IN VITRO PROTEOLYSIS OF A LARGE SUBUNIT OF *DROSOPHILA MELANOGASTER* RNA POLYMERASE B

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1. Introduction

Eukaryotic RNA polymerase B (or II) has often been found to exist in two or three forms which differ from one another in the molecular weight of their largest subunits [1–4]. It has been suggested that the form of the enzyme containing the highest molecular weight subunit might be the only genetically specified enzyme species, serving as a precursor from which the other forms are derived by specific proteolysis [5]. An alternative suggestion is that the largest subunits of each form of the enzyme are unique gene products [1].

In extracts of *D. melanogaster* third instar larvae we had previously found only one form of RNA polymerase B, containing as its largest polypeptide a species of 174 000 daltons (polypeptide-1) [6]. Using specific immunoprecipitation we have now investigated the polypeptide composition of *D. melanogaster* RNA polymerase B in extracts from embryos and from a line of cultured *Drosophila* cells. We find that the enzyme from embryos also contains the 174 000 dalton polypeptide-1, whereas the enzyme from cultured cells contains instead a larger polypeptide of approximately 215 000 daltons (polypeptide-0). Furthermore, a mixing experiment demonstrates that embryo extracts contain an activity that degrades polypeptide-0 to give rise to a polypeptide that migrates in a dodecyl sulfate polyacrylamide slab-gel at the position of polypeptide-1. Recently a similar in vitro conversion of a 220 000 dalton polypeptide of yeast RNA polymerase B was reported [7]; these findings and our results suggest that the different largest polypeptides of RNA polymerase B subclasses are derived from a common precursor and are not the products of separate genes.

2. Materials and methods

The preparation of antibodies directed against purified larval RNA polymerase B, the specific immunoprecipitation of the enzyme from partially fractionated extracts, and the analysis of the antibody precipitates by dodecyl sulfate slab-gel electrophoresis have been previously described [6].

Larvae were grown as before [6]; embryos were collected 12 to 18 h after egg deposition, dechorionated in half-strength 'Clorox', rinsed thoroughly with water, and stored at −70°C. Cultured cells (Schneider line 1W [8]) were grown at 25°C in Schneider's Drosophila medium [9] supplemented with 10% fetal calf serum (Gibco) either in monolayer culture (T-flasks) or in suspension (spinner flasks).

For radioactive labeling, mid-log phase cells grown in suspension were harvested by centrifugation, washed 3 times with phosphate buffered saline and resuspended in Shields and Sang Drosophila medium [10] lacking leucine and fetal calf serum to a final cell density of $6–8 \times 10^6$ cells/ml. After a 1 h preincubation, [^{3}H] leucine (L-[4,5-^{3}H]leucine, Amersham, 53 Ci/mmol) was added and labeling performed for 3 h at 25°C. The cells were pelleted, washed once, and stored frozen in liquid nitrogen.

RNA polymerase B was partially purified as follows (all steps at 0–4°C). Cells or embryos were suspended in 2 vol of buffer A [6] containing 0.5% (v/v) Nonidet P40 (Fluka). A freshly prepared phenylmethyl-

sulfonyl fluoride ($PhMeSO_3F$) solution (10 mg/ml in 100% ethanol) was added to this and all other buffers used in the purification just prior to use to give a final $PhMeSO_3F$ concentration of 1.72 mM. The suspension was homogenized in a Dounce homogenizer (tight fitting glass plunger for cells, motor driven Teflon plunger for embryos), an equal volume of buffer A/50 (buffer A containing 50% (v/v) glycerol) containing 0.60 M ammonium sulfate was added, and the suspension was sonicated 8 × 15 s with a MSE sonicator. Two volumes of buffer A/25 (buffer A containing 25% (v/v) glycerol) were added and the solution centrifuged 1 h at 120 000 × *g*. The supernatant was poured through 2 layers of Miracloth (Calbiochem) and applied over a 4 h period to a 2 ml heparin-Sepharose column [11] previously equilibrated with buffer A/25 containing 0.10 M ammonium sulfate. The column was washed with 10 vol of the same buffer and then RNA polymerase activities were eluted stepwise with 0.60 M ammonium sulfate in buffer A/25. The fractions containing peak RNA polymerase activity (assayed as described earlier [6] except that the specific activity of the tritiated UTP was increased 20-fold), about 50% of which was inhibited by 2 μg/ml α-amanitin, were pooled, diluted with buffer A/25 to a final ammonium sulfate concentration of 0.09 M, and applied to a 1 ml DEAE–cellulose column (Whatman DE-52) previously equilibrated with buffer A/25. After washing the column with 10 vol of buffer A/25 containing 0.12 M ammonium sulfate (to elute α-amanitin-resistant RNA polymerase activity), the RNA polymerase B was eluted with 0.30 M ammonium sulfate in buffer A/25; the RNA polymerase activity in the 0.3 M salt step was entirely of the B type since it was completely inhibited by 1 μg/ml α-amanitin. Aliquots of this partially purified B enzyme were used for the antibody precipitation experiments.

3. Results and discussion

RNA polymerase B purified from third instar larvae of *D. melanogaster* contains two large subunits of 174 000 daltons and 137 000 daltons (polypeptides 1 and 2), and several small subunits of less than 50 000 daltons (fig.1A); it thus resembles calf thymus RNA polymerase form BII [1]. We found no enzyme in larvae analogous to calf thymus polymerase BI, containing a subunit greater than 200 000 daltons, even by antibody precipitation of the larval polymerase at an early stage in the purification and in the presence of $PhMeSO_3F$ [6].

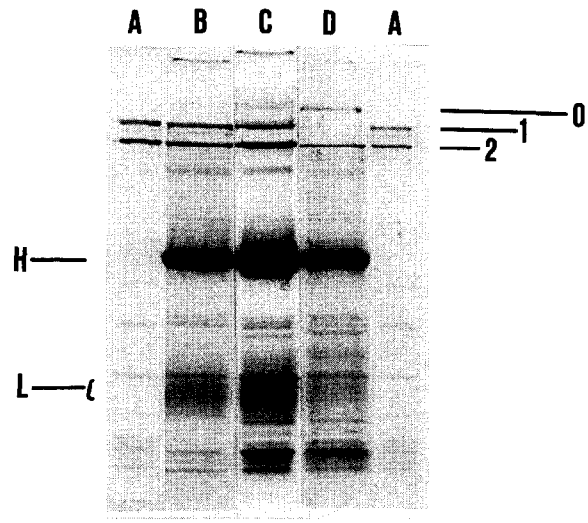

Fig.1. Polypeptide composition of RNA polymerase B from *D. melanogaster* larvae, embryos, and cultured cells. Purified enzyme or antibody precipitates were solubilized and analyzed as described [6] in a dodecyl sulfate slab-gel containing an acrylamide gradient of 7.5–12% (w/v). (A) RNA polymerase B purified from third instar larvae as previously described [6]. (B) Antibody precipitate of purified larval enzyme. (C) Antibody precipitate of embryo RNA polymerase B partially purified as described in Materials and methods. (D) Antibody precipitate of cultured-cell RNA polymerase B partially purified as in Materials and methods. H and L indicate heavy and light immunoglobulin chains, respectively.

To see if other forms of RNA polymerase B might exist at a different growth stage or in a particular cell type, we isolated the B enzyme from partially fractionated extracts of embryos or cultured cells by antibody precipitation (Materials and methods), and analyzed the composition of the precipitates by dodecyl sulfate slab-gel electrophoresis. $PhMeSO_3F$ was added to all buffers to inhibit any serine-proteases that might be present. We found that the embryo enzyme closely resembled the larval polymerase in polypeptide composition; in particular its two largest polypeptides corresponded to those found in the larval enzyme (fig.1C). In contrast, the RNA polymerase B from the

cultured cells differed markedly from the larval enzyme; instead of the 174 000 dalton polypeptide-1, it contained as its largest polypeptide a new species of approximately 215 000 daltons (polypeptide-0; fig.1D). Thus the cultured cell enzyme is analogous to calf thymus RNA polymerase BI [1]. The antibody precipitates from both the embryo and cultured cell extracts contained a few low molecular weight polypeptides not found in the purified larval enzyme; these remain to be investigated.

The presence of only RNA polymerase BII in extracts of embryos and larvae could indicate that form BI is not synthesized at these growth stages, or that it is synthesized but then converted to form BII by proteolytic alteration of polypeptide-0. To test whether embryos contain an activity capable of converting polypeptide-0 into polypeptide-1 we performed the following mixing experiment. We prepared a [^{3}H]leucine-labeled extract of cultured cells, mixed it with an embryo extract, precipitated the RNA polymerase B from the mixed extract with anti-polymerase B γ-globulins, and analyzed the precipitated polypeptides by dodecyl sulfate slab-gel electrophoresis. As a control we performed a similar mixing experiment using unlabeled cultured cells instead of embryos.

Fluorography [12] of the slab-gel indicated that in the control experiment (radioactive cells mixed with unlabeled cells), the radioactive cellular enzyme contained polypeptides 0 (approximately 215 000 daltons) and 2 (137 000 daltons) in approximately equal amounts, as expected from the experiment of fig.1, while no species corresponding to polypeptide-1 (174 000 daltons) was evident (fig.2A). In contrast, the radioactive cellular enzyme that had been exposed to the embryo extracted contained, relative to polypeptide-2, very little label in polypeptide-0 but approximately stoichiometric amounts of label at the position of polypeptide-1 (fig.2B). These results indicate that embryos contain an activity that, even in the presence of $PhMeSO_3F$, can proteolytically degrade cellular polypeptide-0, to give rise to a polypeptide having the same molecular weight as polypeptide-1.

The proteolytic activity responsible for the degradation of *Drosophila* polypeptide-0 is apparently different from that responsible for the degradation of the largest polymerase B subunit in yeast [7], since it is not inhibited by $PhMeSO_3F$. It remains to be determined if the proteolysis of RNA polymerase B we observe in

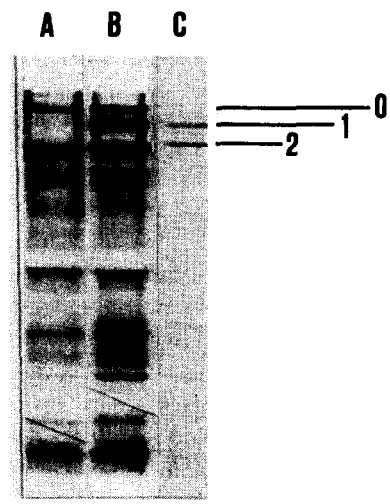

Fig.2. Polypeptide composition of radioactive cultured-cell RNA polymerase B after mixing with non-radioactive cultured cells or embryos. Approximately 0.2 g of Schneider 1W cells, labeled with [^{3}H]leucine (Materials and methods), were mixed either with 1.8 g unlabeled 1W cells or with 5 g embryos, and RNA polymerase B partially purified and antibody-precipitated as described in Materials and methods. The solubilized antibody precipitates were analyzed by dodecyl sulfate slab-gel electrophoresis as in fig.1, and the radioactive polypeptides were visualized by fluorography [12]. (A) Radioactive polypeptides precipitated from a portion of the cell–cell extract. (B) Radioactive polypeptides precipitated from a portion of the cell–embryo extract. (C) Stained gel of purified larval RNA polymerase B, for comparison.

Drosophila extracts occurs only in vitro, or whether it might also occur in vivo.

The fluorographs shown in fig.2 indicate the presence in the antibody precipitates of radioactive polypeptides that do not correspond to species found in the purified larval enzyme. The significance of the presence of these polypeptides is not yet known; however, the low molecular weight species present in the precipitate from the mixing experiment and not present in the control experiment might, for example, be cleavage products derived from polypeptide-0.

We have shown that in vitro proteolysis of polypeptide-0 (215 000 daltons) can lead to formation of a species with the same molecular weight as polypeptide-1 (174 000 daltons), suggesting that the larger species serves as a precursor for the smaller one. This would imply that the two polypeptides are products of the same gene. Another explanation could be that in

vitro proteolysis of polypeptide-0 only fortuitously results in the formation of a protein of 174 000 daltons, but that this protein is not polypeptide-1; by this explanation polypeptides 0 and 1 would be products of different genes. Further experiments, such as a comparison of tryptic digests, are required to determine whether in fact polypeptide-1 and the 174 000 daltons species created in vitro during the mixing experiment are identical or not. In the absence of those experiments, the fact that an apparent conversion of one form of RNA polymerase B to another by proteolysis has now been observed in organisms as divergent as fruit flies and yeast [7], suggests to us that the first explanation is more likely and that the two subunits are coded by the same gene. Similar conversions might explain the multiplicity of B enzymes found in other eukaryotes.

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