The EMBO Journal vol.6 no.7 pp.1921-1927, 1987

The properties of a new polymerase III transcription factor reveal that transcription complexes can assemble by more than one pathway

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Communicated by F.W.Stahl

We have resolved a previously unidentified factor (TFIIID) that is required for *in vitro* transcription of polymerase III templates. Our ability to resolve factor D from each of the other components of the transcription machinery (polymerase and transcription factors IIIB and IIIC) allowed us to test the capacity of these separated components to form stable complexes with tRNA genes. We find that none of the individual components binds detectably to tRNA genes, but that certain combinations of transcription factors do bind. Our results show that TFIIID is essential for binding and that formation of a full transcription complex can proceed by either of two different pathways.

Key words: protein-protein interactions/RNA polymerase III/transcription complex assembly

Introduction

Recently it has become clear that specific transcription in eukaryotes depends upon a group of components known as transcription factors. Such factors are distinct from the three eukaryotic nuclear RNA polymerases themelves, but are absolutely required for correct initiation by each of these enzymes (reviewed in Lassar *et al.*, 1983; Dynan and Tjian, 1985). In particular, three factors have been described that direct specific transcription by eukaryotic RNA polymerase III. Two of these (TFIIIB and TFIIIC) are required for all templates that have been tested whereas a third factor (TFIIIA) is required, in addition to factors B and C, for a subset of polymerase III templates - namely, 5S RNA genes (Lassar *et al.*, 1983). These three factors have been shown to act by forming very stable complexes with template DNA. Such complexes persist through multiple rounds of transcription and are able to withstand challenge by excess template (Bogenhagen *et al.*, 1982).

It has been observed that among factors required for transcription of a particular template, one factor is capable of binding to the template by itself, whereas the others bind detectably only as a consequence of this primary interaction. For example, in the case of templates that require only factors B and C (genes encoding tRNAs and the adenovirus RNAs VAI and VAII), factor C binds to the templates in the absence of factor B or RNA polymerase (Lassar *et al.*, 1983; Baker and Hall *et al.*, 1984; Ruet *et al.*, 1984; Carey *et al.*, 1986). Binding by factor B is detectable only when factor C is also present. In the case of 5S RNA genes, which require an additional factor for transcriptional activity, that factor (TFIIIA) can bind to 5S RNA genes without the other components of the transcription apparatus (Lassar *et al.*, 1983; Bieker and Roeder, 1984; Setzer and Brown, 1985; Carey *et al.*, 1986).

These observations have led to the idea that there is a strict temporal order in which the components of the transcription apparatus must be assembled into active complexes on genes. In this view, assembly is triggered by the stable interaction of one factor with the gene, and addition of other components then occurs in a linear step-wise fashion. In the case of tRNA genes, the pathway for assembly is thought to comprise three steps: a complex between the gene and factor C is formed first; then factor B becomes stably associated with this complex. Finally, polymerase joins the complex. The linearity of this pathway has led to the suggestion that the level of the factor that binds first is critical in determining whether transcription complexes will actually form - and hence whether particular genes will be expressed (Lassar *et al.*, 1983; Brown, 1984).

Our analysis of the silkworm (*Bombyx mori*) polymerase III transcription apparatus suggests a different picture of the process by which transcription complexes are assembled. The results we report here indicate that there is not a single linear pathway for the assembly of transcription complexes on tRNA genes. Our conclusions are based on the properties of a previously unidentified polymerase III transcription factor. This factor (called TFIIID) is absolutely required, in addition to the two known factors (TFIIIB and TFIIIC), for tRNA gene transcription. Moreover, although factor D does not bind detectably to genes by itself, it plays a critical role in the assembly of other factors into transcription complexes. Only in combination with factor D do factors B and C bind to tRNA genes. The key finding is that combinations of factors B and D or of factors C and D bind equally well to tRNA genes, and that formation of complexes between tRNA genes and either of these combinations is an effective first step in assembly of the full transcription complex.

Results

Fractionation of the Bombyx polymerase III transcription apparatus

Using standard chromatography on DEAE Sephadex and on phosphocellulose, we separated the *Bombyx* transcription machinery that acts on tRNA genes into two fractions similar to those described previously (Segall *et al.*, 1980; Fuhrman *et al.*, 1984). These contain polymerase III as well as the transcription factors, TFIIIB and TFIIIC. The discovery of TFIIID upon further purification of these fractions was facilitated by two considerations. One was the use of chromatography on a Mono S cation exchange resin to separate transcription factor activities that are not easily resolved by the standard fractionation methods. Another was the use of a general assay that required only complementation of transcription, not DNA binding, to reveal activity. The generality of this assay made it easy to follow individual transcription components even when separation from other components had rendered them incapable of binding to

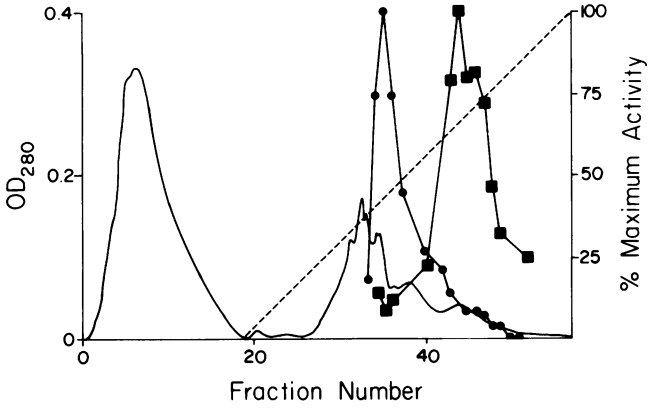

Fig. 1. Resolution of TFIIIC and TFIIID by Mono S chromatography. A typical elution profile from a Mono S cation exchange resin is illustrated. The unbroken line devoid of symbols indicates total protein concentration as estimated by absorbance at 280 nm, the dashed line (- - - - -) is the KCl concentration of the elution buffer, plotted as per cent of the maximum, and the symbols represent TFIIIC (small circles) and TFIIID (large squares) activity plotted as per cent of the maximum activity detected.

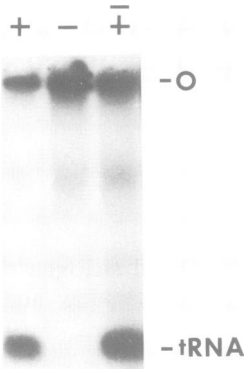

Fig. 2. Pre-incubation of subsets of the *Bombyx* polymerase III transcription apparatus does not impair transcriptional activity. The symbols (+,−) at the top of the figure indicate whether factor C was present (+) or absent (−) during both the pre-incubation and the transcription reactions, or whether factor C was present only during the transcriptional reaction (∓). Transcripts in the lane marked (+) came from reactions in which all four transcription components (RNA polymerase III plus factors B, C and D) were present during both pre-incubation and transcription. Transcripts in the two rightmost lanes came from reactions in which factor C was omitted from the mixture of transcription components during pre-incubation. Pre-incubation reactions were carried out under standard transcription conditions (Wilson *et al.*, 1985) for 40 min in the absence of template and radioactive nucleotides. After pre-incubation, transcription was initiated by the addition of template DNA (6.4 fmol of the Bmt11 $tRNA^{Ala}$ gene) and allowed to proceed for 80 min in the presence of [α-^{32}P]UTP. Factor C was either never added to the reactions (−), or was added only at the beginning of the transcription reaction (∓). The products of transcription were fractionated by polyacrylamide gel electrophoresis and detected by autoradiography. The position of the $tRNA^{Ala}$ primary transcript (tRNA) relative to the gel origin (O) is shown.

templates. An example of the resolution of factors C and D by Mono S chromatography is shown in Figure 1. We will refer to the transcriptional activities in these and other fractions as 'factors' with the caveat that since none of the factors has been purified to homogeneity, multiple components could contribute to each activity. Factor D can be obtained from either of the phosphocellulose fractions that contain transcription factors but it is usually more abundant in the fraction that contains TFIIIC.

Two criteria establish that factor D is distinct from either of the known transcription factors (B and C) and from polymerase III. First, the chromatographic properties of D distinguish it from any of the other transcription components. As shown in Figure 1, elution from Mono S achieves a clear separation of TFIIID from TFIIIC. Chromotography on this resin also separates TFIIID from TFIIIB, and chromatography on heparin Sepharose separates TFIIID from polymerase (not shown). It is likely that efforts to purify the previously described transcription factors, TFIIIB and TFIIIC, have been confounded by the presence of TFIIID in various fractions of the polymerase III transcription apparatus. We decided which Mono S fractions should retain the names TFIIIB and TFIIIC and which one should be called TFIIID on the basis of the properties of the two phosphocellulose factor fractions. These two fractions were derived by standard procedures known to separate two transcription factors, B and C (Segall *et al.*, 1980). Since the third factor can be obtained from either fraction, it does not functionally distinguish them. Therefore, we called the common factor TFIIID, and used the names TFIIIB and TFIIIC to describe the two transcription factors that do distinguish the two phosphocellulose fractions.

The second criterion that distinguishes factor D from factors B or C and from polymerase is a functional one. TFIIID was distinguished from polymerase on the basis of direct measurements of the ability of the polymerase fraction by itself to catalyze non-specific transcription (Jaehning *et al.*, 1975). By this test, our polymerase fraction had the properties of the *Bombyx* polymerase III described previously (Sklar *et al.*, 1976; data not shown). In contrast, the TFIIID fraction was inactive in the non-specific transcription assay. That is, non-specific transcription catalyzed by TFIIID was indistinguishable from the background level of 3%. We also tested the possibility that factor D might be functionally equivalent to polymerase, or to one of the other factors, by measuring specific transcription in reactions where individual factors were systematically omitted and the amounts of the remaining factors were varied. These experiments (not shown) established that increasing the amounts of TFIIIB, TFIIIC or polymerase cannot compensate for the absence of TFIIID. Moreover, since the requirement for each of the four separated fractions was tested directly and individually in this manner, these experiments allow the more general conclusion that none of the transcription components we have resolved can substitute for one another.

We have considered the possibility that some of the components that appear to be required for transcription might not act in a positive sense, but might instead function to suppress the effect of a negative activity (a nuclease or a protease, for example) that is extraneous to the normal transcription reaction. Such a deleterious activity should be exposed in subsets of the transcription apparatus. To test for it, we first allowed the hypothetical negative effect to occur by incubating all combinations of fractions containing only three transcription components under the conditions of a typical transcription reaction. To learn whether the transcription apparatus had been damaged as a consequence of this incubation, we determined whether transcription could be rescued by addition of the missing component. For comparison, we measured the transcription rate in a parallel reaction mixture that contained all four transcription components throughout the entire experiment. We found that pre-incubation of subsets of the transcription apparatus does not reduce subsequent transcription. All combinations gave the same result, an example of which is shown in Figure 2. The data in Figure 2

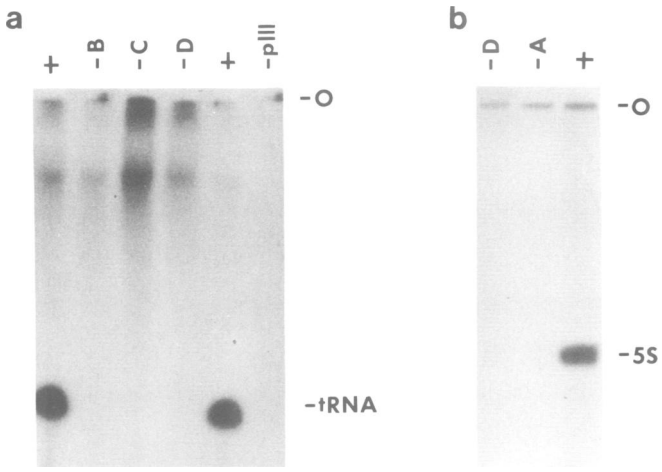

Fig. 3. (**a**) Transcription of tRNA genes requires TFIIID. Transcription reactions were carried out as in Wilson *et al.* (1985) using 0.032 pmol of the Bmt11 $tRNA^{Ala}$ gene as template and the amounts of transcription components as determined in Materials and methods. Transcription was catalyzed by the fully reconstituted *Bombyx* transcription apparatus (+) or by a partially reconstituted apparatus lacking TFIIIB (−B), TFIIIC (−C) or TFIIID (−D) or RNA polymerase III (−pIII). The products of transcription were analyzed as in Figure 2. (**b**) Transcription of 5S RNA genes requires TFIIID. Transcription reactions were carried out as described in (a), using 0.24 pmol of a *Bombyx* 5S RNA gene (Morton and Sprague, 1984) as template. Reactions were catalyzed by the *Bombyx* transcription components described in (a) supplemented with *Xenopus* TFIIIA (6 μl) kindly provided by the R.Roeder laboratory. Shown are the transcripts (5S) produced by the fully reconstituted transcription apparatus (+) and by subsets lacking TFIIID (−D) or TFIIIA (−A).

were obtained from experiments in which template DNA was omitted from the pre-incubation reactions, but was added later along with the missing transcription component. The same results were obtained if the template was included during the pre-incubation reaction. By incubating pre-formed transcripts with subsets of the transcription machinery, we have also established that a nuclease capable of degrading the transcript is not exposed by omission of one transcription component (data not shown). We conclude, therefore, that the *Bombyx* polymerase III transcription machinery consists of at least four distinct, positively acting components.

TFIIID is required for transcription of both tRNA and 5S RNA genes

As shown in Figure 3a, transcription of a *Bombyx* $tRNA^{Ala}$ gene requires TFIIID. In the absence of factor D, transcription is undetectable. That is, given the sensitivity of our assay, transcription is reduced at least 50-fold. Figure 3a also shows that each of the previously identified components of the polymerase III transcription machinery, polymerase itself, factor B and factor C is essential for transcription.

To determine whether factor D acts specifically on tRNA genes, or whether it is a more general polymerase III transcription factor, we tested the requirement for factor D in transcription of 5S RNA genes. Since transcription of 5S genes was already known to require a special factor, TFIIIA, in addition to the general factors, B and C (Engelke *et al.*, 1980; Segall *et al.*, 1980), we anticipated that demonstrating the dependence of *Bombyx* 5S RNA gene transcription on factor D would require the presence of factor A. Since we have not yet isolated TFIIIA from silkworms and since it is not present in our other transcription fractions, we took advantage of the fact that TFIIIA from another organism efficiently complements the partially purified silkworm components and allows transcription of *Bombyx* 5S RNA genes. As shown in Figure 3b, transcription of *Bombyx* 5S RNA genes requires a factor A-like activity, but this activity can be supplied by TFIIIA from *Xenopus* frogs. The use of this mixed transcription system enabled us to test the requirement for *Bombyx* factor D in 5S RNA gene transcription. The data in Figure 3b show that 5S RNA genes, like tRNA genes, are absolutely dependent on factor D for transcriptional activity.

What is the function of TFIIID?

To investigate the possibility that TFIIID plays a role in the formation of transcription factor complexes on tRNA genes, we performed template exclusion experiments similar to those used previously to demonstrate stable complex formation (Lassar *et al.*, 1983; Baker and Hall, 1984; Fuhrman *et al.*, 1984; Ruet *et al.*, 1984). In experiments of this sort, one kind of gene is pre-incubated with a subset of the transcription apparatus, and then a second kind of gene is added to compete for unbound factors. In one version of the template exclusion experiment, the two different templates are allowed to compete for a period of time, after which the remaining components of the transcription apparatus are added and transcription is allowed to proceed. If stable complexes have formed on the first gene under conditions of template excess, then little or no transcription from the second gene shuld be observed. A variant procedure, in which the second template is added simultaneously with the remainder of the transcription machinery, has been used to detect weak ('metastable') interactions (Lassar *et al.*, 1983). Metastability may correspond to interactions that are either less stable or that occur more slowly in the absence of additional transcription components.

For the initial characterization of interactions between $tRNA^{Ala}$ genes and the *Bombyx* transcription apparatus, we wished to use an assay capable of revealing even the weakest interactions that had been detected previously. Therefore, we used a protocol in which the second template was added simultaneously with the remainder of the transcription apparatus. We report interactions detected by this assay without discriminating between stable and metastable types. To distinguish transcription events directed by the two templates, we used two *Bombyx* $tRNA^{Ala}$ genes that give transcripts of different lengths. We were concerned that the relative transcriptional efficiency of the two $tRNA^{Ala}$ genes might vary when they were pre-incubated with different subsets of the transcription machinery. Therefore, we determined the ratio of transcripts from simultaneously added genes for each combination of transcription components we tested. In fact, we found that this ratio (gene 1/gene 2 = 1.1 ± 0.2) was not affected by pre-incubation with different transcription components. In addition, we established that the absolute rate of transcription is not greatly influenced by pre-incubation of subsets of the transcription apparatus. The total number of transcripts produced in individual reactions did not vary by more than a factor of two within any complete experiment.

The ability of the full *Bombyx* transcription apparatus, and subsets of it, to exhibit binding to $tRNA^{Ala}$ genes by this assay is summarized in numerical form in Table I. Each value is the ratio of the transcription rate from the gene added first (gene 1) to that of the gene added second (gene 2), after normalization to the ratio of these transcription rates when the two genes are added simultaneously. We interpret these data as follows. Ratios of transcription of gene 1 to transcription of gene 2 that are near

Table I. Binding of transcription components to tRNA genes[a]

Components incubated with gene 1	Preferential transcription from gene 1
One component	
pIII	1.0
B	1.0
C	0.9
D	1.3
Two components	
pIII, B	0.9
pIII, C	1.1
pIII, D	1.3
B, C	1.0
B, D	4.4
C, D	2.8
Three-components	
pIII, B, C	1.1
pIII, B, D	2.9
pIII, C, D	3.3
B, C, D	3.8
Four components	
pIII BCD	3.6

[a]Binding of transcription components to tRNA genes. The data are expressed in the form of ratios: for each reaction, the ratio of the transcription rates for gene 1 to gene 2 was computed for the case in which gene 1 was allowed to interact with transcription component(s) before gene 2 was added. This ratio was then divided by the ratio of the transcription rates from the same two genes (gene 1/gene 2) for the case in which both genes were pre-incubated with transcription components simultaneously. The data shown are the averages of 2–4 experiments. The average standard deviations were 0.2 for reactions that revealed no binding to gene 1 and 1.0 for reactions where binding was observed.

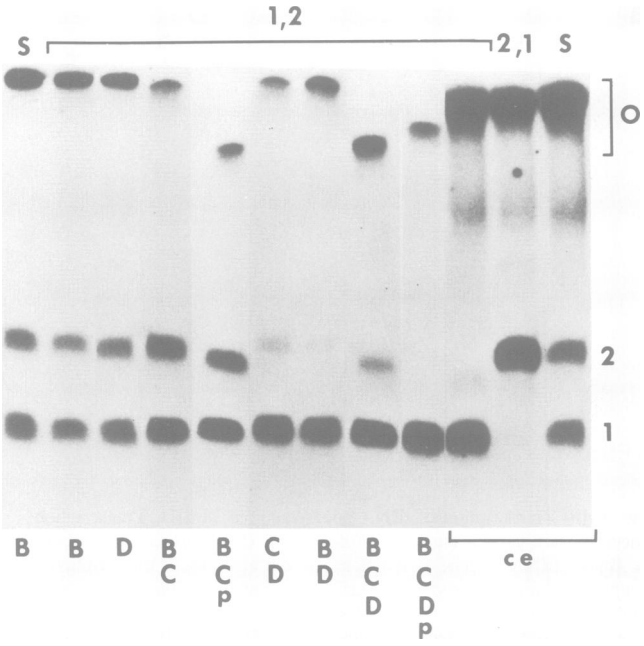

Fig. 4. Representative data showing binding of transcription components to $tRNA^{Ala}$ genes. Conditions for the binding and transcription reactions are described in the Materials and methods. Shown are the primary transcripts from gene 1 (1) and gene 2 (2). Symbols across the bottom of the figure indicate which transcription components were included in each binding reaction: TFIIIB (B), TFIIID (D), TFIIIC (C), RNA polymerase III (p) or crude nuclear extract (ce). Symbols across the top of the figure indicate that both genes were incubated with these components simultaneously (S) or that gene 1 (1,2) or gene 2 (2,1) was added to the reaction first. The lanes shown have been aligned with respect to the position of the primary transcript from gene 1. The positions of the gel origins and the primary transcripts from gene 2 do not line up because the lanes were taken from three different gels. In all cases, the rates of total specific transcription (from genes 1 and/or 2) were similar. All gels were autoradiographed under the same conditions (15 h at −55°C with an intensifying screen).

unity mean that transcription components are not sequestered by the template added first. That is, factors are free to equilibrate between the two genes at the time the second gene is added. In contrast, ratios of gene 1/gene 2 transcription that are greater than unity indicate that at least one transcription component forms a complex with the first gene that is sufficiently stable to prevent subsequent interaction with the second gene. To eliminate possible bias caused by the choice of a particular gene as the first template, another series of experiments was carried out in which the identity of the genes added first and second was reversed. These data are not shown, but they are not different from those in Table I. In addition to the numerical values given in Table I, the raw data from representative experiments are shown in Figure 4.

The data in Table I reveal a clear pattern of interactions between tRNA genes and components of the *Bombyx* transcription apparatus. No single fraction of the transcription machinery interacts detectably with $tRNA^{Ala}$ genes under the conditions of this assay. In contrast, certain combinations of transcription factors do interact. Specifically, combinations of two factors are able to bind – provided that one of the factors is D. Thus, combinations of B plus D or of C plus D exhibit binding, but B plus C does not. The degree to which transcription components associate with the first template is not increased when all three factors are combined. Moreover, polymerase does not contribute appreciably to any of the interactions we have observed.

Discussion

We have shown that the transcription factor we have discovered (TFIIID) is a general component of the *Bombyx* polymerase III transcription machinery in that it is necessary for transcription of both tRNA and 5S RNA genes. For two reasons, we think it likely that TFIIID is also found in other organisms. (i) The chromatographic properties of fractions of the *Bombyx* transcription machinery before resolution on Mono S resins are indistinguishable from the properties of comparable fractions from other organisms. Thus it is reasonable to suppose that the fractions from other organisms contain mixtures of factors C and D, as the *Bombyx* fractions do. Indeed, recent work independent of ours shows that the classical phosphocellulose fraction containing TFIIIC from human cells can be divided into two fractions that are both required for transcriptional activity (Yoshinaga *et al.*, 1987). One of these fractions may be equivalent to TFIIID. (ii) Since purified TFIIIA from *Xenopus* frogs can function efficiently in a TFIIID-dependent *Bombyx* transcription system, it is likely that *Xenopus* TFIIIA normally acts in a context that includes a D-like transcription factor.

It is intriguing that TFIIID appears to be critical for the assembly of other factors into transcription complexes. The key result is that none of the single transcription components we have resolved interacts with genes by itself. Our assays would have detected even weak interactions of the type that have been called 'metastable' (Lassar *et al.*, 1983). We find that combinations of two transcription factors can bind to tRNA genes, but that binding is detectable only when factor D is one of the pair of factors. Our results contrast with others showing that TFIIIC

alone can bind to tRNA genes (Lassar *et al.*, 1983; Baker and Hall, 1984; Ruet *et al.*, 1984; Carey *et al.*, 1986). A likely explanation for this discrepancy is the presence of TFIIID in the TFIIIC factor fractions that were tested previously. Indeed, our crude TFIIIC-containing phosphocellulose fraction is sufficient for binding to tRNA genes (data not shown). The common finding that factor B does not bind to templates by itself (Lassar *et al.*, 1983; Carey *et al.*, 1986) is consistent with our observation that crude factor B fractions contain less factor D than do crude factor C fractions.

How does TFIIID act? The challenge now is to understand how factor D stimulates factor binding to tRNA genes. Our results do not discriminate between models in which TFIIID acts in a kinetic sense to stimulate the overall rate of formation of factor–gene complexes as compared with models in which TFIIID acts in a thermodynamic sense to increase the affinity of factors for these complexes. In either case, however, preliminary experiments suggest that factor D acts stoichiometrically rather than catalytically. That is, TFIIID appears to remain stably associated with the template during complex formation and transcription (D.Rivier, unpublished). Thus it is unlikely that factor D promotes the binding of other transcription factors, but does not itself form part of the transcription complex.

It is worth considering how the fully interacting complex of transcription factors and genes might be formed. The traditional view is that complex formation is initiated by a single transcription factor binding to a specific DNA sequence. Additional components then join the complex by binding sequentially to other DNA sites, to a previously bound protein, or to a site comprised of specific contacts with both DNA and protein. Our results show that if transcription complexes are assembled on tRNA genes in such a stepwise fashion, there is flexibility as to which interactions can initiate the process. Combinations of factors B and D or of C and D serve equally well as starting points for assembly.

These results also emphasize the importance of protein–protein interactions in initiating the formation of transcription complexes. Successful binding is achieved only when multiple factors are allowed to act in concert. This fact suggests that it may be useful to consider alternative assembly modes. It is possible, for example, that transcription factors form complexes with each other in the absence of templates, and that it is these pre-formed complexes that then bind to genes.

Three considerations make the idea of pre-existing complexes of transcription factors appealing. First, the formation of specific complexes between individual components of the polymerase III transcription machinery is detectable even in the absence of template (Burke *et al.*, 1983; Wingender *et al.*, 1986). Second, some unusual features of tRNA gene control elements could be understood if the critical interaction between template and transcription apparatus involved a multifactor complex stabilized by protein–protein contacts. In particular, our laboratory has recently shown that such transcriptional control elements occupy a much larger region than was previously supposed. The sequences required for full transcriptional activiy of a silkworm $tRNA^{Ala}$ gene occupy a stretch of ~160 bp that includes the coding region and sequences both upstream and downstream from it. Much of this large region (~125 bp of it) is involved in binding transcription factors (Wilson *et al.*, 1985). The large size of the factor binding region is surprising. It clearly exceeds the size of the classical targets of individual prokaryotic regulatory proteins. What is particularly intriguing, however, is the observation that although deletion of part of the factor binding region profoundly reduces transcriptional activity, even severely truncated genes can be transcribed at high rates if the concentration of templates relative to the concentration of transcription factors is sufficiently high (Larson *et al.*, 1983; Wilson *et al.*, 1985). At first glance, such behaviour seems paradoxical since we expect severely deleted tRNA genes to have lost specific binding sites for some transcription factors. Since all of the factors are essential for transcriptional activity, we expect that loss of the DNA binding site should exclude the corresponding factor from the transcription complex and thus abolish transcriptional activity regardless of the template concentration. This paradox could be resolved if protein–protein contacts among factors were sufficient to allow the full set of factors to participate in transcription even in the absence of the normal set of protein–DNA contacts. Finally, a multi-factor complex with the potential to bind to tRNA genes through many specific contacts could reduce the problem of target site selection during the early stages of transcription complex formation. The problem, as delineated by von Hippel and colleagues (von Hippel *et al.*, 1974) and by Lin and Riggs (1975), is that a regulatory protein must bind specifically to one site, or to a small number of sites, in the midst of a vast excess of non-specific sites for which it also has appreciable affinity. Conceivably, interactions between multifactor complexes and large DNA segments could provide the high degree of specificity required for regulators to find their targets in large eukaryotic genomes.

Materials and methods

Fractionation of the Bombyx polymerase III transcription apparatus

Preparation of RNA polymerase III. Nuclear extract (Morton and Sprague, 1984; Wilson *et al.*, 1985) (60 ml; 4 mg/ml) was loaded onto an 80 ml DEAE Sephadex (Sigma, A-50-120) column 2.5 cm in diameter equilibrated in Buffer B [50 mM Tris–HCl pH 7.5, 20% (v/v) glycerol except where noted, 1 mM EDTA, 1 mM 2-mercaptoethanol] plus 250 mM KCl, and was washed through with the same buffer. Protein-containing fractions (detected by the method of Bradford (1976) having an $A_{260}nm/A_{280}nm$ ratio of <0.7 (measured in a Beckman DU7 spectrophotometer) were pooled and called DEI. Subsequent fractions having an $A_{260}nm/A_{280}nm$ ratio between 0.7 and 1.5 were pooled and called DEII. The DEI pool was loaded onto a 24-ml phosphocellulose (Whatman P-11) column. After washing with 1.1 column volumes of Buffer B plus 300 mM KCl, a fraction containing polymerase III, TFIIIB and some TFIIID was obtained by elution with one column volume of Buffer B plus 450 mM KCl. The peak protein-containing fractions from this step were pooled and dialyzed against buffer A [50 mM Tris–HCl pH 7.5, 20% (v/v) glycerol except where noted, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol] plus 75 mM KCl, and were stored at −70°C. Aliquots of the 450 mM phosphocellulose pool that contained a total of 5 mg of protein were diluted to 1.0 mg/ml protein and dialyzed for 1 h against Buffer H [50 mM Tris–HCl pH 7.7, 20% (v/v) glycerol, 0.25 mM EDTA, 0.5 mM dithiothreitol, 0.5 μM leupeptin] plus 20 mM KCl and loaded onto a 1 ml heparin Sepharose column (Pharmacia) equilibrated in Buffer H. Protein was then eluted in the following steps: 10 column volumes Buffer H plus 0.25 mg/ml BSA, 7.5 column volumes Buffer H plus 100 mM KCl and 0.25 mg/ml BSA, 5 column volumes Buffer H plus 200 mM KCl and 0.25 mg/ml BSA, 5 column volumes Buffer H plus 300 mM KCl and 5 column volumes Buffer H plus 400 mM KCl. Fractions that eluted at 400 mM KCl and that contained the peak polymerase III activity were pooled and dialyzed against Buffer H plus 50 mM KCl after the addition of 0.2 mg/ml BSA. Immediately before use in transcription reactions, polymerase fractions were adjusted to 5 mM $MgCl_2$ and 0.5 mg/ml BSA.

Fractionation of TFIIIC and TFIIID

Fractions containing TFIIIC and TFIIID came from the same fractionation of the DEI pool that was used to prepare polymerase III. TFIIIC and TFIIID activities were obtained by elution of the phosphocellulose column with Buffer B plus 600 mM KCl, after prior elution at 450 mM KCl had removed polymerase III and TFIIIB. Fractions containing TFIIIC and TFIIID free of polymerase and TFIIIB were pooled, dialyzed against Buffer A plus 180 mM KCl and stored at −70°C.

To separate TFIIIC from TFIIID, 2 ml of the TFIIIC plus TFIIID fraction that eluted from phosphocellulose at 600 mM KCl was dialyzed against Buffer B plus 180 mM KCl and then loaded onto an analytical HR 5/5 Mono S column (Pharmacia) pre-equilibrated in 0% CS buffer [175 mM KCl, 25 mM Hepes

pH 6.7, 10% (v/v) glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol]. Protein was eluted with a linear gradient (25 mM/ml KCl) from 0 to 100% CS buffer [565 mM KCl, 40 mM Hepes pH 7.1, 10% (v/v) glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol]. Individual fractions of 350 μl were collected in the presence of BSA to achieve a final protein concentration of 1 mg/ml, dialyzed against Buffer A plus 125 mM KCl at a glycerol concentration of 10% and assayed for transcriptional activity. TFIIIC activity eluted at 44% of the gradient, whereas TFIIID activity eluted at 65% of the gradient.

Fractionation of TFIIIB

We have obtained fractions containing TFIIIB activity in two ways. One of these relies on phosphocellulose chromatography to produce the standard fractions that originally distinguished TFIIIB from TFIIIC (Segall *et al.*, 1980). Since the TFIIIB in this fraction is significantly contaminated with polymerase III (Segall *et al.*, 1980; Lassar *et al.*, 1983; Yoshinaga *et al.*, 1986) and with TFIIID, we have developed an alternative method based on fractionation of the DEII pool described above. This method yields TFIIIB that is functionally indistinguishable from conventionally prepared TFIIIB, and is free of detectable polymerase III and TFIIID activities.

The DEII fraction was dialyzed against Buffer A until the KCl concentration reached 125 mM (~1 h) and was then divided into two equal parts. Each part was loaded onto a separate 1.5 ml DEAE Sepharose (CL-6B) column equilibrated in Buffer A containing 10% glycerol and 50 mM KCl. Each column was washed with 10 column volumes of Buffer A containing 10% glycerol and 75 mM KCl and was eluted with Buffer A containing 10% glycerol and 550 mM KCl. The fractions containing protein from both runs were pooled (~2 ml) and split into three parts, each of which was fractionated on a Superose 6 gel filtration column (Pharmacia, HR 10/5) equilibrated in Buffer A containing 10% glycerol, 75 mM KCl and 50 μM EDTA. Individual fractions were assayed for TFIIIB activity and the active fractions from the three runs were pooled, loaded onto a 1-ml DEAE Sepharose column pre-equilibrated in Buffer A plus 50 mM KCl, and eluted with Buffer A plus 550 mM KCl. BSA (0.5 mg/ml) was added to the pooled protein-containing fractions, which were then dialyzed against Buffer A plus 75 mM KCl.

General procedures used during fractionation

During purification, the activities of individual transcription components were detected by complementation of the activities present in appropriate subsets of the full transcription apparatus. These assays typically employed fractions of intermediate purity in order to avoid unnecessary use of the purest fractions. Although the cruder fractions were sometimes contaminated with low levels of the activity being followed, they none the less permitted convenient and reliable monitoring of chromatographic separations. The assay for specific transcription by polymerase III consisted of complementation of the 600 mM KCl phosphocellulose fraction (TFIIIC and TFIIID) plus purified TFIIIB (prepared as described above). Polymerase III activity was also measured by its ability to catalyze non-specific transcription of poly(dA).(dT) (Jaehning *et al.*, 1975). The assay for TFIIIB consisted of complementation of the 600 mM KCl phosphocellulose fraction (TFIIIC and TFIIID) plus purified polymerase III (prepared as described above). The assay for TFIIIC consisted of complementation of a single crude fraction that contains polymerase, TFIIIB and TFIIID, but only low levels of TFIIIC. This crude fraction is derived from the supernatant of the first centrifugation step during preparation of the nuclear extract used as starting material (Morton and Sprague, 1984; Wilson *et al.*, 1985). The assay for TFIIID consisted of complementation of purified TFIIIC (prepared as described above) plus a fraction containing both TFIIIB and polymerase III that was generated by chromatography of the 450 mM KCl phosphocellulose fraction on Mono S.

All column buffers contained 1 μM leupeptin as a protease inhibitor. The starting extract was made 0.1 μM in phenylmethylsulfonyl fluoride. The DEI, phosphocellulose, and Mono S fractions containing TFIIIC, TFIIID or polymerase were sometimes frozen before assay, pooling or subsequent fractionation. In contrast, preparation of TFIIIB was carried out without interruption by freezing. All fractionation was performed at 4°C.

Extent of cross-contamination of fractions of the transcription apparatus

The purity of the fractions with respect to other components of the polymerase III transcription apparatus was determined from a series of transcription reactions in which individual components were systematically omitted. These assays showed that the extent of cross-contamination among the four fractions required for tRNA gene transcription is very low. The level of contaminating polymerase or TFIIIB contributed by the other three fractions is undetectable (<1%). Contaminating TFIIIC or TFIIID in the remainder of the transcription apparatus is detectable with some preparations, but only at a level of 1–2%. The apparently higher levels of cross-contamination between TFIIIC and TFIIID (~10%) suggested by the elution profile in Figure 1 reflect the use of crude fractions as the source of complementing transcription activities for the TFIIIC and TFIIID assays used during purification.

Although the transcription factors we have resolved are not homogeneous, it is likely that they are highly enriched. We estimate that the concentration of total protein in the D factor fraction is ~0.001 of the protein concentration in the original nuclear extracts. The rates of transcription catalysed by crude extracts and by combining these separated components were 30 and 15 transcripts/gene/h respectively.

Binding of transcription components to tRNA genes

Binding reactions were carried out in the same conditions used for transcription reactions: 600 μM ATP, CTP, GTP, 25 μM UTP, 65 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl pH 7.5, and 6% (v/v) glycerol, at room temperature (22°C). Each reaction contained 0.01 μg (3.2 fmol) of gene 1 and 0.01 μg (5.9 fmol) of gene 2 plus non-specific (pBR322) DNA to bring the total mass of DNA to 0.03 μg. Gene 1 was the *Bombyx* $tRNA^{Ala}$ gene originally named Bmt 11 (Hagenbüchle *et al.*, 1979). Gene 2 was a derivative of Bmt 11 (see 3′+89 in Larson *et al.*, 1983) that produces a longer transcript because removal of the normal termination site allows termination at a downstream site. A complete experiment consisted of binding reactions carried out in parallel for all possible combinations of single and multiple transcription components. To allow the simultaneous addition of multiple components to individual reaction mixtures, we devised the following protocol: the appropriate buffers, salts and unlabeled nucleotides were mixed in the bottom of each of a series of microfuge tubes. Then, gene 1 and the transcription component(s) to be tested were deposited as separate drops on the walls of these tubes. The binding reactions were started by forcing the drops to the bottom of the tubes (~5 s centrifugation in a microfuge) and then mixing the tube contents manually. While the binding reactions proceeded, separate drops containing gene 2, 5 μCi [α-^{32}P]UTP (800 Ci/mmol) plus appropriate buffers, and the remaining transcription components were placed on the walls or lids of the same tubes. These components were mixed, and radioactive labeling of transcripts was initiated, exactly as described for the binding reactions. The final reaction volume was 40 μl. The initial incubation with gene 1 was carried out in 15, 20 or 25 μl for reactions with one, two or either three or four transcription components respectively. The time allowed for binding to gene 1 was held constant for all reactions within a single experiment, but varied from 15 to 18 min among experiments. In all cases, transcription in the presence of a labeled nucleotide proceeded for 2 h. Transcription reactions were stopped by the addition of SDS, and the products were fractionated directly by polyacrylamide gel electrophoresis (Morton and Sprague, 1984). Transcripts were detected by autoradiography and were quantitated by scintillation counting of excised gel pieces.

The amounts of the transcription components used in these experiments were determined by titration of individual fractions against each other and against a fixed amount of template (0.05 or 0.1 μg of gene 1) previously shown to be saturating for crudely titrated transcription components. Crude titrations of fractions relative to each other were used to determine approximate limiting amounts of different components. The component available to us in shortest supply was then chosen as a reference. The amount of this component was fixed at a convenient level and each of the other components was titrated with respect to it in a detailed fashion. The amount of each fraction used in binding and transcription experiments was that amount needed to just saturate the transcription rate when the reference component was limiting and the other two components were in excess. These titrated amounts varied for different preparations, but were within the following ranges: 1.5 μl of polymerase, 1.5–2.0 μl of TFIIIB, 2–5 μl of TFIIIC and 6–7.5 μl of TFIIID. In reactions catalyzed by crude extract, 3 μl was used. The experiments reported were carried out with one preparation of polymerase, two preparations of TFIIIB and three preparations each of TFIIIC and TFIIID. The stability of all fractions of the transcription apparatus to incubation under the conditions used for binding to templates was tested. No deleterious effects of such incubation were detected.

Acknowledgements

We are grateful to Bob Roeder for the generous gift of *Xenopus* TFIIIA and to Peter von Hippel, Rick Dahlquist, Eric Selker and Tim Smith for their comments on the manuscript. This work was supported by National Institutes of Health Research Grants GM 25388 and GM 32851 and Career Development Award HD00420 to K.U.S., a Damon Runyon-Walter Winchell postdoctoral fellowship to G.M.D. and a Genetics Training Grant pre-doctoral fellowship (GM 07413) to D.H.R.

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Received on April 14, 1987