II after 3 min of recovery. In (c) anti-DNA-RNA staining after 3 min at 25°C. In (d) after 15 min at 25°C. In (a) control chromosomes from larvae grown at 25°C

tion at 25°C for longer periods of time we think that the amount of chromosomal transcripts synthesized in 15 min, able to hybridize with their templates, is the same as that existing over the chromosomes in a steady state. Thus, shortly during recovery at 25°C the repression caused by the heat shock is released and chromosomal transcription is restored. The data indicate, moreover, that all of the chromosomal subdivisions which accumulate RNA molecules during larval growth are open for RNA transcription at recovery. Since an identical distribution of hybrids was observed in homologous chromosomes of all the cells after transfer to 25°C, we think that the reversibility to the transcriptional condition occurs at the same time in all nuclei of the gland.

That the epitopes detected during recovery represent DNA-RNA hybrids was demonstrated by treatment of the chromosomes for 30 min at 37°C with DNase I free pancreatic RNase A (50μg/ml in 2 x SSC) and with RNase free DNase I (0.2 units/20μl per slide in 2 x SSC, 10 mM $CaCl_2$) for 10 min before and after the hybridization step, and with RNase H (0,2 units/20 μl per slide in 25 mM Hepes-KOH, 50 mM KCl and 1 mM dithiothreitol) for 30 min after DNA-RNA annealing. If the RNA molecules had been removed by a RNase A treatment and if the DNA had been digested with DNase I before melting, anti-hybrid reactivity was not observed. On the other hand, a RNase treatment of chromosomes in which the DNA:RNA hybrids had been formed did not influence antibody binding. However, anti-hybrid reactivity was not observed on these chromosomes after RNase H digestion (results not shown).

Complexity of transcriptional domains during recovery

In order to have an estimation of the number of transcribing domains relative to the number of structural band-interband units, we counted the number of bright and diffuse fluorescent bands of the 4th and 5th chromosomes in autoimmunograms of salivary glands obtained 15 min after the larvae were transferred to 25°C. The number of resolved fluorescent bands of different intensities in the 4th chromosomes is 260±20 and 320±32 in the 5th chromosome (20 chromosomes were examined). Most of the bright fluorescent bands could be resolved in several bands when they were analyzed in stretched chromosomes. At the same microscopic resolution the number of band-interband structures is about 300±15 in the 4th and 370±30 in the 5th chromosome. These numbers are about 50% of those detected by EM [9]. In particular regions of the 4th chromosome (4th-73D-78, 4th-81C and 4th-92-93; Fig. 5) where the analysis could be carried out with greater detail we observed that

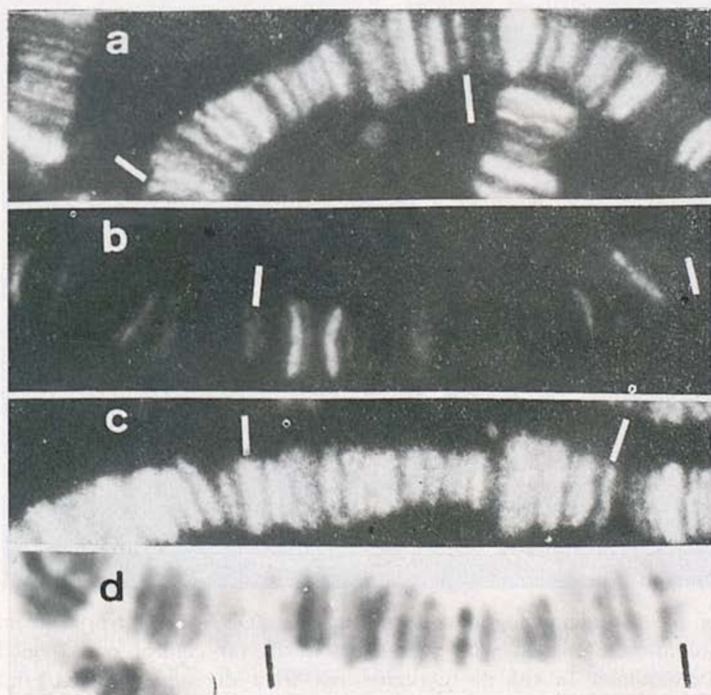

Fig. 5 — DNA-RNA autoimmunograms of a fraction of the 4th chromosome (90D-94). (a) DNA-RNA hybrids from animals grown at 25°C. (b) DNA-RNA hybrids from animals heat shocked at 37°C for 50 min. (c) DNA-RNA hybrids from animals after 15 min recovery from a heat shock for 50 min at 37°C and (d) acetoorcein stained chromosome

the ratio of fluorescent bands to DNA bands is close to 1:1. Also, in subregion 4th-81C (heat inducible), formed by 5 DNA bands, 4 distinct thin fluorescent bands could be detected in non induced form.

The RNA forming hybrids is bound to the DNA by a protein molecule

The O order-like kinetics of hybrid formation [21] suggests that the molecules involved in the hybridization reaction behave like inverted repeats and that, therefore, a molecule should link the RNA to the DNA. We think that this molecule is a protein and that the hybrids could not be formed and that they could not reconstitute upon melting if it were removed. In order to test that hypothesis we digested the chromosomes with DNase-RNase free proteinase K. Fig. 6 shows that when the chromosomes were treated with proteinase K (10μg/ml) for 10 min before DNA denaturation DNA-RNA hybrids could not be formed (b) and that once the hybrids had been formed digestion of chromosomal proteins did not have any effect on the distribution of the anti-hybrid

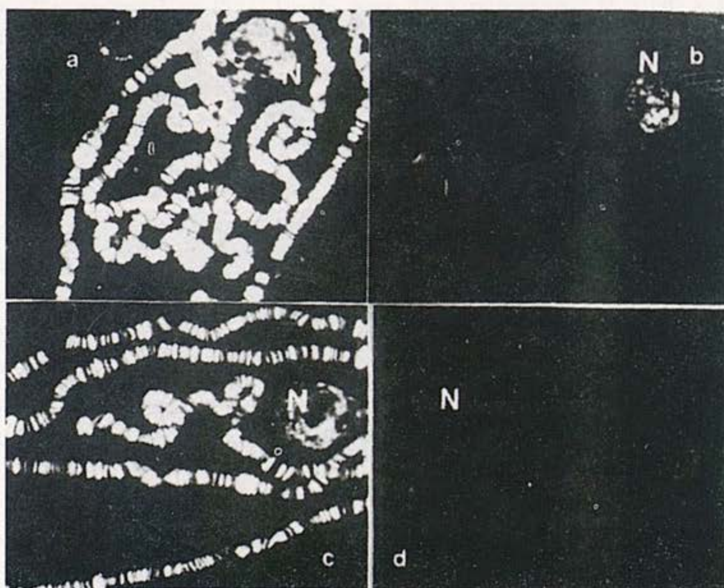

Fig. 6 — Autoimmunograms of chromosomal DNA-RNA hybrids from chromosomes treated with proteinase K. In (a) control non treated chromosomes. In (b) the chromosomes were digested with 10µg/ml of the enzyme for 15 min before DNA denaturation. (N): nucleolus. In (c) the chromosomes were digested with the enzyme after the hybrids had been induced. In (d) the chromosomes in which hybrids had been induced were treated with 10µg/ml of the enzyme and subsequently melted in 0.1 x SSC at 85°C. After each treatment the chromosomes were incubated in the annealing buffer and with the antihybrid antibodies

reactivity (c) which was identical to that observed in non treated chromosomes (a). As expected, in chromosomes treated with proteinase K, after the first in situ hybridization, DNA:RNA epitopes could not be detected if the hybrids were melted and allowed to reanneal (d). Fig. 7 (a and b) shows, on the other

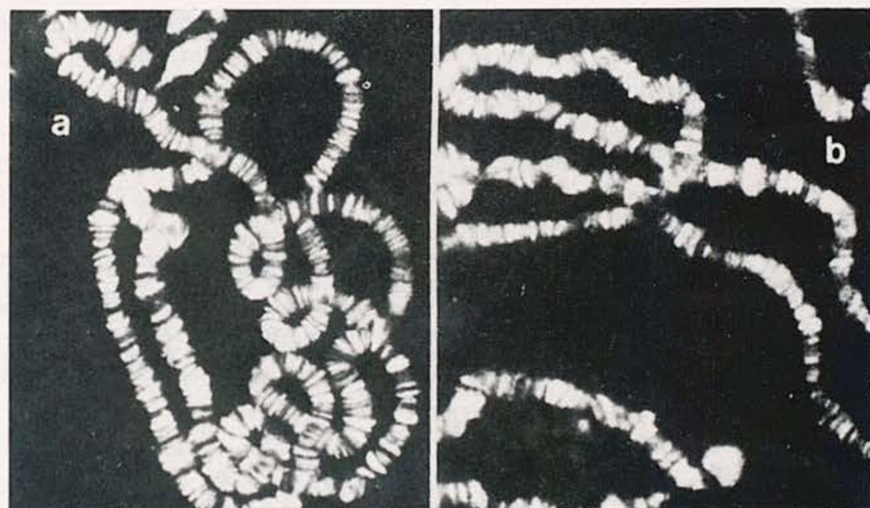

Fig. 7 — Autoimmunograms of chromosomal DNA-RNA hybrids. In (b) the induced DNA-RNA hybrids after melting and reannealing were incubated with the antihybrid antibody. In (a) control chromosomes in which tre induced hybrids were not melted

hand, that when the hybrids, in non treated chromosomes, were melted and further incubated in the annealing buffer they could be reconstituted and that their distribution was identical to that found in control glands. The process of denaturation and reannealing could be repeated several times, but the overall fluorescence lowers after each denaturation and annealing step probably due to RNA and DNA extraction [25, 32].

Thermal stability of the DNA-RNA hybrids

To test whether the hybrids formed in situ behave termally as expected for DNA-RNA molecules we studied their melting and reannealing behavior. Since the chromosomal RNA molecules annealed to the DNA within 1-3 seconds, melting of hybrids was performed in the presence of formaldehyde to block reannealing. Table 1 indicates that the Tm of the hybrids formed in situ is similar to that of chromosomal DNA. The number of DNA:RNA epitopes decreases upon denaturation but the absence of hybrids occurs only when they were melted at 85°C.

TABLE 1. **Thermal stability of the induced DNA-RNA hybrids**

Temperature of melting (°C)	Fluorescence
(a) 55	+++++
(b) 60	+++++
(c) 65	+++++
(d) 70	++++
(e) 75	+++ (++)
(f) 80	++
(g) 85	+ (−)
(h) 90	+ (−)

The chromosomes were denatured in 0.1xssc, and incubated in the annealling buffer to induce the formation of hybrids. Then the chromosomes were heated at each temperature − in 0.1 x SSC-2 % formaldehyde. (+++++): maximal fluorescence. (−): no fluorescence. The overall intensity of fluorescence was estimated by determination of the time needed to eliminate the fluorescence upon illumination at 450 nm using a 40 x oil immersion objective lens. In (a) the time needed was 25 min. In (h) it was 1-3 min.

DISCUSSION

The results presented in this paper show that ideed the DNA:RNA epitopes detected over the chromosomes following a drastic repression of RNA synthesis, reveal actual transcription since the transcripts forming the observed DNA:RNA hybrids are due to annealing to their templates of endogenous growing nascent RNA molecules synthesized de novo. The experiments show, moreover, that a protease removable molecule links the RNA to the chromosomes and that this fact may explain the 0-order like kinetics of hybridization. It is likely, therefore, that these RNA molecules form transcriptional ternary complexes with their templates. Based on the Christmas tree model of transcription of polytene chromosomes [33] and in the fact that after a prolonged heat shock treatment and during recovery the same loci which were stained by DNA:RNA antibodies were also stained by anti RNA polymerase II IgG, we think that the linker molecule is the RNA polymerase molecule. This molecule would maintain the RNA molecule in the vicinity of the template allowing the unimolecular-like type of DNA-RNA hybridization to take place and the reconstitution of the hybrids after their melting.

Since 3 min after recovery from the heat shock RNA polymerase II molecules bind to the chromosomes and DNA:RNA epitopes are detected we think that immediately after transfer to 25°C repression of RNA synthesis stops and binary RNA polymerase-DNA complexes are formed with subsequent initiation and elongation of RNA chains. Since the intensity of fluorescence reaches a

plateau after 15 min and it is the same as that observed during normal growth, we think that after 15 min of recovery the autoimmunograms reveal a steady--state level of transcription. Assuming that the length of each transcriptional domain has an average of 25 to 50 kb in length we calculated that the rate of RNA chain elongation in the chromosomes would be of 1.7-3.3 kb per min. In Balbiani rings of Chironomus tentans the rate of RNA elongation is 1.8 kb per min [33]. Rates of RNA elongation between 1.3 and 3 kb per min have been reported for T7 and 5 kb per min in T. cruzi respectively [34, 35]. Since a significative repression of RNA synthesis was only detected after 30 min at 37°C we think that the heat shock does not affect elongation but that it inhibits reinitiation [19]. The rapid rate of the RNA initiation reaction at recovery suggest that during repression the molecules involved in the transcriptional machinery were not drastically damaged since the block is readily reversible. It is not surprising that chromosomal transcription reassumes shortly after the larvae were transferred to the temperature of growth since recovery depends on the novo synthesis of mRNA [36].

We observed that after recovery from the heat shock repression the complexity of transcribing domains is almost as high as the number of band-interband units because DNA:RNA epitopes could be found in 85-90 % of the chromosomal subdivisions. In particular regions the proportion of transcribing domains and units of band-interband structure is 1:1, suggesting that at that time most chromosomal domains are expressed. Because the pattern of DNA:RNA epitopes is the same in chromosomes after recovery from RNA repression and in chromosomes during normal growth, at different developmental stages (early second to late third instar), we think that our results speak in favor of a constitutive expression of most domains. As suggested by Davidson and Britten [5] the complex and moderately prevalent class of mRNAs are transcribed continuously at more or less similar rates being ubiquitously represented in mRNAs.

In particular subdivisions, however, (during puffing, but also in other non puffed subdivisions) the intensity of fluorescence differs in various developmental stages [21]. If the relative intensity of fluorescence between loci reflect differential amount of DNA:RNA epitopes we suggest that at the moment of recovery initiation complexes are formed in all domains but that the distribution of the amount of transcripts is not uniform in all of them [37]. Thus, other factors besides binding of the RNA polymerase may also control the growing of the RNA chains. The low level of fluorescence observed in some subdivisions may suggest, in addition, that transcription is initiated without significant elongation. In fact, the most intensely stained bands accumulate large amounts of silver grains during pulse labeling with ^{3}H-uridine revealing the most actively expressed regions [22]. Subdivisions which show low fluorescence are only revealed as active in autoradiograms after long exposure times or long incubation periods with the labeled precursors (unpublished observations). It is obvious that

the autoimmunograms can not resolve whether all the genes of a given domain are expressed at a given time and whether the identical distribution of DNA:RNA epitopes observed at different developmental stages, reflect the synthesis of the same species of transcripts or of new ones, but we think that they do indicate that most of these domains are open for RNA polymerase II binding and that upon binding they are transcribed.

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PROTEIN AND ESTERASE ISOENZYME PATTERNS AND ESTERASE HISTOCHEMICAL LOCALIZATION IN ANTHERS OF PARTIALLY MALE STERILE APRICOT CULTIVARS

MARIA CLARA MEDEIRA * AND MARIA EDUARDA GUEDES **

RESUMO

Observaram-se três bandas de proteínas (pI: 7.65, 7.30, 5.60) e cinco bandas de polipéptidos (15,000, 35,000, 65,000, 73,000, 89,000 daltons) em anteras de damasqueiro «CNEFFC», contendo 50 % de pólen estéril. Na cultivar «CNEFF1», com 20 % de esterilidade polínica, não foram observados estes polipétidos e proteínas. É possível que esta ocorrência esteja relacionada com um aumento do nível de esterilidade em «CNEFFC».

A perda de dezassete polipéptidos durante os estádios de tétrada e de micrósporo uninucleado em anteras de «CNEFFC» significa um bloqueio prematuro da síntese de proteínas. A diminuição rápida dos níveis da esterase, nesta cultivar, após a meiose II, deve resultar da prematura degenerescência do tapete confirmada por estudo histoquímico da esterase.

A expressão da esterilidade em «CNEFFC» está relacionada com a alteração do processo secretor da esterase no tapete e meiócitos. Grandes glóbulos corados como esterase aparecem frequentemente em extrusão nos meiócitos, tétradas e células do tapete degenerescentes. No desenvolvimento normal do pólen fértil a esterase acumula-se sob a forma de precipitado fino nas células referidas.

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ABSTRACT

Three protein (pI: 7.65, 7.30, 5.60) and five polypeptide (15,000, 35,000, 65,000, 73,000, 89,000 daltons) bands were detected in «CNEFFC» anthers, with 50 % sterility. The fact that this was not observed in «CNEFF1», 20 % sterile, suggest that these polypeptides can be involved in the manifestation of higher levels of pollen sterility observed in «CNEFFC».

The loss of seventeen polypeptides during tetrad and uninucleated microspore stage in «CNEFFC» points to a premature blockage of protein synthesis in «CNEFFC» anthers. A rapid decrease of esterase levels in «CNEFFC», after meiosis II can result from premature degeneration of the tapetum, which was confirmed by the histochemical study of the esterase.

Sterility expression is related to a disturbed secretory process of esterase in tapetum and meiocytes. Big globules that stained like esterase seem to be extruded from degenerescent meiocytes, tetrads and tapetum cells. In the normal pollen development, esterase accumulates as a thin precipitate in the mentioned cells.

INTRODUCTION

Partial male sterility occurs in most of the apricot (*Prunus armeniaca* L.) varieties cultivated in Portugal and studied until now. «CNEFF1» and «CNEFFC» belong to different varietal groups. «CNEFF1» produces morphological regular pollen, with an annual pollen fertility ranging from 70 to 80 %, «CNEFFC» is a more labile variety, with an annual pollen fertility of 30 % to 70 %, producing variable ammounts of giant and abnormal pollen. Male sterility observed in this cultivar is associated with a deficient post-meiotic tetrad cytokinesis, irregular formation of exne and abnormal tapetum development (MEDEIRA AND GUEDES 1989). «CNEFFC» male sterility expression varies not only from year to year but also from the flowers of the same tree the anthers within a flower and the locules of the same anther. Anatomical and cytological aspects of apricot anthers development are similar to the cases of genic male sterility described in *Glycine max* (STELLY and PALMER 1982; GRAYBOSCH and PALMER 1985; 1987), *Hordeum vulgare* (HERD and STEER 1984; AHOKAS, 1978; 1982), *Impatiens sultani* (TARA and NAMBOODIRI,1974; 1975) and to those of cytoplasmic male sterility of *Triticum timopheevi* (RAI and STORKOFF 1974; YOUNG *et al*, 1979), *Secale cereale* (LA VEGA and LACADENA, 1979), *Capsicum annum* (SHIFRISS and GURI, 1979), *Petunia hibrida* (IZHAR, 1978; BINO, 1985), *Brassica napus* (GRANT *et al*, 1986), *Medicago sativa* (BARNES and GARBOUCHEVA, 1973; MCCOY and SMITH, 1986).

Esterase isoenzyme patterns have been used in differentiating sterilizant cytoplasms (ABBOT *et al*, 1984; KARIN *et al*, 1984; MARREWIJK *et al*, 1986) and in the characterization of anther developmental stages (NAVE and SAWHNEY, 1986).

In the present work we tried to determine the relation between the changes in the esterase isoenzymes and soluble protein patterns and histological events in the anthers of «CNEFF1» and «CNEFFC» apricot, during microsporogenesis.

MATERIAL and METHODS

Plant material:

Anthers were collected from flower buds inserted in short branches of six five years old apricot trees cv. CNEFF1 and cv. CNEFFC, growing in field conditions, at Pegões.

Methods:

Anthers collected in four developmental stages (1-meiosis, 2-tetrads, 3-uninucleated microspores, 4-young pollen) were homogenized for 1 minute in cold (0-4°C) 0.05 M Tris — HC1 buffer, pH 6.9, containing 0.01% 2-mercaptoethanol (20 μl/mg). The homogenate was centrifuged at 10,000 g for 10 minutes. The supernant was used for protein and isoenzyme analysis.

SDS — Page proteins patterns:

SDS, β-mercaptoethanol in buffer 0.01 M Tris HC pH 8 and EDTA were added to the samples to the final concentratrions of respectively: 25%, 5% and 0.01 M. The samples were heated at 100°C for 5 to 10 minutes. Electrophoresis was done with 0.5 μl of the samples in phastgel gradient 10-15 programed into the separation method file of phastsystem (Pharmacia). The thin-layer polyacrilamide gels were silver stained.

Proteins and esterase isoenzymes patterns:

Polyacrilamide isoelectric focusing of the samples extracts was carried out in the phastsystem separation and development apparatus (Pharmacia). Thin-layer polyacrilamide gels in the pH ranges of 3-9 were used. The esterase was stained as described by SAWHNEY and NAVE (1986) and proteins were silver stained.

Histochemical localization of esterase:

Anthers in the same developmental stages were preparated for cryosectioning at 10 μm- 15 μm (KNOX 1970) and were stained in the same solution used for esterase isoenzymes (NAVE and SAWHNEY, 1986). Control sections were stained with the reaction solution minus substrate α — and β-naphthyl acetate.

RESULTS

Soluble protein patterns obtained by isoelectric focusing:

Three bands pI: 7.65, 7.30, 5.60 were detected in «CNEFFC» and were not observed in «CNEFF1» (Fig. 1). The bands pI 5.95 and 6.04 correspond the esterase bands. In CNEFF1», at stage 4, the band PI 6.04 is still detected. A decrease in the intensity of all the bands was observed at tetrad stag in CNEFFC and after pollen mitosis in CNEFF1.

SDS — Page protein patterns:

Five polypeptides with molecular weights of 15,000, 35,000, 65,000, 73,000, 89,000 daltons were detected in «CNEFFC» anthers and were no observed in «CNEFF1» (Fig. 2).

Seventeen polypeptides were lost during stages 2 and 3 in «CNEFFC», while «CNEFF1» maintains the polypeptides detected at stage 1. In this cultivar the intensity of the bands increased at stages 2 and 3 (Fig. 2).

Appearance of two new polypeptides with molecular weights of 93,000 and 98,000 daltons at stages 3 and 4 in «CNEFF1» not observed in «CNEFFC» (Fig. 2).

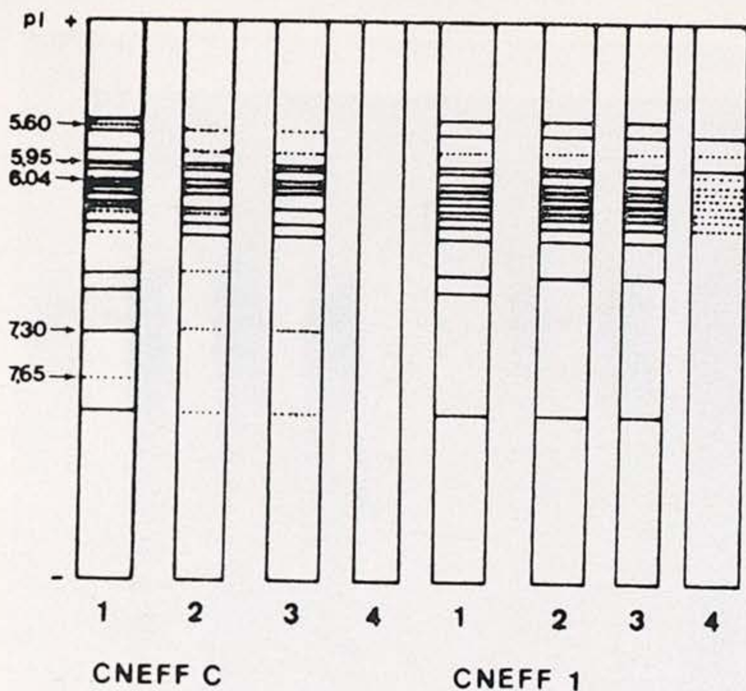
Esterase isoenzyme pattern

The band pI 5.95 was observed in «CNEFFC», only at stage 1 (Fig. 3). The band pI 6.04 was detected from stage 1 to stage 3, disappearing after that. In «CNEFF1», the same two bands were observed between stage 1 and 3. Only onde band (pI. 6.04) persists in stage 4.

Histochemical localization of esterase

In «CNEFFI» anthers, at meiosis I and II (Fig. 4, 6) the enzyme reaction product concentrates mainly in the tapetum (T) and meiocytes (M). Slight esterase activity can be seen in the parenchymatous cells of the middle layers (ML). Staining of epidermis (E) and connectivum (C) is due to non specific binding of the dye, and was also observed in the control, which had been treated with the dye minus substrate (Fig. 5).

At tetrad stage, a slight homogenous staining in the tetrad cytoplasm can be observed (Fig. 7). At the young pollen stage in the anther locule stained for



1

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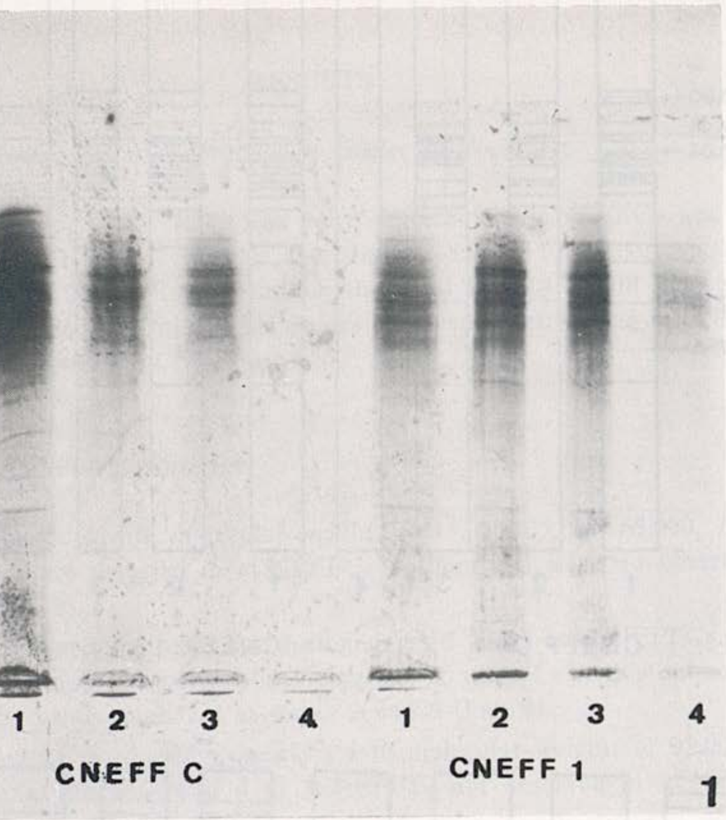
65,000
73,000
89,000
93,000
98,000
-
1 2 3 4 1 2 3 4
CNEFF C
CNEFF 1

2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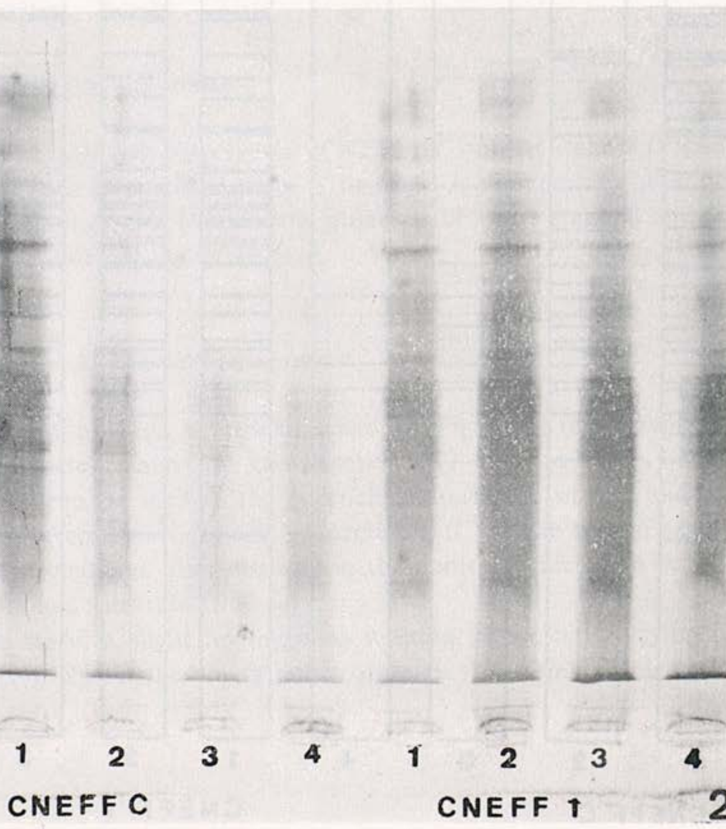
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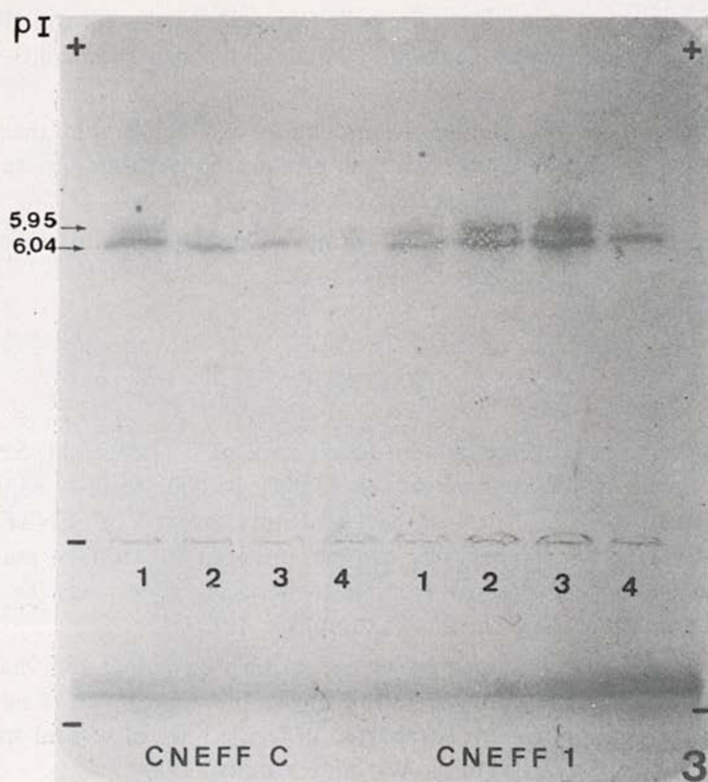


Fig. 1 — Isoelectric focusing gel of proteins from anthers of partially male sterile apricot cultivars. 1 — meiosis; 2 — tetrads; 3 — uninucleated microspores; 4 — young pollen

Fig. 2 — SDS-page, gradient 10-15 of proteins extracted from anthers of partially male sterile apricot cultivars. 1 — meiosis; 2 — tetrads; 3 — uninucleated microspores; 4 — young pollen

Fig. 3 — Esterases isoenzymes pattern obtained on isoelectric focusing gels from anthers of partially male sterile apricot cultivars. 1 — meiosis; 2 — tetrads; 3 — uninucleated microspores; 4 — young pollen

esterase activity, the degenerating tapetum and pollen exine concentrates the enzyme reaction product (Fig. 8). Some exine and anther epidermis staining may be due to non specific binding of the dye, and was also observed in the control (Fig. 9).

Big globules esterase like stained (Fig. 10, rust colour-arrows) in the meiocytes and in premature degenerating tapetum of «CNEFFC» are frequently observed. Unstained globules were observed in the control (Fig. 11). In tetrads and in microspores releasing from callose wall (Fig. 14) similar globules stained

like esterase seem extruded from the cells (arrows). In the control (Fig. 15) the globules appeared unstained. Variability in the cytoplasm stainability of esterase is observed (Fig. 14).

A different kind of esterase accumulation can be seen in the anthers of the same cultivar. The enzyme reaction product concentrates in tapetum and meiocytes or tetrads as a fine precipitate (Fig. 12).

Delayed tapetal degeneration at young pollen stage, with no evidence of esterase activity was also observed (Fig. 16).

DISCUSSION

The appearance of three protein bands with pI of 7.65, 7.30, 5.60 and five polypeptides with molecular weight of 15,000, 35,000, 65,000, 73,000, 89,000 daltons detected in «CNEFFC» anthers and not observed in «CNEFF1» led us to suspect that these polypeptides can be involved in sterility manifestation. Unusual polypeptides expressed from mitochondrial genes may be the causal agents of cytoplasmic male sterility (LONSDALE, 1987).

Two new polypeptides, with molecular weights of 93,000 and 98,000 daltons were detected in «CNEFF1», after tetrad stage. The appearance of new proteins, possibly pollen specific proteins is reported in fertile lines of several species, after microsporogenesis, e.g. in Petunia WU and MERRY, 1985).

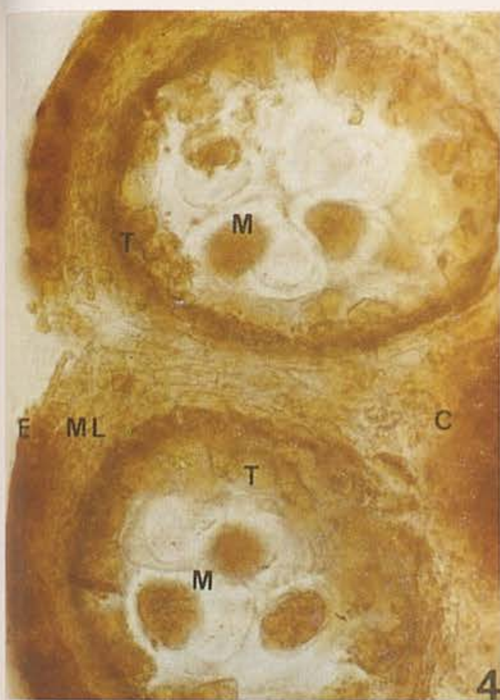
The loss of seventeen polypeptides during the tetrad and uninucleated microspore stages and the fact that no new polypeptides were found in «CNEFFC», after tetrad stage, suggest a premature blockage of protein synthesis in these anthers.

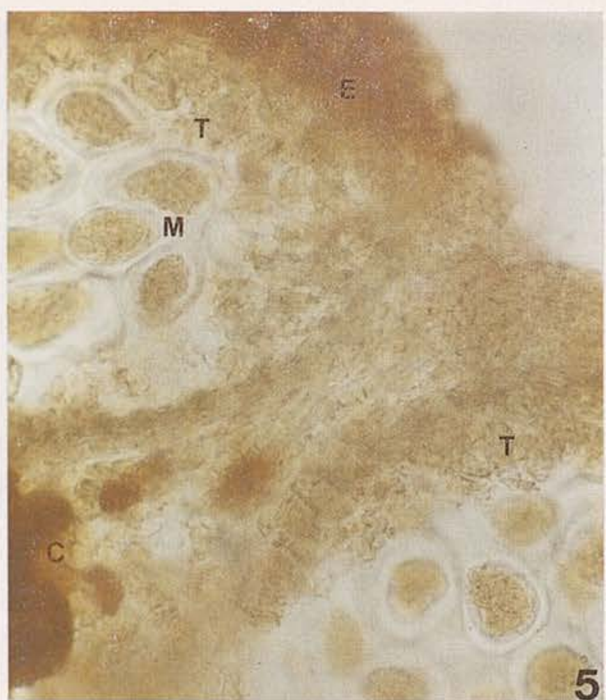
In fertile anthers esterase accumulates in the tapetum after meiosis I until tapetal breakdown, decreasing after microspore release and increasing in exine during pollen maturation (VITHANAGE and KNOX, 1976; SAWHNEY and NAVE, 1986; VAN MARREWIJK *et al,* 1986).

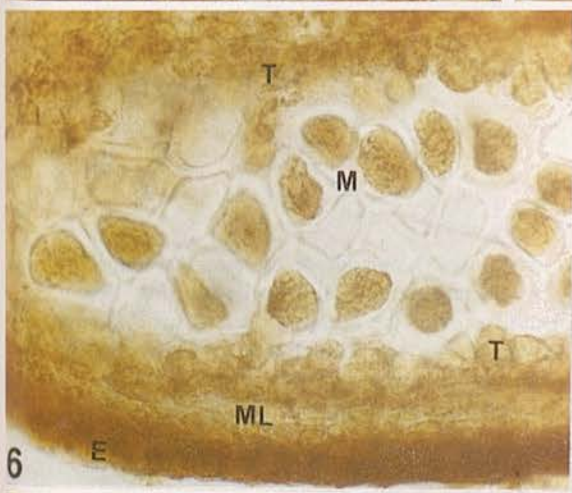
A rapid decrease in esterase levels in the labile cultivar CNEFFC, after meiosis II can be related to a premature degeneration of the tapetum confirmed by the histochemical study of esterase.

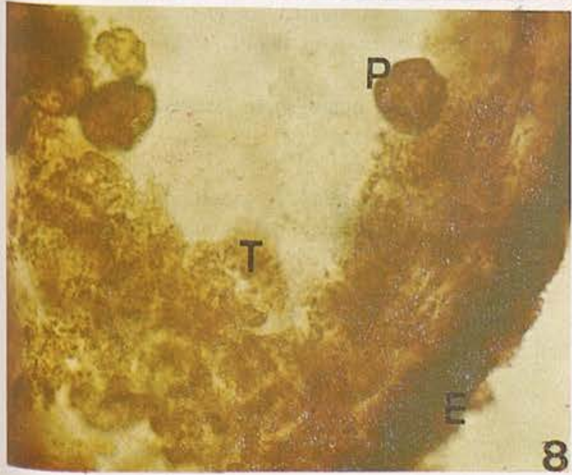
Accumulation of esterase as a thin precipitate in meiocytes, tetrads and microspores was observed in «CNEFF1» and in normal «CNEFFC» anthers. These observations are in agreement with those of SAWHNEY and NAVE (1986) and VAN MARREWIJK *et al,* (1986) on Petunia hybrida.

Globules stained like esterase in the meiocytes, tetrads and in the desorganizing tapetum were frequently observed in some anthers of «CNEFFC». These globules seem to be originated by a disturbed secretory process in the mentioned cells.









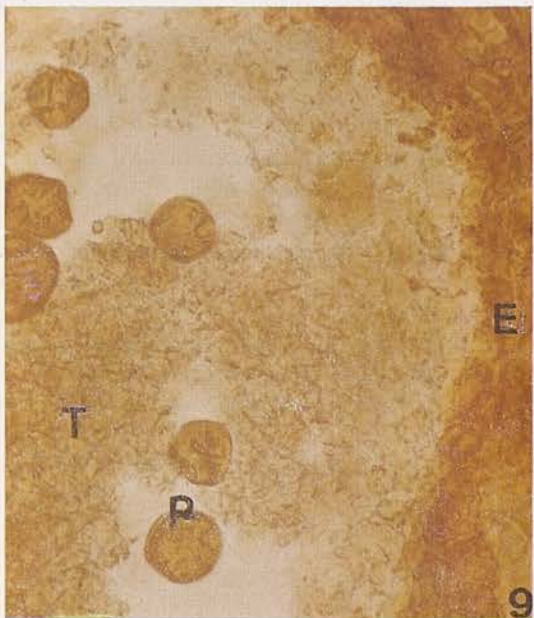


Fig. 4 to 9 — «CNEFF1» anthers. Histochemical localization of esterase

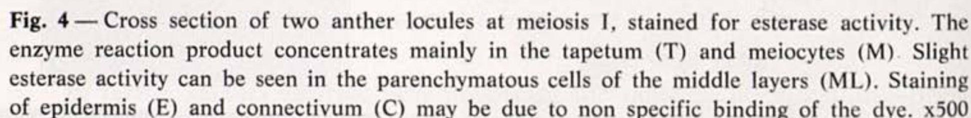


Fig. 4 — Cross section of two anther locules at meiosis I, stained for esterase activity. The enzyme reaction product concentrates mainly in the tapetum (T) and meiocytes (M) Slight esterase activity can be seen in the parenchymatous cells of the middle layers (ML). Staining of epidermis (E) and connectivum (C) may be due to non specific binding of the dye. x500

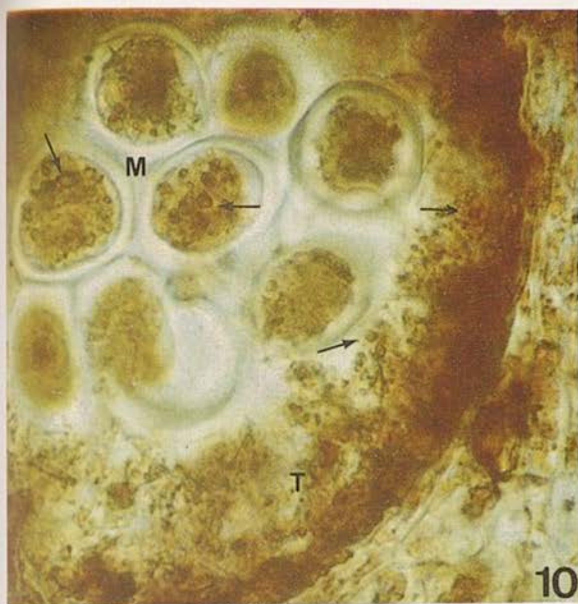
Fig. 5 — Cross section of two anther locules, at meiosis, incubated in the control staining solution for esterase activity. x800

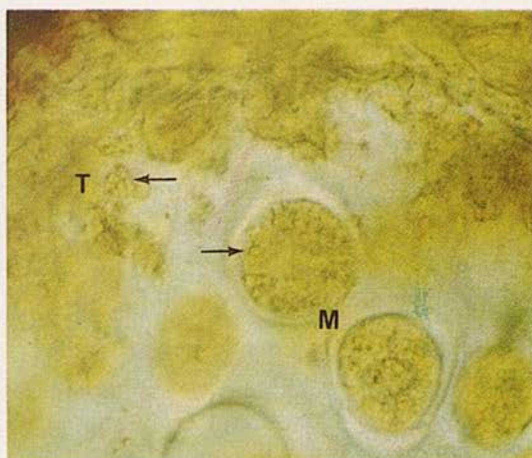
Fig. 6 — Longitudinal section of an anther locule at meiosis II stained for esterase activity Esterase activity distribution is similar to that described in Fig. 1. x500

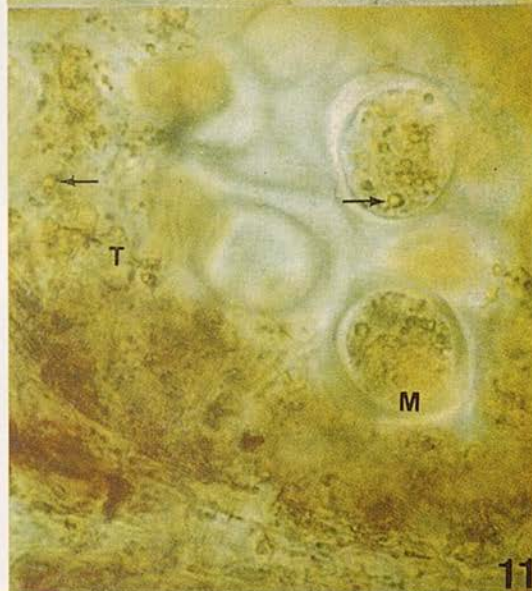
Fig. 7 — Normal tetrad stained for esterase activity. Slight homogenous staining in the cytoplasm can be observed. x1000

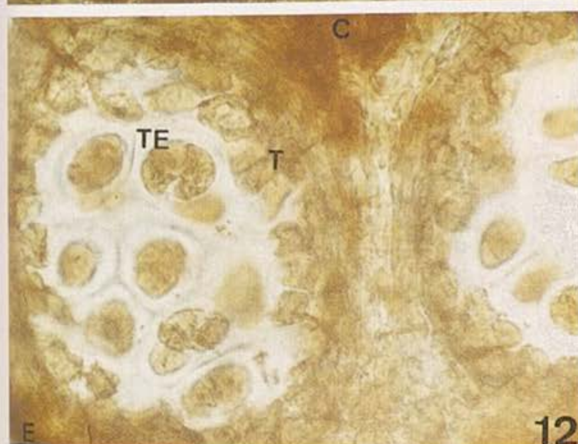
Fig. 8 — Young pollen in the anther locule stained for esterase activity. Degenerating tapetum and pollen exine concentrates the enzime reaction product. Some exine and epidermis staining may be due to non specific binding of the dye. x800

Fig. 9 — Young pollen in the anther locule, incubated in the control staining solution for esterase activity. x800









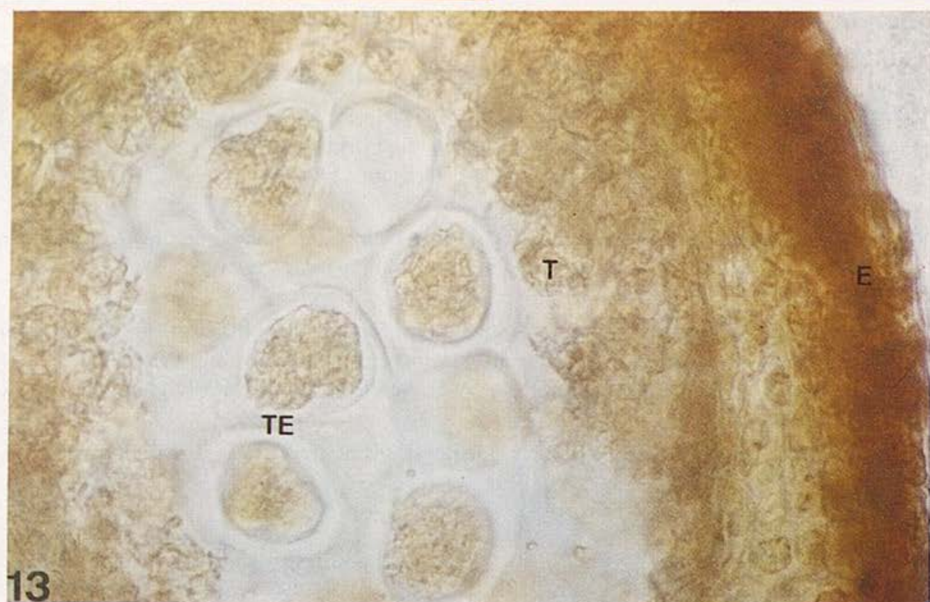


Fig. 10 to 13 — «CNEFFC» anthers. Histochemical localization of esterase

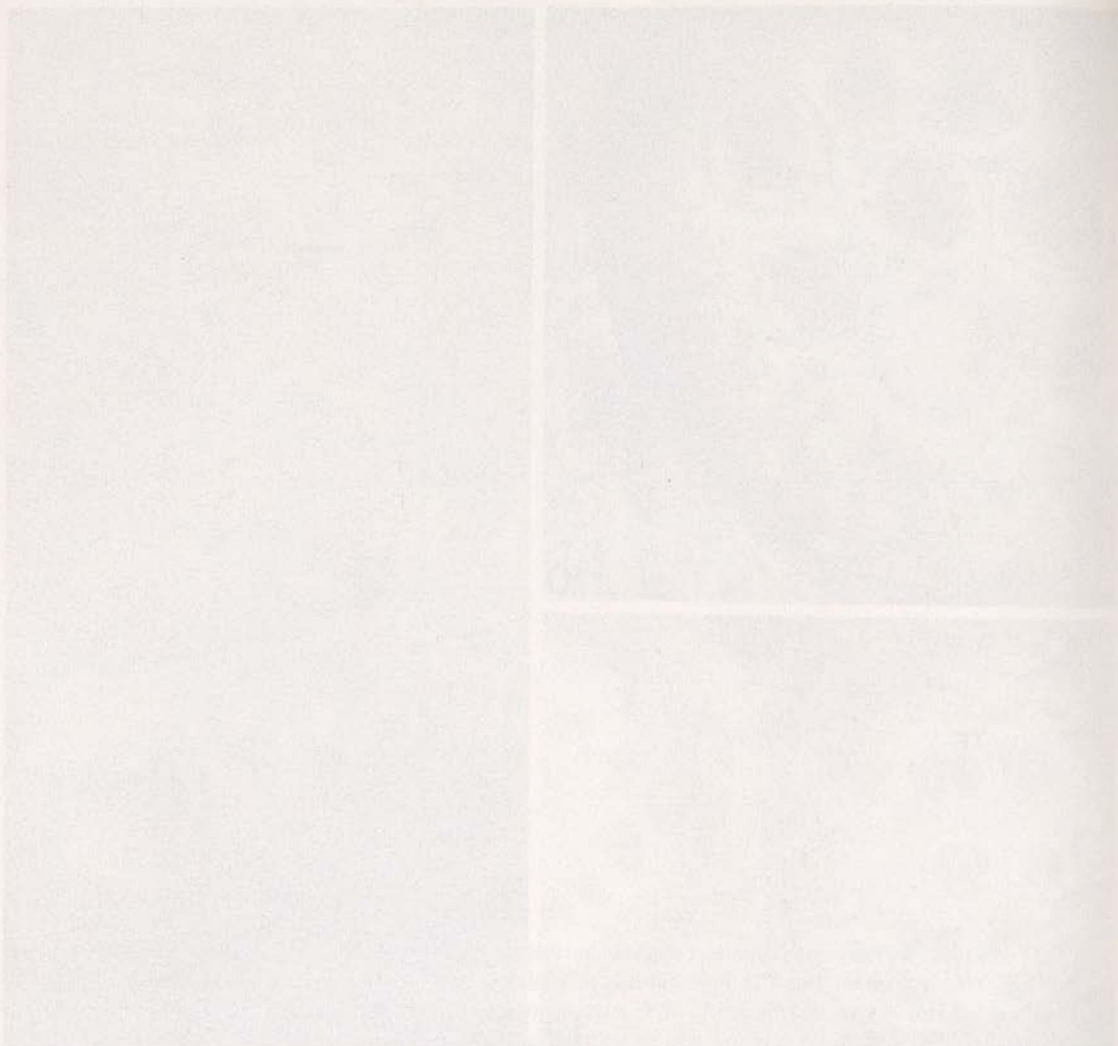
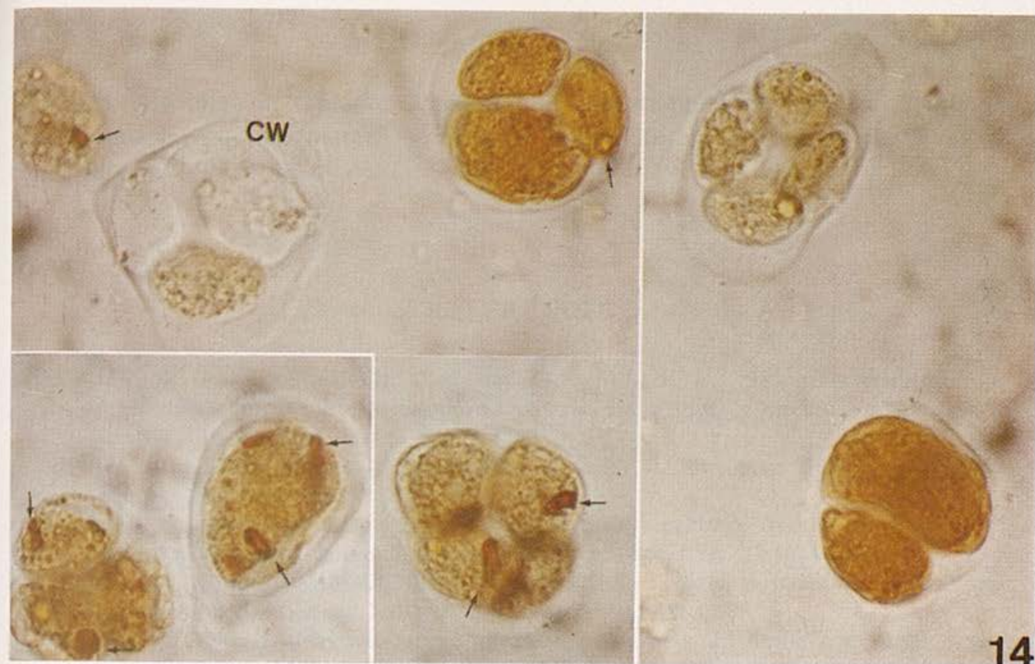


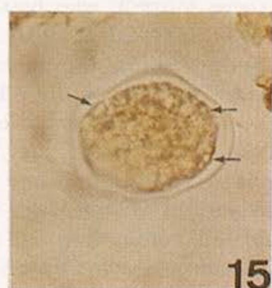
Fig. 10 — Cross section of an anther locule, at meiosis, stained for esterase activity. Premature tapetal degeneration. Globules stained like esterase (rust colour-arrows) in the meiocytes (M) and tapetum (T). x800

Fig. 11 — Cross section of two anther locules, at meiosis, incubated in the control staining solution for esterase activity. No staining was observed. x800

Fig. 12 — Cross section of two anther locules, at tetrad cytokinesis, stained for esterase activity. The enzyme reaction product concentrates in tapetum (T) and tetrads (TE). Colour or the epidermis (E) and connectivum (C) may be due to non specific binding of the dye. x500

Fig. 13 — Cross section of one anther locule. Control for esterase activity reported in Fig. 12. x800





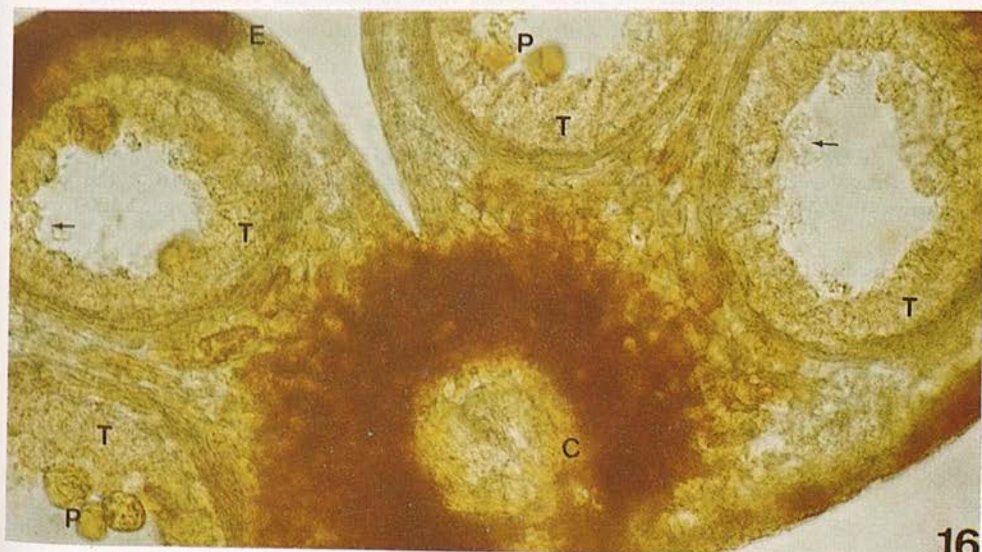


Fig. 14 to 16 — «CNEFFC» anthers. Histochemical localization of esterase

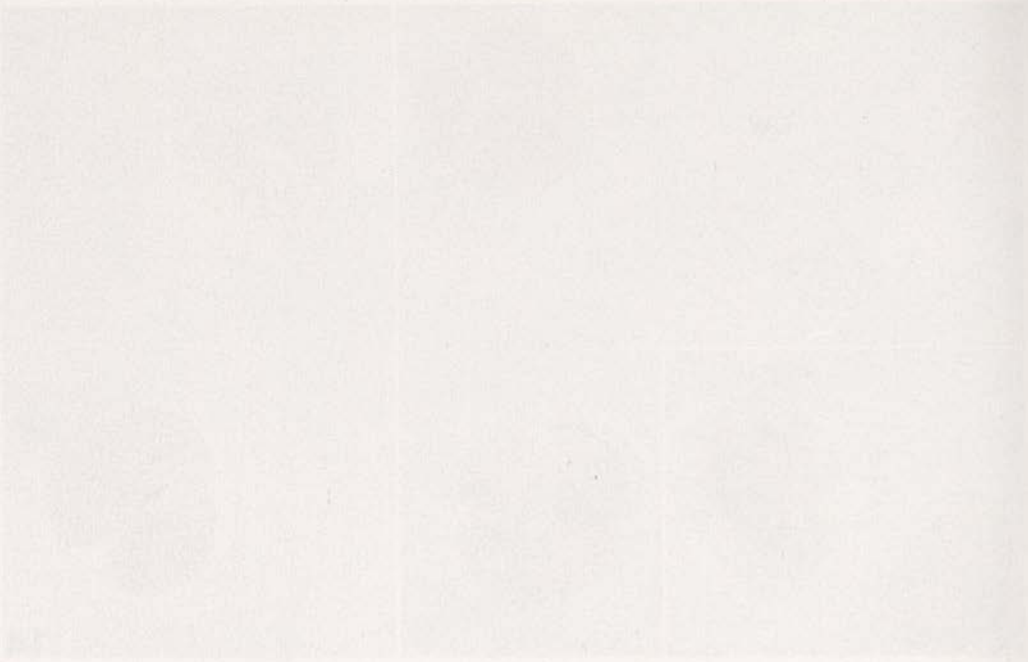


Fig.14 — Tetrads and microspores releasing from callose wall (CW), stained for esterase activity. Irregular cytokinesis. Globules stained like esterase seem extruded from the cells (arrows). Variability in the cytoplasm stainability for esterase is observed. x1000

Fig. 15 — Tetrad non segmented, incubated in the control staining solution. No staining was observed. x1000

Fig. 16 — Cross section of an anther, at young pollen stage. Delayed tapetal degeneration (arrows), without staining for esterase activity. Pollen (P) is slightly coloured. Colour of the epidermis and connectivum may be due to non specific binding of the dye. x300

A mistiming in tapetum degeneration, a blockage of the protein synthesis and unusual esterase pattern seem to be related to an increase in the level of sterility in «CNEFFC».

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RESTRICTION MAPS FOR THE GENOMES OF *BACILLUS SUBTILIS* PHAGES IG1, IG3 AND IG4

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SUMMARY

The sites for the action of nine restriction endonucleases in the 130 kb-DNA of IG1 and of six restriction endonucleases in the 140 kb-DNAs of IG3 and IG4 were localized.

Bacillus subtilis temperate phages were divided into four groups on the basis of host range, morphology, serology and immunity (Dean *et al.*, 1978). IG1, IG3 and IG4 are large bacteriophages isolated in our laboratory (Fernandes *et al.*, 1986) belonging to group III which includes as well SPβ, SPR, Z, H2, ∅3T and Rho11. Several interesting features have been attributed to some of these phages, such as: lysogenic conversion to thymine prototrophy; an endonuclease specific for *Bacillus* DNA; DNA methyltransferases multispecific; production and tolerance to betacin; and specialized transduction (for a review see Zahler, 1988). They contain single linear DNA molecules with sizes over 114 kb. Physical maps were published for ∅3T (Cregg and Ito, 1979), Rho11 (Mizukami *et al.*, 1980), SPβ (Fink and Zahler, 1982) and H2 (Zahler *et al.*,

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1987) genomes. We have previously reported a detailed restriction analysis and comparison of three other group III phages, IG1, IG3 and IG4 (Cardoso *et al.*, 1986). By a combination of several approaches, including double digestions, restriction of *Bal*31 nuclease-treated DNAs and, in some cases, using pulsed-field gel electrophoresis (transverse alternating field electrophoresis — TAFE) to resolve large fragments, the restriction analysis was further extended. This lead to the construction of the physical maps for IG1, IG3 and IG4 presented in Fig. 1.

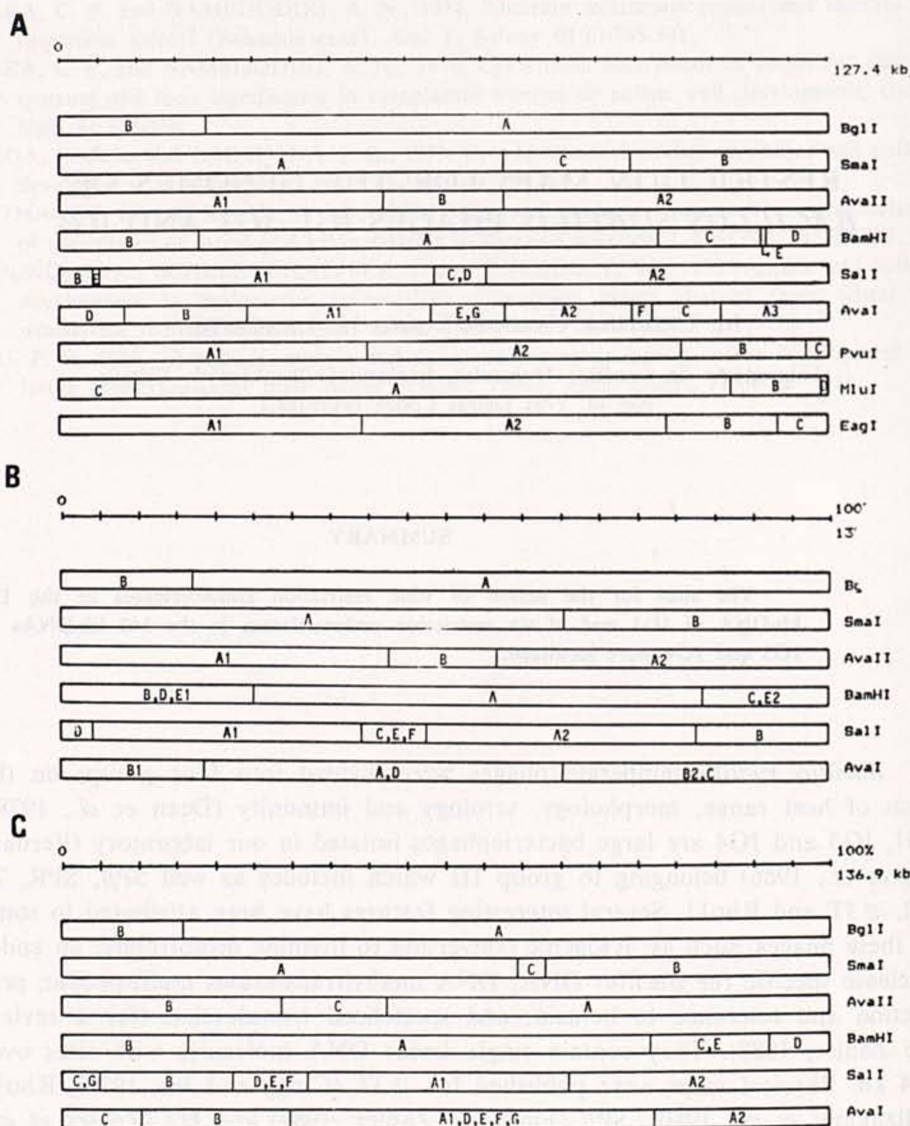

Fig. 1 — Restriction maps of IG1 (A), IG3 (B) and IG4 (C) genomes. The top lines represent the entire length of the genomes. Comigrating fragments are randomly distributed.

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