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Quantification of DNA-dependent RNA polymerase subunits and initiation factor(s) by antibody-linked polymerase assays

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The antibody-linked polymerase assay is a method which allows one to assign RNA polymerase activity to SDS-denatured polypeptides on nitrocellulose membranes using antibodies which were raised against only partially purified polymerase preparations. Here we show that with this method not only enzyme subunits but also initiation factor(s) can be determined in crude homogenates. Moreover the determination is quantitative. Therefore changes in the amount of individual polymerase subunits and factor(s) can be visualized within different crude homogenates.

RNA polymerase; Immunoblotting; *(E. coli)*

1. INTRODUCTION

E. coli RNA polymerase core enzyme is composed of 2 large ($\beta\beta'$) and 2 smaller identical (α) subunits. The holoenzyme is formed by association of the σ-factor to the core enzyme. The σ-factor is released again from the core enzyme after addition of the eighth or ninth nucleotide to the nascent RNA chain. In contrast to the rather simple subunit structure of the well-characterized *E. coli* enzyme (review [1]), isolated plant nuclear and chloroplast RNA polymerases contain 7–14 different polypeptides, and attempts of reversible dissociation of subunits have not yet been successful. Therefore it is still questionable to define all polypeptides associated with the enzyme(s) after purification as true subunits or factors. In a first attempt to overcome this unsatisfactory situation we adapted the antibody-linked polymerase assay of Van der Meer et al. [2] to an oligomeric enzyme like DNA-dependent RNA polymerase from

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Abbreviations: ALPA, antibody-linked polymerase assay; NC, nitrocellulose

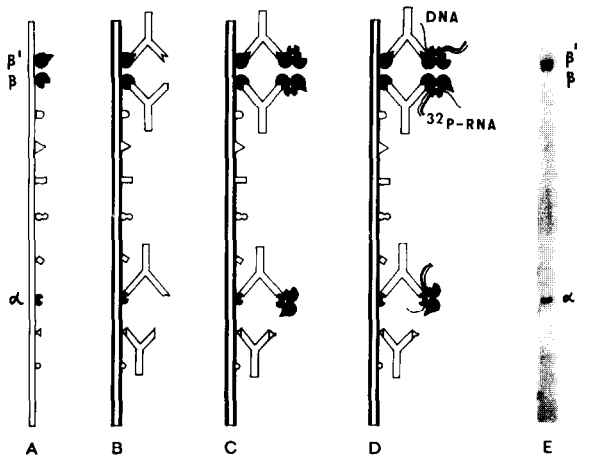

Fig.1. The principle of the ALPA. Proteins of crude homogenates are separated by SDS-polyacrylamide gel electrophoresis and transferred onto NC paper (A). After saturation of the free binding sites of the NC filter with bovine serum albumin antibodies raised against polymerase, also other polypeptides are linked to the NC-bound polypeptides via one Fab-site (B, first binding reaction). Subsequently native polymerase molecules are bound via their corresponding subunits to the other Fab-sites of the antibody molecules. Equally non-polymerase polypeptides present in the partially purified enzyme preparation are bound (C, second binding reaction). Transcription is performed with the NC-bound native polymerase molecules using a radioactively labelled precursor and the transcription products are precipitated on the NC filter (D). Polymerase subunits are revealed by autoradiography (E).

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spinach chloroplasts [3]. This method enables us to determine RNA polymerase polypeptides in crude homogenates after SDS-polyacrylamide gel electrophoresis using antibodies which are raised against only partially purified polymerase preparations. The principle of the ALPA is demonstrated in fig.1. From our previous experiments we could exclude artefactual binding of polypeptides during the isolation procedure of the chloroplast enzyme as source of ALPA reactivity, but it remained unclear whether transcription factors which are associated with the core enzyme only during a limited period of the transcription cycle are revealed by this assay. Here by using the well-characterized *E. coli* polymerase as a model system we show that the ALPA can also be used to determine initiation factors like the σ-factor and moreover to quantify polymerase subunits and factors within crude homogenates.

2. MATERIALS AND METHODS

The *E. coli* polymerase holoenzyme was purchased from Boehringer (Mannheim); the core enzyme was a gift from Dr M.A. Gratchev (Novosibirsk). *E. coli* polymerase antibodies were kindly supplied by Professor Dubert (Paris). Protein blotting and transcription reactions were performed as described in [3].

3. RESULTS AND DISCUSSION

The time course of the transcription reactions on NC strips is represented in fig.2A. For both core and holoenzyme it is linear up to 15 min. Therefore in the following experiments transcription was performed for 15 min. Addition of the σ-factor to the *E. coli* core enzyme leads to about 3-fold stimulation of the transcription of native and denatured calf thymus DNA [4]. The same holds true when the tests are performed on NC strips (cf. core and holoenzyme in fig.2A and B). The amount of transcription products correlates with the amount of polymerase polypeptides which in these first orientating experiments for easiness were spotted onto the NC filters (fig.2B). Separated subunits and the σ-factor were examined after SDS-polyacrylamide gel electrophoresis of the *E. coli* polymerase holoenzyme and blotting of the polypeptide (fig.2C and D). The capacity of the NC membrane to bind SDS denatured protein was determined to be 27 μg/cm^2 [5]. As revealed by the

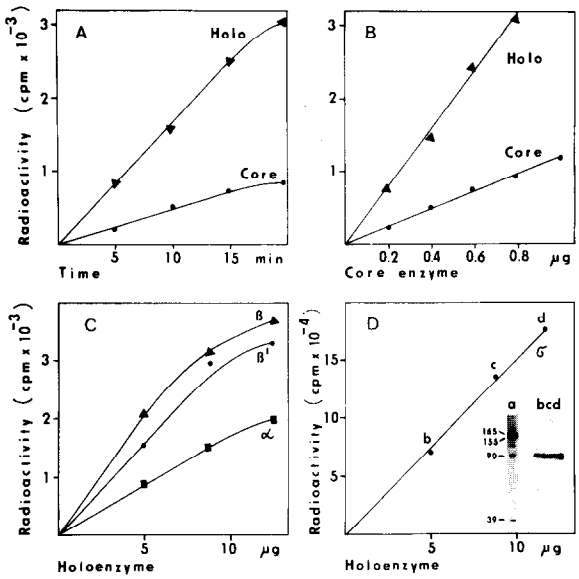

Fig.2. Quantification of polymerase, its subunits and the σ-factor. 0.6 μg (A) or 0.2; 0.4; 0.6; 0.8 and 1 μg of polymerase core enzyme (B) were mixed with 5 μg bovine serum albumin and spotted onto the NC filter. In the second binding reaction the *E. coli* core or holoenzyme was linked to the free Fab-sites. Subunits and σ-factor were analysed after separation of 5, 8.75 and 12.5 μg of polymerase holoenzyme by SDS-polyacrylamide gel electrophoresis and transfer of the proteins to NC sheets. Antibodies raised against the core enzyme of *E. coli* (A–C) or the σ-factor (D) were linked to the proteins on the NC filters. The *E. coli* core (C) or holoenzyme (D) was bound in the second binding reaction. Transcription was performed using calf thymus DNA as template and [U-^{14}C]uridine-5′-triphosphate (A–C) or [α-^{32}P]adenosine-5′-triphosphate as labelled precursor. After autoradiography spots (A,B) and bands (C,D) were cut out from the NC filters and radioactivity was counted. The small inset in D shows the stain of the *E. coli* holoenzyme used (a) and the autoradiograph of the ALPA experiment before the bands were cut out for counting (b–d).

corresponding autoradiographs the polymerase subunits occupy 6 mm^2 on the NC sheets, e.g. with 1.62 μg of each subunit the membrane is 'saturated'. With a composition of $\alpha_2\beta\beta'\sigma$ this critical amount should be reached if more than 8.1 μg of holoenzyme is separated by SDS-polyacrylamide gel electrophoresis. The validity of this calculation is reflected by the curves of the α-, β- and β'-subunits (fig.2C). The analysis of the σ-factor differs from that of the subunits as follows: the transcription assay has to be performed under conditions in which the antibody-fixed σ-factor does not separate from the transcribing core en-

zyme, i.e. the nascent RNA should not extend the length of nine nucleotides (see above). Therefore we used [α-^{32}P]ATP (3000 Ci/mmol, 2.7 pmol) as labelled precursor to limit substrate supply and to arrest transcription before the σ-factor is released from the enzyme. In all other experiments we used [^{14}C]UTP (340 Ci/mmol, 1.27 μmol).

The [^{32}P]ATP incorporation into nascent RNA increases linearly with increasing amounts of σ-factor on the NC filter up to 12 μg of separated holoenzyme (fig.2D). This means that the σ-factor of the separated holoenzyme does not saturate the NC filter as do the $\beta\beta'$- and α-subunits (see fig.2C) probably because commonly the σ-factor is not present in stoichiometric amounts in the enzyme preparations.

In summary our results show that in ALPA assays the amounts of transcription products correlate with the amounts of polymerase polypeptides (subunits and initiation factors) on NC membranes. In the case of a well-characterized enzyme like *E. coli* polymerase, where antibodies against the pure enzyme or even isolated subunits and factors can be raised, quantification would be performed by binding of antibodies and ^{125}I-protein A. However, if we think of the more complex organization of plant nuclear and chloroplast enzymes, the ALPA could be the method of choice to investigate problems of transcriptional regulation which are based on changes of polymerase composition or changes in polymerase synthesis itself. With antibodies raised against partially purified enzyme preparations only one is able to determine changes in the amounts of polymerase polypeptides in crude extracts prepared from different tissues or from material grown under different physiological conditions.

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