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QUANTITATIVE SYNTHESIS OF FULL-LENGTH GLOBIN GENES: DEPENDENCE ON TEMPLATE

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SUMMARY:

An efficient two step method for the synthesis of full-length globin gene is described. T4 DNA polymerase directs the conversion of greater than 70% of input globin cDNA template into duplex DNA. A majority of these molecules are full-length double stranded molecules. This duplex DNA was characterized with respect to size by analytic 4.0% polyacrylamide - 0.6% agarose composite gel electrophoresis. The contour length of the molecules and the integrity of the duplex DNA were also evaluated by direct visualization using electron microscopy. A mean number of 588 base pairs was determined for the approximately 400 molecules measured. Globin mRNA transcription in the absence of the antibiotic actinomycin D was shown to be less efficient in template utilization and less effective in synthesizing complete gene copies.

Previously two enzymatic techniques have been described for the *in vitro* elongation of cDNA[1] into DNA duplexes (1-3). Avian myeloblastosis virus (AMV) DNA polymerase has been used to synthesize the globin, ovalbumin and insulin genes (1,3-7). DNA polymerase I isolated from *E. coli* has been used for globin and ovalbumin gene synthesis (5,8-10). Efstratiadis et al (2), using the latter enzyme and molecular cloning of this DNA, conserved 98% of the information contained in the base sequence of rabbit β-globin mRNA (11). However the yield of full-length duplex DNA was low (2) and this extent of sequence conservation has not been duplicated in any other clone (1,3-10).

[1] Abbreviations: DNA complementary to an mRNA template, cDNA; avian myeloblastosis virus, AMV; covalently attached second strand DNA which is anti-complementary to the cDNA, sDNA.

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Recently, E. coli DNA polymerase I has been shown to synthesize molecules which do not correspond to the template (12). AMV DNA polymerase requires specific in vitro reaction conditions to transcribe different templates (10,13) and transcripts have been demonstrated which do not correspond to the sequence of the template.[2] We report a method that was developed to synthesize full-length globin gene in good yield using T4 DNA polymerase. With globin cDNA as template this method resulted in the copying of more than 70% of the template, with a majority of the product being full-length duplex DNA.

EXPERIMENTAL PROCEDURES

Materials: DNA polymerase (EC 2.7.7.7) lot no. G-876 (reverse transcriptase) was purified from avian myeloblastosis virus strain BAL by Drs. J. and D. Beard and G. Houts of Life Sciences, Inc. and was generously supplied by the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute. The AMV DNA polymerase had 13,580 U per ml [where 1 unit of enzyme activity is expressed as the incorporation of 1 nmol of dTMP into an acid-insoluble product in 10 min at 37° in the presence of 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 8.0 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM [^{3}H]dTTP, 100 mg/ml of bovine serum albumin, and 0.2 mM poly ($rA_n \cdot dT_{13}$)] and a specific activity of 54,320 U per mg protein. T4 DNA polymerase (EC 2.7.7.7) was purified by the method of Lo and Bessman (14) and was a kind gift of Dr. M. Bessman, Johns Hopkins University. S_1 nuclease was purchased from Miles Labs. Inc.

Primers and Templates: Oligo thymidylic acid primer was obtained from Collaborative Research, Inc. Rabbit globin mRNA was isolated and characterized as described elsewhere (15). The mRNA was transcribed in vitro under standard conditions previously optimized for full length transcription which are: 50 mM Tris-HCl, pH 8.5, 70 mM KCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 100 µg per ml actinomycin D, 70 µg of globin mRNA per ml, 20 µg of oligo$(dT)_{12-18}$ primer per ml, 320 µM nonradioactive deoxyribonucleoside triphosphates, 100 µM radionuclide and 120 U of AMV DNA polymerase per ml. The reaction mixture was incubated at 37° for 1 hr. A second cDNA template was synthesized as above except that actinomycin D was omitted from the reaction mixture. The cDNA was fractionated by 5-25% linear alkaline sucrose velocity sedimentation and fractionated into the putative full-size fraction (25-35% of the input material). The globin cDNA was further characterized by S_1 nuclease digestion (6-12% resistant) and by analytic gel electrophoresis. This full-length cDNA was used as template for sDNA synthesis. The cDNA template synthesized in the absence of actinomycin D, however demonstrated 60% resistance to S_1 nuclease digestion and was approximately 30% larger than full-length globin cDNA as determined by analytic gel electrophoresis.

T4 DNA Polymerase Globin sDNA Elongation: The 90 µl reaction mixture for T4 polymerase directed elongation contained: 67 mM Tris-HCl, pH 8.5; 7 mM $MgCl_2$; 17 mM $(NH_4)_2SO_4$; 50 µg per ml of nuclease free bovine serum albumin, 0.7 mM β-mercaptoethanol and 700 µM of each unlabeled deoxynucleoside triphosphate and 460 µM radionuclide [^{125}I]iododCTP (Amersham-Searle), 1400 Ci/mmol. Iododeoxycytosine triphosphate was not inhibitory to the transcription reaction at <1% of 460 µM. The [^{125}I]iododCTP was convenient for the subsequent product characterizations. Full length globin cDNA was used at a concentration of 20 µg per ml. The reaction was incubated at 40° for 20 min.

[2] L.J. Krueger and W.F. Anderson, Manuscript in preparation.

Analytic Composite Polyacrylamide-agarose Gel Electrophoresis: The reaction products were denatured by heating 90° for 5 min and layered onto the electrophoretic gels. The material was stacked at 1.0 mA per gel for 1 hr and electrophoresed under denaturing conditions at 1.8 mA per gel for 20 hr on the 4.0% acrylamide - 0.6% agarose in 98% formamide (buffered in 20 mM barbitol and 20 mM NaCl) composite gels. The gels were fractionated automatically into 2 nm slices and the radioactivity quantitated by scintillation spectrophotometry.

Contour Length Measurements of Duplex DNA: The reaction product duplex DNA and the input cDNA templates were measured by direct visualization at a 20,000 X magnification on a JOEL JEM 100B electron microscope. The reaction products were extracted and prepared for electron microscopy by the aqueous procedure of Davis et al (16). Contour lengths were measured with a K&E map measurer at an additional 20X magnification on a Nikon Shadograph. This procedure selects for contour length $\geq$ 150 base pairs (which can be distinguished above background). Bacteriophage ΦX-174 double stranded DNA (1.69 μm = 5,375 base pairs) was used to calibrate the contour length measurements. Errors due to inadvertent selection were limited by using random fields for photography and measuring all observable fragments.

RESULTS AND DISCUSSION

The covalently linked anti-complementary second strand DNA (sDNA) to globin cDNA is copied from the self-primed (2,9) template by T4 DNA polymerase. Approximately 70% of the globin cDNA template was copied. This represents an efficient utilization of the cDNA template in the preparation of globin duplex DNA. The size distribution of the resultant DNA molecules was characterized by analytic electrophoresis on denaturing composite agarose-polyacrylamide gels as shown in Fig. 1. The majority of the sDNA-cDNA duplex synthesized was nearly twice the length of the input cDNA template. These results are consistent with the synthesis of the complete duplex globin gene.

Globin cDNA synthesized in the absence of the antibiotic actinomycin D showed an increase in double stranded DNA as evaluated by: 1) an increased resistance to S_1 nuclease of 40-60% and 2) the direct visualization by electron microscopy as shown in Figure 2 and in Table 1. The average contour length of the partial duplex DNA (within this heterogeneous mixture) was 356 base pairs. T4 DNA polymerase synthesized a significant number of full-length double stranded DNA molecules from the isolated partially duplexed DNA template as shown in Fig. 2A. However, when full-length cDNA (S_1 resistance of 5-12%) was used as template, T4 DNA polymerase copied 60% of the template. The contour length of this duplex DNA synthesized was an average of 588 base pairs.

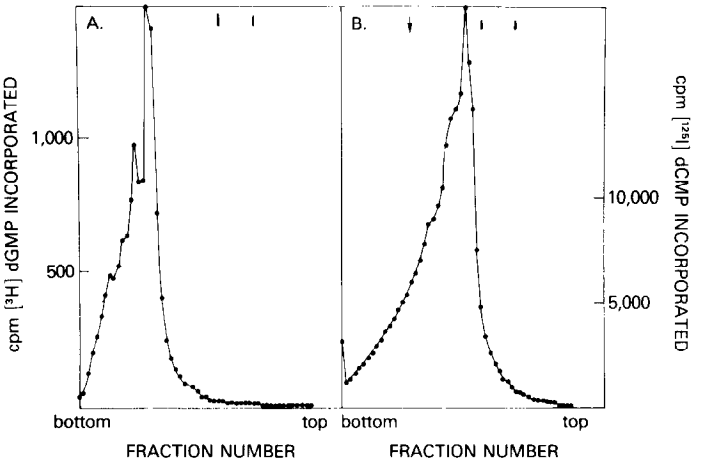

Fig. 1. Size determination of globin cDNA template and the reaction product isolated from the T4 DNA polymerase directed elongation. Full-length globin cDNA was synthesized in the presence of actinomycin D as described. The T4 DNA polymerase directed reaction was initiated by the addition of 2.5 U per ml of enzyme (polymerization was linear for approximately 5 min., although the reaction was active for several hr.). The reaction was terminated, the reaction product extracted and purified. The DNA samples were denatured, stacked at 1.0 mA per gel for 1 hr. and electrophoresed under denaturing conditions in 98% formamide on 4.0% polyacrylamide- 0.6% agarose gels for 20 hr. at 1.8 ma per gel. A) Full-length globin cDNA template, B) The T4 DNA polymerase directed sDNA-cDNA complex. Rabbit globin full-size cDNA (arrow) and rabbit 18S and 28S polysomal RNA (vertical marks) were electrophoresed as standards.

Table 1. Template Effect on in vitro sDNA Synthesis*

Molecules Analyzed	DNA Polymerase Assayed	Mean Base Pair Length of Product	Molecules Between 540-740 base pairs (per cent)
Partial duplex DNA template	AMV	356	12
sDNA	T4	516	24
Full-length cDNA template	AMV	-	-
sDNA	T4	588	44

*See legend to Figure 2 for experimental details

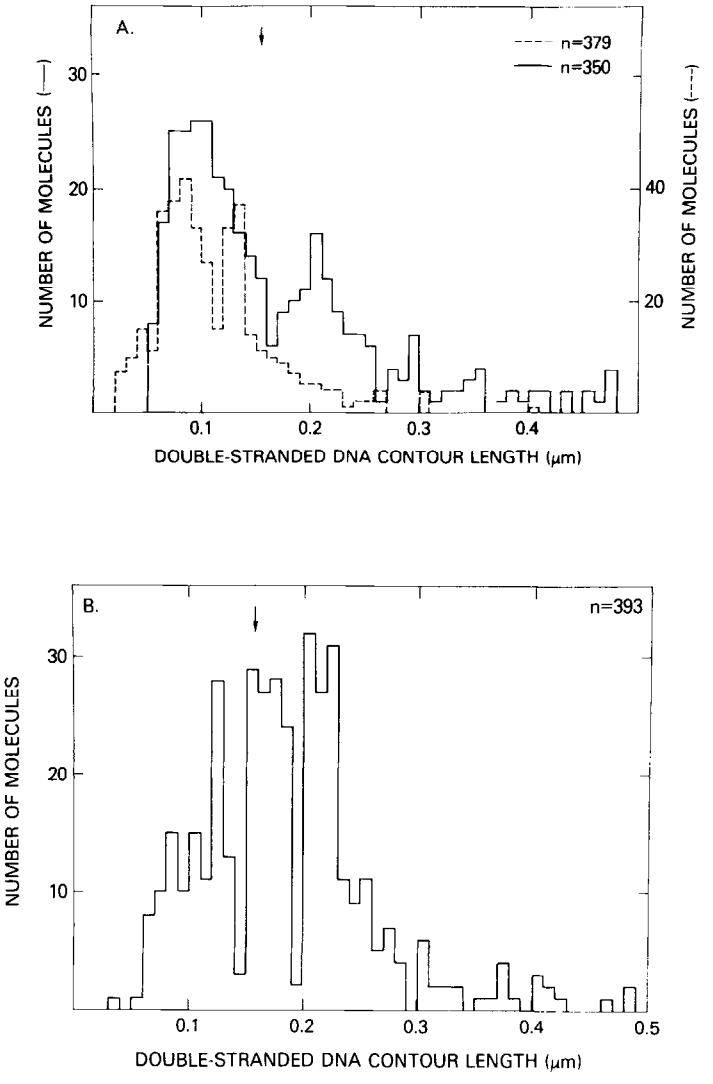

Fig. 2. Contour length measurements of duplex DNA by electron microscopy. The reaction products were synthesized as described, extracted and prepared for electron microscopy by the aqueous procedure of Davis et al. (16)). Contour lengths of the double strand regions of the partial and complete globin gene products were determined by measurement of individual molecules. A) Contour length measurements of partial duplex DNA template synthesized in the absence of actinomycin D (----) and T4 DNA polymerase directed elongation of the partial duplex DNA template (——). B) Contour length measurements of the T4 DNA polymerase directed reaction product synthesized on globin full-length cDNA. The input cDNA template exhibited fewer than one per cent double strand DNA molecules compared to the partial duplex DNA template synthesized in the absence of actinomycin. Contour length measurements of each reacton product are summarized in Table 1.

The distribution of the molecules as shown in Fig. 2B was consistent with a majority of the molecules being full-length (9-11,17,18).[3] Thus, T4 DNA polymerase is shown to be a useful enzyme in the synthesis of complete duplex DNA.

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