Interaction of nucleic acids with the DNA-dependent RNA polymerases of *Drosophila*

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The interaction between the three *Drosophila* DNA-dependent RNA polymerases (EC 2.7.7.6) and the DNA template or the RNA product was investigated by photochemical cross-linking and binding studies, using RNA polymerase subunits immobilized on nitro-cellulose filters. It can be shown that the two largest subunits are responsible for the binding of the enzymes to both template and newly-synthesized RNA.

Western blotting *UV cross-linking* *DNA–protein interaction* *RNA polymerase* *Subunit function* *RNA–protein interaction*

1. INTRODUCTION

Elucidation of the mode of interaction of RNA polymerase with the DNA template and the nascent RNA chain is essential for understanding the structure of the active transcription complex and the mechanism of RNA synthesis. In prokaryotes these types of studies have been successful, mainly because the *Escherichia coli* RNA polymerase works in vitro as well as in vivo. This allowed reconstruction experiments in which the interaction could be studied in detail [1–3]. In eukaryotes this type of approach is hindered by the lack of specificity of in vitro transcription carried out with purified enzymes. In some cases the use of a nicked template improves the rate of transcription, making the template–enzyme interaction even less specific (see [4]). As a result very little is known about the mechanism of RNA synthesis in eukaryotes.

Here, I have attempted to study the interaction between *Drosophila* RNA polymerases I, II and III and nucleic acids under different conditions, dissecting the enzymes into their protein subunits.

Abbreviation: ad2, adenovirus type 2

The results show that by analogy with what is found in *E. coli*, the two large RNA polymerase subunits can tightly bind to the DNA in a non-specific complex. It is, however, possible to show that in the presence of elongation complexes the DNA-binding is clearly shifted to one of the subunits, while the other seems to bind mainly to the RNA chain.

2. MATERIALS AND METHODS

2.1. *Enzymes and template*

Drosophila hydei RNA polymerases I, II and III were purified as in [5,6]. Nick translation of adenovirus 2 (ad2) DNA (a gift from Dr Ernst Spindler) was performed with an Amersham Buchler nick translation kit, using [^{3}H]dCTP (80 Ci/mmol) as labelled nucleotide.

2.2. *Protein blotting and DNA-binding to isolated subunits*

To determine the DNA-binding capacities of individual RNA polymerase subunits these were separated on SDS–polyacrylamide gels and transferred to nitrocellulose filters (Sartorius type SM 11306) as in [7]. Binding of labelled DNA to

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the separated subunits was carried out as described in [8]. The polypeptides capable of binding DNA were identified by fluorography [9].

2.3. *Formation of enzyme–DNA complexes*

Stable ternary complexes between *Drosophila* RNA polymerases I, II and III and tritium-labelled ad2 DNA were formed by incubating the enzymes with the DNA in 0.05 M Tris–HCl (pH 7.4), 10% (v/v) glycerol, 2 mM $MnCl_2$, 0.01 M thioglycerol, 0.1 mM dithiothreitol, 0.025 M ammonium sulphate, and 0.3 mM each of ATP, GTP, and CTP for 10 min at 30°C. To investigate transcriptionally active complexes of RNA polymerase II the above binding buffer was supplemented with 10 μM UTP. After 5 min incubation, $(NH_4)_2SO_4$ was added to block further initiation at a final concentration of 0.25 M and RNA synthesis was continued for another 5 min [10]. In order to investigate the attachment of RNA polymerase II subunits to RNA, RNA synthesis was carried out on mechanically nicked ad2 DNA using [α-^{32}P]UTP (Amersham Buchler). *E. coli* tRNA (Boehringer) was added to the reaction mixture before UV irradiation to prevent non-specific attachment of free RNA polymerase to the newly-synthesized RNA.

2.4. *UV irradiation and analysis of cross-linkage-products*

Photochemical cross-linking was carried out exposing the reaction mixture for 8 min at 0°C to the UV light from two 15 W Sperisol lamps (254 nm). The dose of irradiation was 4.10^5 erg/mm^2 for protein–DNA and 10^5 erg/mm^2 for protein–RNA cross-linking [11].

After UV exposure the unbound DNA was removed by digestion with DNase I (Worthington, 20 μg/μg DNA) for 2.5 h at 37°C in the presence of 0.01 M $MgCl_2$. In some experiments this treatment was followed by digestion with snake venom phosphodiesterase (Boehringer), alkaline phosphatase (Boehringer, from calf intestine), and micrococcal nuclease (Boehringer) essentially as in [1,12]. Samples containing newly-synthesized RNA were additionally treated with RNases A and T1 in order to remove free RNA [13].

Electrophoresis of the nucleic acid–protein complexes was in SDS–polyacrylamide gels [14]. After fluorography [15], the dried gels were exposed to Kodak XR5 X-ray films. The intensity of the bands was determined by scanning the films with a Joyce Loebl Microdensitometer.

3. RESULTS

3.1. *Binding of DNA to RNA polymerase subunits after transfer of the proteins to nitrocellulose filter*

We have previously shown that only the two largest subunits of RNA polymerase I of *Drosophila* can tightly bind DNA [6]. The same result is obtained using *Drosophila* RNA polymerase II and III. Fig.1 shows the blotting of the electrophoretically separated subunits of *Drosophila* RNA polymerases after incubation of the nitrocellulose filters with nick translated ad2 DNA (fig.1, B lanes). Only two major bands of radioactivity are visible in all samples tested and their position corresponds to that of the respective RNA polymerase subunits shown in the stained gels (fig.1, A lanes). The results suggest that of the several subunits found in RNA polymerases only two, and consistently the largest ones, have affinity for DNA. Similar binding properties have been implied by the experiments in [16] for yeast RNA polymerase I.

3.2. *Photo-cross-linkage of* Drosophila *RNA polymerase to ad2 DNA and to the synthesized RNA chain*

In order to study the DNA-binding of the RNA polymerases under more physiological conditions, I have analyzed RNA polymerase–DNA ternary complexes, obtained incubating the *Drosophila* enzymes with ad2 DNA in the presence of 3 of the nucleoside triphosphates. These complexes are known to be very stable and should contain mainly initiation complexes [17]. DNA and proteins were covalently linked by UV irradiation, extensively digested by nucleases, and the products of digestion were analyzed in SDS–polyacrylamide gels.

The results of such experiments are shown in fig.2. The radioactive DNA seems to be exclusively bound to the large subunits of RNA polymerase I, II and III (fig.2b, B lanes), although the cross-linkage-products are migrating in the gel somewhat slower than the corresponding protein subunits. This is probably due to the DNA binding which increases the size of the subunits as compared to that

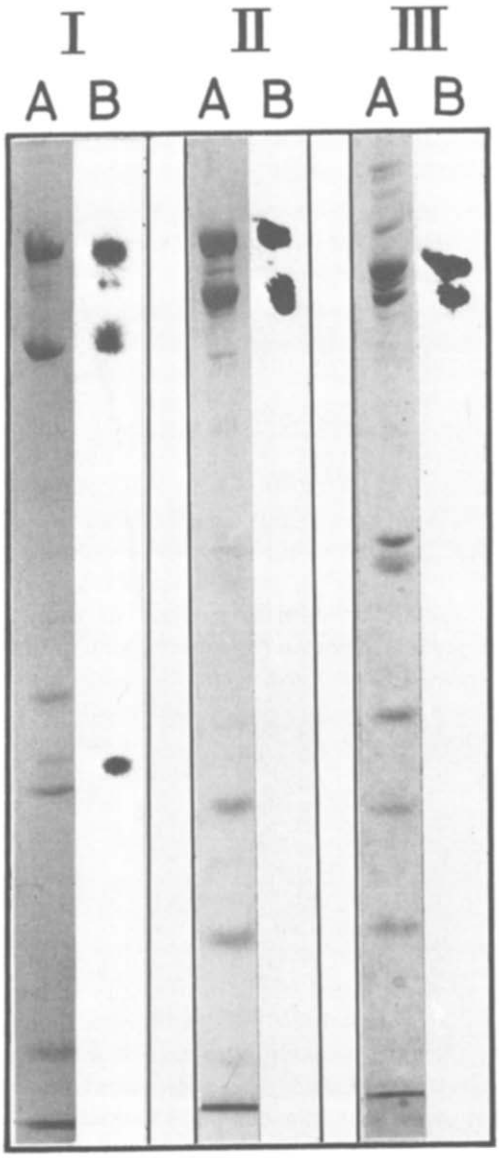

Fig.1. Binding of ad2 DNA to *Drosophila* RNA polymerase subunits. 10–15 μg of each of RNA polymerase I (I), II (II) and III (III) were separated on 9 or 10% SDS–polyacrylamide gels and blotted onto nitrocellulose filters [7]. Binding of 2.5 μg ^{3}H-labelled ad2 DNA (spec. act. 4×10^{6} cpm/μg) was carried out at room temperature for 2–3 h in 10 ml binding buffer containing 0.05 M NaCl [8]. The filters were washed in the same buffer twice for 15 and 30 min, dried, fluorographed [9] and exposed to a Kodak X-ray film (x-omat XR5). A lanes: reference gels stained with Coomassie brilliant blue; B lanes: corresponding fluorographs.

of the control. In experiments where the cross-linkage was carried out in the absence of DNA, but in the presence of labelled nucleotides, the protein subunits were not labelled.

The separation between the cross-linkage-products is poor, especially for class II and III enzyme (fig.2, lane IIB,IIIB), even after extensive digestion with several nucleases [1,12] (lane IIC). The products derived from the RNA polymerase I complex are better separated even after DNase I digestion only. The most likely reason for the smear is the wide range of size variability of the protected DNA segments. This has been confirmed in experiments where the size of the protected DNA has been examined after digestion of the proteins with proteinase K (not shown). On average, the size of the fragments varies between 20 and 50 bp, which is similar to the values found for *E. coli* and wheat germ enzymes [12,19].

The results of the experiment of fig.1 and 2 are consistent and suggest that the two large polypeptides are involved in DNA-binding during RNA synthesis. However, the formation of a ternary complex may still allow non-specific binding of the proteins to DNA (see below). I have thus carried out binding studies on the elongation complex, assuming that such a complex reflects more accurately the situation in vivo. Ad2 DNA was incubated with RNA polymerase II and the 4 nucleoside triphosphates, and RNA synthesis was allowed to proceed. After 5 min the formation of initiation complexes was blocked by addition of ammonium sulphate to 0.25 M [10] and the incubation was continued for another 5 min. The complexes were then cross-linked, nuclease-digested, and examined as described above.

The tracing of the radioactivity bands obtained after gel electrophoresis of these cross-linkage-products is compared with that of the ternary initiation complex products (fig.3). The comparison shows that in the absence of initiation complexes the majority of the radioactive DNA is associated with the faster migrating of the two subunits, emphasizing a difference which is also suggested in the RNA polymerase I ternary complex (fig.2a, lane IB).

The experiment of fig.4 was carried out as in fig.3, except that ad2 DNA was cold and one of the nucleotides was labelled. Nuclease digestion of the cross-linked RNA polymerase II complex followed

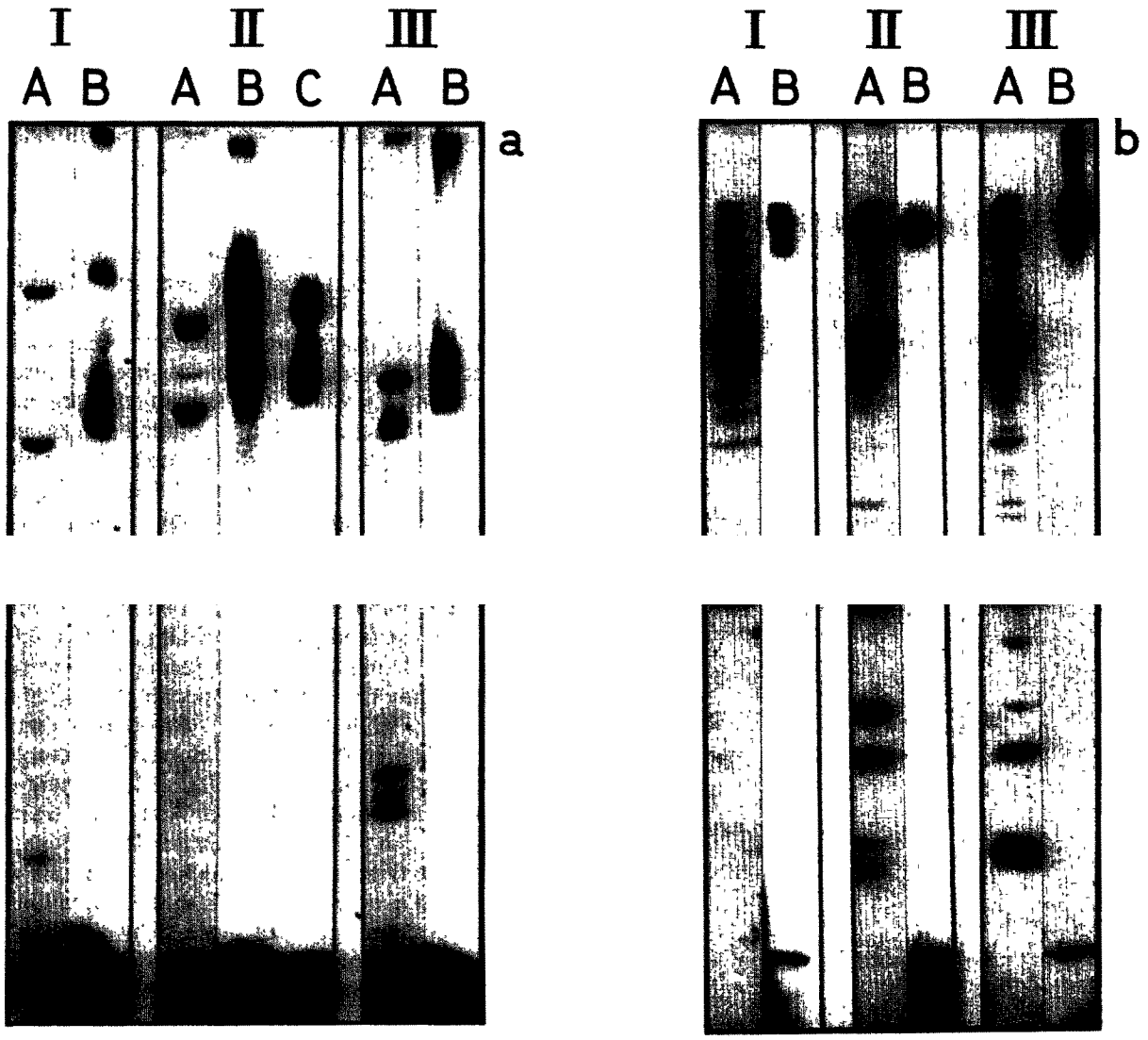

Fig.2. RNA polymerase subunits UV cross-linked to ad2 DNA: 20 μg of RNA polymerase I (I) and 50 μg each of RNA polymerase II (II) and III (III) were incubated for 10 min with 0.5 μg ^{3}H-labelled ad2 DNA (spec. act. 1.6×10^7 cpm/μg) in the presence of ATP, GTP and CTP. The complexes were cross-linked by UV irradiation. Unbound DNA was removed either with DNase I (B lanes) or by a combined nuclease digestion [1,12] (lane IIC). One fifth volume of each sample was analyzed on 7.5% (a) or 15% SDS–polyacrylamide gels (b). Oligonucleotides cross-linked to RNA polymerase subunits were detected by fluorography [15]. Reference gels were stained either with Coomassie brilliant blue (a, A lanes) or by silver staining [18] (b, A lanes).

by gel electrophoresis shows that the nascent RNA chain is protected by the enzyme subunits, but the situation is reversed as compared with DNA. Radioactive RNA is bound mainly to the slower migrating of the two large subunits. The experiments shown here suggest that both large protein subunits of *Drosophila* RNA polymerases may be involved in the binding to nucleic acids. The

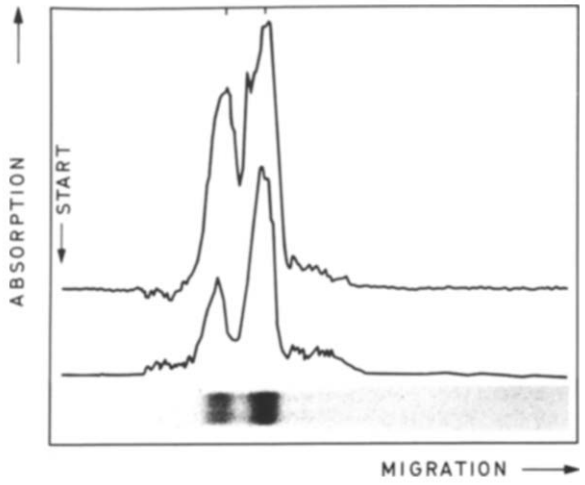

Fig.3. UV cross-linking of RNA polymerase II elongation complexes. Elongation complexes between 20 μg of RNA polymerase II and 0.5 μg ^{3}H-labelled ad2 DNA (spec. act. 1.6×10^{7} cpm/μg) were formed (see section 2). A fluorograph obtained after UV irradiation, combined nuclease digestion, and separation in a 7.5% SDS–polyacrylamide gel was scanned with a Joyce Loebl microdensitometer (lower tracing). For comparison ternary initiation complexes (upper tracing), obtained under similar conditions but in the absence of UTP, are shown (compare fig.2a, lane IIC).

larger of the two proteins seems to bind to the nascent RNA chain while the smaller binds better to the DNA template.

So far the best evidence for RNA polymerase–DNA interaction comes from experiments carried out in *E. coli*, where it has been clearly shown that non-specific binding can occur with the large protein subunits β and β' and with the o-factor [1,2]. Promoter-specific interaction is however observed only with the β-subunit and with o [2,3]. The binding pattern observed with eukaryotic RNA polymerases is consistent with the findings for prokaryotes. Of the two large subunits, the faster migrating one, comparable to the β-subunit of *E. coli*, binds preferentially to the template in the functional complex. The predominant binding to RNA observed in the other subunit suggests a functional diversification of the two polypeptides implying a role of the largest protein as carrier of the synthesized product.

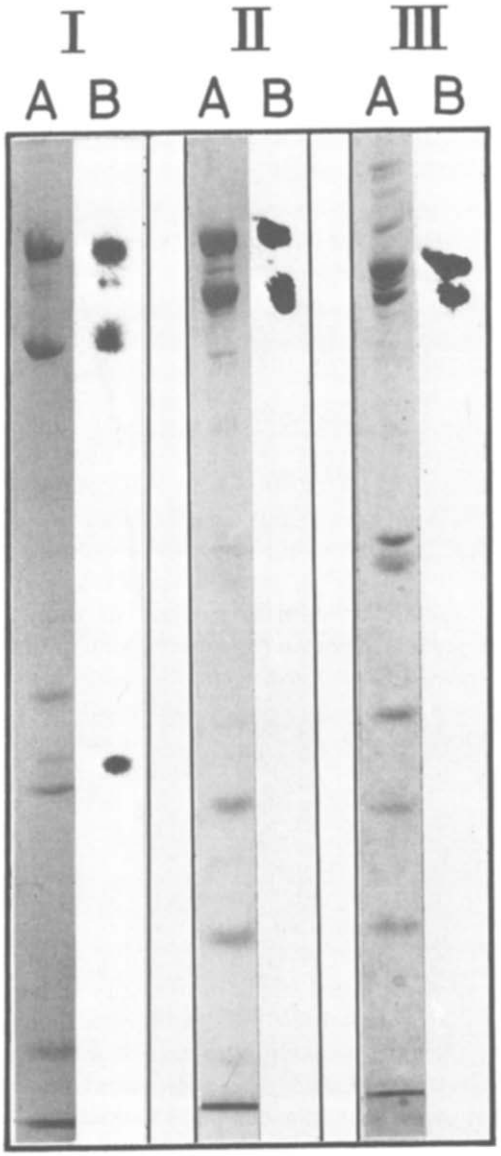

Fig.4. UV cross-linking of RNA polymerase II to the newly-synthesized RNA. RNA synthesis was carried out with 12 μg RNA polymerase II on 1.4 μg cold, mechanically nicked ad2 DNA in the presence of [α-^{32}P]UTP (spec. act. 20 Ci/mmol). UV irradiation and analysis of cross-linkage-products were carried out as described in section 2. Lane A: 7.5% SDS–polyacrylamide gel of RNA polymerase II subunits stained with Coomassie brilliant blue; lane B: autoradiograph of RNA-enzyme photoproducts analyzed in the same gel as in A; lane C: analysis of the photoproducts on a 15% gel (the position of the size markers are given by arrows). The 70 kDa-labelled band represents alkaline phosphatase.

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