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Rapid Enrichment of HeLa Transcription Factors IIIB and IIIC by Using Affinity Chromatography Based on Avidin-Biotin Interactions

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Plasmid DNA containing the adenovirus type 2 genes for VA RNA was linearized at a site distal to the gene, end labeled with a biotin-nucleotide analog of TTP, and incubated with avidin to form an avidin-biotinylated DNA complex. HeLa cell S100 extracts containing crude RNA polymerase III and transcription factors (TFs) IIIB and IIIC were programmed with the avidin-biotin-VA DNA to allow stable complex formation (A. B. Lassar, P. L. Martin, and R. G. Roeder, Science 222:740–748, 1983). Chromatography of the programmed extract over a biotin-cellulose affinity resin resulted in the selective, and virtually quantitative, retention of one of two stable preinitiation complexes, either VA-IIIC or VA-IIIC-IIIB, depending on the length of template incubation in the S100 extract. After washing the resin with 0.10 M and 0.25 M KCl to remove RNA polymerase III and nonspecifically bound proteins, respectively, TFIIIC was eluted from the VA-IIIC complex by the addition of 1.5 M KCl. The VA-IIIC-IIIB complex exhibited a higher salt stability. Most of TFIIIB and some TFIIIC were released by the addition of 1.5 M KCl; however, the majority of TFIIIC activity was recovered only after a subsequent 3.0 M KCl elution. The specific activity of the TFIIIC in the 3.0 M KCl fraction was 770-fold higher than that in the S100 extract, while the protein content of the 1.5 and 3.0 M KCl fractions was reduced 7,500- and 100,000-fold, respectively.

The elucidation of the molecular mechanisms underlying eucaryotic gene expression has been facilitated by the development of soluble cell extracts which direct the accurate transcription of purified DNA templates. Studies analyzing the transcription of class I, II, and III genes by RNA polymerases I, II, and III (pol III), respectively, have identified both specific DNA sequences and cellular factors as key elements of these processes (16). Our present study is focused on the development of rapid procedures for the isolation of those cellular factors involved in the transcription of class III genes, which include the tRNA, 5S RNA, and adenovirus VA RNA genes.

Functional dissections of class III genes have identified intragenic sequences as essential elements for the initiation of transcription. The promoter of tRNA genes is split into two regions of about 10 nucleotides each (termed the A and B blocks), which are separated by 30 to 40 base pairs and are highly conserved in all tRNA genes (12, 17). The VA RNA gene promoter appears to share the same structure (11). The 5S RNA genes contain a 34-nucleotide internal promoter consisting of two separable but contiguous elements (4, 26); the first 11 nucleotides are homologous to the A block of tRNA genes, and the remainder are specific to 5S genes (7).

Chromatographic fractionation of crude cellular extracts has shown that in addition to pol III, tRNA and VA RNA gene transcription requires at least two separate components (IIIB and IIIC), whereas 5S RNA gene transcription requires these two together with another gene-specific factor (IIIA) (27, 28). Although factor IIIA, a 38,000-molecular-weight protein, has been purified to homogeneity from *Xenopus* oocytes (9), the purification and characterization of factors IIIB and IIIC has proven to be much more difficult, in large part owing to their relatively low abundance and instability during purification.

Detailed analyses of gene-factor interactions (3, 20) have led to a model for the ordered interaction of these transcription factors (TFs) with specific gene sequences. TFIIIA binds to the promoter region of the 5S RNA gene in the absence of other components (9), yielding a metastable complex of 1:1 stoichiometry (2). The addition of TFIIIC converts the 5S-IIIA complex into a stable conformation (5S-IIIA-IIIC) that persists for many rounds of transcription, withstands challenge by competing templates (5, 20), and requires ionic strengths exceeding 1 M KCl for its dissociation (30). The stable sequestration of TFIIIB by the 5S-IIIA-IIIC complex requires both an extended incubation period and the presence of ATP. The subsequent association of pol III forms the rate-limiting complex 5S-IIIA-IIIC-IIIB-pol III, necessary for the synthesis of 5S RNA. It appears that pol III does not remain stably sequestered, but is cycled between rounds of transcription (3).

TFIIIC binds to the B-sequence block of tRNA and VA RNA genes first, with TFIIIB and pol III binding second and third, respectively. The binding of TFIIIC to the VA RNA genes is necessary and sufficient for the formation of a stable complex, while the tRNA gene requires the presence of both TFIIIB and TFIIIC for stable association of TFIIIC (20). There is currently no evidence for the stable sequestration of TFIIIB by either the VA-IIIC or tRNA-IIIC stable complex, nor for a stable association of RNA polymerase III in the VA-IIIC-IIIB-pol III or tRNA-IIIC-IIIB-pol III rate-limiting complex.

The extreme stability of the preinitiation complexes suggested a novel approach for their isolation and analysis. By substituting biotin-labeled dUTP (Bio-dUTP) in sequences distal to a cloned gene, we isolated DNA-protein complexes that are formed when the affinity-labeled template is incubated in a crude cellular extract. Here we demonstrate the selective enrichment of TFIIIB and TFIIIC by virtue of their

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tight association with the adenovirus type 2 (Ad2) VA RNA genes.

MATERIALS AND METHODS

Recombinant plasmid DNA. The plasmid pA2-wt was obtained from T. Shenk (Princeton University) and contains the VAI and VAII genes on an Ad2 DNA segment (from 26.5 to 31.5 map units) inserted between the *Sal*I and *Hin*dIII sites of pBR322. The plasmid phH2D was obtained from M. Zasloff (National Institutes of Health) and contains a human $tRNA_i^{Met}$ gene on a 3.7-kilobase *Hin*dIII fragment inserted in the *Hin*dIII site of pBR322. The plasmids were propagated in *Escherichia coli* LE392 in accordance with National Institutes of Health guidelines (P2/EK1).

The plasmid pVA20x contains 20 copies of the VAI RNA gene. The 523-base-pair *Hin*fI-*Bal*I fragment of pA2-wt, containing the VAI RNA gene, was subcloned into the *Eco*RI site of pARA1, kindly provided by J. Hartley, and excised with *Ava*I as described previously (15). The ligation of multiple copies of the *Ava*I-ended VAI fragment into the *Ava*I site of plasmid pBR322 was by the method of J. Hartley (personal communication). pBR322 DNA was digested with *Bam*HI and *Ava*I, and the two fragments (1,050 and 3,313 base pairs, respectively) were purified by agarose gel electrophoresis. The small pBR322 fragment was ligated with a large molar excess of the *Ava*I-ended VAI fragment; the large pBR322 fragment was then added to the ligation mixture. DNA was transformed into *E. coli* HB101, selecting for tetracycline resistance. Individual clones were analyzed for the insertion of multiple copies of the VAI gene-containing fragment.

Transcription reaction and analysis of products. HeLa cell S100 extracts containing pol III activity were prepared as described by Weil et al. (29). Aliquots were frozen in liquid nitrogen and stored at −80°C. The standard in vitro reaction mixtures (20 μl) contained 15 to 20 μg of DNA per ml, 8 μl (40% [vol/vol]) of cell extract, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 600 μM each ATP, CTP, and UTP, 25 μM GTP, and 10 μCi of [α-^{32}P]GTP (410 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). After incubation at 30°C for 60 min, reactions were terminated by the addition of 150 μl of stop buffer (10 mM Tris [pH 7.9], 10 mM EDTA, 7 M urea, 100 mM LiCl, 0.5% sodium dodecyl sulfate (SDS), 350 μg of carrier tRNA per ml), extracted with phenol-chloroform, precipitated with ethanol, and suspended in 10 mM Tris–1 mM EDTA. An equal volume of loading dye (10 M urea, 1 mg of bromphenol blue per ml, 1 mg of xylene cyanol per ml) was added; and samples were boiled for 5 min and electrophoresed on 10% polyacrylamide–8 M urea slab gels (0.6 mm) in 0.1 M Tris borate–2 mM EDTA for 1 h at 20 mA. Gels were dried under vacuum and used to expose Kodak XAR5 film with or without Du Pont Cronex intensifying screens.

Competition-complementation assay. Transcription reactions were as described above with the following modifications: initial reaction mixtures (20 μl) contained 0.03 μg of phH2D DNA, 2 μl (10% [vol/vol]) of cell extract, 10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 600 μM each ATP, CTP, and UTP, and 25 μM GTP. After incubation of the reaction mixture at 30°C for 20 min, 0.03 μg of pA2-wt DNA was added. [α-^{32}P]GTP (10 μCi; 410 Ci/mmol) and the indicated fraction(s) were added at 30 min with adjustment of KCl to 100 mM. Reactions were terminated at 75 min by the addition of stop buffer and processed for gel electrophoresis as described above. Note: The sensitivity of this assay was achieved by performing the reactions at 10% (vol/vol) cell extract rather than the standard 40% (vol/vol); the DNA concentration indicated was optimal for transcription under these conditions.

Transcription reconstitution assay. The reaction mixtures (20 μl) contained 10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 600 μM each ATP, CTP, and UTP, 25 μM [α-^{32}P]GTP (100 Ci/mmol), and 400 ng of pVA20x DNA. Pol III {~100 dAT units per reaction; one unit catalyzes the incorporation of 1 pmol of UMP into RNA in 20, using poly[(dA-dT)·(dA-dT)] as a template} kindly provided by M. Van Dyke (Rockefeller University), and the VA DNA eluates (4 μl per reaction) were present as indicated in the legend to Fig. 7. After incubation at 30°C for 60 min, reactions were terminated and processed as described above.

Preparation of biotinylated templates. The plasmids pA2-wt and pBR322 were linearized with *Eco*RI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), extracted with phenol-chloroform and chloroform, ethanol precipitated, suspended in TE (10 mM Tris [pH 7.5], 1 mM EDTA), and chromatographed over a G-50 spin column. The DNA was end labeled with Bio-11-dUTP (6) and [α-^{32}P]dCTP by using T4 polymerase (P-L Biochemicals, Inc., Milwaukee, Wis.) as described previously (21) with slight modifications. The standard exonuclease reaction contained 0.4 mg of DNA per ml, 33 mM Tris acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, 0.5 mM DTT, and 100 μg of bovine serum albumin (BSA). The reaction mixture was brought to 37°C; T4 DNA polymerase was added (0.5 U/μg of DNA), and the reaction mixture was incubated at 37°C for 7 min. An equal volume of mixture containing 300 μM each dATP, dGTP, and Bio-11-dUTP, 20 μM dCTP, [α-^{32}P]dCTP, 33 mM Tris acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, 0.5 mM DTT, and 100 μg of BSA per ml was added; incubation at 37°C was continued for 30 min. The reaction was then chased with cold dCTP at a final concentration of 150 μM for 60 min at 37°C. The reaction was stopped by addition of 0.1% SDS–10 mM EDTA, and the mixture was heated at 65°C for 10 min. The DNA was then ethanol precipitated, suspended in TE, and spun through a 1.0-ml G-50 column equilibrated with TE to remove unincorporated nucleotides. Typically, these reaction conditions resulted in the introduction of 10 to 15 biotinylated nucleotides at each terminus of the linearized plasmid DNA.

Synthesis of biotin-cellulose. Biotin-cellulose was prepared by a modification of the general bisoxirane coupling method of Porath (24). For each 3.0 ml of biotin-cellulose resin, 1.0 g of dried cellulose powder (Whatman CC31, microgranular) was suspended in 20 ml of 1 M NaOH containing 2 mg of $NaBH_4$ per ml, and 20 ml of 1,4-butanediol diglycidyl ether (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added. The reaction mixture was stirred at room temperature for 2 h. The activated cellulose was thoroughly washed with double-distilled water (ddH_2O), first by centrifugation and then, after all traces of organic solvent were removed, by filtration on a 0.45-μm-pore-size filter unit (Nalgene Labware Div., Nalge-Sybron Corp., Rochester, N.Y.). Centrifugation was performed at room temperature with 50-ml polypropylene tubes in an IEC clinical centrifuge for four times for 4 min each at setting 5. The cellulose was transferred to a 0.45-μm-pore-size filter unit, washed three times with 100 ml of ddH_2O, and dried by suction. The activated cellulose was mixed with a suspension of 500 mg of biotin hydrazide (Calbiochem-Behring, La Jolla, Calif.) in 6 ml of

0.2 M Na_2CO_3 (pH 11.0 to 11.5), and the mixture was gently stirred at 45°C for 16 h. The biotin-cellulose product was sequentially washed by filtration as above, three times with 100 ml of ddH_2O, three times with 100 ml of 1 M NaCl, and then three times with 100 ml of ddH_2O. The resin was dried by suction, suspended in 1 M triethanolamine (pH of ~11), and stirred at 37°C for 16 h to block residual reactive groups. After the blocking step, the resin was sequentially washed by filtration as above with three times 100 ml each of ddH_2O, 1 M NaCl, ddH_2O, and phosphate-buffered saline (PBS). The biotin-cellulose resin was stored in PBS at 4°C. Biotin coupling was verified by a colorimetric assay with *p*-dimethylaminocinnamaldehyde essentially as described by McCormick and Roth (22). A 100-μl sample of the resin was dried by suction and mixed with 100 μl each of 2% (vol/vol) concentrated H_2SO_4 in absolute ethanol and 0.2% (wt/vol) *p*-dimethylaminocinnamaldehyde in absolute ethanol, followed by 800 μl more of the solvent. The presence of biotin is indicated by the development of a reddish orange color.

The capacity of the biotin-cellulose resin was determined to be 0.2 μg of avidin-biotinylated DNA per μl of resin under the conditions described below.

Biotin-cellulose chromatography. Biotin-cellulose was poured into silanized 1-ml tuberculin syringes plugged with polyethylene disks. A minimum of 5 μl of biotin-cellulose was used for each microgram of biotinylated DNA present in the programmed transcription extract. The resin was washed sequentially with 5 column volumes each of 1 M NaCl, PBS, 0.2 M acetic acid, PBS, 50 mM NaOH, PBS, 0.2 M acetic acid, PBS, and transcription buffer. This series of sequential washes should be done just before (1 to 2 h) using the resin as their omission results in significantly higher nonspecific retention of biotin-free DNA and lower recoveries of active transcription factors during the salt elution steps.

Biotinylated DNA probes were complexed with Avidin-DN (Vector Laboratories) at a 10-fold molar excess of avidin to biotin by incubation at room temperature for 10 min. Avidin-complexed DNA was incubated in S100 extract under basic transcription conditions for 45 to 120 min as indicated below and loaded directly onto a biotin-cellulose column. In the experiments reported here, 2.0 ml of a transcription reaction containing 15 to 20 μg of DNA (as an avidin-DNA complex) per ml was chromatographed over 200 μl of biotin-cellulose resin. The column was washed with 10 column volumes of transcription buffer, and DNA-bound proteins were eluted with 4 column volumes of transcription buffer containing KCl as indicated below. Salt-eluted fractions were concentrated 20-fold with Centricon microconcentrators (Amicon Corp., Lexington, Mass.). The 1.5 and 3.0 M KCl fractions were then reduced to 0.5 M KCl by a three- to sixfold dilution, respectively, with transcription buffer containing no KCl, followed by a three- or sixfold concentration, respectively, with microconcentrators. The total protein content of the fractions was determined by a modified Lowry procedure (1).

SDS-polyacrylamide gel electrophoresis. Column fractions were electrophoresed on 0.8-mm polyacrylamide slab gels (19) and silver stained (23).

RESULTS

Biotin forms a tight and essentially irreversible complex with avidin ($K_D = 10^{-15}$ mol/liter), a tetrameric glycoprotein with four biotin-binding sites per molecule (10). We developed a novel procedure for the isolation of TFIIIB and TFIIIC which capitalizes on the biotin-avidin interaction.

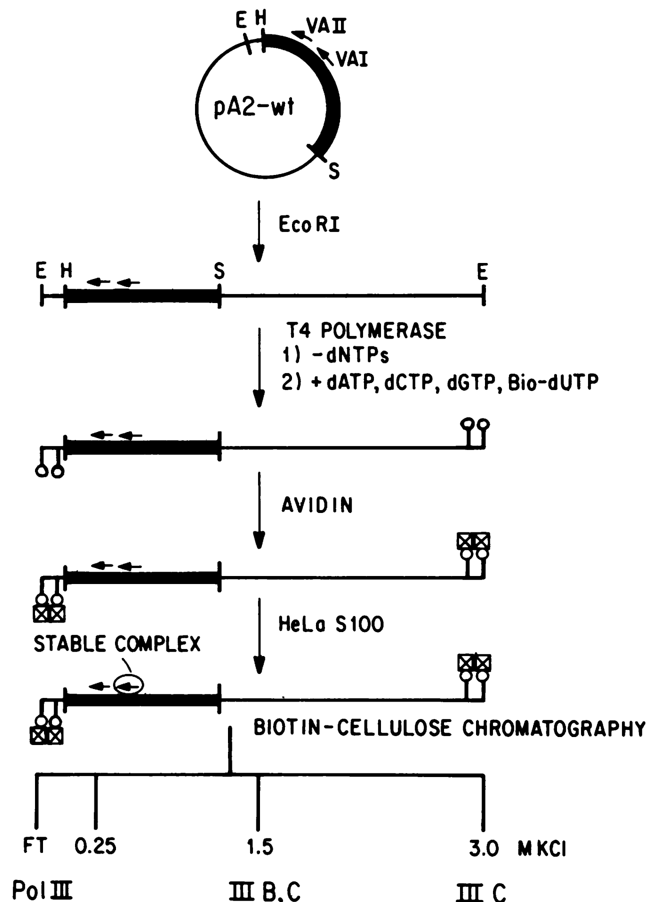

FIG. 1. Selective enrichment of pol III TFs from HeLa cell extracts. The purification steps are described in detail under Materials and Methods. The presence of pol III and TFIIIB and TFIIIC in the various fractions is indicated. FT denotes flowthrough; the KCl concentrations indicate the salt concentration at which protein was loaded onto or eluted from the column. E, H, and S are recognition sites for restriction endonucleases *Eco*RI, *Hin*dIII, and *Sal*I, respectively. ○ and ⊠ represent Bio-dUTP and avidin, respectively. dNTPs, deoxynucleoside triphosphates.

The general outline of this affinity selection procedure is schematically illustrated in Fig. 1. Plasmid pA2-wt containing the Ad2 genes for VAI and VAII RNAs was linearized with the restriction enzyme *Eco*RI which cuts at a unique site distal to the genes. The linearized template was biotinylated by substitution with Bio-11-dUTP (6) and incubated with avidin to form an avidin-biotin-DNA complex. Soluble extracts (S100) from HeLa cells were programmed with the affinity-labeled DNA to allow the formation of stable complexes (20). Stable complexes were isolated by chromatography on biotin-cellulose; TFIIIB and TFIIIC were eluted from the VA DNA template with KCl. The successful development of this general protocol required that several experimental parameters be optimized, as outlined below.

Choice of affinity resins for selective retention of biotinylated DNA. Several affinity resins are currently available which bind either avidin or biotin. We showed that biotin-DNA can be retained by direct chromatography on avidin-conjugated resins, such as avidin-Sepharose and streptavidin-Sepharose, or on antibody columns, e.g., anti-biotin immunoglobulin G-Sepharose. Alternatively, biotin-DNA can be complexed with free avidin and the

TABLE 1. Relative efficiency of affinity resins in specific retention of biotinylated DNA

Affinity resin	Sample[a]	% Retention	
		DNA	Biotin-DNA
Avidin-agarose	TE	6	65–70
	S100	<1	35
Avidin-Sepharose	TB	15–20	>95
	S100	20–30	90–95
Anti-biotin immunoglobulin G-Sepharose	TB	3–5	60–70
	S100	4–8	50–65
Biotin-BSA-Sepharose	TE + avidin	17	97
	S100 + avidin	15–20	70–90
Biotin-cellulose	TE + avidin	<1.0	>99
	TB + avidin	<0.1	>99
	S100 + avidin	2–3	95–99
Biotin-Sepharose	TE + avidin	2	75
	S100 + avidin	5	60

[a] ^{32}P-labeled pA2-wt or ^{32}P-labeled biotin-pA2-wt (1 to 2 μg) containing 50,000 to 100,000 cpm (Cerenkov) was added to TE, TB (transcription buffer containing 10 mM HEPES [pH 7.9], 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT), or a HeLa S100 reaction mixture (40% [vol/vol] S100 extract in TB). Where indicated, Avidin-DN (60 μg/ml, final concentration) was added to the sample, and the solution was maintained at room temperature for 10 min. Samples (50 μl) were applied to columns packed with 50 μl of the indicated resin. Flowthrough fractions were collected, and columns were washed with 4 column volumes each of sample buffer and 2 M KCl. The distribution of ^{32}P-labeled DNA in each fraction was determined by Cerenkov counting.

avidin-complexed DNA retained by chromatography on biotin-conjugated resins, such as biotin-Sepharose or biotin-BSA-Sepharose. These resins yielded high levels of selective retention of biotin-DNA from both TE buffer and transcription buffer (10 mM HEPES [pH 7.9], 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT) (Table 1). However, the selective retention of biotin-DNA from a standard transcription reaction mixture was significantly reduced. Furthermore, these resins exhibited various degrees of nonspecific retention of nonbiotinylated DNA or S100 proteins essential for transcriptional activity or both. This nonspecific retention can be best attributed to the presence of charged groups on the resident protein (i.e., avidin, streptavidin, BSA, or immunoglobulin G). In addition, molecular sieving effects of the Sepharose appeared to reduce the transcriptional activity of the S100 extract.

To circumvent these problems, we synthesized a biotin-cellulose resin (see Materials and Methods). The resin is electrically neutral and exhibits no apparent molecular sieving. The biotin-cellulose selectively retained avidin-complexed DNA from both TE buffer and transcription buffer as well as from a standard transcription reaction mixture, with negligible retention of normal DNA (Table 1). Furthermore, chromatography of the HeLa S100 extract on biotin-cellulose had no effect on its transcriptional activity (data presented below).

Are biotinylated templates faithfully transcribed? The transcriptional fidelity of the VA RNA genes was unaltered in the linearized and biotinylated templates (Fig. 2, compare lanes 4, 8, and 9). In addition, the presence of avidin in the S100 extract did not interfere with the transcription process (Fig. 2, compare lanes 10 and 11).

Effect of ionic strength on transcription. It was important to establish the ionic strength optima with respect to transcription of the VA RNA and tRNA genes for two reasons. First, we wanted to establish the KCl optimum for stable complex formation. Second, the TFs were to be eluted from their DNA templates by the addition of KCl and subsequently assayed by their addition to transcription reactions.

Weil et al. (29) reported that pol III transcription of Ad2 DNA was optimal at 60 to 100 mM KCl; we found that pA2-wt and its linear derivative were transcribed efficiently from 50 to 125 mM KCl, with optimal transcription at 85 to 100 mM KCl (Fig. 2, lanes 1 to 7). Similar results were obtained with the human $tRNA_i^{Met}$ gene (data not shown). We therefore set our KCl optimum for stable complex formation at 100 mM. Add-back assays were designed to initiate at a low KCl level such that the resulting increase in ionic strength upon the addition of the salt-eluted fractions would not inhibit transcription.

Establishment of optimum template concentration for stable complex formation. The incorporation of affinity-labeled genes into stable complexes was performed at the minimum template concentration necessary to bind the high-affinity TF(s) present in the reaction mixture. The purpose of this was twofold: we wanted to (i) eliminate any flowthrough of activity owing to free TFs during the subsequent biotin-cellulose chromatography procedure and (ii) minimize the

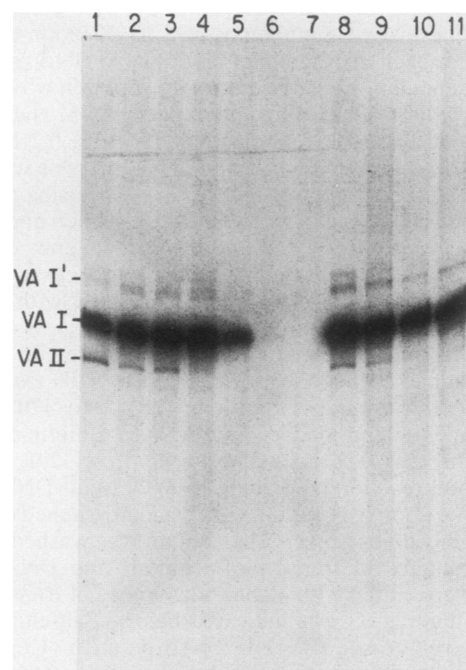

FIG. 2. Effects of KCl concentration, linearization and biotinylation of DNA template, and Avidin-DN on transcription of the VA RNA gene. An autoradiograph of a polyacrylamide gel of in vitro transcription products is shown. Lanes 1 to 7, Transcripts produced from 20 μg of supercoiled VA DNA (pA2-wt) per ml in the presence of 50, 70, 85, 100, 125, 150, or 200 mM KCl, respectively. The VAI' species results from the polymerase occasionally reading beyond the normal termination site for the VAI RNA to a second site approximately 200 nucleotides from the transcription initiation site. The VAI and VAII RNAs migrate differently in the gels (containing 8 M urea), although both are 160 nucleotides in length (11). Lane 8, Transcripts produced from 20 μg of linear VA DNA (pA2-wt/*Eco*RI) per ml. Lane 9, Transcripts produced from 20 μg of biotinylated linear VA DNA (biotin-pA2-wt/*Eco*RI) per ml. Lanes 10 and 11, Transcripts produced from 20 μg of biotinylated, linear VA DNA per ml with the addition of buffer or 4.5 μg of Avidin-DN, respectively, after 15 min. In these two reactions, [α-^{32}P]GTP was added after 25 min for a 1-h pulse. The KCl concentration in the reactions shown in lanes 8 to 11 was 70 mM.

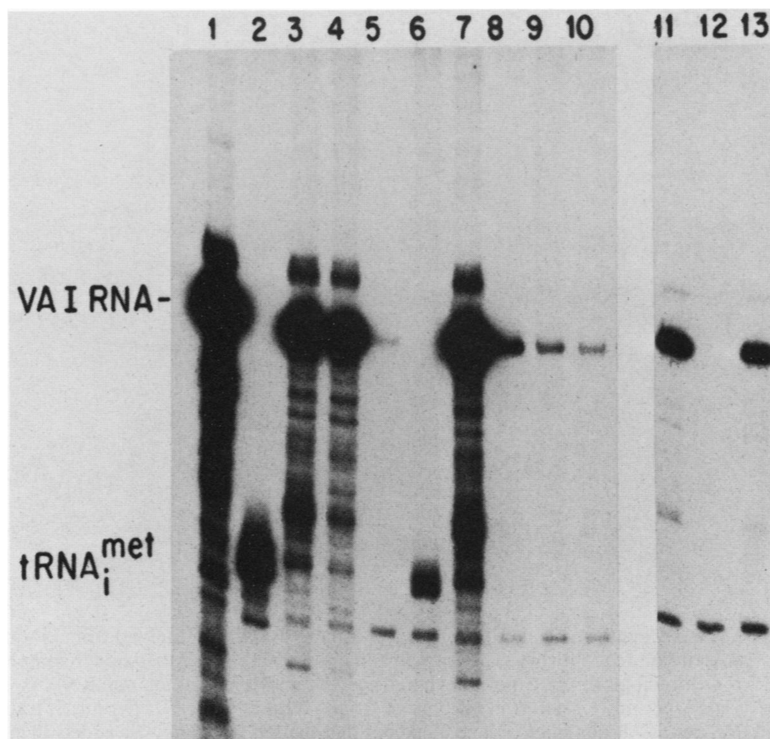

FIG. 3. Biotin-cellulose chromatography of avidin-biotin-VA DNA stable complexes. An autoradiograph of a polyacrylamide gel of in vitro transcription products is shown. Lanes 1 to 7 and 11 show products synthesized by extracts not subjected to biotin-cellulose chromatography; lanes 8 to 10 and 12 and 13 show products synthesized by chromatographed extracts. Lanes: 1 and 2, transcripts produced from 20 μg of VA DNA and tRNA$_i^{Met}$ DNA per ml, respectively, by the initial HeLa S100 extract; 3, transcripts produced by the S100 extract after a 45-min preincubation with 15 μg of avidin-biotin-VA DNA per ml; 4, duplicate of lane 3, except that 10 μg of tRNA$_i^{Met}$ DNA per ml was added after the preincubation; 5, transcripts produced by the S100 extract after a 45-min preincubation with no DNA; 6, duplicate of lane 5, except that 10 μg of tRNA$_i^{Met}$ DNA per ml was added after the preincubation; 7, transcripts produced by a sample of the programmed extract held at room temperature during the chromatography procedure; 8 to 10, transcripts produced by the programmed flowthrough fraction from endogenous VA DNA alone, 10 μg of exogenously added VA DNA per ml, or 10 μg of exogenously added tRNA$_i^{Met}$ DNA per ml, respectively; 11, transcripts produced by a sample of the unprogrammed extract held at room temperature during the chromatography procedure. (VA DNA [10 μg/ml] was added); 12 and 13, transcripts produced by the unprogrammed flowthrough fraction from endogenous DNA alone or 10 μg of exogenously added VA DNA per ml, respectively. All reactions were pulse-labeled for 1 h with [α-^{32}P]GTP.

level of nonspecific DNA-binding proteins retained on the column as a result of interaction with vector sequences. The optimum template concentration was determined by using the template competition assay developed by Bogenhagen et al. (5). This assay measures the ability of one template, when incubated in an extract, to inhibit transcription of a second template added subsequently. A series of competition experiments with various concentrations of pA2-wt (VA RNA gene) and phH2D (human tRNA$_i^{Met}$ gene) demonstrated that 15 μg/ml was the optimum plasmid template concentration for stable complex formation (data not shown).

Isolation of stable complexes on biotin-cellulose. The biotinylated VA RNA gene was incubated with avidin to form the avidin-biotin-DNA complex. HeLa cell S100 extracts were programmed with 15 μg of the affinity-labeled VA RNA gene per ml for 45 min at 30°C to allow for the formation of stable preinitiation transcription complexes. The transcription mixture was then chromatographed over a biotin-cellulose affinity column. The results are shown in Fig. 3. A small sample of the S100 extract was removed for assay before the addition of the VA RNA gene probe as a reference for initial transcriptional activity of the extract (lanes 1 and 2). Assay of a small sample of the programmed extract after stable complex formation indicated that the template was being efficiently transcribed (lane 3) and had stably bound all available TFIIIC, since addition of the tRNA$_i^{Met}$ template resulted in no synthesis of the corresponding tRNA$_i^{Met}$ transcription product (lane 4). Analysis of the biotin-cellulose flowthrough fraction (lane 8) showed that >95% of the transcriptional activity was retained on the column. Biotin-cellulose chromatography of an unprogrammed extract (i.e., containing no DNA) resulted in complete recovery of transcriptional activity in the flowthrough fraction (compare lanes 11 and 13).

To monitor the loss of transcriptional activity solely owing to time, a sample of the programmed extract was held at 30°C for the 45-min incubation period and at room temperature for the duration of the chromatography procedure. The transcriptional activity remained stable (Fig. 3, compare lanes 1, 3, and 7). However, in the absence of DNA, the activity of the S100 extract decayed significantly with time (compare lanes 2 and 6 and lanes 1 and 11). This is consistent with previous results showing that the addition of template to an S100 extract stabilizes the transcriptional activity of the extract (data not shown), presumably owing to the sequestration of the transcription factors into stable complexes with the template DNA.

The retention of the affinity-labeled VA DNA on the biotin-cellulose resin was measured by Cerenkov counts, as the gene was colabeled with [α-^{32}P]dCTP in the biotinylation

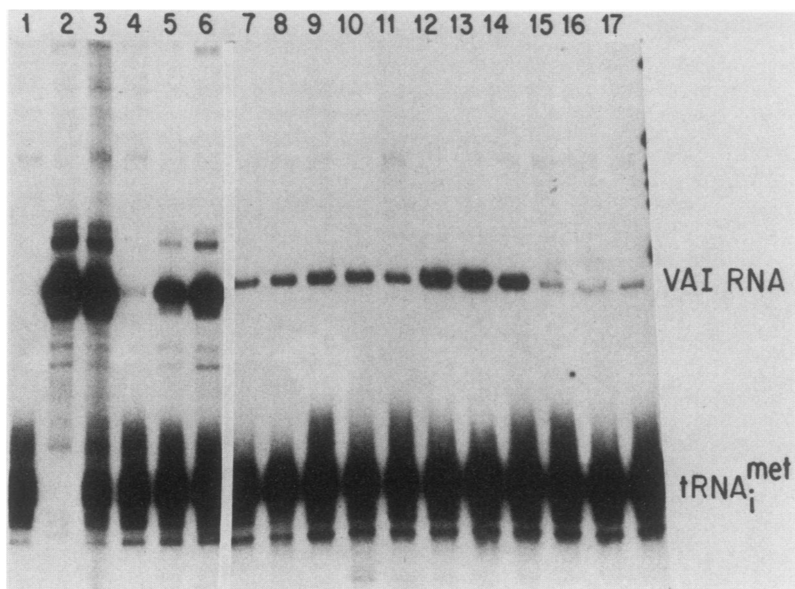

FIG. 4. Complementation for VA RNA synthesis in factor-depleted extract by fractions obtained from biotin-cellulose chromatography of VA gene programmed extract. Transcription reactions were performed and processed as described under competition-complementation assay (see Materials and Methods). An autoradiograph of a polyacrylamide gel of in vitro-synthesized RNA is shown. Lanes: 1, transcripts produced from 1.5 μg of $tRNA_i^{Met}$ DNA per ml; 2, transcripts produced from 1.5 μg of VA DNA per ml; 3, transcripts produced from 1.5 μg each of $tRNA_i^{Met}$ DNA and VA DNA per ml; 4, duplicate of lane 3, except that the VA template was added 20 min after the $tRNA_i^{Met}$ template (this constitutes the control reaction for determining the extent of transcriptional activation by added fractions). Lanes 5 to 17 are duplicates of lane 4, except that the fractions indicated below were added 10 min after the VA template addition: 5 and 6, 2 and 4 μl, respectively, of S100 extract; 7 and 8, 1.5 and 3.0 μl, respectively, of a 0.5 M KCl eluate from a VA gene-programmed extract chromatographed on biotin-cellulose; 9 and 10, 1.5 and 3.0 μl, respectively, of a 1.0 M KCl eluate obtained from a VA gene-programmed extract chromatographed on biotin-cellulose (the KCl concentration of the eluate was reduced to 0.5 M); 11, 1.5 μl of the 1.0 M KCl eluate described above, before desalting; 12 and 13, 1.5 and 3.0 μl, respectively, of a 1.5 M KCl eluate obtained from a VA gene-programmed extract chromatographed on biotin-cellulose (the KCl concentration of the eluate was reduced to 0.5 M); 14, 1.0 μl of the 1.5 M KCl eluate described above, before desalting; 15 to 17, duplicates of lanes 12 to 14, except that an unprogrammed extract was chromatographed.

procedure. In this experiment, 95% of the DNA was retained on the column. The activity in the flowthrough fraction (lane 8) thus resulted from the 5% unbound biotin-VA RNA gene which had already formed stable complexes since addition of [α-^{32}P]GTP alone yielded transcription. Addition of VA or tRNA template DNAs yielded no additional transcription (lanes 9 and 10), indicating the absence of free TFs in the flowthrough fraction. We therefore concluded that we had selectively and virtually quantitatively retained VA RNA gene stable complexes on a biotin-cellulose affinity column.

Elution of TFs from stable complexes. To determine the ionic strength necessary to release TFs from the VA DNA, we chromatographed the DNA-protein complexes over three separate biotin-cellulose columns. After the resins were washed extensively with transcription buffer (0.1 M KCl), the columns were eluted by the addition of transcription buffer containing 0.5, 1.0, or 1.5 M KCl. Fractions were concentrated 20-fold, and the KCl concentration of the 1.0 and 1.5 M fractions was reduced to 0.5 M (see Materials and Methods). The fractions were then analyzed for transcriptional activity by a competition-complementation assay. The results (Fig. 4) demonstrate that the $tRNA_i^{Met}$ gene is an effective competitor for a factor(s) required for transcription of the VA RNA gene, completely inhibiting VA RNA synthesis when preincubated for 20 min at 30°C in an S100 extract (lane 4). This inhibition was fully relieved by complementation with S100 extract (lanes 5 and 6). The addition of the 0.5 M (lanes 7 and 8), 1.0 M (lanes 9 to 11), or 1.5 M fraction (lanes 12 to 14) resulted in partial complementation for VA RNA synthesis, with the activity of the fractions increasing markedly as the ionic strength of the eluant was increased.

To test whether the activity was indeed eluted from DNA-protein complexes bound to the affinity resin and was not due to proteins sticking nonspecifically to the resin, we chromatographed a transcription mixture containing no DNA over biotin-cellulose. A 1.5 M KCl fraction was obtained, as above, and shown to contain no activity (Fig. 4, lanes 15 to 17). We conclude that the active fractions contain TF which are released from the column-bound DNA upon the addition of high-ionic-strength buffer. The majority of the activity was not released until the KCl concentration reached 1.5 M, indicating that the factor(s) is very tightly bound to the VA DNA template.

The VAI and VAII RNA genes made up only 6% of the biotin-VA DNA template used to program the S100 extract; the remainder consisted of flanking Ad2 sequences (27%) and the pBR322 vector sequences (67%). These additional sequences might be expected to be capable of associating nonspecifically with DNA-binding proteins since pol III transcribes a pBR322 "template" in a random fashion (data not shown). To determine the extent of nonspecific DNA-protein complex formation and its salt stability relative to the gene-specific interaction, we programmed the S100 extract with an avidin-biotin-pBR322 DNA probe. The pBR322 DNA-protein complexes were chromatographed over five biotin-cellulose columns. Four of the columns were step eluted with a first step of (i) 0.3 M KCl, (ii) 0.4 M KCl, (iii) 0.5 M KCl, or (iv) 1.0 M KCl, respectively, followed by a

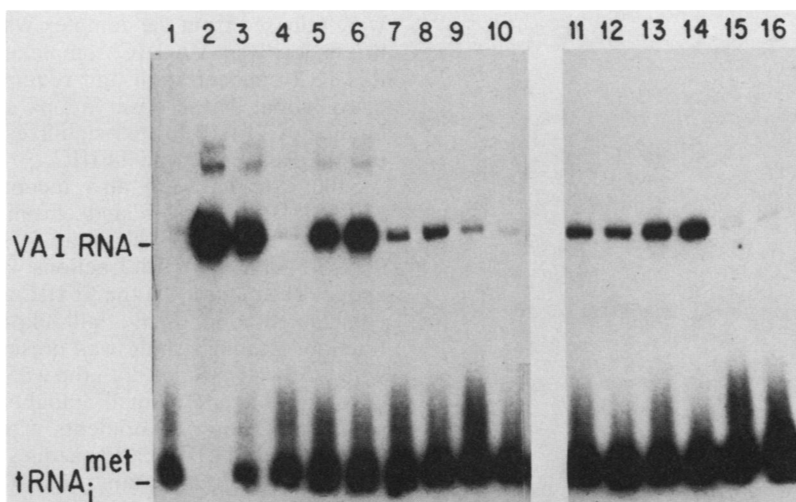

FIG. 5. Complementation for VA RNA transcription in factor-depleted extract by biotin-cellulose chromatographic fractions of pBR322-programmed S100. Conditions are as described in the legend to Fig. 4. An autoradiograph of a polyacrylamide gel of in vitro-synthesized RNA is shown. Lanes: 1 and 2, transcripts produced from 1.5 μg of tRNA$_i^{Met}$ DNA and VA DNA per ml, respectively; 3, transcripts produced from 1.5 μg each of tRNA$_i^{Met}$ DNA and VA DNA per ml; 4, duplicate of lane 3, except that the VA template was added 20 min after the tRNA$_i^{Met}$ template. Lanes 5 to 16, duplicates of lane 4, except that the fractions indicated below were added 10 min after VA template addition: 5 and 6, 2.0 and 4.0 μl, respectively, of S100 extract; 7 and 8, 1.5 and 3.0 μl, respectively, of a 0.3 M KCl eluate obtained from biotin-cellulose chromatography of a pBR322-programmed extract; 9 and 10, 1.5 and 3.0 μl, respectively, of a 1.5 M KCl eluate obtained subsequent to the 0.3 M KCl eluate described above (the KCl concentration of the 1.5 M KCl fraction was reduced to 0.5 M); 11 and 12, 1.5 and 3.0 μl, respectively, of a direct 1.5 M KCl eluate obtained from biotin-cellulose chromatography of a pBR322-programmed extract (the KCl concentration was reduced to 0.5 M); 13 and 14, duplicates of lanes 11 and 12, except that the extract was programmed with the VA gene; 15 and 16, duplicates of lanes 11 and 12, except that an unprogrammed extract was chromatographed.

second step of 1.5 M KCl. The fifth column was eluted directly with 1.5 M KCl. The competition-complementation assay of the salt-eluted fractions (Fig. 5) indicated that TFs do associate with pBR322 DNA but that those associations are relatively weak, being disrupted at ionic strengths as low as 0.3 M KCl (compare lanes 7 and 8 to lanes 9 and 10). In addition, the one-step 1.5 M KCl fraction (lanes 11 and 12) was significantly less active than an analogous VA DNA 1.5 M KCl fraction (lanes 13 and 14), suggesting that the VA gene-containing plasmid bound more factors than the plasmid alone. These results indicate that in experiments with the VA DNA probe, the less-active 0.5 M KCl fraction contained factors eluted from vector sequences alone, while the 1.5 M KCl fraction, which exhibited 7- to 10-fold greater activity, contained factors eluted preferentially from the VA RNA gene sequences.

On the basis of the data presented above, we predicted that we could discriminate between gene-specific and nonspecific DNA-protein association by performing an intermediate salt step before elution with 1.5 M KCl. To test this hypothesis, we programmed the S100 extract with the affinity-labeled pA2-wt DNA, chromatographed the reaction over a biotin-cellulose column, and eluted fractions by the addition of 0.25 M KCl, followed by 1.5 M KCl. The competition-complementation assay of these fractions (Fig. 6) showed that some activity was released from the DNA at 0.25 M KCl (lane 6); however, the majority of the activity was released at 1.5 M KCl (lane 7), as predicted. Combination of the two fractions did not enhance or diminish the degree of complementation; the effects were strictly additive (lane 8). To further determine the efficacy of 1.5 M KCl in releasing factors from the complex, we conducted the same experiment, adding a third salt step of 3.0 M KCl. No activity was recovered in the 3.0 M KCl eluate (data not shown), indicating that 1.5 M KCl was sufficient for total disruption of the complex. We conclude that we selectively retained transcriptional activity owing to the specific association of TFs with the VA RNA gene. Nonspecific DNA-binding proteins can be eluted by an intermediate salt step (0.25 to 0.40 M KCl), leaving primarily gene-specific material on the column. This specific factor(s) is most effectively released upon the addition of 1.5 M KCl, further exemplifying the stability of the gene-factor(s) complex.

TFIIIB and TFIIIC both can be stably sequestered on, and eluted from, the VA RNA gene. Since only TFIIIC is stably bound by the tRNA$_i^{Met}$ template during a 20-min preincubation period (20), the competition-complementation assay described above was only capable of detecting the presence of TFIIIC in the affinity-selected fractions, i.e., the presence of TFIIIB was indiscernible by this assay method. The stable sequestration of TFIIIB by a tRNA-IIIC or VA-IIIC stable complex has not been previously demonstrated. Furthermore, an extended incubation (80 to 100 min for maximal effect) was required for the stable binding of TFIIIB to the 5S-IIIA-IIIC stable complex (3, 8). If the kinetics of factor IIIB association with the VA-IIIC stable complex are analogous to those for the 5S-IIIA-IIIC complex, one would not expect to see any IIIB activity in the KCl eluates since the incubation period allowed for stable complex formation before biotin-cellulose chromatography was only 45 min. Indeed, this was the case, i.e., all attempts to reconstitute transcription with the purified fractions and purified pol III failed (data not shown). However, when the incubation period was increased to 120 min, the stable association of TFIIIB with the VA-IIIC complex was effected. Reconstitution experiments with the subsequent KCl-eluted fractions showed that the 1.5 M KCl eluate supported transcription of the VA RNA gene in the presence of exogeneous pol III (Fig. 7, lane 4), indicating that this fraction contained both IIIB and IIIC. Likewise, the 0.25 M KCl fraction transcribed

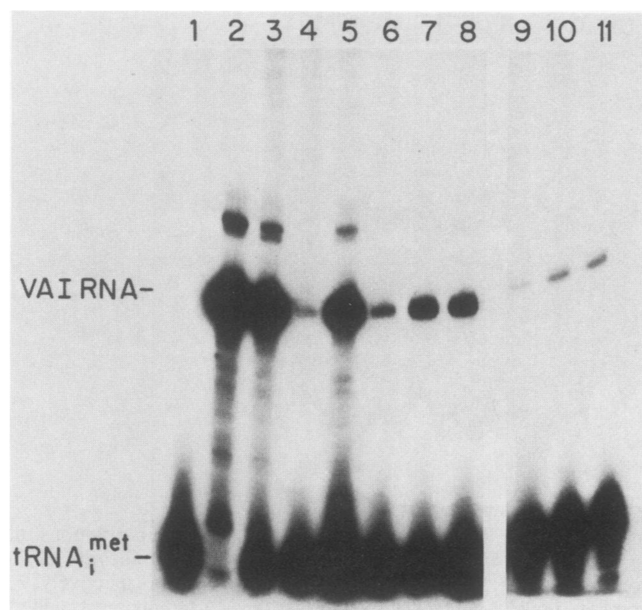

FIG. 6. Analysis of KCl step-eluted fractions obtained from biotin-cellulose chromatography of extracts programmed with VA DNA. Conditions are as described in the legend to Fig. 4. An autoradiograph of a polyacrylamide gel of in vitro transcription products is shown. Lanes: 1 and 2, transcripts produced from 1.5 μg of tRNA$_i^{Met}$ DNA and VA DNA per ml, respectively; 3, transcripts produced from 1.5 μg each of tRNA$_i^{Met}$ DNA and VA DNA per ml; 4, duplicate of lane 3, except that the VA template was added 20 min after the tRNA$_i^{Met}$ template. Lanes 5 to 14, Duplicates of lane 4, except that the following additions were made 10 min after VA template addition: 5, 2.0 μl of S100 extract; 6, 2.0 μl of a 0.25 M KCl eluate obtained from biotin-cellulose chromatography of a VA gene-programmed S100 extract; 7, 2.0 μl of a subsequent 1.5 M KCl eluate obtained from the column described above; 8, 1.5 μl each of the 0.25 and 1.5 M KCl eluates described for lanes 6 and 7; 9 to 11, duplicates of lanes 6 to 8, except that an unprogrammed extract was chromatographed.

the VA gene in the presence of pol III (lane 2), although to a lesser extent. The level of transcription effected by the 3.0 M KCl fraction was minimal (lane 6). Pairwise combinations or a mixture of the three fractions resulted in strictly additive effects, i.e., no synergism or inhibitory effects were observed (data not shown). In addition, no transcription was observed in the absence of exogenous template (data not shown) and pol III (compare lanes 1, 3, and 5 with lanes 2, 4, and 6).

The reconstitution experiment described above verified the presence of TFIIIB and TFIIIC in the 0.25 and 1.5 M KCl eluates, with the 1.5 M KCl fraction possessing the greatest activity. To determine whether the binding of IIIB altered the elution profile of IIIC from the VA complex, we analyzed the salt fractions described above for IIIC activity using the competition-complementation assay. The results (Fig. 8) demonstrated that little, if any, activity was released from the VA DNA at 0.25 M KCl (lane 6) but that activity was released at 1.5 M KCl (lane 7), as expected. Strikingly, the majority of the activity remained associated with the VA RNA template at 1.5 M KCl and was released only upon the addition of 3.0 M KCl (lane 8). All possible combinations of the three fractions resulted in strictly additive effects (data not shown). Collectively, the results demonstrated that incubation for 120 min at 30°C was sufficient for VA-IIIC-IIIB stable complex formation. TFIIIB and some TFIIIC were released from the complex with 1.5 M KCl. However, the majority of VA-IIIC complexes were not disrupted at this KCl concentration and required 3.0 M KCl for thier dissociation. These observations strongly suggest that the binding of TFIIIB further stabilizes the interaction between the VA RNA gene and TFIIIC.

S100 extracts were also incubated with avidin-biotin-pBR322 DNA for 2 h and chromatographed over biotin-cellulose, and the distribution of TFIIIC and TFIIIB in the 0.25, 1.5, and 3.0 M salt fractions was analyzed as described above. The majority of the TFIIIC activity, measured by the complementation assay, still eluted in the 0.25 M KCl fraction, although some was present in the fraction eluted with 1.5 M KCl. Since both the 0.25 and 1.5 M KCl fractions gave approximately equal amounts of VA transcription in the reconstitution experiments with exogenous pol III, we conclude that TFIIIB can be sequestered on pBR322-TFIIIC complexes and that its binding also stabilizes the interaction of TFIIIC with nonspecific DNA (data not shown).

Protein profiles of affinity-selected TFs. The protein composition of the fractions obtained from the biotin-cellulose chromatography of programmed S100 extracts is shown in Fig. 9. The majority of the proteins in the extract were recovered in the flowthrough (Fig. 9A, lane FT) and initial wash (Fig. 9A, lanes 1 to 4) fractions. After the fifth wash, no protein was observed in the wash fractions (Fig. 9A, lanes 5 and 6). The protein profiles of the fractions eluted from the VA DNA (Fig. 9B, lanes 1 to 6) and pBR322 DNA (lanes 11 and 12) demonstrate the selective enrichment of several proteins. These represent bona fide DNA-binding proteins as they were not present in fractions eluted from an unprogrammed (no DNA) column (Fig. 9B, lanes 7 to 9). However,

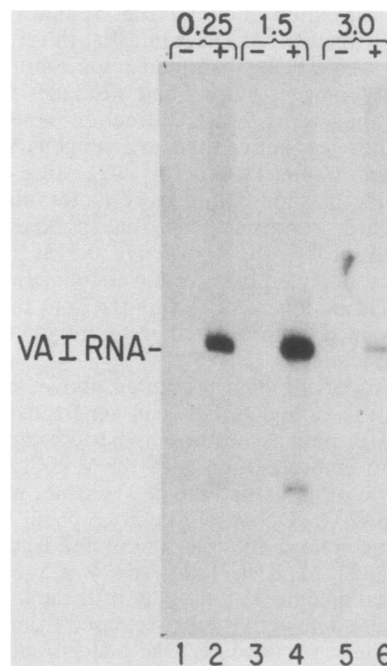

FIG. 7. Reconstitution of transcription with purified fractions. Transcription reactions were performed and processed as described under transcription reconstitution assay (see Materials and Methods). An autoradiograph of a polyacrylamide gel of in vitro-synthesized RNA is shown. The component(s) being assayed in each is the 0.25 M KCl fraction (lanes 1 and 2); the 1.5 M KCl fraction (lanes 3 and 4); and the 3.0 M KCl fraction (lanes 5 and 6). Lanes 2, 4, and 6 also contained purified pol III.

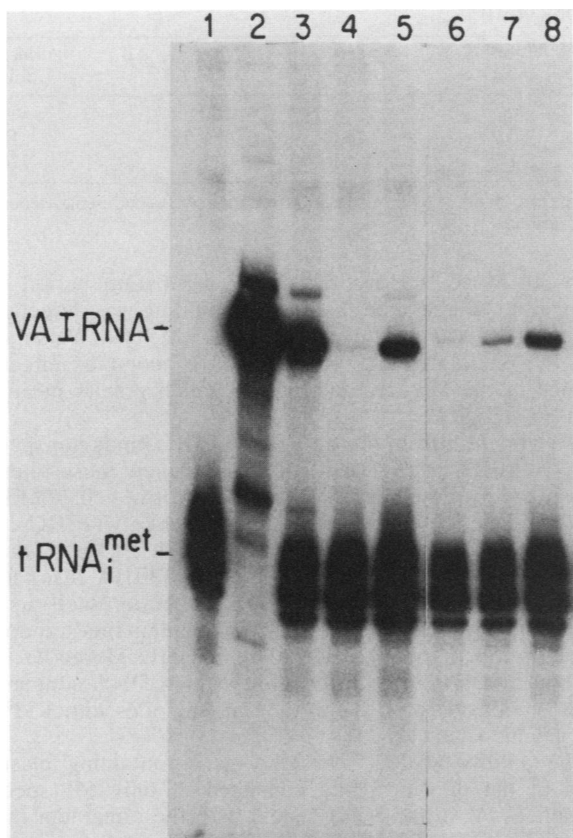

FIG. 8. Analysis of KCl step-eluted fractions obtained from biotin-cellulose chromatography of extracts incubated for VA DNA for 120 min. Conditions are as described in the legend to Fig. 4. Autoradiograph of a polyacrylamide gel of in vitro transcription products is shown. Lanes: 1 and 2, transcripts produced from 1.5 μg of tRNA$_i^{Met}$ DNA and VA DNA per ml, respectively; 3, transcripts produced from 1.5 μg each of tRNA$_i^{Met}$ DNA and VA DNA per ml; 4, duplicate of lane 3, except that the VA template was added 20 min after the tRNA$_i^{Met}$ template. Lanes 5 to 14, duplicates of lane 4, except that the following additions were made 10 min after VA template addition: 5, 1.0 μl of S100 extract; 6, 2.5 μl of a 0.25 M KCl eluate obtained from biotin-cellulose chromatography of a VA gene-programmed S100 extract; 7, 2.5 μl of the subsequent 1.5 M KCl eluate obtained from the column described above; 8, 2.5 μl of the subsequent 3.0 M KCl eluate from the same column.

there is a dramatic difference between the elution profiles of the VA DNA and pBR322 DNA columns. All the proteins associated with pBR322 DNA appear to be released at 0.3 M KCl (lane 12) with no additional protein recovered in a 1.5 M KCl eluate (lane 11), consistent with the activity profile of these fractions (data presented above). Some protein is also eluted from the VA DNA at 0.25 M KCl (lanes 1 and 4); however, in contrast to the pBR322 profile, several species remain associated with the VA DNA and are preferentially released at 1.5 M KCl (lanes 2 and 5) or 3.0 M KCl (lanes 3 and 6). It is also apparent that by increasing the template-extract incubation period from 1 to 2 h before biotin-cellulose chromatography, one can detect several proteins in the 3.0 M KCl eluate (compare lanes 3 and 6 in Fig. 9B) that did not appear in the 0.25 and 1.5 M KCl fractions (lanes 1, 2, 4, and 5). Furthermore, since these proteins were not detected in either a 1.5 M (Fig. 9B, lane 11) or a 3.0 M (data not shown) KCl eluate obtained after programming the HeLa cell extract with biotinylated pBR322 DNA, we conclude

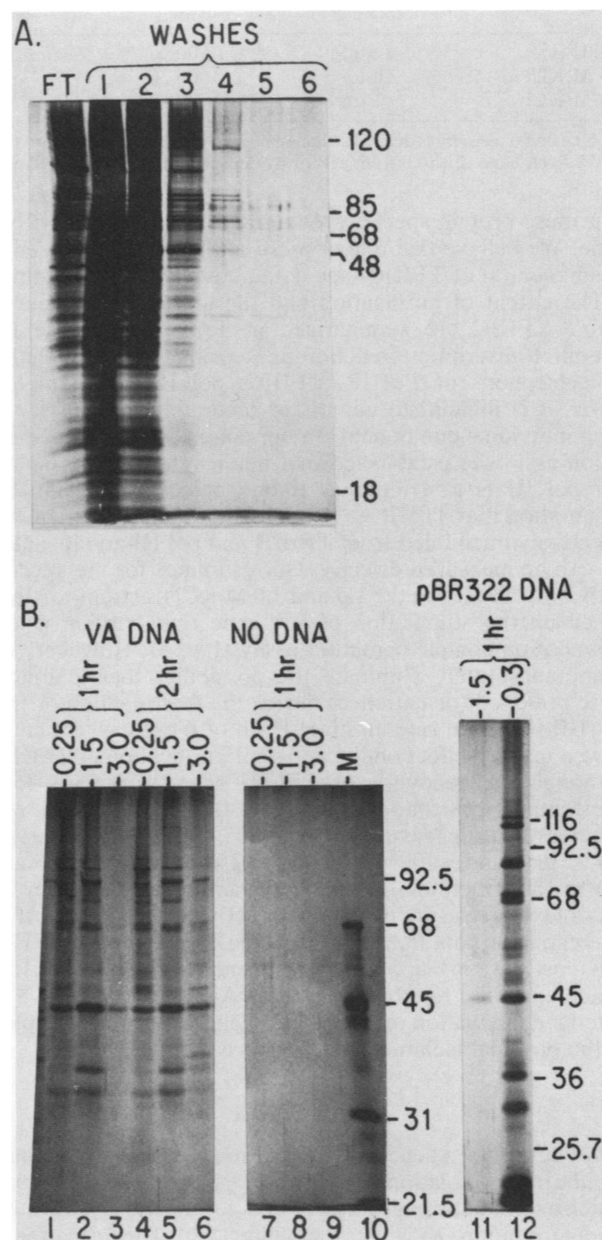

FIG. 9. Fractionation of HeLa S100 on biotin-cellulose. Polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis on 15% (A) or 10% (B) gels followed by staining with silver as described in Materials and Methods. (A) Lanes: FT, 1 μl of the flowthrough fraction; 1, 8 μl of the first 0.10 M KCl wash; 2 to 6, 16 μl of each of the second through sixth 0.10 M KCl washes, respectively. (B) Lanes 1, 2, and 3, The 0.25, 1.5, and 3.0 M KCl fractions, respectively, eluted from a VA DNA-programmed column. The DNA was incubated in the extract for 1 h before chromatography. Lanes 4, 5, and 6 are the same as lanes 1 to 3 except that the incubation period was increased to 2 h. Lanes 7, 8, and 9 are the same as lanes 1 to 3, except that the DNA was omitted. Lane 10, Molecular weight standards (numbers on right [$\times10^3$]). Lanes 11 and 12, 1.5 and 0.3 M KCl fractions, respectively, eluted from a column programmed with biotin-pBR322 DNA. The DNA was incubated in cell extract for 1 h.

TABLE 2. Purification of TFIIIC

Fraction	Protein concn (per ml)	Total vol (ml)	Total protein	Total units[a]	Sp act (U/mg)	Yield (%)	Purification (fold)
S100	7.5 mg	0.800	6 mg	90	15	100	1
1.5 M KCl	20 μg	0.050	1 μg	0.23	230	0.25	15
3.0 M KCl	2 μg	0.050	0.1 μg	1.2	11,600	1.3	774

[a] One unit of activity is defined as the number of specific transcripts synthesized per gene in a 45-min reaction at 30°C. Quantitation by direct Cerenkov counting of VA RNA bands excised from gels or by densitometric analysis of films gave similar results.

that these protein species are interactive with the VA RNA gene. We believe that one or more of these may represent a component(s) of TFIIIC (see data above and Discussion).

The extent of purification and the approximate yield of active TFIIIC are summarized in Table 2. Because the overall transcription reaction is sensitive to the relative concentrations of TFIIIB, TFIIIC, pol III, and template DNA, it is difficult to quantitate accurately the activity of each individual component. In our competition-complementation assay we established that neither the DNA template nor pol III is rate limiting. If one makes the reasonable assumption that TFIIIC is the sole limiting factor, then it is effectively uncoupled from TFIIIB and pol III and its activity can be measured directly. Our estimates for the specific activity of TFIIIC in the 1.5 and 3.0 M KCl fractions are thus based on the stimulation of VA gene transcription in the competition-complementation assay (Fig. 8). However, we cannot absolutely eliminate the possibility that inhibitors were removed (or enriched) during the fractionation or that TFIIIB is indeed rate limiting. Each of these caveats could have a marked effect on the apparent purification of TFIIIC. Although the recovery of TFIIIC activity appears low, substantial purification of both TFIIIB and TFIIIC was effected. In a single chromatographic procedure, we selected 1.3×10^{-4} and $\sim 10^{-5}$ of the starting protein in the 1.5 and 3.0 M KCl fractions, respectively, and achieved nearly an 800-fold increase in the specific activity of TFIIIC. It is interesting to note that this extensive enrichment of TFIIIC was possible only upon the stable sequestration of TFIIIB, which appeared to strengthen the VA-IIIC interaction. The extended incubation may well be a general avenue to exploit in the physical isolation of other class III TFs.

DISCUSSION

We described a general affinity chromatography procedure for the rapid isolation of specific DNA-protein complexes which exploits the high affinity of the tetrameric glycoprotein avidin for biotin ($K_D = 10^{-15}$ mol/liter) (10). The formation of stable complexes between eucaryotic class III genes and protein factors in crude cellular extracts (3, 5, 20) makes it feasible to isolate and characterize components required for the transcription of a specific gene, using a single biotin-cellulose chromatography step. This study demonstrates the isolation of TFIIIB and TFIIIC from a HeLa S100 extract as a result of their high affinities for the VA RNA gene. Previous studies have shown that IIIC binds to the VAI RNA gene within several minutes to form a stable complex (20). We demonstrate here the stable sequestration of TFIIIB after an extended incubation period (120 min). The integrity of VA-IIIC-IIIB stable complex was further illustrated by the requirement of high KCl concentrations for its disruption. TFIIIB was effectively released from the VA-IIIC-IIIB complex by 1.5 M KCl. Although some IIIC activity was also released at 1.5 M KCl, the majority remained bound to the VA DNA and was finally dissociated with 3.0 M KCl. However, if the incubation period was reduced to 60 min such that no IIIB binding was detectable, the VA-IIIC was fully disrupted by 1.5 M KCl. We suggest that the binding of IIIB to the VA-IIIC complex effects a change in the VA-IIIC interaction which results in an increased stability.

It is apparent from our data that TFIIIC binds nonspecifically to pBR322 DNA sequences. However, these interactions were disrupted at ionic strengths as low as 0.30 M KCl (Fig. 5, lanes 7 to 10). The interaction of TFIIIC with pBR322 DNA is not surprising since TFIIIC is a strong DNA-binding protein. The 5S-specific TFIIIA also binds nonspecifically to pBR322 DNA (14) as well as to bacteriophage λ DNA (30). This could explain the "random" transcription of pBR322 DNA by pol III. Moreover, the inhibition of specific transcription at high DNA concentrations is believed to be due to the trapping of essential TFs at nonspecific sites (13, 29).

The affinity-labeled VA RNA gene-containing plasmid used in the present study consisted of only 6% specific sequences (VAI and VAII genes) with the remainder made up of flanking Ad2 DNA and pBR322 vector sequences. Nevertheless, this probe was extremely effective in the selection of TFIIIB and TFIIIC from a crude cytoplasmic extract. While nonspecific interactions of DNA-binding proteins with either vector or flanking sequences might be expected to be reduced further by eliminating these nonspecific sequences, we have observed a similar degree of purification after programming the extract with either a 20-copy tandem VAI RNA gene clone containing 22% specific sequences or a 523-base-pair VAI RNA gene-containing fragment consisting of 31% specific sequences (data not shown). It will be interesting to repeat these experiments with a nonspecific competitor DNA such as poly(dI-dC).

Our studies demonstrate that after a single affinity enrichment step we achieved approximately an 800-fold increase in the specific activity of TFIIIC, coupled with a 100,000-fold decrease in total protein. This translates into a effective yield for biologically active TFIIIC of only 1 to 2% relative to the activity present in the original S100 extract. However, it is well known to those who have worked on the purification of TFIIIC and TFIIIB that both proteins are relatively unstable, especially when at low protein concentrations. Indeed, their apparent instability in the absence of template DNA and their relatively low cellular abundance are the major reasons why they have not as yet been purified to homogeneity. In a recent paper (18), Klekamp and Weil described a highly successful scheme for the partial purification of TFIIIB from *Saccharomyces cerevisiae* and reported a net increase of 130-fold in specific activity after fractionation over five columns, a yield of 1.3%, and an overall protein enrichment of 10,000-fold. Our data on the purification of TFIIIC are thus quite comparable. Furthermore, since our affinity fractionations were all done on a microscale, losses of biological activity may be reduced markedly upon scale-up.

The SDS-polyacrylamide gel electrophoretic analysis of a 3.0 M KCl fraction containing TFIIIC showed several prominent protein bands (Fig. 9B, lane 6), raising the possibility that the HeLa factor may be a multimeric species. This is consistent with the report by Ruet et al. (25) that the yeast pol III transcription factor, τ, which resembles HeLa TFIIIC in many respects, is a large macromolecule, sedimenting at a molecular weight of ~300,000 on a nondenaturing glycerol gradient. Alternatively, TFIIIC may be a single polypeptide which becomes stably associated with other TFs or pol III subunits. Our present data do not discriminate between these two possibilities. Nonetheless, since the 3.0 M KCl fraction is so highly enriched, it could serve as an immunogen to generate antibodies that might lead to the positive identification of the TFIIIC polypeptide(s).

The affinity chromatography procedure described in this report has potentially broad applications by which any nucleotide sequence can be employed as an affinity probe for the components of a specific DNA- or RNA-protein complex in which it resides, e.g., DNA replication proteins, eucaryotic class I and class II gene TFs, enhancer-sequence-binding proteins, RNA splicing or RNA transport proteins, provided these interactions are sufficiently stable to withstand the chromatographic conditions employed.

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