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A general method for cloning eukaryotic structural gene sequences

(complementary DNA/messenger RNA sequencing/gene isolation/terminal transferase)

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ABSTRACT **Complementary DNA, transcribed *in vitro* from purified rabbit globin messenger RNA and made double-stranded, has been inserted into *Escherichia coli* plasmids pSC101 and pMB9 by the poly(dT)/poly(dA) "tailing" and annealing technique. *E. coli* transformants given by this DNA preparation have been shown to contain globin sequences by the hybridization of globin RNA to DNA from clones grown and lysed *in situ* on nitrocellulose filters. An estimate of the amount of inserted globin sequences has been provided by fingerprint analysis of globin mRNA sequences hybridized to the purified plasmid chimeras. Inserted sequences so far subjected to detailed analysis have been ascribed to the rabbit beta globin chain. The susceptibility of inserted beta globin sequences to the restriction endonuclease *Eco*RI confirms the existence of a site already found through previous nucleotide sequence analysis.**

The recent exciting advances in recombinant DNA cloning technology (1–3) encouraged us to undertake the development of methods for the conversion of polyadenylylated messenger RNA into double-stranded DNA which could then be inserted into bacterial plasmids and cloned. This would provide large quantities of pure structural gene sequences in the form of duplex DNA, permitting the facile application of new rapid sequence techniques employing restriction endonucleases. In addition, cloned mRNA sequences in recombinant plasmids would provide specific probes for selecting mRNA and the nuclear RNA precursors to mRNA, as well as for isolating structural genes together with adjacent sequences from eukaryotic DNA.

As summarized in recent review articles (4, 5), we have been involved in the development of general methods for the sequence analysis of eukaryotic mRNAs with the immediate goal of elucidating the sequences of the rabbit globin messenger RNAs. In this report we describe our approach for the creation of bacterial plasmids containing inserted structural gene sequences for rabbit globin and present some preliminary characterization of the globin inserts by fingerprint analysis and restriction enzyme cleavages. The methods developed with rabbit globin mRNA should be generally applicable for the insertion of any polyadenylylated mRNA sequence into plasmid DNA and, because of the power of cloning techniques, should allow the experimenter to obtain in pure form the duplex DNA sequence corresponding to any one of a complex mixture of mRNAs without need for biochemical purification. Concurrent with the work reported here, Maniatis *et al.* (6), using a similar procedure, and Rougeon *et al.* (7) and Rabbitts (8), using different approaches, have also inserted rabbit globin gene sequences into *Escherichia coli* plasmid DNA and cloned the chimeras.

MATERIALS AND METHODS

Enzymes, Bacterial Strains, and Plasmids. Purified RNA-dependent DNA polymerase (reverse transcriptase) from avian myloblastosis virus (AMV) (9) was provided by the Office of Program Resources and Logistics, Viral Cancer Program, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. 20014. DNA polymerase I, fraction 7, was prepared by K. Fry (10). *Aspergillus* nuclease S1 was purified by the methods of V. Vogt (11). Bacteriophage lambda exonuclease (12) and restriction endonuclease *Eco*RI were provided by R. Firtel. Calf thymus deoxynucleotidyl terminal transferase (13) was prepared in the laboratory of R. L. Ratliff by J. Isaacson, C. Manske, and W. Salser. *E. coli* RNA polymerase was a gift of R. Burgess (14). *E. coli* K12 strains HB101 and C600 have been described (15, 16), as has plasmid pSC101 (17). Plasmid pMB9 was developed by M. C. Betlach and H. W. Boyer (personal communication).

Purification of mRNA. Rabbit globin mRNA was prepared by a new large-scale method‡ from reticulocytes from phenylhydrazine-treated rabbits (18). Polysomes were prepared from a post-nuclear supernatant and nuclear wash by magnesium precipitation (19). The 10S globin mRNA was then purified by repeated phenol/$CHCl_3$ extractions, poly(U)-Sepharose selection of poly(A)-containing mRNA, and two sodium dodecyl sulfate/sucrose gradient velocity sedimentations.

Synthesis of cDNA. When cDNA of maximal length was desired, the reaction was carried out with 1000 μM concentrations of each deoxyribonucleoside triphosphate. This results in an increase of about 15% over the level of synthesis observed at the 500 μM nucleotide concentration used by Efstratiadis *et al.* (20). The cDNAs synthesized both with and without actinomycin D were compared by fingerprint analysis of RNA transcribed *in vitro* from each template. The patterns obtained were the same in either case, but the yields of cDNA were about two times higher when actinomycin D was omitted. Reactions were carried out for 2 hr at 37° in 0.05–1.00 ml volumes with $(dT)_{12-18}$ primer present at 20 μg/ml; 60 μg/ml of globin mRNA, 120 units/ml of reverse transcriptase in 50 mM Tris·HCl, pH 8.3, 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol. After treatment with 0.3 M NaOH at 90° for 20 min, the reaction mixtures were neutralized with HCl, phenol extracted, and passed through a Sephadex G-100 column (1 × 50 cm for the 1.00 ml preparations).

Synthesis of Double-Stranded Globin DNA. The reaction was carried out in a 120 μl volume for 15 min at 37° in 66 mM KH_2PO_4 at pH 7, 6.6 mM $MgCl_2$, 1 mM 2-mercaptoethanol with 1 μg of globin cDNA and 5 μg of DNA polymerase I (7.6 units). Deoxyribonucleoside triphosphates were present, 4 nmol each, with 20 μCi of ^3H label in one of the dNTPs such that acid-precipitable radioactive material could be followed. No primer was required for reasons discussed below. Ten microliters of 1 M EDTA was added to stop the reaction, and after phenol extraction the aqueous layer was passed over a 0.6 × 15

Abbreviations: cDNA, DNA complementary to RNA; Hb, hemoglobin; AMV, avian myeloblastosis virus.

‡ S. Lipman, K. Toth, N. Fedoroff, and R. Wall, manuscript in preparation.

cm Bio-Gel P-60 column, and the excluded peak was precipitated with ethanol.

Cleavage of Hairpin Structures. Earlier studies involving bacteriophage T4 endonuclease IV digestions of ribosubstituted DNA synthesized from a cDNA template revealed that the product consisted of rapidly renaturing material presumed to have a hairpin structure (N. Fedoroff and W. Salser, ref. 5, page 948, and unpublished results). This and the atypical kinetics of DNA synthesis from a cDNA template and the lack of utilization of exogenous primer suggested that reverse transcriptase may produce a "fold-back" region which is effective as a primer for synthesis by *E. coli* DNA polymerase I. Such hairpin structures are formed even when actinomycin D is added during the synthesis of cDNA in attempts to inhibit double-strand synthesis by reverse transcriptase (5). Efstratiadis *et al.* (6) have recently confirmed these results. S1 nuclease was used to cleave the hairpin loop as well as to remove any remaining single-stranded DNA. The conditions of Schenk *et al.* (21), designed to minimize cleavage of one strand ("nicking") of duplex DNA, were employed with 135 units of S1 per μg of duplex DNA (as calculated from the amount of label incorporated during synthesis of the second strand) incubated for 1 hr at room temperature in 200 μl of 0.28 M NaCl, 0.0045 M $MgSO_4$, 0.03 M sodium acetate, pH 4.6.

Attachment of Homopolymers to Double-Stranded Globin and Plasmid DNA. Bacteriophage lambda exonuclease was used under conditions described by Lobban and Kaiser (1) to remove approximately 30 nucleotides from the 5'-OH ends of the double-stranded globin and linear plasmid molecules. The reaction was terminated by phenol extraction. Addition of poly(dA) to exonuclease-treated globin DNA with terminal transferase was carried out in 150 μl of 0.05 M KH_2PO_4, pH adjusted to 6.9 with KOH, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol with globin DNA at 3.3 μg/ml. One microcurie of ^{32}P was present as the alpha phosphate in 8 nmol of dATP. One hundred fifty units of terminal transferase were added to initiate the reaction at 37°. Gel electrophoresis in 98% formamide showed that the globin DNA was 400 base pairs in length on the average. Thus, in this reaction there were 4 pmol of free 3'-OH termini, such that the addition of 0.40 nmol of dATP, or 5% of the total radioactivity present, would give poly(dA) "tails" of an average of 100 nucleotides in length. The reaction can be halted on ice at 5-min intervals and acid-precipitable radioactive material can be measured. If the desired percentage of dATP incorporation (corresponding to 50–100 bases) has not been reached, the reaction can be reinitiated at 37° by adding an additional 150 units of terminal transferase. Poly(dT) was added to lambda-exonuclease-treated plasmid DNA similarly, except in 0.2 M cacodylic acid, pH adjusted to 7.2 with KOH, 1 mM $CoCl_2$, and 2.5 mM 2-mercaptoethanol made fresh prior to each use. The reactions were terminated by phenol extraction. The aqueous layers were passed over Bio-Gel agarose A-5m columns (100 ml/mg of DNA), and the products were precipitated with ethanol.

Annealing and Transformation. Equimolar quantities of poly(dA)-tailed globin DNA and poly(dT)-tailed plasmid DNA were resuspended at 7 nM each (4 μg/ml of pSC101) in 10 mM Tris·HCl at pH 8.1, 100 mM $CaCl_2$, 1 mM EDTA, and were incubated at 51° for 0.5 hr, then allowed to cool slowly to room temperature over 3 hr. Transformation was carried out as described by Cohen *et al.* (22) with modifications: to the annealed DNA mixture was added an equal volume of 0.01 M Tris·HCl at pH 7.5, 0.02 M $CaCl_2$, 0.02 M $MgCl_2$; twice this total volume of *E. coli* cells treated with 0.05 M $CaCl_2$ was then added, and the mixture sat on ice for 15 min, was taken to 37° for 4 min, and was left standing at room temperature for 10 min; one-half this total volume of a 3× concentrate of Luria broth was then added and the mixture was incubated 0.5 hr at 37° before being spread onto Luria broth agar plates containing 15 μg/ml of tetracycline.

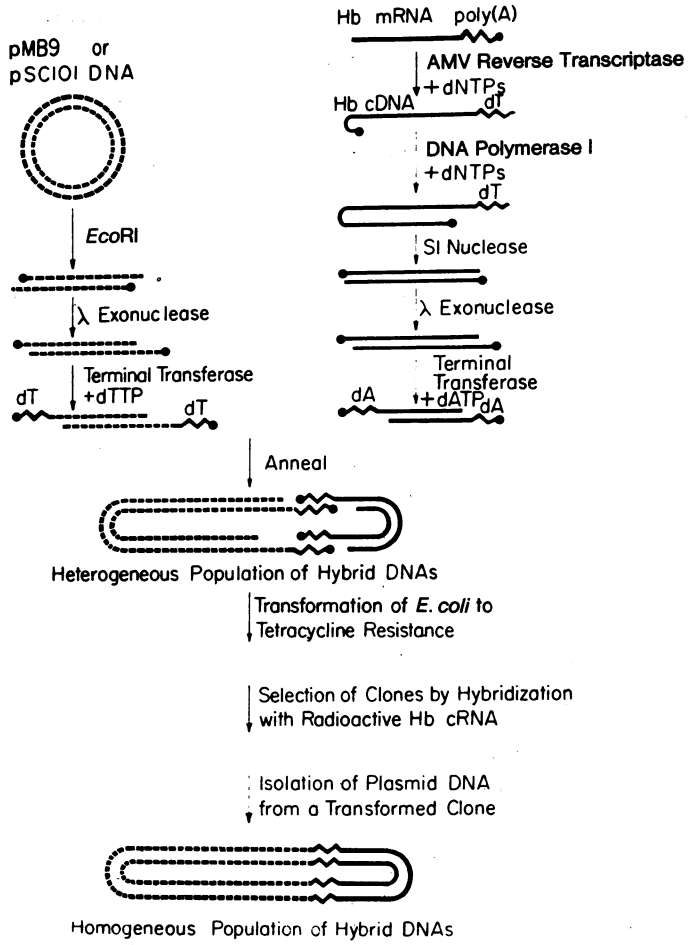

FIG. 1. Depiction of enzymatic steps leading to the creation of duplex globin DNA and its insertion into a plasmid.

Biohazard Considerations. The hemoglobin mRNA preparations were estimated to be of greater than 90% purity. To guard against potential biohazards, especially those from unknown contaminating sequences, the transformations and all subsequent manipulations with live bacteria which might carry chimeric plasmids were carried out in P3 physical containment (23). In the initial experiments EK1 hosts were used. Subsequently, as bacterial strains with reduced capabilities for growth outside the laboratory have become available, we have employed them in our work (examples are strains X1849 and X1776 prepared by Dr. Roy Curtiss III and his collaborators).

RESULTS

The basic strategy by which globin cDNA was synthesized from the mRNA with AMV reverse transcriptase and made double-stranded with DNA polymerase I is shown in Fig. 1. Since cDNA acts as a self-priming template for DNA polymerase I (see *Materials and Methods*), Fig. 1 shows a small double-stranded "hook" at the 3'-end of the cDNA. After S1 nuclease treatment, approximately 30 nucleotides were removed from the 5'-ends of both synthetic double-stranded globin and *Eco*RI-cleaved plasmid molecules with bacteriophage lambda exonuclease (1). Terminal transferase was used to add poly(dA) tails to the free 3'-ends of the globin DNA molecule and po-

Table 1. Transformation of *E. coli* HB101 by recombinant globin DNA/pSC101 hybrids

DNA	No. of tetracycline-resistant colonies per μg of DNA
pSC101 intact circles	8000
Poly(dT)-tailed pSC101	0
Poly(dT)-tailed pSC101 annealed with poly(dA)-tailed double-stranded globin DNA	47

ly(dT) tails to the 3'-ends of each strand of the linear plasmid (1). Equimolar amounts of tailed plasmid and globin DNA were then annealed and used to transform $CaCl_2$-shocked *E. coli*. Transformants were selected by the tetracycline resistance carried by pSC101 or pMB9.

Transformation. Since the poly(dT)-tailed plasmid DNA has no way to efficiently cyclize, it should be unable to transform *E. coli* cells except when allowed to anneal and cyclize with the poly(dA)-tailed globin DNA. Table 1 shows the number of tetracycline-resistant colonies obtained when the various DNAs were used to transform $CaCl_2$-treated HB101 *E. coli*. Forty-seven transformants were obtained with mixed globin and pSC101 DNAs, while no transformants were observed with the tailed linear plasmid alone.

In a later experiment utilizing the chloramphenicol-amplifiable plasmid pMB9 and the *E. coli* strain C600, the data in Table 2 were obtained.

Colony Hybridization Screening. In order to show that these transformants do indeed carry globin sequences, we employed the colony hybridization technique of Grunstein and Hogness (3), in which clones of bacteria to be screened are grown on nitrocellulose filters lying over nutrient agar plates, and then are lysed and their DNA is denatured *in situ* by alkali treatment. Transformant DNA thus immobilized and localized to the spot on the filter upon which the colony grew was then challenged under hybridizing conditions with ^{32}P-labeled globin RNA transcribed *in vitro* from the original single-stranded globin cDNA with RNA polymerase in the presence of a [^{32}P]NTP precursor. Approximately 60% of the different transformants gave an autoradiographic signal greater than control colonies of cells carrying non-hybrid plasmids, also screened on the same filters.

Analysis of Inserted Sequences. Clones giving positive autoradiographic signals were reisolated twice on tetracycline-containing agar plates and cultured, and their plasmid DNA was isolated as described above. These plasmids have been named pHb*n*, where *n* denotes the isolate number. Previous sequencing studies (4) predicted an endonuclease *Eco*RI site at the nucleotide sequence corresponding to beta globin amino

Table 2. Transformation of *E. coli* C600 by recombinant globin DNA/pMB9 hybrids

DNA	No. of tetracycline-resistant colonies per μg DNA
pMB9 intact circles	190,000
pMB9 *Eco*RI cleaved	617
pMB9 poly(dT)-tailed	406
pMB9 poly(dT)-tailed annealed with poly(dA)-tailed double-stranded globin DNA	3,336

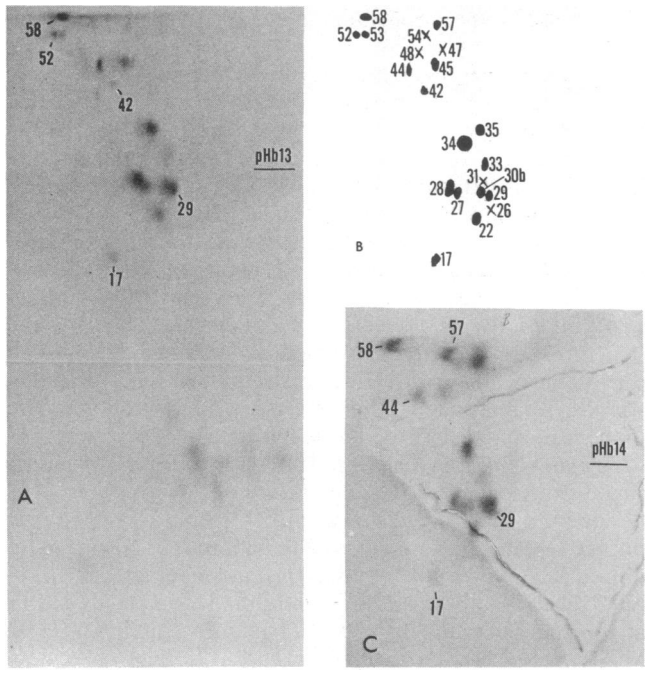

FIG. 2. (A) Fingerprint of combined ribonuclease U1 and bacterial alkaline phosphatase digest of RNA synthesized *in vitro* from globin cDNA and found to be hybridizable to pHb13. [α-^{32}P]CTP was the labeled precursor. pHb13 DNA was immobilized on a 24 mm Schell and Delaumer nitrocellulose membrane filter (25) after linearization by *Eco*RI and alkali denaturation. The labeled RNA transcript was treated with RNase-free DNase (4 μg in 100 μl) for 1 hr at room temperature to eliminate the cDNA template and extracted with phenol before desalting on a Bio-Gel P-60 column. After evaporation, the RNA was resuspended into 0.01 M Tris·HCl at pH 7.3, 0.33 M KCl, 0.002 M EDTA. Plasmid DNA (10–20 μg) was typically bound to filters and challenged with up to 0.1 μg of labeled RNA. Hybridization was for 16 hr at 65° in 0.5 ml of the RNA solution. Filters were then treated with heat-treated RNase at 1 mg/ml in 0.30 M NaCl, 0.030 M sodium citrate, pH 7.0 (2× SSC) for 20 min at 37° and were washed again with 2× SSC. Elution of the hybridized RNA from the filters was as described by Barnes (25), using iodoacetate-treated DNase. The eluent was passed with suction through another nitrocellulose membrane and precipitated in ethanol before digestion and fingerprinting as previously described (26). (B) Line drawing depicting beta globin RNA spots present and alpha globin RNA spots absent in the fingerprint of pHb13 shown in (A). ×s indicate the positions of absent spots. (C) Top half of fingerprint of pHb14 as done in (A) except the plasmid was linearized with endonuclease *Hin*dIII rather than *Eco*RI before denaturation. As discussed in the *text*, spot 57 spans the *Eco*RI site in the beta globin DNA sequence.

acid residues 120 and 121 and alpha globin residues 116 and 117. By 0.5% agarose gel electrophoresis, plasmids from seven of eight clones tested were shown to be *Eco*RI-sensitive and larger than the parent pSC101 molecules. Since the single *Eco*RI site of pSC101 is destroyed by our insertion technique, the *Eco*RI site of these plasmids must lie in the inserted DNA.

To obtain an accurate estimate of the fraction of the globin mRNA sequence inserted into a given hybrid plasmid, the plasmid DNA was denatured onto nitrocellulose filters and ^{32}P-labeled RNA synthesized *in vitro* from globin cDNA was allowed to hybridize (24). After pancreatic ribonuclease treatment to digest any RNA not hybridized to the inserted globin DNA sequences, the remaining RNA was eluted from the filter with DNase (25), digested with ribonuclease U1 and bacterial alkaline phosphatase, and fingerprinted as shown in Fig. 2. These fingerprints could then be compared with earlier fingerprints of *in vitro* RNA transcripts of globin cDNA prepared as part of the mRNA sequencing efforts of this laboratory

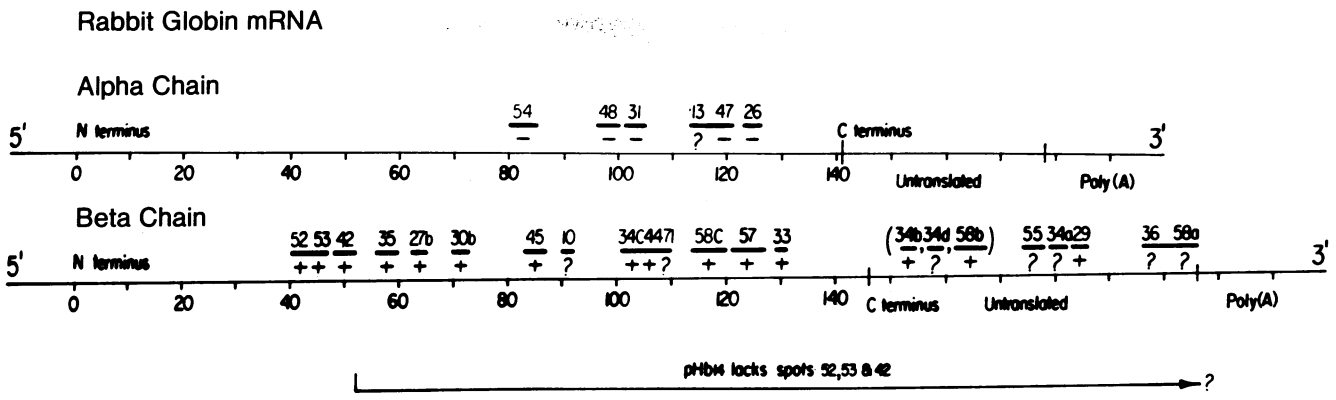

FIG. 3. Nucleotide sequences of T_1 digestion fragments assigned to either the alpha or beta globin message by prior sequencing data (4, 26) are represented. Those fragments found in fingerprints of pHb13 are indicated with a +; those absent, with a −; and those uncertain, with a ?. The extent of the globin insertion of pHb14 is also indicated. Numbers under the lines refer to amino acids.

(4, 26). Control filters carrying pSC101 DNA showed no hybridization above background.

Examination of the fingerprint pattern shown in Fig. 2A suggests that this plasmid, pHb13, carries most or all of the nucleotide sequences corresponding to the beta globin structural gene and untranslated region, since all of the spots already uniquely assigned to the beta chain by our previous sequencing work (4) and none of those assigned to the alpha chain are detectable. Secondary digestion of the spots with RNase U2 and/or RNase A confirmed the presence of 14 out of 19 possibly unique beta assignments, with the remaining five possible present; these five represent fragments that are not labeled when the radioactivity is introduced as [α-^{32}P]CTP, as in this experiment, or fragments that occur as part of a complex mixture. Fig. 2B shows the beta globin RNA spots present and alpha spots absent in this fingerprint. Notice that spot 57 is present in low yield. This is expected, since the spot 57 oligonucleotide spans the *Eco*RI cleavage site mentioned above and *Eco*RI was used in this experiment to open the twisted circles of pHb13 prior to denaturation. Thus, the spot 57 sequence should be protected little if at all from the RNase treatment. In a later experiment using *Hin*dIII to open pHb13, spot 57 appeared in high yield.

Fig. 3 summarizes the beta globin sequences confirmed by this method to be carried by pHb13. Thus, at least 420 nucleotides and possibly the entire beta globin mRNA sequence has been inserted into the plasmid. Since RNA sequences corresponding to the first 40 amino acids of the beta globin chain have not yet been assigned, it cannot be precisely determined how near to the 5′-end of the message the inserted sequences of pHb13 approach. In contrast, Fig. 2C shows a fingerprint obtained from hybrid plasmid pHb14. Here spots 52, 53, and 42 corresponding to beta globin amino acid residues 40 through 51 are absent, whereas spot 35 (corresponding to amino acid residues 55 through 60) and the other spots characteristic of beta chain sequences are present, suggesting that the inserted sequences of this plasmid terminate somewhere in the region corresponding to beta globin amino acid residues 49–55. Both pHb13 and pHb14 fingerprints contain spot 29, so the inserted sequences of both continue at least to within 44 nucleotides of the 3′-end of the message, well into the untranslated region (the exact position of the spot 29 sequence within the untranslated region is known from ref. 27).

Fingerprint patterns have also been obtained for several other pHb plasmids: pHb23, which is identical to pHb13 in this test; other plasmids that give simpler fingerprints; and still others that appear to bear alpha chain mRNA sequences. We have not yet carried out the necessary secondary digestions required to identify these latter fingerprint patterns conclusively.

We have also begun to utilize digestion with restriction endonucleases in order to characterize the inserted sequences and to provide discrete sized fragments of duplex globin DNA suitable for sequencing by the rapid new high percentage polyacrylamide gel techniques (W. Gilbert and A. Maxam, unpublished). Fig. 4 shows an 8% polyacrylamide gel electro-

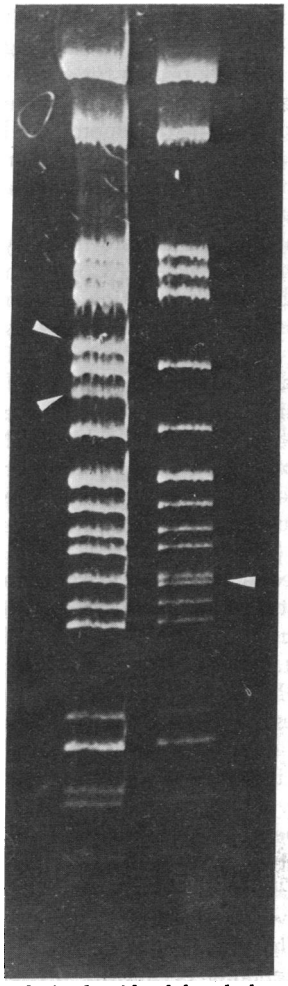

FIG. 4. An 8% polyacrylamide slab-gel electrophoresis of *Hae* III digests of pSC101 (right tract) and pHb23 (left) carried out by J. Browne and H. Heindell. The arrows indicate bands described in the text. The direction of electrophoresis is from top to bottom.

phoresis of a pHb23 *Hae* III digest paralleled by a parent pSC101 *Hae* III digest. Note the disappearance of a low-molecular-weight fragment from the pSC101 tract and the appearance of two larger fragments in the pHb23 tract. These new fragments must carry globin gene sequences, and in fact the larger of the two has been found to carry the inserted *Eco*RI site. Digestion of this fragment with *Eco*RI yields a 51-base-pair fragment which has now been completely sequenced using the Gilbert and Maxam technique (ref. 28 and J. Browne, P. Clarke, and W. Salser, manuscript in preparation). The sequence obtained agrees completely with the rabbit beta globin amino acid sequence from residue 120 (*E*coRI site) through 138 (*Hae* III site).

DISCUSSION

The methods presented in this paper should be applicable to any polyadenylylated RNA; in collaboration with others, we have since cloned sequences from immunoglobulin, chick globin, and ovalbumin mRNAs and total mRNA from *Dictyostelium discoideum*. Further, the ability to add poly(A) sequences *in vitro* to RNAs which are not normally polyadenylylated (29) makes the approach a truly general one. The method does not depend on a highly purified mRNA preparation; it is itself a powerful method of sequence purification since any one transformant should contain sequences originating from a single mRNA molecule. Thus, the technique can be applied to picking out a single message species from a mixture of mRNAs, assuming that there exists a suitable means of identifying the desired clones. Such purification should be especially useful in the study of developing tissues where one may wish to obtain pure probes for each of the different tissue-specific mRNAs present at any stage of development. Cloning should permit the developmental biologist to resolve complex mixtures of mRNAs or study minor mRNAs for which pure probes are otherwise unobtainable by any direct approach previously available.

We have compared the transformability of *E. coli* HB101 and C600 and have found that C600 generally gives about twenty-fold higher transformation efficiencies than HB101. In a typical experiment, 1 μg of tailed plasmid DNA was annealed to about 0.2 μg of duplex globin DNA; we obtained several thousand transformants with *E. coli* C600.

Concurrent with our studies, several other laboratories have also cloned cDNA from rabbit globin mRNA. Maniatis *et al.* (6) using an approach similar to that reported here, have obtained high globin cDNA cloning efficiencies. Also using *E. coli* C600, but with substantially different experimental approaches, Rougeon *et al.* (7) and Rabbitts (8) obtained transformants bearing globin sequences at an efficiency much lower than that reported here. It is possible that the low efficiencies observed by Rougeon *et al.* and Rabbitts may be at least in part due to their failure to take advantage of the self-priming properties of the globin cDNA (5, 6) for the second-strand synthesis. Both their approaches involve the addition of a homopolymer sequence to the 3'-end of the cDNA, a reaction which should be very inefficient if this sequence is base-paired as suggested by ourselves and by others.

An obvious benefit of working with recombinant plasmid DNA is the ability to make milligram amounts of a given DNA sequence in absolute purity. Thus, large amounts of globin DNA probe are now available that we hope to use to obtain sequences extending in either direction from the globin structural genes in the rabbit genome, permitting investigation of possible control mechanisms in the globin gene family and the obtaining of new information on the organization and function of mammalian genes.

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