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The BN51 Protein Is a Polymerase (Pol)-Specific Subunit of RNA Pol III Which Reveals a Link between Pol III Transcription and Pre-rRNA Processing

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The three eukaryotic nuclear RNA polymerases (Pol) contain common and unique subunits. Cloning of the unique Pol III subunit genes in yeast cells has revealed a potential homolog in the mammalian system, the BN51 gene. The human BN51 gene was originally isolated as a suppressor of a temperature-sensitive cell cycle mutant of BHK cells (tsBN51). Although tsBN51 cells have a marked decrease in RNA Pol III activity at the nonpermissive temperature, direct biochemical evidence for the BN51 protein being a human Pol III subunit was lacking. Using antibodies directed against the BN51 protein, we show the following: (i) the BN51 protein copurifies with Pol III activity, (ii) Pol III activity can be specifically immunoprecipitated from HeLa nuclear extracts, and (iii) the immunopurified BN51 complex is active in restoring both nonspecific and promoter-specific Pol III activity. Our findings provide direct biochemical evidence for BN51 being a Pol III-specific subunit. Despite the fact that BN51 is not a subunit of Pol I, the production of mature Pol I transcripts is inhibited in tsBN51 cells at the nonpermissive temperature. tsBN51 cells appear defective in processing the 32S precursor rRNA into mature 5.8S and 28S rRNA at the nonpermissive temperature. We surmise that ribosome assembly has halted because of the loss of Pol III transcripts. Thus, there is regulation of the synthesis of mature Pol I transcripts by a posttranscriptional mechanism based on the availability of Pol III transcripts.

The eukaryotic nuclear genome is transcribed by three distinct multisubunit RNA polymerases. RNA polymerase I (Pol I) synthesizes pre-rRNA, which is processed in the developing ribosome into 5.8S, 18S, and 28S rRNAs. Pol II transcribes protein-encoding and small nuclear RNA (snRNA) genes. Pol III synthesizes a wide variety of small RNAs, including tRNAs, 5S rRNA, and U6 snRNA. Despite these differences in transcriptional activity, all three polymerases are composed of a homologous core of subunits which appear to be evolutionarily related to the core prokaryotic RNA polymerase (reviewed in reference 25). In addition to the polymerase core, there are five subunits that are shared between the polymerases. Each polymerase also has a set of subunits that are unique to that particular polymerase. These polymerase-specific subunits presumably endow each polymerase with the specificity necessary to transcribe the different classes of genes. How polymerase-specific subunits direct each polymerase to the appropriate promoter remains a fundamental unanswered question.

In the yeast *Saccharomyces cerevisiae*, for which polymerase subunits have been well characterized, four subunits (C31, C34, C53, and C82) are unique to Pol III (or Pol C). The genes for each subunit have been cloned and are required for yeast viability (3, 16, 17, 27). The gene which encodes the C53 (53-kDa) subunit was found to have 23% identity to a previously cloned human gene encoding the BN51 protein (10, 16). The BN51 gene was originally isolated by its ability to complement a temperature-sensitive cell cycle mutant of BHK cells (tsBN51) which arrest in G_1 at the nonpermissive temperature (39.5°C). Pol III activity is markedly reduced in tsBN51 cells at 39.5°C and in S100 extracts of these cells incubated at the nonpermissive temperature (10), but indirect effects leading to loss of Pol III activity cannot be excluded since direct biochemical evidence that the BN51 protein is a subunit of Pol III was lacking. In mammalian cells, polypeptides of 49 and 53 kDa were originally reported to be subunits of Pol III (26) and thus potential BN51 candidates. However, a subsequent study reported that both were contaminants, since their presence was variable from preparation to preparation (13). Thus, the issue as to whether the BN51 protein is a subunit of Pol III remains unresolved.

A further observation that limits the potential assignment of the BN51 protein as a unique subunit of Pol III is that tsBN51 cells, at the nonpermissive temperature, are defective in producing mature Pol I-synthesized 18S and 28S rRNAs (10). 5.8S and 28S rRNAs are derived through processing of a 32S precursor rRNA, which originates from a 45S primary rRNA transcript. Cleavage of the 45S transcript in the nucleolus not only results in the production of the 32S pre-rRNA but also yields the mature 18S rRNA. Maturation of rRNAs takes place in the context of ribosomal proteins, which ensures that RNA cleavage and ribosome subunit assembly are coordinated.

Since Pol I and Pol III transcript levels decrease when the BN51 protein is inactivated in vivo, the BN51 protein might be shared between Pol I and III, or BN51 might be a general regulator of tRNA and rRNA synthesis. Alternatively, the inability of BN51 cells to accumulate 18S and 28S rRNAs might be an indirect consequence of the absence of Pol III transcripts (such as 5S rRNA or snRNAs) which might be essential for the developing ribosomes to process pre-rRNA into mature rRNA.

This study addresses biochemically whether the BN51 protein is a subunit of Pol III or any other RNA polymerase. Using antibodies directed against the BN51 protein, we tracked the copurification of the BN51 protein with the three nuclear RNA polymerases. In addition, the anti-BN51 antibod-

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ies were used to immunoprecipitate BN51 from HeLa cell extracts and partially purified fractions. The depleted supernatants and the resulting immunoprecipitates were tested for promoter-specific and nonspecific Pol I, II, and III transcription activity. In all cases, the BN51 protein copurified with Pol III activity but not with Pol I or II activity. Our findings indicate that the BN51 protein is a Pol III-specific subunit. To reconcile the concomitant loss of the 18S and 28S rRNAs with the 5S and tRNAs in temperature-restricted tsBN51 cells, we examined the steady-state level of 28S's immediate precursor, the 32S pre-rRNA. When tsBN51 cells were placed at the nonpermissive temperature, the 32S pre-rRNA accumulated at the expense of the 28S and 5.8S rRNAs, whereas control BHK cells showed no such accumulation of precursor or loss of mature rRNA. This in vivo analysis reveals a tight coordinate regulation in the processing and assembly of Pol I- and III-transcribed rRNAs into the developing ribosome.

MATERIALS AND METHODS

DNA, antibodies, and buffers. The following plasmid templates have been previously described: pS-G_6TI (synthetic Sp1-responsive Pol II promoter), pBRVA_1 (adenovirus VA_1 RNA promoter), pXBS1 (*Xenopus* 5S rRNA promoter), pU6 (human U6 snRNA promoter), and pSBr208 (human rRNA promoter) (1, 20, 32). Rabbit polyclonal anti-BN51 antibodies were affinity purified by using pure recombinant BN51 protein as an immobilized ligand. Rabbit polyclonal anti-TAF250 antibodies were affinity purified by using pure recombinant TAF250 (248 C-terminal amino acids) as an immobilized ligand. H buffer contained 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8), 10% glycerol, 2 mM $MgCl_2$, 0.05 mM EDTA, 1 mM dithiothreitol, and KCl. For all buffers in the H series, the number after the H denotes the molar KCl concentration. TSB buffer contained 20 mM Tris-acetate (50% cation [pH 8]), 20% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 200 mM potassium glutamate, and 100 μM phenylmethylsulfonyl fluoride. Dialysis buffer contained 50 mM Tris-Cl (50% cation [pH 8]), 20% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and 240 mM KCl.

HeLa nuclear extract fractionation. HeLa nuclear extracts (22 mg) dialyzed to a conductivity equivalent to that of H.15 buffer were loaded on a 2-ml phosphocellulose column equilibrated with H.15 (4, 28). Sequential protein fractions, as monitored by UV spectroscopy, were eluted in an equal volume (6.5 ml) with H.15 (the flowthrough), H.3, H.5, H.7, and H1.0. The resulting phosphocellulose fractions were designated P.15, P.3, P.5, P.7, and P1.0, respectively. Each fraction was dialyzed against 4 liters of dialysis buffer for 7 h at 4°C.

Western blot (immunoblot) analysis. An equal volume of each phosphocellulose fraction or depleted phosphocellulose fraction was precipitated with 10% trichloroacetic acid. Samples were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (10%), transferred to nitrocellulose, and probed with 100 ng of affinity-purified rabbit anti-BN51 antibody and with alkaline phosphatase-conjugated secondary antibody.

Nonspecific polymerase assay. The following conditions were used in nonspecific polymerase assays: 12.5 mM Tris-Cl (50% cation [pH 8]), 60 mM KCl, 0.5 mM $MgCl_2$, 5% glycerol, 0.02 mM EDTA, 1 mM $MnCl_2$, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 10 μM UTP, 5 μCi of [α-^{32}P]UTP, 1.25 μg of poly(dA-dT) template, and phosphocellulose fractions as indicated in each figure legend, in a total volume of 20 μl. Three identical reactions, with either no α-amanitin, 0.5 or 200 μg of α-amanitin per ml, were set up. A fourth reaction, with no DNA and no α-amanitin, was also set up to measure background. In reactions which contained immunoprecipitates, the tubes were briefly vortexed every 3 min during the incubation period. All reaction mixtures were incubated at 30°C for 60 min, and then the reactions were terminated with an equal volume of 90% formamide–2% SDS. Five microliters of each terminated-reaction mixture was electrophoresed on a 7 M urea–6% polyacrylamide gel (20 cm wide, 15 cm high, and 0.4 mm thick) for 10 min at 1,000 V. Gels were dried, and the radioactivity migrating greater than 100 nucleotides was quantitated with a Beta-scope imaging system. Nonspecific polymerase activity for each polymerase ($A_{Pol\ x}$) was determined as follows: $A_{Pol\ I} = cpm_{200} - cpm_{bkd}$, $A_{Pol\ II} = cpm_0 - cpm_{0.5}$, and $A_{Pol\ III} = cpm_{0.5} - cpm_{200}$, where cpm denotes radioactivity, subscripts denote α-amanitin concentration, and bkd refers to the background reaction.

Promoter-specific transcription assays. Except where noted, all Pol I, II, and III promoter-specific transcription reactions were performed under identical conditions: 5 mM HEPES (50% anion [pH 7.5]), 1 mM Tris-Cl (50% cation [pH 8]), 1% polyvinyl alcohol, 60 mM KCl, 60 mM potassium glutamate, 1 mM spermidine (G_6TI required 2 mM spermidine), 3 mM $MgCl_2$, 10 μg of poly(dG-dC) per ml, 10 μg of DNA (containing the promoters indicated in each figure) per ml, 6% glycerol, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM GTP, 0.5 mM CTP, and one of the following two nucleotide combinations: 0.5 mM ATP–10 μM UTP–5 μCi of [α-^{32}P]UTP or 0.5 mM UTP–10 μM ATP–5 μCi of [α-^{32}P]ATP. (U6 required 20 μg of DNA template per ml and 3 ng of recombinant human TATA-binding protein.) Reaction mixtures also contained protein fractions as indicated in each figure. Reactions were performed in a total volume of 20 μl and were preincubated for 20 min at 30°C before the addition of nucleoside triphosphates. Reactions were allowed to proceed for 30 min and then terminated with 80 μl of stop mix (3.125 M ammonium acetate, 125 μg of tRNA per ml). In reactions which contained immunoprecipitates, the tubes were briefly vortexed every 3 min during both the preincubation and elongation periods. The RNA was extracted with a phenol-chloroform mixture, precipitated with ethanol, resuspended in 90% formamide, and electrophoresed on 7 M urea–6% polyacrylamide gels. Gels were dried and subjected to autoradiography with an intensifying screen. In these promoter-specific assays, Pol III terminates at the normal terminator of the gene, yielding transcripts of 164 nucleotides (nt) for VA_1, 120 nt for 5S, and 106 nt for U6. For G_6TI, Pol II terminates by running off the end of a linearized template, yielding a transcript of 175 nt. For the human rRNA promoter, pSBr208 was restricted with *Pvu*II, allowing Pol I to run off the template to generate a 290-nt transcript.

Immunodepletion. Affinity-purified anti-BN51 or anti-TAF250 antibodies were bound to protein A-Sepharose at a density of 0.1 mg of antibody per ml of resin. The resins were washed with H.7 buffer and equilibrated with H.15 buffer. Mock resins contained only protein A-Sepharose. Nuclear extract or protein fractions were mixed with the protein A-Sepharose/antibody resin in amounts indicated in each figure for 1.5 h at 0°C. Immunoprecipitates were centrifuged, and the supernatant was used as a source of depleted or mock-depleted (control) extract. The immunoprecipitates were washed with either H.15 buffer or TSB.

Cell culture. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum. tsBN51 and ts11 were routinely maintained at 32°C, while BHK cells were maintained at 37°C. The nonpermissive temperature was 39.5°C for ts11 and tsBN51 cells.

In vivo labeling and analysis of RNA transcripts. Cells were plated at 3×10^5 cells per 60-mm-diameter dish at 32°C. At specified times, plates were switched to 39.5°C. After the indicated incubation at 39.5°C, cells were labeled with either $^{32}P_i$ or [^{3}H]uridine. To label with $^{32}P_i$, cells were incubated for 1 h in phosphate-free minimal essential medium with 2% serum and then for 2 h in minimal essential medium with 2% serum containing 40 μCi of $^{32}P_i$ per ml. Total RNA was isolated as previously described (22). ^{32}P-labeled RNA transcripts were then analyzed on 7 M urea–6% acrylamide sequencing gels followed by autoradiography or separated on 1.2% agarose–formaldehyde gels (11), transferred to nylon membranes by capillary action, and subject to autoradiography. To label with [^{3}H]uridine, cells were incubated for 1 h in Dulbecco's modified Eagle's medium with 10% calf serum and 50 μCi of [^{3}H]uridine per ml. RNAs were extracted as described above. Equal counts per minute of ^{3}H-labeled RNAs were separated on 0.8% agarose–formaldehyde gels. Following electrophoresis, RNAs were transferred to nylon membranes as previously described (15). The membranes were sprayed with En^{3}Hance (Dupont NEN), and fluorography performed with X-ray film at −70°C.

RESULTS

The BN51 protein copurifies with RNA Pol III. To determine which RNA polymerase, if any, the BN51 protein copurified with, HeLa nuclear extracts were fractionated over phosphocellulose. This resin has been traditionally used to separate and purify the basal initiation factors of the various RNA polymerases (4, 21, 23, 28). Phosphocellulose is particularly advantageous for purification since most of the protein present in nuclear extracts flows through the resin, while all three RNA polymerases are efficiently retained. Nuclear extracts were applied to phosphocellulose in buffer containing 0.15 M KCl. The flowthrough was designated P.15. The column was step eluted with buffers containing 0.3, 0.5, 0.7, and 1.0 M KCl. The respective fractions (designated P.3, P.5, P.7, and P1.0) were assayed for the ability to nonspecifically transcribe poly(dA-dT) DNA. To distinguish the three polymerases, reactions were performed in triplicate, with each containing either 0, 0.5, or 200 μg of α-amanitin per ml. Only Pol II is inhibited by 0.5 μg of α-amanitin per ml. Both Pol II and Pol III are inhibited by 200 μg of α-amanitin per ml, while Pol I is resistant to α-amanitin. RNA transcripts were generated by using radiolabeled nucleotides and size fractionated on polyacrylamide gels. Size fractionation permitted the exclusion of artifactual low-molecular-size RNAs (<~100 nucleotides) from quantitative analysis. Radiolabeling of low-molecular-size RNAs occurred in the absence of the DNA template and most likely arises

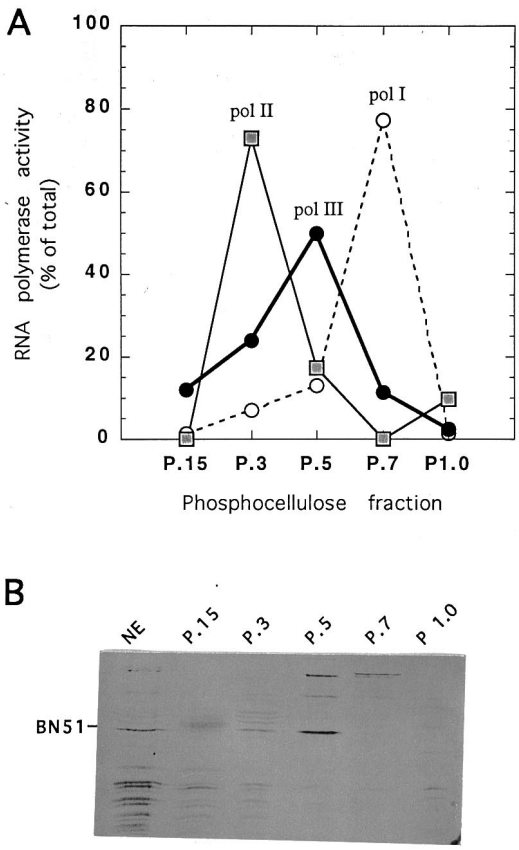

FIG. 1. The BN51 protein and RNA Pol III activity copurify. HeLa nuclear extracts (NE) were fractionated over phosphocellulose and step eluted with increasing KCl concentrations, as described in Materials and Methods, to generate the indicated fractions. (A) Each fraction was assayed for nonspecific Pol I (○), II (■), and III (●) activities as described in Materials and Methods. The activities in each fraction are reported as a percentage of the total activity in all fractions for that polymerase. The data represent an average of several experiments performed within the linear range of the assay. (B) Each fraction (130 μl) was assayed for the BN51 protein by standard Western blot analysis as described in Materials and Methods.

from RNA end-labeling activities present in the impure fractions.

As shown in Fig. 1A, Pol I, II, and III activities peaked in distinct fractions: 77% of the measurable Pol I activity was present in the P.7 fraction, 73% of the Pol II activity fractionated into P.3, and 50% of the Pol III activity fractionated into P.5. Thus, a single resin, phosphocellulose, allowed all three RNA polymerases to be chromatographically resolved. Simply probing each fraction for the presence of the BN51 protein should discern which polymerase, if any, the BN51 protein copurified with.

Equal proportions of each phosphocellulose fraction, as well as the input nuclear extract, were subjected to Western blot analysis using affinity-purified anti-BN51 antibodies to probe for the BN51 protein (Fig. 1B). A 53-kDa band corresponding to the BN51 protein was detected predominantly in the P.5 fraction. Lower levels were also detected in the P.3 fraction. However, no BN51 protein was detected in the P.15, P.7, or P1.0 fraction. The BN51 polypeptide cofractionated precisely with the Pol III activity but not with either Pol I or Pol II. From these data, we conclude that the BN51 protein is not a major component of either Pol I or Pol II, although at this point we cannot rule out that it is a minor component of these enzymes. In addition, while the activities reported in Fig. 1A were all within the linear range of the assay, we cannot rule out the presence of polymerase-specific inhibitors (or stimulators) in particular fractions which could potentially skew the results significantly. These concerns are addressed further below. The cofractionation of the BN51 protein with Pol III is consistent with the BN51 protein being a subunit of Pol III. However, the BN51 protein might nevertheless be distinct from Pol III, having fortuitously copurified in the P.5 fraction.

Anti-BN51 antibodies deplete Pol III activity from the P.5 fraction. If the BN51 protein is a subunit of Pol III, then anti-BN51 antibodies should immunodeplete Pol III activity from the P.5 fraction. The P.5 fraction was incubated with anti-BN51 antibodies coupled to protein A-Sepharose. The mixtures were then centrifuged briefly to separate the resin from the depleted supernatant. Control immunodepletions included either no antibody or anti-TAF250 antibodies, which recognize a subunit of the unrelated Pol II basal factor TFIID. As shown by Western blot (Fig. 2A), the BN51 protein was fully depleted from the P.5 fraction by anti-BN51 antibodies, while the control immunodepletions had no effect. When Pol III activity was assayed, anti-BN51 antibodies depleted nearly all of the Pol III activity whereas the control immunodepletions had no effect on Pol III activity (Fig. 2B). Of the low levels of Pol I and II activity present in P.5, no loss of activity relative to control depletions was detected when the BN51 protein was depleted. The ability of anti-BN51 antibodies to specifically deplete Pol III activity strongly suggested that the BN51 protein is a subunit of Pol III.

To provide further evidence for the BN51 protein being a Pol III subunit, we examined the immunoprecipitates for Pol III activity. As shown in Fig. 2C, surprisingly, the BN51 immunoprecipitates contained significant Pol III activity. Control mock and TAF250 resins lacked any detectable Pol III activity. Thus, despite the BN51 protein being bound to an antibody and immobilized to solid support, it was nevertheless active for Pol III transcription. This finding further substantiated our conclusion that the BN51 protein is indeed a Pol III subunit and not a distinct factor that fortuitously copurified with Pol III. In addition, we were unable to detect any nonspecific Pol I or II activity in any of the immunoprecipitates (data not shown), which provides further evidence that the BN51 protein is not a component of Pol I or II.

Depletion of promoter-specific transcription. The evidence thus far, from nonspecific polymerase assays, suggests that the BN51 protein is a subunit of Pol III but not a subunit of either Pol I or Pol II. However, we were concerned that depletion of the BN51 protein with anti-BN51 antibodies might potentially dissociate BN51 from Pol I or II, and if the BN51 protein was not required for nonspecific Pol I or II transcription, its potential association with Pol I or II would go undetected. To further substantiate our conclusions, we examined whether the BN51 protein was present during promoter-directed transcription by all three polymerases.

HeLa nuclear extracts provide a highly active source of initiation factors essential for promoter-directed transcription by all three RNA polymerases. Extracts were either mock depleted or depleted with immobilized anti-BN51 antibodies. The extracts were then tested for the ability to initiate transcription from an rRNA Pol I promoter, the synthetic G_6TI Pol II promoter, and three distinct types of Pol III promoters: VA_1, 5S, and U6. VA_1 contains an internal promoter composed of A and B boxes which bind TFIIIC. The 5S gene contains internal A and C boxes which bind both TFIIIC and TFIIIA. The U6 snRNA promoter lacks internal control elements but instead contains an upstream TATA box and prox-

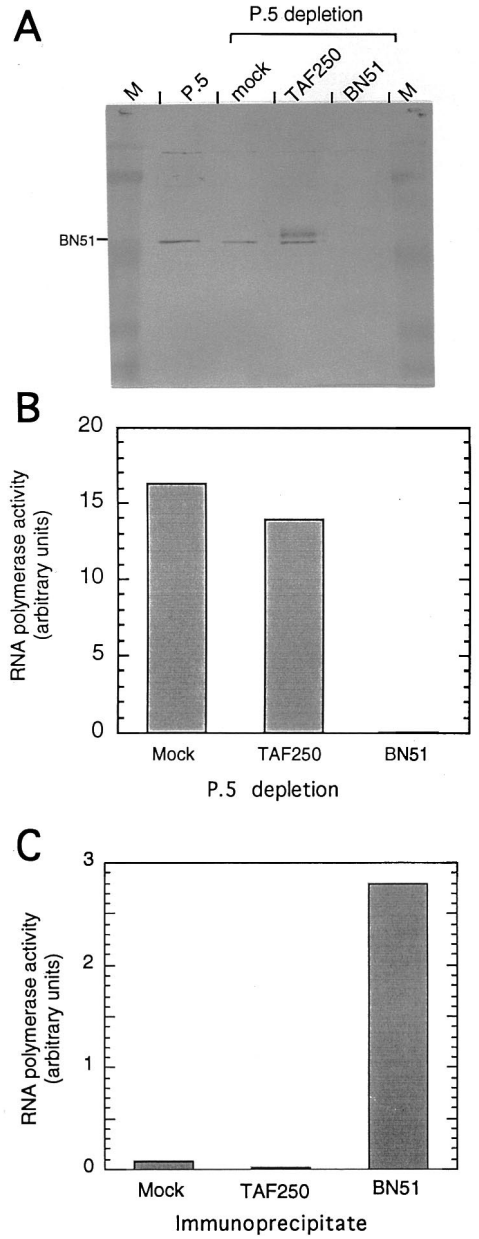

FIG. 2. Immunoprecipitation of Pol III activity with anti-BN51 antibodies. Immunodepletions with protein A-Sepharose (5 μl) containing either no antibody (mock), control anti-TAF250 antibodies, or anti-BN51 antibodies were performed on the P.5 fraction (160 μl) as described in Materials and Methods. (A) Western blot of the BN51 protein in immunodepleted P.5 fractions (130 μl). The higher-molecular-weight band in the TAF250 lane corresponds to contaminating TAF250 antibody cross-reacting with anti-immunoglobulin G secondary antibody. Lane M, size markers. (B) Nonspecific Pol III assay of the indicated depleted P.5 fraction (5 μl). (C) Nonspecific Pol III activity of 1-μl bed volume of the indicated P.5 immunoprecipitates.

imal sequence element (PSE). In nuclear extracts, these promoters direct the assembly of distinct polymerase-specific transcription preinitiation complexes which initiate at a define start site. In this system, the transcribing RNA polymerase either terminates at a terminator, as in the case of Pol III, or is artificially terminated when it comes upon the end of the linearized template, as in the case of Pol I and II. RNAs are detected by the incorporation of ^{32}P-radiolabeled nucleotides

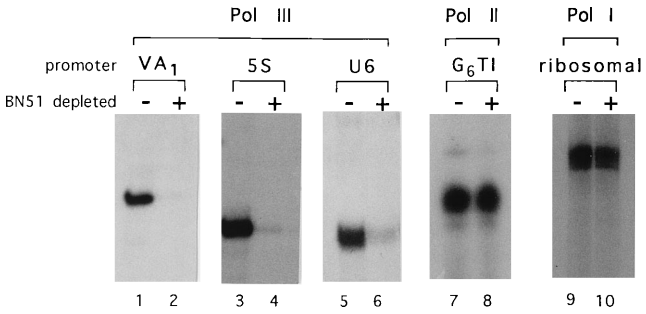

FIG. 3. Immunodepletion of promoter-directed transcription with anti-BN51 antibodies. HeLa nuclear extracts (0.4 mg, 18 μl) were immunodepleted with protein A-Sepharose (3 μl) containing either no antibody (mock) or anti-BN51 antibodies as described in Materials and Methods; 4 μl (or 6 μl for G_6TI) of BN51-depleted (+) or mock-depleted (−) extracts were then tested for the ability to initiate promoter-specific transcription in a runoff assay. Reactions shown in lanes 9 and 10 also contained 200 μg of α-amanitin per ml to eliminate any potential nonspecific Pol II or III transcription. The following promoters, as indicated above each set of lanes, were used: adenovirus VA_1RNA, *Xenopus* 5S rRNA, human U6 snRNA, synthetic G_6TI, and human rRNA.

into nascent RNA transcripts. As shown in Fig. 3, transcription from all three Pol III promoter templates was severely reduced in extracts immunodepleted of the BN51 protein. However, promoter-directed transcription arising from the Pol II G_6TI template or the Pol I ribosomal template was unaffected by the loss of the BN51 protein. We conclude from these data that the BN51 protein is a component of the Pol III transcription initiation machinery and is not involved in Pol I or II initiation.

Reconstitution of promoter-directed transcription with immobilized BN51 protein. In Fig. 2, we demonstrated that immunoprecipitated BN51 contained nonspecific Pol III activity. The ability of immobilized Pol III to nonspecifically transcribe DNA was surprising in that neither the anti-BN51 antibody nor immobilization of Pol III seemed to totally inhibit its activity. Apparently, Pol III is still accessible to DNA when immobilized in this manner. To follow up on this observation, we tested whether Pol III, immobilized via anti-BN51 antibodies, could reconstitute VA_1 promoter-specific transcription to BN51-depleted fractions. In this system, we used partially purified TFIIIB/Pol III and TFIIIC/Pol III fractions as sources of initiation factors. The fractions were depleted either with anti-BN51 antibodies or with control anti-TAF250 antibodies. As expected, the anti-BN51 antibodies depleted VA_1 transcription (Fig. 4, lanes 2 and 3), while the control antibodies (lanes 4 and 5) had little effect. When the control TAF250 or the BN51 immunoprecipitates were added back to the BN51-depleted fractions, only the BN51 immunoprecipitate restored VA_1 transcription (lanes 6 and 7). Thus, immobilized Pol III is capable of restoring promoter-directed Pol III transcription to BN51-depleted fractions. This finding further establishes that BN51 is a subunit of Pol III and a component of all three types of Pol III initiation complexes.

Relative accumulation of the 32S pre-rRNA in tsBN51 cells at 39.5°C. While the data thus far demonstrate that the BN51 protein is not a subunit of Pol I, cell lines containing a tsBN51 allele show a slight decrease of total 18S and 28S rRNA at the nonpermissive temperature (39.5°C) (10). However, total rRNA synthesis, as judged by run-on experiments with isolated nuclei, is unabated under these conditions (10). To address the nature of the defect in production of the 18S and 28S rRNAs, we compared the production of precursor and mature rRNAs at the nonpermissive temperature with that of normal BHK cells or tsBN51 cells grown at the permissive temperature.

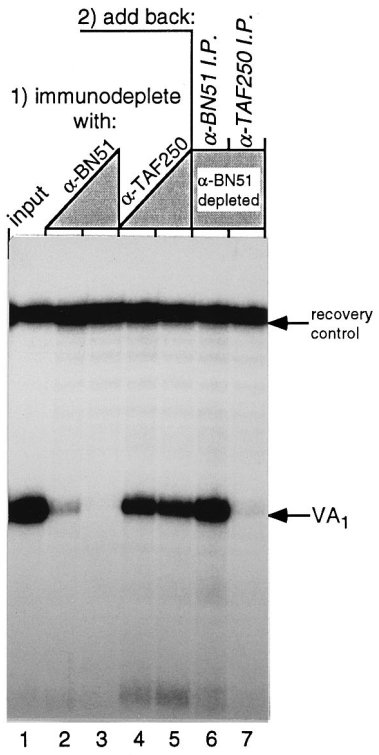

FIG. 4. Reconstitution of promoter-specific VA_1 RNA transcription with immunopurified BN51/Pol III complex. P.3 (0.01 mg, 0.5 μl) and P.5 (0.02 mg, 2.0 μl), which contain TFIIIB, TFIIIC, and RNA Pol III, were combined and immunodepleted with protein A-Sepharose (lanes 2 and 4, 1 μl; lanes 3 and 5 to 7, 7 μl), containing either anti-TAF250 (lanes 4 and 5) or anti-BN51 (lanes 2, 3, 6, and 7) antibodies as described in Materials and Methods. In reactions shown in lanes 6 and 7, the immunoprecipitates obtained from immunodepletions shown in lanes 2 and 4, respectively, were added back to a BN51-immunodepleted reaction (equivalent to that shown in lane 3).

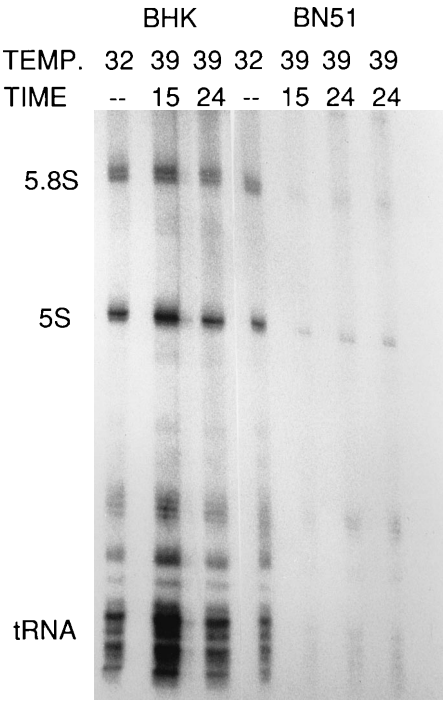

FIG. 5. Synthesis of 5.8S, 5S, and tRNAs by tsBN51 cells at 39.5°C. BHK or tsBN51 cells were cultured at 3×10^5 cells per 60-mm-diameter dish and incubated at 32°C for 24 h or 39.5°C for 15 or 24 h, as indicated above each lane. RNA was then labeled with $^{32}P_i$ and extracted as described in Materials and Methods. Labeled RNA, corresponding to 3×10^4 cells, was separated on 7 M urea–6% acrylamide gels, and autoradiography was performed. The first three lanes correspond to RNA isolated from BHK cells, while the last four lanes are from tsBN51 cells. Duplicate samples from tsBN51 cells kept at 39.5°C for 24 h were loaded to rule out artifactual loss of RNA.

Control BHK cells and tsBN51 cells were plated at 32°C, and the next day some were switched to 39.5°C while others were left at 32°C. After an additional 15 or 24 h of incubation, newly synthesized RNA was labeled with $^{32}P_i$. Total RNA was extracted, and small RNAs were analyzed by electrophoresis on a 7 M urea–6% acrylamide gel followed by autoradiography (Fig. 5). Each lane corresponds to RNA from equal numbers of cells as determined by counting of cells in parallel plates. As expected, there was a marked loss in production of 5S rRNA and tRNAs in the tsBN51 cells at 39.5°C. However, there was an equally dramatic loss of 5.8S rRNA production. Other experiments showed a similar decrease in production of 5.8S, 5S, and tRNAs as early as 7 h after the shift-up to 39.5°C (data not shown). In addition, similar results were obtained when cells were labeled with [^{3}H]uridine instead of $^{32}P_i$ and under different medium conditions (data not shown), which indicated that the phenomenon was reproducible under a number of different growth conditions. It should be noted that up to 48 h after the shift-up to 39.5°C, tsBN51 cells remained viable, although by 48 h cells are arrested in the G_1 phase of the cell cycle (19). Production of larger Pol I transcripts was assessed in similar ^{32}P-labeled RNA extracts separated on 1.2% agarose–formaldehyde gels (Fig. 6). Again, each lane corresponds to RNA from equal numbers of cells. As expected, there was a marked decrease in production of small RNAs, which are primarily Pol III transcripts, in tsBN51 cells after 24 h at 39.5°C. In addition, a decrease in production of both 18S and 28S RNAs was observed at the nonpermissive temperature. Thus, temperature-restricted tsBN51 cells appeared to be unable to accumulate mature Pol I transcripts.

Failure to process the 32S pre-rRNA could account for loss of both the 5.8S and 28S rRNAs in temperature-restricted tsBN51 cells. To investigate this possibility, labeling experiments were performed with [^{3}H]uridine, which provides higher resolution of bands and a stronger signal-to-noise ratio. As an additional control, the ts11 cell line was also analyzed. ts11 cells are temperature-sensitive cell cycle mutants of BHK cells that arrest in G_1 after 48 h at 39.5°C (29). ts11 cells contain a temperature-sensitive asparagine synthetase (7). Thus, use of these cells would detect any changes in rRNA transcript processing due to G_1 arrest at 39.5°C. To enhance the visibility of the larger RNA transcripts in tsBN51 cells at 39.5°C, equal amounts of total [^{3}H]uridine-labeled RNA were loaded in each lane. Since total RNA production is decreased at 39.5°C, it was necessary to use five times as many cells at the restrictive temperature than at the permissive temperature. Thus, absolute RNA levels should not be compared from lane to lane. Instead, the accumulation of the 32S pre-rRNA relative to the 28S (or 18S) rRNA should be compared. As shown in Fig. 7A, there was a clear accumulation of 32S RNA in tsBN51 cells after 24 or 48 h at 39.5°C relative to the 28S rRNA. Quantitative densitometry confirmed that there was a fivefold increase in the relative amount of 32S RNA compared with 28S RNA after 48 h at 39.5°C (Fig. 7B). No change in the 32S/28S ratio was seen in either BHK or ts11 cells incubated at 39.5°C for a similar period. Thus, in tsBN51 cells at 39.5°C, there is a

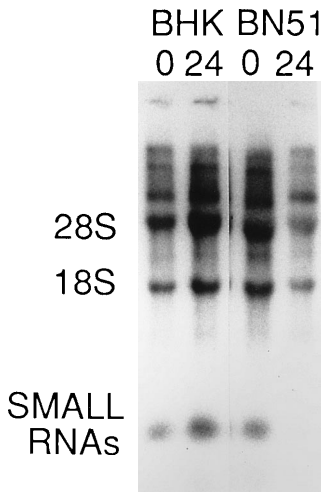

FIG. 6. Synthesis of RNA species by tsBN51 cells at 39.5°C. BHK and tsBN51 cells were plated, and ^{32}P-labeled RNAs were extracted as described for Fig. 5 after incubation at either 32°C (0) or 39.5°C for 24 h (24). Labeled RNA corresponding to 3×10^4 cells was separated on a 1.2% agarose–formaldehyde gel, transferred to a nylon membrane by capillary action, and subjected to autoradiography.

marked loss of production of mature Pol I transcripts accompanied by a relative accumulation of precursor 32S rRNA and, to a much lesser extent, 45S RNA (data not shown). The increased accumulation of 32S relative to 28S implies a defect in posttranscriptional processing of precursor rRNA in temperature-restricted tsBN51 cells, which presumably is a consequence of the loss of Pol III transcripts.

DISCUSSION

In this report, we provide direct biochemical evidence that the BN51 protein is a subunit of RNA Pol III. This conclusion is based on the following observations: (i) the BN51 protein copurifies with Pol III but not Pol I or Pol II, (ii) immobilized anti-BN51 antibodies deplete nonspecific Pol III activity from partially purified Pol III fractions, (iii) anti-BN51 antibodies deplete promoter-directed Pol III transcription but not promoter-directed Pol I or II transcription, and (iv) immunopurified native BN51 complexes can reconstitute both promoter-directed and nonspecific Pol III activity in a BN51-depleted transcription system. This work confirms and extends previous indications that the BN51 protein is the human homolog of C53, the 53-kDa subunit of yeast RNA Pol III. The BN51 gene is therefore the first gene of mammalian Pol III to be cloned.

Early purification strategies variably reported the presence of a 53-kDa subunit in mammalian Pol III (26), with the more recent report concluding that it was not a subunit (13). Strikingly, the BN51 protein migrates in SDS-polyacrylamide gels as a 53-kDa protein. Since our studies have not compared, side by side, the BN51 protein with Pol III subunits purified by the early methods, we cannot unequivocally assign the BN51 protein to the previously defined subunits. However, if the BN51 protein does correspond to the previously reported 53-kDa subunit of mammalian Pol III, then these reports shed light on potential functions of the BN51/53-kDa subunit. First, the variable presence of the 53-kDa subunit in different Pol III preparations suggests that the BN51 protein might be easily dissociated from Pol III. It has been reported that in *S. cerevisiae*, C53 dissociates quite readily from Pol III. In addition, C53 is highly susceptible to proteolysis and as a consequence is often

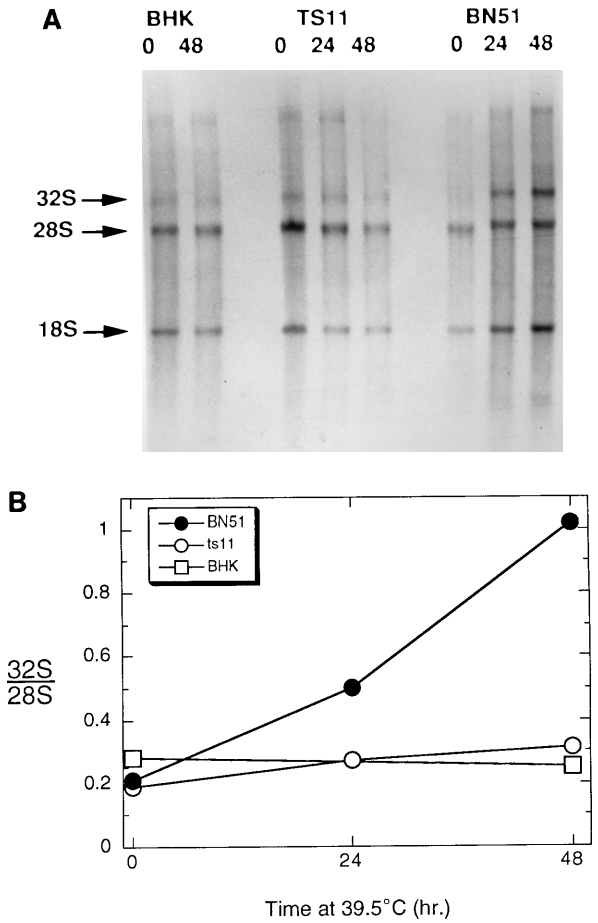

FIG. 7. Accumulation of 32S RNA in tsBN51 cells at 39.5°C. (A) BHK, ts11, and tsBN51 cells were plated at 3×10^5/60-mm-diameter dish and incubated at either 32°C (0) or 39.5°C for 24 or 48 h, as indicated above each lane. Cells were cultured with [^{3}H]uridine, and RNA was extracted as described in Materials and Methods; 2×10^4 cpm of ^{3}H-labeled RNA was loaded in each lane of a 0.8% agarose–formaldehyde gel and, following electrophoresis, transferred by capillary action to nylon membranes. Fluorography was then performed after spraying of membranes with En^{3}Hance (Dupont NEN). From left to right on the gel, each lane contained 5.2, 1.2, 3.3, 1.8, 1.0, 2.2, 4.0, and 10.0 (each $\times 10^5$) cells. (B) The intensities of the 32S and 28S bands shown in panel A were quantitated by densitometry, using an LKB Ultrascan XL densitometer. The ratio of the peak area of the 32S to 28S RNA in each sample is indicated as a function of time at 39.5°C.

seen as a doublet on protein gels (9). Second, since mammalian Pol III is apparently active without the 53-kDa subunit (13), then BN51 protein might not be required for RNA polymerization. Being a polymerase-specific subunit, the BN51 protein might be involved in promoter recognition, transcription initiation, or transcription termination.

Previous work on the C53 subunit of yeast Pol III has some similarity to the work reported here. Huet et al. were able to inhibit promoter-specific transcription of a tRNA gene with antibodies to C53 (9). Higher concentrations of antibody were needed to inhibit nonspecific transcription of poly(dA-dT). Since promoter-specific transcription is more sensitive to the inhibition with anti-C53 antibodies than nonspecific transcription, the C53 subunit was speculated to interact with initiation factors (2). While the C34 subunit of yeast Pol III interacts with the 70-kDa subunit of TFIIIB, there is no evidence that C53 interacts with an initiation factor (31). Thus, it remains

unresolved as to whether C53 or its mammalian counterpart, the BN51 protein, targets initiation factors.

In vivo, studies on the temperature-sensitive BN51 cell line reveal a surprising pleiotropic effect of decreased Pol III transcription. Not only is the production of tRNAs and 5S rRNA shut down at the restrictive temperature, but there is also a concomitant loss of Pol I-transcribed 5.8S, 18S, and 28S rRNAs. Loss of these rRNAs is not due to a general drop in Pol I transcription. Instead, we find that 32S and to a lesser extent the 45S precursor rRNA accumulate as the levels of mature 5.8S, 18S, and 28S rRNAs dwindle. Apparently, tsBN51 cells are unable to process precursor rRNAs at the restrictive temperature.

Cleavage of the 32S pre-rRNA occurs in the nucleolus during assembly of the 60S large ribosomal subunit. Assembly of the 60S subunit not only depends on the presence of ribosomal proteins but also requires the proper complement of rRNAs, including the Pol III transcript 5S RNA. At the restrictive temperature, tsBN51 cells do not synthesize 5S rRNA. One possible consequence is that without the 5S rRNA, assembly of the 60S ribosomal subunit is arrested. The partially assembled subunit either would be unable to incorporate the 32S rRNA or if 32s rRNA is incorporated, would be unable to cleave it. The failure to cleave the 32S rRNA would account for the loss of mature 5.8S and 28S rRNAs. These findings demonstrate that the stepwise assembly of the ribosome is tightly controlled.

The reason for the decrease in mature 18S rRNA is less clear. Loss of 28S rRNA production in itself does not lead to loss of 18S rRNA production, as shown by the ts422E mutant of BHK cells, which has a temperature-sensitive defect in rRNA processing that leads to loss of 28S but not 18S rRNA production (30). It is possible that one or more of the uncapped small RNAs located in the nucleolus, which are thought to play a role in rRNA splicing, is critical for 18S RNA production (reviewed in reference 6). Of the four uncapped small nucleolar RNAs, the U14 RNA appears to be produced by an unusual splicing of intron sequences within the heat shock 70 gene (14). The 7-2 RNA appears to be transcribed by Pol III (30). Temperature-sensitive mutants of 7-2 RNA in yeast cells show a decreased rate of rRNA processing with decreased production of both 18S and 28S RNA and accumulation of an aberrant 5.8S RNA (24). RNA X and RNA Y may also play a role in rRNA processing (30).

Given that the mature Pol I transcripts give rise to ribosomes in association with the 5S Pol III transcript and carry out translation, which is dependent on the presence of tRNAs, it is logical that there should be coordinate regulation of Pol I and Pol III activities. At the transcriptional level, Pol I and Pol III activities increase in parallel in proliferating cells (5, 12). Our data indicate a close posttranscriptional control of production of mature Pol I transcripts based on the availability of Pol III transcripts. A similar phenomenon has recently been found in yeast cells, in that temperature-sensitive mutants of Pol III show decreased production of 5.8S RNA and accumulation of larger precursors (8). The decrease in production of mature Pol I transcripts is much less apparent in yeast than in mammalian cells, possibly because of the relative preservation of 5S RNA transcription in such mutants, even though tRNA production is dramatically reduced. The reason for the preservation of 5S production in yeast Pol III mutants is unclear but may be related to the unique location of the 5S genes within the yeast ribosomal DNA (18). The correlation between the degree of loss of 5S and 5.8S rRNAs in yeast and mammalian cells argues that aberrant assembly of the 60S subunit (and associated abnormal processing of 32S rRNA) is the primary reason for loss of 5.8S RNA production in temperature-sensitive Pol III mutants. Given that accumulation of increased amounts of mature rRNA is characteristically seen during the G_1 phase of the cell cycle, it is possible that the G_1 arrest seen in both yeast (16) and mammalian (10, 19) temperature-sensitive Pol III mutants is due to the abnormal rRNA processing described above. However, other direct or indirect effects of decreased Pol III activity could also play a role in G_1 arrest.

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