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A novel method for the purification of the *Xenopus* transcription factor IIIA

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ABSTRACT

We have developed a quick and simple purification method that yields large quantities of the *Xenopus* zinc finger protein transcription factor IIIA (TFIIIA). The protein is purified in the form of the 7S storage particle (TFIIIA/5S RNA complex) found in the ovaries of immature *Xenopus* . Our method yields 0.5 to 1 mg of pure 7S particle per ovary. It involves a high speed centrifugation step, fractionation on a gel filtration column and a precipitation step using calcium chloride. TFIIIA purified using this protocol retains full DNA binding activity and has the expected zinc ion content.

INTRODUCTION

The protein transcription factor IIIA (TFIIIA) is required for the correct initiation of transcription of *Xenopus* 5S RNA genes by RNA polymerase III (1). Proteolytic studies showed that the 40 kD TFIIIA protein consists of a 30 kD DNA binding domain that binds to about 50 base pairs of DNA within the coding region of the 5S RNA gene and a 10 kD domain that is necessary for transcriptional activation (2). Further proteolytic studies (3,4) revealed that the 30 kD domain may consist of nine 3 kD sub-domains. This observation, together with the discovery of a 30 amino acid sequence unit repeated nine times in tandem in the sequence of TFIIIA (5,6) and a measured zinc concentration of 7−11 atoms, led Miller *et al.* (3) to propose that each 30 amino acid repeat is folded around a central zinc atom into an independent 3 kD domain. The proposed tetrahedral zinc coordination to the invariant pair of histidines and cysteines was later substantiated by an extended x-ray absorption fine structure study (EXAFS)(7). This 30 amino acid sequence motif was called the 'zinc finger'. In the last few years it has emerged that zinc finger motifs found within the sequence of many regulatory proteins serve as specific nucleic acid binding domains [reviewed in reference (8)].

TFIIIA, in addition to binding to DNA, has the unusual property of also forming a complex with its own gene product, 5S RNA (9,10). The ability of TFIIIA to bind both DNA and RNA presents an interesting structural problem which can be studied because TFIIIA is present in large amounts (15% of the total protein) in the oocytes of immature *Xenopus laevis*. However, published purification methods (9,11), developed before it was known that zinc is an essential component of TFIIIA, employ buffers containing chelating agents which lead to the dissociation of the 7S particle (3), inhibition of DNA binding activity and inactivation of transcription (12).

Here we report a novel purification method which yields 10 mg quantities of TFIIIA in the form of the 7S particle. TFIIIA purified by our method retains its full complement of zinc that is required for specific and efficient nucleic acid binding activity. The 7S particle

preparation involves three purification steps: (a) a high speed centrifugation to remove cell debris and some high molecular weight components of oocytes; (b) fractionation on a gel filtration column which gives 50% pure 7S particle; and (c) a precipitation step using calcium chloride which produces pure 7S particle.

MATERIALS AND METHODS

Buffers

The same buffer was used throughout the purification. It contained: 20 mM MES (pH 6.0), 20 mM KCl, 15 mM NaN_3, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.5 mM phenylmethylsulphonyl flouride (PMSF) and 50 μM zinc acetate [$Zn(OAc)_2$]. Zinc acetate was added last from a fresh 10 mM solution because precipitates of mixed zinc aqua, hydroxo and oxo complexes form with time. PMSF was made up as a 50 mM stock solution in isopropanol. 1.0 M stock solutions of DTT and benzamidine were stored as aliquots at −20°C. All manipulations were carried out at 4°C except for gel electrophoresis.

Xenopus laevis

Xenopus laevis of 5−6 cm body length were obtained either from Nasco, Fort Atkinson, Wisconsin, USA. or from the African Reptile Park, Roggebaai, South Africa.

Preparation of high speed homogenate

The ovaries from approximately 50 immature *Xenopus laevis* were dissected and collected in the above buffer on ice. Only ovaries of pink or yellow appearance were used. The ovaries were washed twice with fresh buffer and then homogenised in a Dounce glass homogeniser with 5 up and down strokes. The homogenised ovaries were centrifuged at 40,000 rpm. for 40 minutes in a Beckman SW 60 rotor at 4°C. The yellow yolk found at the top of the tube was discarded and the pink supernatant that contains the 7S particle collected.

Fractionation on a Sephacryl S-400 column

A Sephacryl S-400 (Pharmacia) column (2.5×100 cm), equilibrated and run in the above buffer, gives a good fractionation of up to 3000 A_{260} of supernatant from the high speed centrifugation step. The absorbance of the supernatant was measured by dilution into 0.1 M NaOH. The column was run at approximately 30 ml per hour and 5 ml fractions were collected. Fractions were assayed for the presence of 7S particle using particle gels (see below).

Calcium chloride precipitation

Fractions from the Sephacryl S-400 column containing the 7S particle were pooled and concentrated to about 40 A_{260}/ml in a Sartorius vacuum dialysis apparatus with buffer surrounding the collodion bag. 0.5 to 1 ml aliquots of the pooled and concentrated fractions were transferred to siliconised Eppendorf tubes (1.5 ml) and spun for 10 minutes in a microfuge to remove insoluble protein. The supernatant was then transferred to fresh siliconised Eppendorf tubes and $CaCl_2$ was added to 40 mM from a 1.0 M stock solution. Aliquots were left on ice for between 30 and 60 minutes after which time more than 80% of the 7S particle had formed a white, cloudy precipitate. The precipitate was collected by centrifugation for 10 minutes in a microfuge. All supernatant was carefully removed and discarded. In order to remove trapped soluble protein, the 7S particle precipitate was washed with buffer containing 40 mM $CaCl_2$. The precipitate was agitated with a sealed

capillary tube, respun and all of the supernatant removed. The 7S particle precipitate was resolubilised by addition of $^3/_4$ (or more) the original volume of buffer (without $CaCl_2$). To speed up the resuspension the pellet was broken up with a capillary tube and left overnight at 4°C. The cloudy solution was then spun for 10 minutes in a microfuge to remove insoluble proteins. The supernatant containing pure 7S particle was collected and analysed on a protein gel (see below). If inadequate purification was obtained the $CaCl_2$ precipitation step was repeated. The supernatant was then dialysed against buffer and, if required, concentrated in a Sartorius vacuum dialysis apparatus before use. 7S particle concentrations were estimated using 10 A_{260} (measured in 0.1 M NaOH) equivalent to 1 mg.

Particle gel

We have adopted agarose gels, commonly used for the fractionation of DNA restriction fragments, to assay for the presence of 7S particle and the formation of the specific TFIIIA/5S RNA gene complex (4).

Samples were made 4% glycerol, 4 mM Tris/HCl (pH 7.4) 0.02% bromophenol blue and analysed in a 0.7% agarose gel (BRL high gelling temperature). The electrophoresis buffer was 45 mM Tris/borate pH 8.5. *Note* the omission of EDTA from electrophoresis buffer. EDTA would chelate zinc and hence destabilize TFIIIA/nucleic acid complexes. Depending on the purification stage, 0.05 to 0.5 A_{260} of 7S particle were analysed. Electrophoresis was carried out in an 8×10 cm gel at room temperature at 30 mA, for about 45 minutes or until the bromophenol blue had migrated to about $^3/_4$ of the length of the gel. To visualise protein/nucleic acid complexes and nucleic acid, gels were stained with ethidium bromide at 1 μg/ml for about 30 minutes.

Protein gel

Protein samples were made 25mM Tris/HCl (pH 6.8), 0.5% SDS, 5% glycerol, 0.05% bromophenol blue, 0.5% 2-mercaptoethanol and analysed in 12% SDS-polyacrylamide gels. The gels were stained with PAGE blue 83 (BDH Chemicals).

RNA gel

To analyse the 5S RNA composition of the 7S particle samples were made 50% deionised formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol and analysed in 10% polyacrylamide gels. The gel and electrophoresis buffer contained 7 M urea, 90 mM Tris/borate (pH 8.3) and 2.5 mM EDTA . 0.02 to 0.2 A_{260} of RNA were analysed. Gels were first fixed in 50% methanol and then stained with ethidium bromide at 1 μg/ml.

DNA binding activity

The DNA binding activity of TFIIIA was measured by adding increasing amounts of TFIIIA to a fixed amount of binding site DNA. Binding site DNA was either a restriction fragment containing the *Xenopus borealis* somatic type 5S RNA gene, or as used in the experiment shown in Figure 4, a 54 bp fragment comprising the TFIIIA binding site (2,14). Binding site DNA was used at a concentration of 10^{-9} to 10^{-8} M for radiolabelled DNA or 2.5×10^{-6} M for visualisation by ethidium bromide staining. Binding of TFIIIA to its binding site was achieved by RNase treatment of the 7S particle which results in the transfer of the protein from RNA to DNA (13). Binding site DNA is diluted in binding buffer containing 20 mM Tris/HCl (pH 7.4), 2 mM $MgCl_2$, 40 mM KCl, 50 μM $Zn(OAc)_2$, 0.5 mM DTT, 0.1% Nonidet P-40. The 7S particle at 1 mg/ml was treated with RNase A at 10 μg/ml for 15 minutes at room temperature and appropriate amounts were added to the mixture containing DNA. Analysis of protein/DNA complex formation was by band shift assay on particle gels as used for analysis of the 7S particle.

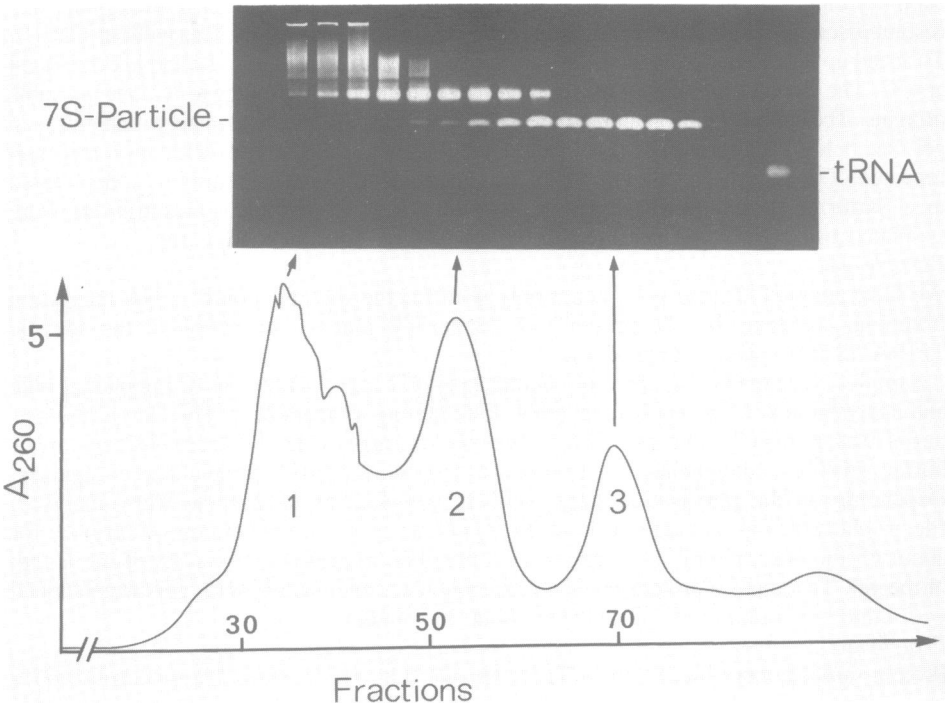

Figure 1 A typical gel filtration profile for the fractionation of the high speed supernatent of homogenised ovaries on a Sephacryl S-400 column and analysis of column fractions on a particle gel. 1500 A_{260} of high speed supernatant in 6 ml volume was applied to a 2.5×100 cm column run at 30 ml per hour. 5 ml fractions were collected. 20 μl volumes every three fractions at the start of the profile and every two fractions in the region of the 7S particle peak were assayed in a 0.7% agarose particle gel. The gel was stained with ethidium bromide.

RESULTS

7S particle purification

The aim of this purification protocol is to purify TFIIIA complexed with 5S RNA in the form of the naturally occuring 7S particle. The first step in the purification, the high speed centrifugation of the homogenised ovaries, results in the removal of cell debris and partial removal of high molecular weight components such as ribosomal RNAs and the other main component of oocytes, the 42S particle (figure 2, track 1). Yields of supernatant from the high speed centrifugation step vary between 40−100 A_{260} per ovary. The second step in the purification is by gel filtration fractionation. Figure 1 shows a typical elution profile of the fractionation of the high speed supernatant on a Sephacryl S-400 gel filtration column together with the analysis of column fractions on a particle gel. Peak 1 contains high molecular nucleic acid and nucleic acid/protein complexes. Peak 2 contains residual 42S particle which breaks down to an approximately 15S component. Peak 3 contains the 7S particle which, as judged from its protein content, is at this stage about 50% pure (Figure 2, track 2). The final step in the purification of the 7S particle was achieved by precipitation with calcium chloride. The protein moiety of the 7S particle prepared by this method was

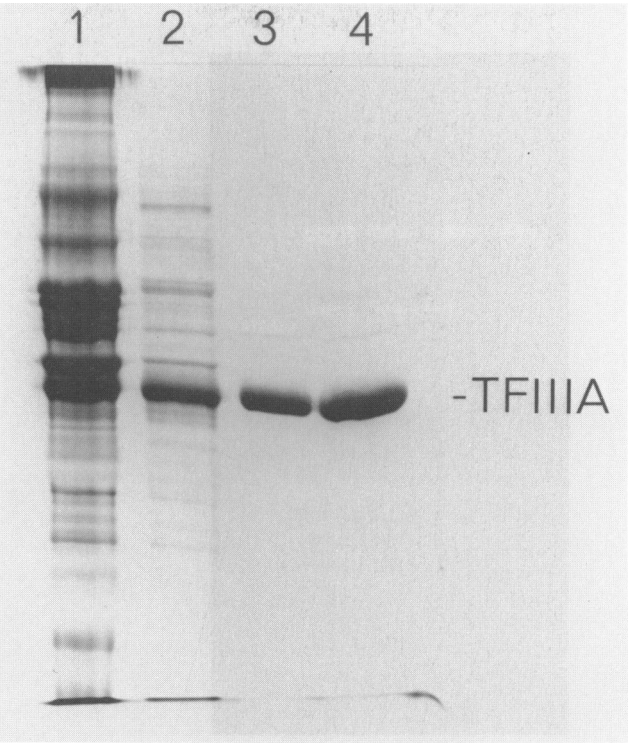

Figure 2 Analysis of protein content at each purification step of the 7S particle. Lane 1 shows 0.5 A_{260} of high speed supernatant. Lane 2 shows 0.4 A_{260} of pooled fractions from peak 3 from the Sephacryl S-400 column (Figure 1). Lanes 3 and 4 show 0.1 and 0.3 A_{260} respectively of pure 7S particle. Analysis was carried out in a 12% SDS-polyacrylamide gel. The gel was stained with PAGE blue 83.

estimated to be greater than 95% pure when assayed on a 12% SDS-polyacrylamide gel stained with PAGE blue 83 (Figure 2, tracks 3 and 4).

The 5S RNA within the 7S particle was estimated to be about 90% intact from analysis in a 10% denaturing polyacrylamide gel stained with ethidium bromide (Figure 3). 10% of 5S RNA molecules are clipped at one specific position resulting in two fragments of about 40 and 80 nucleotides in length. In other preparations the extent of nicking varied from about 5 to 30% and appeared to be dependent on the age of the *Xenopus* used. The inclusion in buffers of ribonuclease inhibitors, such as RNasin, did not prevent this specific RNA cleavage or affect the extent.

Typical yields from 1000 A_{260} of supernatant from the high speed centrifugation step are 10 mg of pure 7S particle. This is equivalent to 0.5 to 1 mg 7S particle per ovary.

DNA binding activity

Figure 4 shows a titration of a 54 bp DNA fragment corresponding to the TFIIIA binding site with increasing amounts of TFIIIA. A 1.3 to 1.6 molar excess of RNase treated 7S particle is sufficient to form a 1:1 complex between the DNA binding site and TFIIIA. In the titration shown, the DNA concentration was 10^{-6} M. In the experiments in which

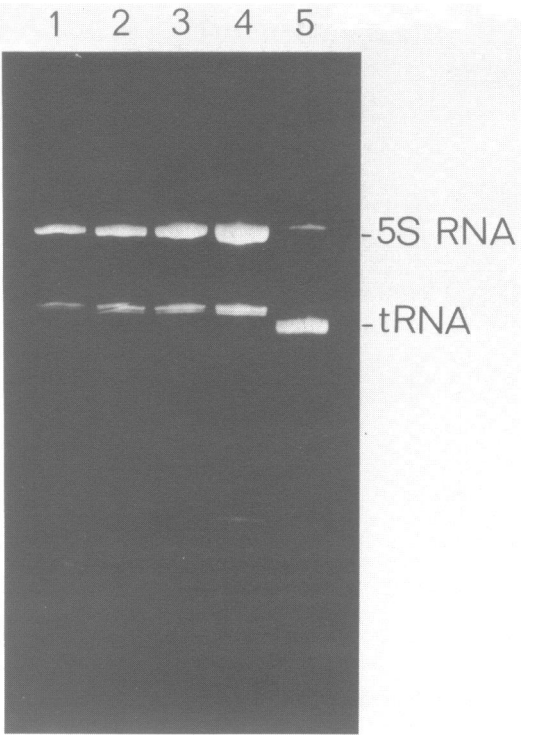

Figure 3 Analysis of the 5S RNA in the pure 7S particle. Lanes 1, 2, 3 and 4 contain 0.02, 0.04, 0.08 and 0.16 A_{260} respectively of extracted 5S RNA. Lane 5 contains 0.05 A_{260} of t-RNA included as size marker. In this 7S particle preparation approximately 90% of the 5S RNA molecules are intact. The analysis was carried out in a 10% denaturing polyacrylamide gel stained with ethidium bromide.

the DNA concentration was dropped to 10^{-9} or 10^{-8} M of 5S RNA gene, an equimolar to 2 molar excess of TFIIIA was required to obtain a 100% complex as judged by band shift and footprinting assay (not shown, but see references 13 and 14).

DISCUSSION

Because zinc is essential for maintaining the tertiary structure of the zinc finger domains of TFIIIA, is required for DNA binding (3) and is also required for transcription of the 5S RNA genes (12), we have developed a new purification method that, in contrast to previous protocols, yields protein which has the expected zinc content (7) and full DNA binding activity. In addition the method is suited for the large scale production of TFIIIA.

We have chosen to purify TFIIIA complexed to 5S RNA in the form of the naturally occurring 7S storage particle for two reasons. First, TFIIIA that remains bound to 5S RNA thoughout the various purification steps is likely to retain the native structure required for nucleic acid binding activity, particularly given that the structure of TFIIIA is extended rather than globular (3,15), and is therefore likely to be more stable bound to nucleic acid than free. Second, many DNA binding proteins and TFIIIA in particular, are highly positively charged which makes them exceedingly difficult to purify because they adhere

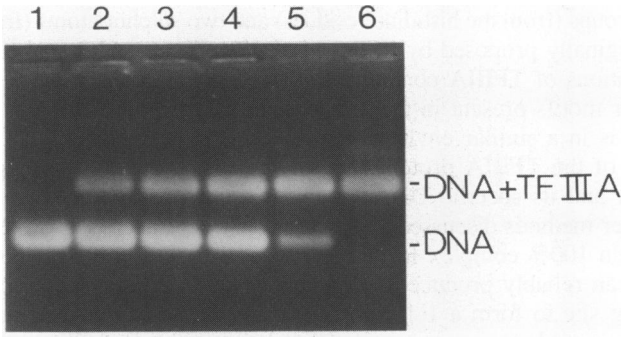

Figure 4 Binding of TFIIIA to its 54 bp DNA binding site. The DNA concentration was 2.5 μM and 20 μl of each binding reaction was applied to each lane. Molar ratios of RNase treated 7S particle: DNA used in the binding reactions were: lane 1, 0:1; lane 2, 0.3:1; lane 3, 0.6:1; lane 4, 1:1; lane 5, 1.3:1; lane 6, 1.6:1. The band shift assay shows that between 1.3 and 1.6 molecules of TFIIIA are required to bind 1 molecule of binding site DNA. Analysis was carried out in a 0.7% agarose particle gel. The gel was stained with ethidium bromide. Staining of the protein/DNA complex is less intense than that of a corresponding amount of DNA, because of interference from the protein.

to glassware and column resins. Our experience suggests that it may be a better approach to purify nucleic acid binding proteins bound to their respective nucleic acids.

Previously published purification methods made use of buffers containing chelating agents such as DTT and EDTA which cause the dissociation of the complex by removing zinc from TFIIIA (11). In addition Engelke *et al.* (11), in their purification method, used ion exchange columns which expose the 7S particle to high salt buffers that can result in the dissociation of the RNA/TFIIIA complex. Another method involved sucrose gradients and preparative electrophoresis, but this method is only suited for the preparation small quantities of 7S particle (9). In contrast to these methods, our purification method is very gentle as it does not contain any ion exchange or high salt steps which may cause dissociation of the 7S particle and in addition strong chelating agents such as EDTA have been omitted from buffers.

The use of the calcium chloride precipitation as a step in the purification is an idea that originates from our work on the crystallisation of the nucleosome core particle in which divalent ions, such as magnesium and manganese, were used as the precipitating agent (16). We suggest that a precipitation step using divalent ions, as used in the 7S particle preparation, might be generally applicable to the purification of other protein/nucleic acid complexes.

The development of the purification method reported here has permitted us to purify the large amounts of TFIIIA required for accurate measurements of zinc content and definition of the ligands to the zinc atom. For these measurements, in order to retain the endogenous zinc, it is not only necessary to use non-chelating buffers but to remove exogenous zinc from glassware, column resins and buffers. Using this procedure Miller *et al.* (3) were able, depending on the preparation, to find 7 to 11 zinc atoms per 7S particle. In contrast, Hanas *et al.* (12) using earlier more disruptive purification methods found only 2 zinc atoms per 7S particle. Subsequently, we carried out an EXAFS study that provided the first direct information for the coordination of the zinc atom in zinc finger domains (7). From this study it was deduced that the zinc is tetrahedrally coordinated to

two imidazole groups (from the histidine residues) and two sulphur atoms (from the cysteine residues), as originally proposed by Miller *et al.* (3). The EXAFS analysis also showed that our preparations of TFIIIA contain 8 to 9 zinc atoms, that is one zinc per each of the 9 zinc finger motifs present in the amino acid sequence of TFIIIA, and further that each zinc atom is in a similar environment.

The integrity of the TFIIIA protein also affects the efficiency of complex formation between TFIIIA and its specific DNA binding site on the 5S RNA gene. For TFIIIA prepared by other methods discussed above, a 10–50 fold excess of protein was usually required to obtain 100% complex formation (10,11,12). Using the method presented in this paper, we can reliably produce TFIIIA that binds in a sequence specific manner to its DNA binding site to form a 1:1 complex, using a one to two fold molar excess of protein. This has enabled us to produce the large quantities of TFIIIA/DNA complex required for biochemical and structural studies.

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REFERENCES

1. Brown, D.D. (1982) The Harvey Lectures, Series 76 (Academic Press) 27–44
2. Smith, D.R., Jackson, I.J. and Brown, D.D. (1984) Cell, **37**, 645–652
3. Miller, J., McLachlan, A.D. and Klug, A. (1985) EMBO J., **4**,1609–1614
4. Miller, J. (1987) PhD. Thesis, University of Cambridge, England.
5. Ginsberg, A.M., King, B.O. and Roeder, R.G. (1984) Cell **39**, 479–489
6. Tso, J.Y., Van den Berg, D.J. and Korn, L.J. (1986) Nucleic Acids Res. **14**, 2187–2200
7. Diakun, G.P., Fairall, L. and Klug, A. (1986) Nature, **324**, 689–691
8. Klug, A. and Rhodes, D. (1987) TIBS., **12**, 464–469
9. Picard, B. and Wegnez, M. (1979) Proc. Natl. Acad. Sci. USA., **76**, 241–245
10. Pelham, H.R.B. and Brown, D.D. (1980) Proc. Natl. Acad. Sci. USA., **77**, 4170–4174
11. Engelke, D.R., Ng, S-Y., Shastry, B.S. and Roeder, R.G. (1980) Cell, **19**, 717–728
12. Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y.-H. and Wu, C-W.(1983) J. Biol. Chem. **258**, 14120–14125
13. Rhodes, D. (1985) EMBO J., **4**, 3473–3482
14. Fairall, L., Rhodes, D. and Klug, A. (1986) J. Mol. Biol., **192**, 577–591
15. Bieker, J.J. and Roeder, R.G. (1984) J. Biol. Chem. **259**, 6158–6164
16. Rhodes, D., Brown, R.S. and Klug, A. (1989) Meth. Enzym., **170**, 420–428

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