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Identification of the genes coding for the second-largest subunits of RNA polymerases I and III of *Drosophila melanogaster*

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Summary. We have isolated cDNA and genomic clones of *Drosophila melanogaster* by cross-hybridization with a 658 bp fragment of the yeast gene coding for the second-largest subunit of RNA polymerase III (*RET1*). Determination of the sequence by comparison of genomic and cDNA regions reveals an ORF of 3405 nucleotides which is interrupted in the genomic sequence by an intron of 48 bp. The deduced polypeptide consists of 1135 amino acids with a calculated molecular weight of 128 kDa. The protein sequence shows the same conserved regions of homology as those observed for all the second-largest subunits of RNA polymerases cloned so far. The gene (*DmRP128*) obviously codes for a second-largest subunit of an RNA polymerase which is different from *DmRP140* and *DmRP135*. We have purified three distinct RNA polymerase activites from *D. melanogaster*. By using specific RNA polymerase inhibitors in enzyme assays and by comparing their subunit composition we were able to distinguish between RNA polymerase I, II, and III. RNA polymerase preparations of *D. melanogaster* were blotted and the second-largest subunits were identified with antibodies raised against polypeptides expressed from *DmRP128* and *DmRP135*. Anti-DmRP135 antibodies react strongly with the second-largest subunit of RNA polymerase I but do not react with the respective subunits of RNA polymerase II and III. The second-largest subunit of RNA polymerase III is only recognized by anti-DmRP128. Previously, we have claimed that *DmRP135* codes for the second-largest subunit of RNA polymerase III. Based on the new biochemical data reported here we show that *DmRP135* codes instead for the second-largest subunit of RNA polymerase I and that *DmRP128* corresponds to the equivalent subunit of RNA polymerase III.

Key words: *Drosophila melanogaster* – RNA polymerase I – RNA polymerase III – Second-largest subunits

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Introduction

DNA-dependent RNA polymerases are complex multisubunit enzymes consisting of two large subunits with molecular weights >100 kDa and several smaller polypeptides which are all present in stoichiometric amounts (Sentenac 1985). In eukaryotes there are three different types of enzymes of aggregate molecular weights of ca. 500 kDa which are responsible for synthesis of different classes of RNA. During the past two decades, RNA polymerases have been purified to homogeneity from a large number of eukaryotes (Sentenac 1985) and have been subjected to extensive studies using biochemical and biophysical methods. In a parallel approach, genes corresponding to the individual subunits of the enzymes were isolated, and their protein sequences were determined at the DNA level in order to understand the function of these enzymes in a physiologically meaningful context, i.e. the ability to participate in regulatory processes by interacting not only with DNA-template and substrate but also with protein factors and molecules modulating enzyme activity. While the primary structures of all subunit genes of the RNA polymerase of *Escherichia coli,* of some other eubacteria and also of some archaebacteria are known (Pühler et al. 1989), not all of the genes encoding the large subunits of RNA polymerases from higher eukaryotes have been identified so far. This is due to the almost complete lack of mutants in these organisms, except for yeast where most of the recent information concerning subunit structure has been generated (Sentenac 1985; Woychik and Young 1990; James and Hall 1990; James et al. 1991; Yano and Nomura 1991; McCusker et al. 1991).

The genes coding for two large subunits of RNA polymerase II (pol II) from *Drosophila melanogaster* have been cloned and characterized (Biggs et al. 1985; Jokerst et al. 1988; Falkenburg et al. 1987; Faust et al. 1986; Sitzler et al. 1991). The gene for another second-largest subunit (*DmRP135*) of an RNA polymerase of *D. mela-*

nogaster was cloned and sequenced (Kontermann et al. 1989). Since at that time RNA polymerases I and III (pol I, pol III) of *D. melanogaster* had not been isolated and biochemically characterized, *DmRP135* was thought to code for the second-largest subunit of pol III, based on data obtained from indirect immunofluorescence on polytene chromosomes. By screening yeast mutants affecting pol III transcription gene coding for the second-largest subunit of pol III of yeast (*RET1*) was recently isolated (James et al. 1991). Amino acid sequence comparisons between *DmRP135* and *RET1* revealed homology (24% overall) only in conserved regions common to all second-largest subunits investigated so far. Given the overall similarity (58%) between the second-largest subunit of pol II of *D. melanogaster* (*DmRP140*) and yeast (*RPB2;* Young and Davis 1983; Sweetser et al. 1987) this result was highly surprising. The homology between these pol II subunits is not restricted to the strongly conserved homologous regions. Due to the evolutionary conservation one would also have expected a higher similarity in amino acid composition among the second-largest subunits of RNA polymerase III. Since *RET1* was clearly identified by its mutant allele, while evidence for the identity of *DmRP135* was obtained primarily on the basis of indirect immunofluorescence studies, we were led to consider the possibility that *DmRP135* might code instead for the second-largest subunit of RNA polymerase I (pol I).

In order to settle the question of subunit gene identity unequivocally, we set out to clone the third of the second-largest RNA polymerase subunit genes and to purify and identify all three RNA polymerases from *D. melanogaster* utilizing specific inhibitors of enzyme activity. Finally, by immunoblot staining using purified RNA polymerases and specifc antibodies raised against bacterially expressed fusion proteins, we identified *DmRP128* as coding for the second-largest subunit of RNA polymerase III while *DmRP135* indeed represents the gene coding for the second-largest subunit of RNA polymerase I.

Materials and methods

Isolation and analysis of cDNA and genomic clones. cDNA clones were isolated from a randomly primed λgt11 library of 0–16 h *D. melanogaster* embryos. Following the method of Benton and Davis (1977) the library was screened with a 658 bp *Hin*dIII-*Kpn*I fragment derived from the 3′ end of *RET1*. Positive clones were subcloned into M13mp18 or M13mp19 vectors, and single-stranded DNA was prepared by standard techniques and sequenced (Sambrook et al. 1989) using T7 sequencing systems (Pharmacia). Reaction products were separated on buffer gradient polyacrylamide gels (Biggin et al. 1983). Genomic clones were isolated by screening a genomic Charon 4 library of *D. melanogaster* (Maniatis et al. 1978) using a 1.7 kb cDNA fragment. Lambda phage and plasmid DNAs were isolated, digested and analysed on Southern blots by standard techniques (Sambrook et al. 1989). All probes were labelled with [α^{32}P]dCTP using random primer labelling systems (Amersham).

RNA, reverse transcription, PCR. RNA was prepared from adult *D. melanogaster* flies according to Barnett et al. (1981). Poly(A)$^+$ RNA was isolated from total RNA using oligo d(T) Sepharose (Pharmacia). Reverse transcription was carried out according to Sambrook et al. (1989) using 10 μg of poly(A)$^+$ RNA, 0.05 pmol antisense primer, and 10 units AMV reverse transcriptase (Stratagene) in a total reaction volume of 100 μl. Annealing conditions were 50° C for 5 h. A 1.2 kb fragment was generated by polymerase chain reaction (PCR) on cDNA derived from poly(A)$^+$ RNA from *D. melanogaster* adult flies using synthetic sense and antisense 30-mer primers (sense, 5′-GCGCGGATCCCTCACCGAGGACTTCAACGG-3′ and antisense, 5′-GCGCAAGCTTTAGATGCATCGCTGGGTGTATG-3′; restriction sites are underlined) derived from the sequence of *DmRP128* (nucleotides 1814 and 3045, respectively). PCR (Innis et al. 1990) was performed in a Thermocycler 60 (Biomed) in 100 μl reaction buffer containing 50 mM KCl, 10 mM TRIS-HCl, pH 8.3, 1 mM $MgCl_2$, 0.01% gelatin (w/v), 40 pmol each of sense and antisense primer, 0.1% volume of the reverse transcription reaction, and 2.5 units Taq polymerase (Pharmacia). DNA amplification was achieved with 35 cycles of denaturation for 1 min at 94° C, primer annealing for 2 min at 50° C, and extension for 2 min at 72° C.

Protein expression and antibody production

DmRP135. Four fusion proteins covering the complete coding region of *DmRP135* were constructed. The following DNA fragments were cloned into pUR (Rüther and Müller-Hill 1983) and expressed in-frame with β-galactosidase; DmRP135/1: 0.99 kb *Eco*RI-*Hin*dIII, amino acids (aa) 23–353; DmRP135/3: 1.5 kb *Sal*I-*Hin*dIII, aa 367-850; DmRP135/5: 0.84 kb *Hin*dIII-*Hpa*II, aa 849-1129; DmRP135/7: 0.3 kb *Pst*I-*Hpa*II, aa 1045-1129.

DmRP128. The 1.2 kb *Bam*HI-*Hin*dIII fragment (nucleotides 589–1819) generated by PCR was cloned into expression vector pDS56/RBSII (Hochuli et al. 1988). All expressed proteins were purified by inclusion body purification (Nagai and Thøgerson 1987). Female New Zealand white rabbits were immunized with 500 μg of expressed protein using ABM-S (Linaris) as adjuvant, boosted 3 times at 2-week intervals with 300 μg protein and bled 1 week after the last boost. Anti-DmRP128 serum was purified by affinity chromatography on protein-G Sepharose (Pharmacia) and on fusion protein blotted onto nitrocellulose (Harlow and Lane 1988).

Western blots and immunostaining. Western blot analysis was performed using 2 μg of purified RNA polymerases I and II, and about 0.5 μg of RNA polymerase III, which were run on 6% polyacrylamide gels, electroblot-

ted onto nitrocellulose, blocked in phosphate-buffered saline (PBS), 0.1% Tween-20 overnight and incubated with the first antibodies (dilutions 1:500 to 1:5000). Binding of antibodies was detected with a 1:5000 dilution of horseradish peroxidase goat anti-rabbit conjugate (Dianova) followed by incubation with 4-chloro-1-naphthol.

Growth of D. melanogaster. *D. melanogaster* strain Oregon R were grown in mass culture. Embryos and larvae were collected according to standard protocols (Ashburner 1989).

Standard RNA polymerase activity assays and inhibition assays. The standard enzyme assay for polymerase activity was performed as described by Greenleaf and Bautz (1975). For pol I the reaction mix contained 80 mM TRIS-HCl, pH 8.0, 0.1 mM dithiothreitol (DTT), 2.5 mM $MnCl_2$, 4 mM thioglycerol, 30 mM ammonium sulphate, 1.5 mM $MgCl_2$, 0.5 mM each of ATP, CTP, GTP, 5 µM [^{3}H]UTP (1 Ci/mmol), 15 µg denatured calf thymus DNA. For pol II and pol III of *D. melanogaster*, assays were performed according to Steinberg et al. (1990) in 0.1 ml of 50 mM TRIS-HCl, pH 7.6, 150 mM KCl, 2 mM $MnCl_2$, 0.5 mM each of ATP, CTP, GTP, 1 mM DTT, 10 µM [^{3}H]UTP (1 Ci/mmol), 15 µg denatured calf thymus DNA. Assays were performed at 30° C for 15 min using 10 µl of enzyme preparation. For inhibition assays α-amanitin was used at final concentrations of 1×10^{-4} and 1×10^{-6} M, and tagetitoxin at 1.2×10^{-5} M (Steinberg et al. 1990).

Purification of RNA polymerase I. All purification steps were carried out at 5° C in buffer A (50 mM TRIS-HCl, pH 8.0, 0.1 mM EDTA, 25% glycerol, 10 mM thioglycerol, 0.1 mM DTT, 0.3 mM phenylmethylsulphonyl fluoride, 2 µg/ml each of leupeptin, aprotinin, pepstatin, and varying concentrations of ammonium sulphate. Fifty grams of frozen *D. melanogaster* embryos were thawed and low- and high-salt extracts were prepared according to Gundelfinger and Stein (1982). Combined high- and low-salt extracts were cleared by centrifugation at $100000 \times g$ for 1 h. The cleared homogenate was adjusted to a final ammonium sulphate concentration of 300 mM and submitted to batch Heparin Sepharose treatment using 65 ml Heparin Sepharose (Pharmacia) equilibrated in buffer A containing 300 mM ammonium sulphate. After adsorption for 1 h under gentle stirring the Heparin Sepharose was washed with a ten-fold volume of the same buffer.

RNA polymerase activity was step-eluted by 600 mM ammonium sulphate in buffer A. Active fractions were pooled and precipitated by addition of solid ammonium sulphate to a final saturation of 60% at 5° C. The precipitate was redissolved in buffer A to a final concentration of 150 mM ammonium sulphate and subjected to an 8 ml Q-Sepharose Fast Flow FPLC column (Pharmacia), equilibrated in the same buffer. After washing the column until the effluent was free of protein, RNA polymerase activity was eluted by a gradient of 150–700 mM ammonium sulphate in buffer A. Pol I activity, resistant to α-amanitin (1×10^{-4} M) was pooled, concentrated with Centricon-30 (Amicon) and dialysed against buffer B (= buffer A, pH 6.5) containing 30 mM ammonium sulphate. The concentrated pool was subjected to a 3 ml S-Sepharose Fast Flow FPLC column (Pharmacia). After washing the adsorbent with buffer B, RNA polymerase I activity was eluted by a gradient of ammonium sulphate from 30 to 500 mM in buffer B. Active fractions were pooled, concentrated as before, and further purified on a 100 ml Superose-6 gel filtration FPLC column (Pharmacia) in buffer A containing 500 mM ammonium sulphate at a flow rate of 0.2 ml/min. RNA polymerase activity was assayed in the presence and absence of inhibitors.

Purification of RNA polymerase II, partial purification of RNA polymerase III. The buffer and conditions used were the same as for purification of RNA polymerase I. About 500 g of frozen larvae of *D. melanogaster* were homogenized and the first two purification steps on DEAE Sepharose and Heparin Sepharose were performed as described by Gundelfinger et al. (1980). Total RNA polymerase activity eluted from Heparin Sepharose was precipitated by addition of solid ammonium sulphate to a final saturation of 60% at 5° C. The precipitate was collected by centrifugaton for 30 min (Sorvall HB-4, 10000 rpm) and redissolved in buffer A to a final ammonium sulphate concentration of 300 mM. The sample was applied to a 100 ml Superose-6 gel filtration FPLC column (Pharmacia) equilibrated in the same buffer and eluted at a flow rate of 0.2 ml/min. Active fractions were pooled and precipitated as described above. A 1 ml Mono Q Sepharose column (Pharmacia) was equilibrated with 50 mM ammonium sulphate in buffer A and loaded with the RNA polymerase activity redissolved in buffer A to a final concentration of 50 mM. Nonadsorbed proteins were removed by washing with the same buffer until no protein was detected in the effluent. Enzymatic activity of all three RNA polymerases was separated by a 50–500 mM gradient of ammonium sulphate in buffer A. Active fractions were characterized by their specific RNA polymerase activity in inhibition assays and were analysed by SDS-polyacrylamide gel electrophoresis (PAGE).

Results

Identification of an RNA polymerase subunit gene (DmRP128)

About 50000 recombinants of an embryonic cDNA library were screened under low stringency conditions with a 658 bp DNA fragment covering the coding region for the C-terminus of *RET1* of yeast. A total of 12 positive clones were rescreened and further analysed by restriction mapping and Southern blots. Hybridization under stringent conditions with complete cDNA constructs of the coding regions of *DmRP140* (Falkenburg et al. 1987; Sitzler et al. 1991) and *DmRP135* (Kontermann et al. 1989) identified one clone as *DmRP140* and

three of them as *DmRP135*. The remaining clones were further analysed, and preliminary sequence data indicated that we had isolated an as yet unidentified RNA polymerase gene coding for a second-largest subunit (*DmRP128*).

To determine the structure of the complete gene a genomic library of *D. melanogaster* was screened with a 1.7 kb cDNA fragment. We isolated two independent clones with *Eco*RI inserts of 8.9 kb (λ-Dm8.9) and 15.3 kb (λ-Dm15.3) of genomic DNA, which hybridized with the cDNA probe. Restriction enzyme mapping and Southern analysis located the region of homology in a 4.2 kb *Eco*RI fragment (λ-Dm8.9) and in a 6.8 kb *Eco*RI fragment (λ-Dm15.3), respectively. The 4.2 kb *Eco*RI fragment and an overlapping 1.7 kb *Sma*I-*Hin*dIII fragment from λ-Dm15.3 were cloned into M13 and sequenced. Comparison of the deduced amino acid sequence of *DmRP128* with *RET1* indicated the existence of a small intron in the 5′ region of the gene. Since none of the cDNA clones isolated spanned the putative intron region, a 1.2 kb DNA fragment was amplified by PCR from poly(A)$^+$ RNA using a set of primers deduced from the genomic sequence. This amplified cDNA fragment was sequenced and the existence of an intron of 48 bp was confirmed. The nucleotide sequence of *DmRP128* and the deduced amino acid sequence are shown in Fig. 1.

The putative protein consists of 1135 amino acids with a calculated molecular weight of 128 kDa and an isoelectric point of 8.68. We find the same regions of homology with *DmRP140* and *DmRP135* which are also conserved between the second-largest subunits of pol II of yeast and *Drosophila* and the β subunit of *E. coli* RNA polymerase (Falkenburg et al. 1987). Therefore, it is evident that we have isolated a gene coding for the so far unknown second-largest subunit of a third RNA polymerase of *D. melanogaster*. Figure 2 shows the amino acid alignment of all second-largest subunits of RNA polymerases of *D. melanogaster*. The overall amino acid sequence homologies of DmRP128 are 36% with DmRP140 and 24% with DmRP135. Compared to C128, the second-largest subunit of RNA polymerase III of yeast, DmRP135 shares 24% identical amino acids whereas DmRP128 shares 56% identical amino acids. The strong homology of *DmRP128* to this *RET1* product suggests that this, rather than *DmRP135*, is the gene coding for the second-largest subunit of RNA polymerase III. *DmRP135* shows more homology to *RPA135* (41%), the gene coding for the second-largest subunit of RNA polymerase I of yeast (Yano and Nomura 1991) than to *RET1* (24%) and, therefore, is more likely to code for the second-largest subunit of RNA polymerase I of *D. melanogaster*.

RNA polymerase subunit-specific antibodies

Specific antibodies were raised against bacterially expressed proteins derived from *DmRP135* and *DmRP128*. For the *DmRP128* construct we chose a fragment from regions of less conserved sequence to minimize possible cross-reactions; the 1.2 kb *Bam*HI-*Hin*dIII cDNA fragment (aa 196–606) was expressed in pDS56/RBS II (Hochuli et al. 1988). A series of fusion proteins was also constructed covering the complete coding region of *DmRP135* (see the Materials and methods). The expressed proteins were purified by inclusion body preparation (Nagai and Thøgerson 1987). Antisera raised against the purified proteins were tested on Western blots with preparations of purified RNA polymerases of *D. melanogaster* and yeast (see below). In addition the antibodies were used for indirect immunofluorescence on polytene chromosomes of *D. melanogaster* (results not shown).

Purification and identification of RNA polymerases of D. melanogaster

RNA polymerase I was isolated from embryos; low- and high-salt extracts were combined and further purified using four FPLC purification steps. RNA polymerase activity is reproducibly eluted from Q-Sepharose in two defined peaks at 290 and 370 mM ammonium sulphate. These activities were clearly distinguished in specific inhibition assays. Neither fraction showed reduced activity with 1.2×10^{-5} M tagetitoxin (Fig. 3), an inhibitor specific for pol III (Steinberg et al. 1990). The RNA polymerase activity eluted at 370 mM ammonium sulphate was completely inhibited by an α-amanitin concentration of only 1×10^{-6} M and, therefore, represents pol II. In contrast, the 290 mM eluate was not inhibited by α-amanitin even at concentrations of 1×10^{-4} M. This pool was subjected to further purification steps and resulted in pol I of high purity.

RNA polymerases II and III were extracted from larvae. Total RNA polymerase activity of the initial homogenate was processed by four FPLC purification steps. On Mono Q-Sepharose four different activities were separated. Pol I and pol II were eluted at the same ammonium sulphate concentration as that observed in the purification procedure from embryos. RNA polymerase III activity eluted at 150 and 220 mM ammonium sulphate resulting in two separate peaks resistant to α-amanitin at concentrations as high as 1×10^{-4} M. Pol III activity can be monitored by specific inhibition with tagetitoxin at a final concentration of 1.2×10^{-5} M. This procedure, optimized for the enrichment of pol III, yielded low levels of pol I but particularly pure pol II. Figure 3 shows a graphic representation of enzyme activities with respect to their specific inhibitors.

SDS-PAGE analysis and immunostaining of RNA polymerases

Purified RNA polymerases I, II and III of *D. melanogaster* and yeast were run on SDS-PAGE to analyse the subunit composition in comparison with the yeast enzymes (Fig. 4). Gels of low polyacrylamide content (6%) were used for optimal separation of the largest and the second-largest subunits represented by the major

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   1 GAATTCACAACGTTGACCACTGTGCCGGGATGCATTAAGTACAAGGGCGCCAAGATCCAGCTGCTGGATTTGCCCGGTATCATTGAGGGCGCTAAGGATGGCAAGGGTCGAGGTCGTCAG
 121 GTGATTGCTGTCGCTCGCACCTGTAACCTCATTTTCATGGTGCTGGATTGCCTGAAACCGCTTGGCCACAAGAAACTCCTCGAGCATGAATTGGAGGGCTTCGGCATCCGGCTTAACAAG
 241 AAACCACCAAATATCTACTACAAGCGGAAGGACAAGGGTGGCATCAATCTGAACAGCATGGTTCCGCAGTCCGAGTTGGACACGGATCTGGTGAAGACCATTCTATCCGAGTACAAGATC
 361 CACAATGCGGACATCACCCTGAGATACGACGCCACTAGTGACGACCTCATTGACGTTATCGAGGGCAACCGCATCTACATACCCCTGCATCTATCTGCTGAACAAGATCGATCAGATCTC
 481 CATCGAGGAGCTGGACGTCATCTACAAGATAAGCATTGCGTGCCCATCTCGGCCCATCACCACTGGAACTTTGACGATCTGCTGGAGCTGATGTGGGAATACCTGCGACTGCAGCGCATC
 601 TACACCAACGCAAGGGCCAGCTGCCCGATTACAACTCGCCCGTGGTACTCCACAACGAGCGCACCAGCATTGAGGATTTCTGCAACAAGCTGCATCGCTCCATTGCCAAGGAATTTAAAT
 721 AGTGAGTGCGCTTCACTTTCCCTCTGTTCCCTTGGTTATAATAACATTATGTATTTCTTCGTAGTGCGCTGGTTTGGGGCTCATCTGTGAAGCATCAGCCGCAGAAGGTGGGCATCGAAC
 841 ACGTTCTCAACGACGAGGATGTGGTCCAGATTGTGAAGAAGGTTTAGACTCTGTGCAACGATAACACTTTGATTATTTGATGGCATTTTTTATTCGAAAATAAAAAACGAATGTGCTTTC
 961 TGTGTGCGTGGATTTAAGGCGGCTTAAGTTTAAGAAAACCCTCTAATTTTTATTTTATCAAATTTTTTAATTGGCGCGCACAGAGGTAATTTAAATAAATTAAAGCTAGATTTAGCGGGA
1081 ATTAATTTCACTGCCAGCTCTATGGGAATAACTTTGCTATCACTTGCCCAAGTATATCGATAACCGATAGGTAAAGAAACCGCATATCGAAGAACCTCATAGCACGCTGTTTTGTTGACA

1201 AACTTAAATTAAATTATTATAACGAA ATGGTGGAGCTAAAGATGGGCGATCACAACGTGGAGGCCACCACCTGGGATCCCGGCGACAGCAAGGACTGGTCGGTGCCCATTAAGCCGCTA
                                M  V  E  L  K  M  G  D  H  N  V  E  A  T  T  W  D  P  G  D  S  K  D  W  S  V  P  I  K  P  L     31

1320 ACGGAGAAGTGGAAATTGGTGCCGGCATTCTTGCAAGTAAAAGGATTAGTGAAGCAGCACATTGATTCCTTCAATCACTTCATCAACGTGGACATAAAAAAGATTGTGAAGGCTAACGAG
      T  E  K  W  K  L  V  P  A  F  L  Q  V  K  G  L  V  K  Q  H  I  D  S  F  N  H  F  I  N  V  D  I  K  K  I  V  K  A  N  E     71

1440 CTGGTTACCAGTGGAGCTGATCCTTTGTTTTACCTAAAGTACTTGGATGTGCGGGTGGGCAAGCCGGACATCGACGATGGCTTCAACATCACCAAAGCGACCACACCGCACGAATGCCGT
      L  V  T  S  G  A  D  P  L  F  Y  L  K  Y  L  D  V  R  V  G  K  P  D  I  D  D  G  F  N  I  T  K  A  T  T  P  H  E  C  R    111

1560 CTGAGGGACACCACGTACTCCGCCCCCATCACCGTGGACATCGAGTACACGAGGGGAACCCAGCGGATCAAGCGGAATAATTTGCTCATCGGAAGGATGCCCCTCATGTTGCGGTCCAAT
      L  R  D  T  T  Y  S  A  P  I  T  V  D  I  E  Y  T  R  G  T  Q  R  I  K  R  N  N  L  L  I  G  R  M  P  L  M  L  R  S  N    151

1680 TGCGCCCTTACCGGCAAGTCGGAGTTCGAGCTGTCCAAACTGAATGAGTGTCCACTAGATCCTGGCGGCTACTTTGTGGTGCGAGGTCAGGAGAAGGTTATCCTCATTCAGGAGCAGCTG
      C  A  L  T  G  K  S  E  F  E  L  S  K  L  N  E  C  P  L  D  P  G  G  Y  F  V  V  R  G  Q  E  K  V  I  L  I  Q  E  Q  L    191

1800 TCATGGAACAAGATGCTCACCGAGGACTTCAACGGTGTGGTGCAATGCCAGGTTACCTCCTCCACACACGAAAAGAAGTCACGTACTTTGGTGCTGAGCAAGCATGGAAAGTACTACCTC
      S  W  N  K  M  L  T  E  D  F  N  G  V  V  Q  C  Q  V  T  S  S  T  H  E  K  K  S  R  T  L  V  L  S  K  H  G  K  Y  Y  L    231

1920 AAGCACAACTCGATGACGGACGACATACCCATTGTGGTGATCTTTAAAGCTCTGGGCGTAGTTTCTGATCAGGAAATTCAATCGCTAATTGGCATAGACAGCAAATCGCAGAATAGATTC
      K  H  N  S  M  T  D  D  I  P  I  V  V  I  F  K  A  L  G  V  V  S  D  Q  E  I  Q  S  L  I  G  I  D  S  K  S  Q  N  R  F    271

2040 GGTGCCTCTCTAATAGACGCCTACAACCTGAAGGTCTTCACACAGCAAAGGGCTTTGGAGTACATGG GTAGGTTTGTTTACCCTAGTTAAATCTTGTTCTTAAAATACCTTTTTAG GC
      G  A  S  L  I  D  A  Y  N  L  K  V  F  T  Q  Q  R  A  L  E  Y  M                                                     G    294

2158 TCCAAGTTGGTGGTCAAGCGTTTTCAGAGTGCCACCACAAAAACGCCATCGGAAGAGGCCCGTGAGCTGCTGTTAACCACCATTTTGGCGCATGTGCCTGTGGACAACTTTAACTTGCAA
      S  K  L  V  V  K  R  F  Q  S  A  T  T  K  T  P  S  E  E  A  R  E  L  L  L  T  T  I  L  A  H  V  P  V  D  N  F  N  L  Q    334

2278 ATGAAGGCCATCTATGTATCCATGATGGTGCGTCGTGTTATGGCCGCCGAGCTGGACAAAACGCTCTTTGACGACCGCGATTACTACGGCAACAAGCGACTGGAGCTGGCAGGTTCTTTG
      M  K  A  I  Y  V  S  M  M  V  R  R  V  M  A  A  E  L  D  K  T  L  F  D  D  R  D  Y  Y  G  N  K  R  L  E  L  A  G  S  L    374

2398 CTCTCCATGATGTTCGAGGATCTGTTCAAGCGCATGAACTGGGAGCTTAAGACCATTGCGGACAAAAATATACCCAAGGTGAAAGCGGCGCAGTTCGATGTGGTAAAGCACATGAGGGCG
      L  S  M  M  F  E  D  L  F  K  R  M  N  W  E  L  K  T  I  A  D  K  N  I  P  K  V  K  A  A  Q  F  D  V  V  K  H  M  R  A    414

2518 GCACAAATCACAGCGGGACTGGAGTCTGCTATCAGTTCCGGCAACTGGACGATCAAGAGATTTAAGATGGAAAGAGCTGGCGTAACGCAGGTTCTGTCACGCTTGAGTTATATCTCAGCG
      A  Q  I  T  A  G  L  E  S  A  I  S  S  G  N  W  T  I  K  R  F  K  M  E  R  A  G  V  T  Q  V  L  S  R  L  S  Y  I  S  A    454

2638 TTGGGAATGATGACGCGTGTAAACTCGCAGTTTGAGAAGACGCGTAAGGTGTCGGGACCCAGATCTCTGCAGCCCAGTCAGTGGGGTATGCTGTGTCCCTCGTACACTCCAGAAGGTGAG
      L  G  M  M  T  R  V  N  S  Q  F  E  K  T  R  K  V  S  G  P  R  S  L  Q  P  S  Q  W  G  M  L  C  P  S  Y  T  P  E  G  E    494

2758 GCCTGTGGTCTTGTCAAAAATCTGGCTTTGATGACGCACATCACCACCGAAGTGGAGGAGCGACCCGTTATGATAGTGGCTTTCAATGCTGGCGTGGAAGACATACGAGAAGTGAGCGGC
      A  C  G  L  V  K  N  L  A  L  M  T  H  I  T  T  E  V  E  E  R  P  V  M  I  V  A  F  N  A  G  V  E  D  I  R  E  V  S  G    534

2878 AACCCCATCAATAATCCGAATGTGTTCCTCGTCTTCATCAATGGTAATGTTCTGGGTCTCACCTTGAACCACAAGCACCTGGTGCGGAACCTTCGTTACATGCGCAGAAAGGGTCGGATG
      N  P  I  N  N  P  N  V  F  L  V  F  I  N  G  N  V  L  G  L  T  L  N  H  K  H  L  V  R  N  L  R  Y  M  R  R  K  G  R  M    574

2998 GGCAGCTACGTGTCTGTGCACACATCATACACCCAGCGATGCATCTACATCCACACGGATGGCGGTCGTCTGTGCCGCCCCTATGTAATTGTGGAGAACCGTCGCCCGCTGGTGAAGCAG
      G  S  Y  V  S  V  H  T  S  Y  T  Q  R  C  I  Y  I  H  T  D  G  G  R  L  C  R  P  Y  V  I  V  E  N  R  R  P  L  V  K  Q    614

3118 CATCACCTGGACGAGCTGAATCGAGGGATTCGCAAGTTCGACGACTTCCTTTTGGACGGACTAATCGAGTATTTAGATGTTAACGAGGAGAACGATTCGTTTATCGCCTGGAACGAGGAT
      H  H  L  D  E  L  N  R  G  I  R  K  F  D  D  F  L  L  D  G  L  I  E  Y  L  D  V  N  E  E  N  D  S  F  I  A  W  N  E  D    654

3238 CAAATAGAGGACCGGACTACACACTTGGAAATCGAAACCTTCACTTTGCTGGGAGTCTGTGCTGGATTGGTGCCCTATCCTCATCACAATCAGAGTCCCAGGAACACCTATCAGTGTGCT
      Q  I  E  D  R  T  T  H  L  E  I  E  T  F  T  L  L  G  V  C  A  G  L  V  P  Y  P  H  H  N  Q  S  P  R  N  T  Y  Q  C  A    694

3358 ATGGGTAAGCAGGCGATGGGAATGATTGGCTATAACCACAACAACAGGATTGACTCGCTGATGTATAATCTGGTGTATCCCCATGCTCCTATGGTGAAATCCAAGACTATCGAGCTTACC
      M  G  K  Q  A  M  G  M  I  G  Y  N  H  N  N  R  I  D  S  L  M  Y  N  L  V  Y  P  H  A  P  M  V  K  S  K  T  I  E  L  T    734

3478 AACTTTGACAAATTGCCTGCTGGTCAGAATGCCACTGTGGCAGTTATGAGTTACTCTGGCTACGATATCGAAGATGCCCTGATCTTAAACAAGGCGTCAATAGATCGTGGATATGGACGG
      N  F  D  K  L  P  A  G  Q  N  A  T  V  A  V  M  S  Y  S  G  Y  D  I  E  D  A  L  I  L  N  K  A  S  I  D  R  G  Y  G  R    774

3598 TGTCTGGTGTACAAAAACTCAAAGTGCACTGTGAAACGGTATGCGAATCAAACCTTCGACAGAATCATGGGTCCCATGAAAGATGCGCTGACGAACAAGGTTATCTTCAAGCACGACGTT
      C  L  V  Y  K  N  S  K  C  T  V  K  R  Y  A  N  Q  T  F  D  R  I  M  G  P  M  K  D  A  L  T  N  K  V  I  F  K  H  D  V    814

3718 TTGGACACTGACGGAATTGTGGCTCCAGGCGAGCAGGTTCAAAACAAACAGATCATGATTAACAAGGAAATGCCCGCGGTAACATCGATGAATCCGCTGCAGGGTCAGTCTGCACAGGTT
      L  D  T  D  G  I  V  A  P  G  E  Q  V  Q  N  K  Q  I  M  I  N  K  E  M  P  A  V  T  S  M  N  P  L  Q  G  Q  S  A  Q  V    854

3838 CCCTACACCGCAGTACCCATTAGCTATAAGGGTCCCGAGCCCAGTTACATCGAACGCGTTATGGTTTCCGCCAATGCCGAGGAAGACTTTCTCATCAAGATCCTGCTGCGCCAGACACGT
      P  Y  T  A  V  P  I  S  Y  K  G  P  E  P  S  Y  I  E  R  V  M  V  S  A  N  A  E  E  D  F  L  I  K  I  L  L  R  Q  T  R    894

3958 ATCCCGAGAGGGGACAAATTCAGCTCTCGACACGGACAAAAAGGCGTGACTGGGTTGATTGTTGAACAGGAGGATATGCCCTTTAATGACTTCGGCATCTGCCCGGATATGATAATGAAC
      I  P  R  G  D  K  F  S  S  R  H  G  Q  K  G  V  T  G  L  I  V  E  Q  E  D  M  P  F  N  D  F  G  I  C  P  D  M  I  M  N    934

4078 CCACACGGATTCCCCTCCCGTATGACAGTGGGTAAAACGTTAGAGCTGCTCGGCGGGAAGGCTGGTCTCCTGGAGGGCAAGTTCCACTATGGAACTGCGTTCGGGGGCTCCAAGGTGGAA
      P  H  G  F  P  S  R  M  T  V  G  K  T  L  E  L  L  G  G  K  A  G  L  L  E  G  K  F  H  Y  G  T  A  F  G  G  S  K  V  E    974

4198 GACATTCAGGCGGAACTGGAGCGCCATGGTTTCAACTACGTGGGCAAGGACTTTTTCTACTCCGGCATCACAGGAACACCACTGGAGGCCTACATCTACTCGGGACCCGTGTACTACCAA
      D  I  Q  A  E  L  E  R  H  G  F  N  Y  V  G  K  D  F  F  Y  S  G  I  T  G  T  P  L  E  A  Y  I  Y  S  G  P  V  Y  Y  Q   1014

4318 AAACTGAAGCATATGGTGCAGGATAAGATGCATGCCCGTGCCCGGGGACCCAAGGCGGTGCTCACCCGTCAGCCGACGCAGGGCAGGAGTCGTGAGGGTGGCCTTCGTCTGGGCGAAATG
      K  L  K  H  M  V  Q  D  K  M  H  A  R  A  R  G  P  K  A  V  L  T  R  Q  P  T  Q  G  R  S  R  E  G  G  L  R  L  G  E  M   1054

4438 GAACGGGATTGTCTCATTTCTTACGGAGCTAGTATGCTGATCATGGAGCGTCTGATGATCTCATCGGATGCCTTTGAGGTGGATGTTTGCCGGACTTGCGGCAGGATGGCCTACTGCTCC
      E  R  D  C  L  I  S  Y  G  A  S  M  L  I  M  E  R  L  M  I  S  S  D  A  F  E  V  D  V  C  R  T  C  G  R  M  A  Y  C  S   1094

4558 TGGTGTCATTTCTGCCAGTCGTCGGCCAATGTCTCTAAGATATCCATGCCGTATGCATGCAAACTGCTCTTCCAGGAGTTGACCAGCATGAATGTGGTGCCGAAAATGATTTTGGAAAAC
      W  C  H  F  C  Q  S  S  A  N  V  S  K  I  S  M  P  Y  A  C  K  L  L  F  Q  E  L  T  S  M  N  V  V  P  K  M  I  L  E  N   1134

4678 TATTAA CGAGCAATGGCCTGAAACCGAATAAAATAATTGTTATATAATAATAATGGTTAATGATGGAAACTAGCTTAGGTTTTCCAATATTTTTGTAAAAACGAATGGTGTGTGAACTC
      Y  *                                                                                                                   1135

4797 TGGCAAAGGCAAAAATCCTACGCATTCATTAAGTTTCCATGACCGCGAGCTGCAAATGTCAAAATGAAGAGGTTCAAACGAAGTCAGCGAAAGAAAGTCCTCTTGAATTTTAACGAGGTC
4917 CTTTTGCATATCAGCTTTCTGTTCGTTAACAGGCAGACGCACGCTCTTACTGAAAAGGACTTAGCCGTGTTAAATCGCTTCTGTTATCCTTTTTTTTTTATTGCGCAAAGGATAATTAGT
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *DmRP128*. The nucleotide sequence starts with the genomic *Eco*RI site. The putative polyadenylation signal and splice junctions are *underlined*. The sequence data will appear in the EMBL Data Library under the accession number X58826 D. melanogaster DM RP 128 cDNA

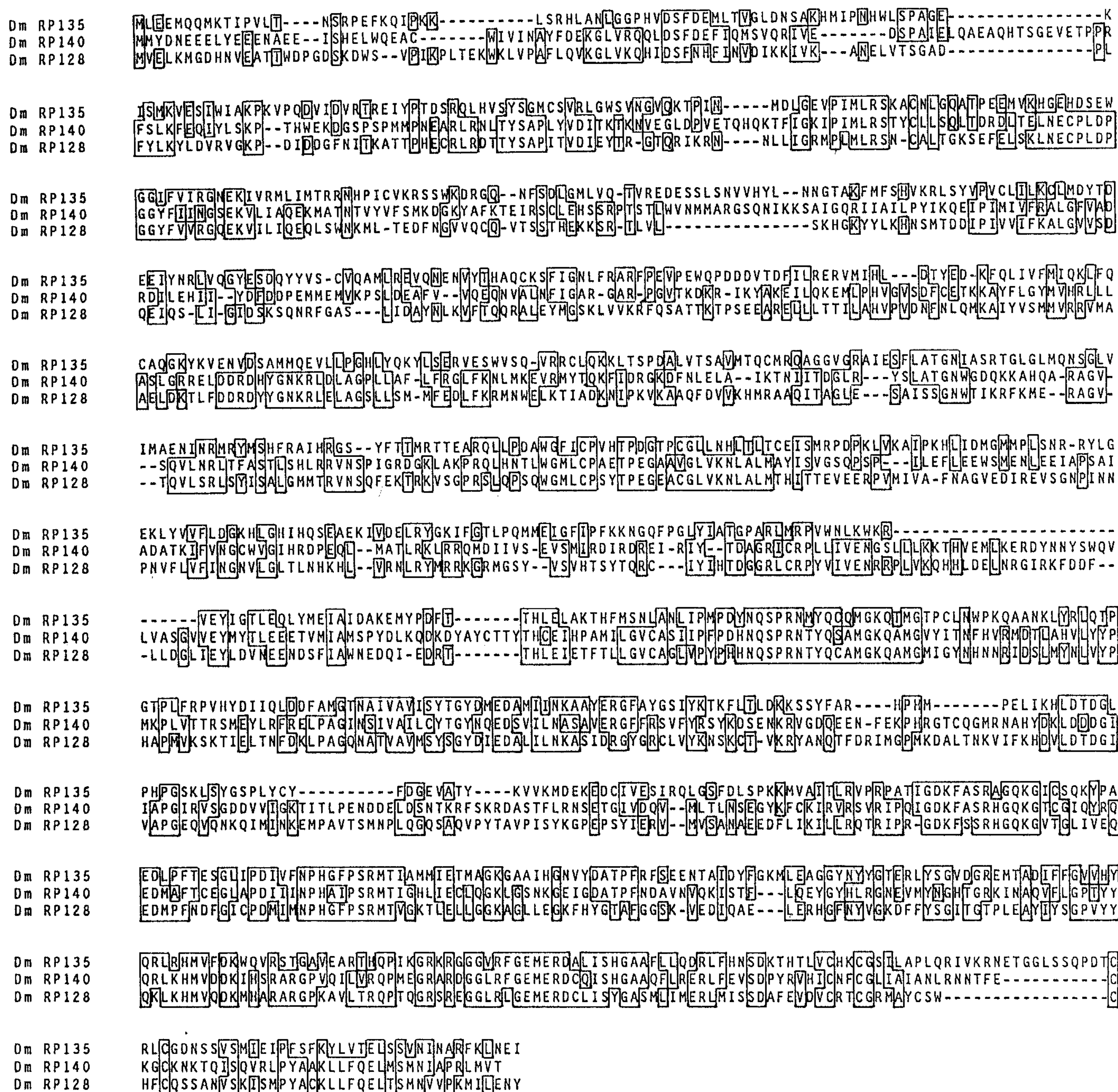

Fig. 2. Amino acid sequence alignment of the second-largest subunits of RNA polymerases I, II, and III of *Drosophila melanogaster*. Identical amino acids are *boxed*

bands in the Coomassie-stained gel. The pol III preparation is not as pure as the preparations of pol I and pol II; however, three major bands can be clearly distinguished which correspond in size to the three largest subunits of the yeast enzyme. The second-largest subunit of pol I of *D. melanogaster* differs in size from the corresponding subunit of yeast.

Pol I, pol II and pol III preparations of *D. melanogaster* and yeast were electroblotted and probed with antibodies directed against all three second-largest subunits of RNA polymerases of yeast: anti-A135, anti-B150, and anti-C128 (Huet et al. 1982, 1985). Antibodies directed against *D. melanogaster* RNA polymerases were anti-DmRPB, directed against the whole pol II enzyme (DmpolB in Krämer and Bautz 1981) and antibodies against the fusion proteins DmRP128/44, DmRP135/1, DmRP135/3, DmRP135/5 and DmRP135/7.

Anti-A135 and anti-B150 recognize their respective subunits and also cross-react with the corresponding subunits of *D. melanogaster*, whereas anti-C128 does not react with any subunit of *D. melanogaster* but only with C128. Besides its specific subunit recognition anti-A135 shows some cross-reactivity with the second-largest subunits of pol I of *D. melanogaster*. Anti-DmRPB reacts strongly with the largest and second-largest subunit of pol II of *D. melanogaster*, albeit rather weakly with the

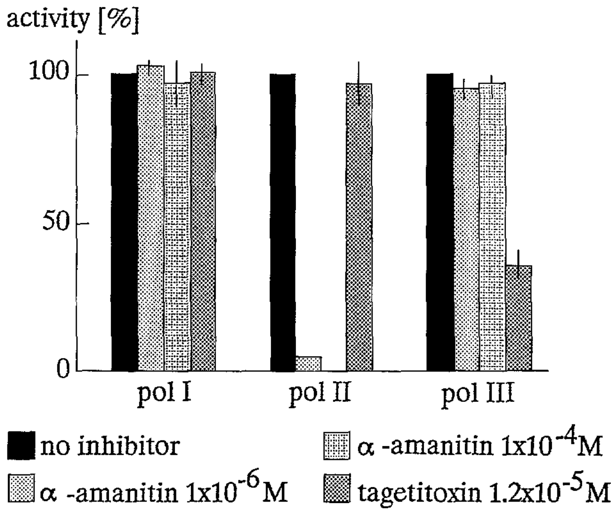

Fig. 3. Inhibitory effects of α-amanitin and tagetitoxin on activity of purified fractions of pol I, pol II, and pol III of *D. melanogaster*. Activity was measured in standard transcription assays (see the Materials and methods) using 10 µl of purified RNA polymerase fractions. Transcriptional activity using inhibitors is shown as percentages of the control activity (without inhibitors)

yeast enzyme. The second-largest subunit of pol I of *D. melanogaster* is specifically recognized by all sera raised against DmRP135. These sera also react with the corresponding yeast subunit A135. Except for some weak, nonspecific cross-reactions, anti-Dm128/44 does not react with any other RNA polymerase subunit but only with the second-largest subunit of *D. melanogaster* pol III. These immunostaining data confirm the conclusions drawn from amino acid sequence comparisons that *DmRP135* codes for the second-largest subunit of pol I and that *DmRP128* codes for the second-largest subunit of pol III of *D. melanogaster*.

Discussion

We had previously reported the cloning of *DmRP135*, a gene that we tentatively identified as coding for the second-largest subunit of pol III of *D. melanogaster* (Kontermann et al. 1989). Since then, *RET1*, the gene coding for the second-largest subunit of pol III of yeast has been clearly identified by means of a mutant allele

Fig. 4. A Coomassie staining and **B, C** immunostaining of purified RNA polymerases (I, II, III) of *D. melanogaster* (Dm) and yeast (y). Equal amounts of purified RNA polymerases were run on 6% polyacrylamide gels, blotted onto nitrocellulose and incubated with the following antibodies: anti-DmRP135/3 (1:500), anti-DMRPB (1:1000), anti-DmRP128 (1:500), anti-A135 (1:5000), anti-B150 (1:1000), anti-C128 (1:1000). No reaction was detected with anti-DmRP128 on yeast RNA polymerases and with anti-C128 on *D. melanogaster* RNA polymerases

(James et al. 1991). Given the overall similarity (58%) between the second-largest subunits of pol II of *D. melanogaster* (*DmRP140*) and yeast (*RPB2*) it was highly surprising to find only 24% homology between the two second-largest subunits of pol III and we had therefore to reconsider the assignment of *DmRP135* to pol III. In order to assigne each subunit gene to it corresponding enzyme we set out to clone the gene coding for the last of the three known second-largest subunits of *D. melanogaster, DmRP128*. In addition, we purified the three RNA polymerases of *D. melanogaster,* pol I, pol II, and pol III. These can be identified by their functional properties, their sensitivity to specific enzyme inhibitors, their salt requirements and their subunit composition as seen on Coomassie-stained gels. Immunostaining of the purified RNA polymerases with specific antibodies was used to correlate each of the two genes, *DmRP135* and *DmRP128*, with its respective product. Antibodies directed against four different fusion proteins covering the coding region of *DmRP135* were found to react with purified pol I of *D. melanogaster* on Western blots. Antibodies directed against *DmRP128* specifically react with pol III. Therefore, it is evident that *DmRP135* codes for the second-largest subunit of pol I and that *DmRP128* codes for the corresponding subunit of pol III.

Our previous assignment of *DmRP135* to the second-largest subunit of pol III was based on the following lines of evidence. First, immunofluorescence on polytene chromosomes using anti-DmRP135 fp antibodies (Kontermann et al. 1989) did not stain the nucleoli but gave the same distinct banding pattern on polytene chromosomes as anti-yeast C128 antibodies. Second, anti-DmRP135 fp antibodies recognized the second-largest subunit of pol III of yeast on Western blots. We cannot explain why both *D. melanogaster* and yeast antisera showed identical antigen distribution on polytene chromosomes or why anti-DmRP135 fp antibodies recognized C128. The observed fluorescence must have been due to non-specific interactions, which, however, were not seen with control sera. The four newly raised anti-*DmRP135* sera as well as anti-yeast A135 and anti-DmRP128 also fail to react with nucleoli and do not show any distinct banding pattern on polytene chromosomes (data not shown). It is possible that these antibodies do not recognize the native protein and/or the second-largest subunits are not accessible in the native enzymes. In addition, the dense structure of nucleoli may interfere with antibodiy recognition in the case of pol I. However, all the antibodies react specifically with purified RNA polymerases separated on SDS-polyacrylamide gels. Since we have been able to isolate and functionally identify all three RNA polymerases of *D. melanogaster,* we can now definitely assign *DmRP135* as coding for the second-largest subunit of pol I and *DmRP128* as coding for the second-largest subunit of pol III.

Comparisons of the deduced molecular weights of all three of the second-largest subunits of RNA polymerases of *D. melanogaster* show that pol I and pol III are of about the same size, 128 kDa, whereas the pol II subunit has a deduced molecular weight of 134 kDa. The size of 128 kDa for the second-largest subunit of pol III is quite unexpected because it differs from the sizes determined for the pol III second-largest subunits of higher eukaryotes (Sentenac 1985). In fact, for *D. hydei,* another closely related species, isolation of pol I and pol III has been described previously (Gundelfinger et al. 1980; Gundelfinger and Stein 1982) and the sizes of the second-largest subunits have been determined on SDS-PAGE to be 135 kDa for the pol III subunit and 125 kDa for the pol I subunit. The similar molecular weights of the second-largest subunits of pol I and pol III of *D. melanogaster* are reflected by their comparable mobility on SDS-polyacrylamide gels; however, both show a lower apparent molecular weight of about 120 kDa. Obviously, SDS-PAGE allows only a rough estimate and does not exactly reflect the actual molecular weights of these proteins.

It remains to be determined whether the RNA polymerase subunits of *D. melanogaster* are different from those of *D. hydei* or whether the discrepancy is due to conditions of the separation on SDS gels. The nomenclature of the second-largest subunits genes of *D. melanogaster* was based on sizes determined on SDS gels. Since pol I and pol III had not previously been purified from *D. melanogaster, DmRP135* was named by analogy to *D. hydei* (Gundelfinger et al. 1980). As it turns out, the second-largest subunits of pol I and pol III of *D. melanogaster* have the same deduced and apparent molecular weight. Comparison of subunit sizes between *D. melanogaster* and yeast for a single RNA polymerase class shows that the deduced molecular weights for the pol II and pol III subunits are almost identical. This is not the case for the pol I subunits where differing sizes of 128 kDa and 135 kDa are shown. The exceptional position of the pol I subunits becomes obvious in the amino acid alignment of all three second-largest subunits of *D. melanogaster. DmRP140* and *DmRP128* share 36% amino acids distributed over the whole sequence; *DmRP135* shows more divergence sharing only 28% amino acid identity with *DmRP140* and 26% amino acids with *DmRP128*. Thus, pol II and pol III subunits seem more closely related to each other than to pol I. The same evolutionary relationship has been observed for the second-largest subunits of yeast (Yano and Nomura 1991) and also for the largest subunits of RNA polymerases (Mémet et al. 1988). RNA polymerase I may have diverged from a common ancestor before separation of pol II and pol III or possibly arose from a different ancestor.

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